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New Excised-Leaf Assay Method to Test Inoculativity of Asian Citrus Psyllid (Hemiptera: Psyllidae) With *Candidatus* Liberibacter asiaticus Associated With Citrus Huanglongbing Disease

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ABSTRACT The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is the primary vector of *Candidatus* Liberibacter asiaticus (Las) associated with huanglongbing, or citrus greening, the most devastating citrus (*Citrus* spp.) disease worldwide. Here, we developed a new "excised-leaf assay" that can speed up Las-inoculativity tests on Asian citrus psyllid from the current 3–12 mo (when using whole citrus seedlings for inoculation) to only 2–3 wk. Young adults of Asian citrus psyllid that had been reared on Las-infected plants were caged on excised healthy sweet orange [*Citrus sinensis* (L.) Osbeck] leaves for a 1–2-wk inoculation access periods (IAP), and then both psyllids and leaves were tested later by quantitative polymerase chain reaction (PCR). When single adults were tested per leaf, percentages of Las-positive leaves averaged 2–6% by using HLBaspr primers and 10–20% by using the more sensitive LJ900 primers. Higher proportions of Las-positive leaves were obtained with 1) higher densities of inoculating psyllids (5–10 adults per leaf), 2) longer IAPs, and 3) incubation of leaves for 1 wk postinoculation before PCR. Logistic regression analysis indicated a positive correlation between Las titer in Asian citrus psyllid adults tested singly and the probability of detecting Las in the inoculated leaves, correlations that can be very useful in epidemiological studies. Comparison between excised leaves and whole seedlings, inoculated consecutively for 1 wk each by one or a group of psyllids, indicated no significant difference between Las detection in excised leaves or whole plants. This new excised-leaf assay method saves considerable time, materials, and greenhouse space, and it may enhance vector relation and epidemiological studies on Las and potentially other Liberibacter spp. associated with huanglongbing disease.

KEY WORDS huanglongbing, citrus greening, *Candidatus* Liberibacter asiaticus, Asian citrus psyllid, *Diaphorina citri*

The bacterium *Candidatus* Liberibacter asiaticus (Las) has been implicated as one of the causative agents of huanglongbing (HLB), also known as citrus greening, currently the most devastating citrus disease in Florida, Brazil, and several other citrus-producing areas worldwide (Halbert and Manjunath 2004, Gottwald 2010, Bassanezi and Montesino 2011). Previously known primarily from Asia and Africa, HLB was detected in the Western Hemisphere in 2004 and was discovered in Florida in 2005 (Bové 2006, Gottwald 2010). HLB disease agent infects many citrus cultivars and causes substantial economic loss by promoting fruit drop, shortening the life span of infected trees, and often rendering fruit inedible (Miyakawa 1980). Yield reduction can reach 30–100% depending on the proportion of the canopy affected and the age of trees during inoculation (Gottwald 2010, Bassanezi and Montesino 2011). There are currently three closely related, phloem-limited fastidious α-proteobacteria associated with this disease that have been provisionally categorized according to the International Code of Nomenclature of Bacteria and named as follows: *Candidatus* Liberibacter asiaticus (Las, isolated from Asia and the Americas); *Candidatus* Liberibacter africanus (Laf, isolated from Africa), and *Candidatus* Liberibacter americanus (Lam, isolated from Brazil) (Garnier et al. 1984, Jagoueix et al. 1996, Gottwald et al. 2007). Although Koch postulates remain to be completed, Tyler et al. (2009) provided evidence, using phloem metagenomic DNA analy-

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sis, that strongly suggested that *Candidatus* Liberibacter asiaticus (Las) is the main, if not the only, phloem microbe present in plants with severe HLB symptoms in Florida.

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), transmits Las in Asia and the Americas and Lam in Brazil, and the African citrus psyllid *Trioza erytreae* (Del Guercio) (Hemiptera: Psyllidae) transmits Laf in Africa (Capoor et al. 1974, Xu et al. 1988, Halbert and Manjunath 2004, Hung et al. 2004, Gottwald 2010). Asian citrus psyllid developing on HLB-diseased trees can acquire Las most efficiently during the nymphal stage, and emerging adults from these nymphs are infected and may immediately be able to transmit the pathogen (Capoor et al. 1974, Inoue et al. 2009, Pelz-Stelinski et al. 2010). Using quantitative polymerase chain reaction (PCR) (qPCR), Inoue et al. (2009) reported that Las apparently multiplies in Asian citrus psyllid when acquired by nymphs but not when acquired by adults. They suggested that multiplication of the bacterium within the psyllids is essential for efficient transmission and that it is difficult for adults to transmit the pathogen unless they acquire it as nymphs. After acquiring the pathogen, Asian citrus psyllid nymphs and adults may remain infective/inoculative throughout their life span. Normally, however, the proportion of Las-infected Asian citrus psyllid individuals, based on PCR assays of the entire body, is much higher than the proportion of psyllids that can transmit Las to healthy plants (i.e., inoculative psyllids) (Inoue et al. 2009, Pelz-Stelinski et al. 2010, Ammar et al. 2011a). Using qPCR and fluorescent in situ hybridization, Ammar et al. (2011a,b) reported that Las systemically invades most of the internal organs and tissues of Asian citrus psyllid, including the alimentary canal, salivary glands, hemolymph, and fat tissue, but the proportion of Las-infected salivary glands was much lower than that of other organs, suggesting that barriers in the salivary glands may be responsible for the lower inoculativity rates reported. For example, Pelz-Stelinski et al. (2010) reported that whereas 40–60% of Asian citrus psyllid that fed on Las-infected plants became PCR positive for the bacterium, successful Las transmission to citrus plants by individual *D. citri* ranged only from 4 to 10%.

Because the proportion of "inoculative" Asian citrus psyllid is normally much lower than that of "Las-infected" psyllids, qPCR detection of Las in Asian citrus psyllid, by itself, may not be a good indicator of the vector pool for epidemiological and resistance studies of Las and HLB. Thus, to understand the epidemiology of HLB, and to test for susceptibility or resistance of cultivars to HLB, inoculativity of Asian citrus psyllid should be experimentally tested. To test their inoculativity with Las, psyllids are usually fed singly or in small groups on young citrus seedlings and the latter assayed by PCR, usually 3–12 mo later, apparently due to the long incubation period of Las in citrus plants (Gottwald 2010, Pelz-Stelinski et al. 2010). To shorten the inoculativity test period for Las and Asian citrus psyllid, we initially tried using leaf disks, a method used previously to test the inoculativity of thrips with some Tospoviruses (Chatzivassiliou et al. 2007, Ciuffo et al. 2010); however, this method did not produce satisfactory results with Las (D.G.H., unpublished data). Thus, here, we report the development of a new "excised-leaf assay" method that can speed up Las-inoculativity tests on Asian citrus psyllid and other psyllids considerably by shortening this period from 3 to 12 mo to 2 to 3 wk.

Materials and Methods

Psyllids. We used adult Asian citrus psyllid from a laboratory colony that has been maintained for several generations on HLB-symptomatic, Las-infected (PCR-positive) citrus plants (rough lemon, *Citrus jambhiri* Lush.). Healthy control Asian citrus psyllid adults were taken from a laboratory colony established during 2000 that has been maintained on healthy orange jasmine [*Murraya paniculata* (L.) Jack] trees, and more recently on healthy citrus trees (*Citrus macrophylla* Wester) in the greenhouse as described by Hall et al. (2007). No wild Asian citrus psyllid were introduced into this colony, and individuals from the colony are PCR assayed every 3 mo to ensure that the colony remains free of Las.

Excised-Leaf Assay Setup. Young adults of Asian citrus psyllid, reared from the egg stage on Las-infected citrus plants, were tested for their inoculativity of Las into citrus leaves by using the excised leaf assay. This was done by caging these adults singly or in small groups (5–10 adults per leaf) on young excised sweet orange leaves [*Citrus sinensis* (L.) Osbeck 'Ridge Pineapple'] of medium size (≈3–4 cm in width and ≈6–8 cm in length, having petioles ≈2–3 cm in length). The excised leaves were washed thoroughly with deionized (DI) water to remove any pesticide or fertilizer residue then air dried before subsequent use in testing. The terminal end of the leaf petiole was then cut diagonally with a sharp clean razor blade, so that it has a wider cut area for better water absorption, and each petiole was immediately inserted into a small (0.6-ml) microcentrifuge tube filled with water (Fig. 1A). A piece of Parafilm membrane (Pechiney Plastic Packaging Company, Chicago, IL) was wrapped around the top of this tube and the petiole to minimize water evaporation and to keep insects from coming into contact with water. The excised leaf with the microcentrifuge tube were then inserted into a larger 50-ml polypropylene tube (3 cm in width and 11.5 cm in length; Thermo Fisher Scientific, Waltham, MA), and the adult psyllids were subsequently added. For ventilation, the screw caps of these "caging" tubes were perforated by a hot needle or, alternatively, we used 50-ml BD Falcon Conical Tubes with Flip-Top Cap (352077; BD Biosciences, San Jose, CA) where we removed the flip portion of the cap and placed a piece of fine-mesh screen cloth under the screw cap which provided better ventilation (Fig. 1A).

These caging tubes were placed on a tube rack (Fig. 1B) and kept either on the benchtop in the laboratory (at 23.7 ± 1.5°C) or in an environmental chamber (at

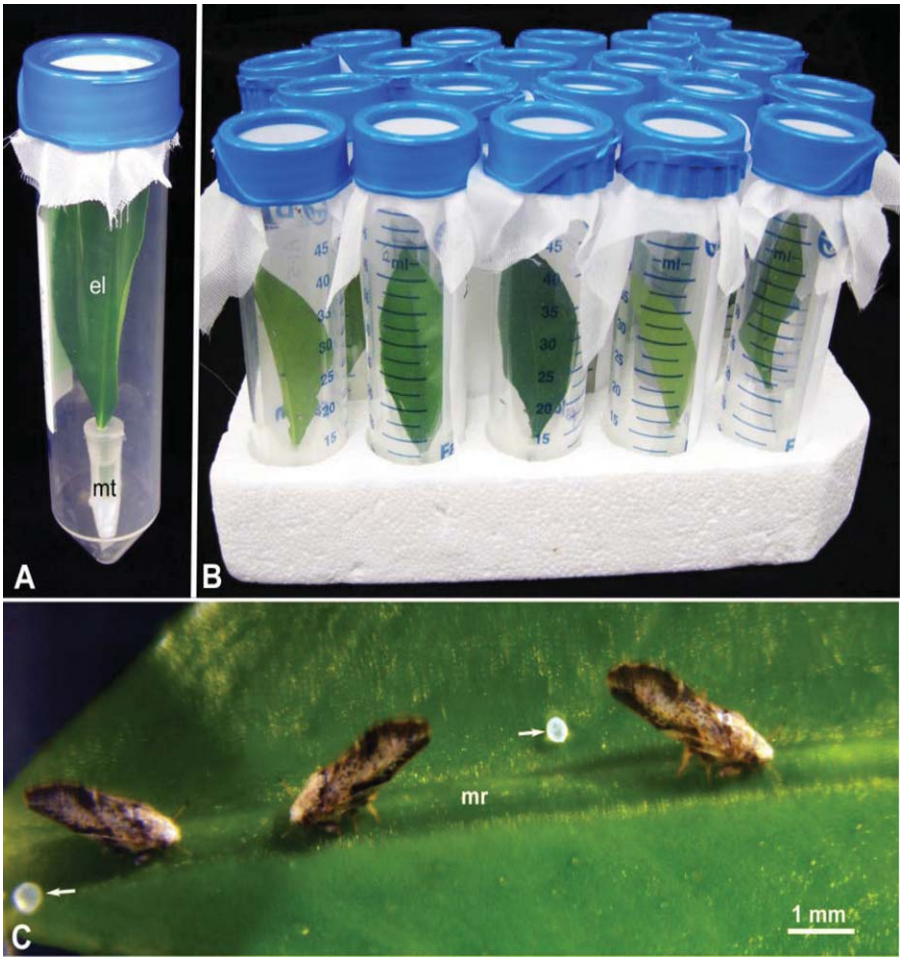

Fig. 1. (A) Polypropylene 50-ml tube used for caging psyllids with an excised citrus leaf (el), the petiole of which is inserted into a small microcentrifuge tube (mt) full of water, with a piece of Parafilm membrane wrapped around the top of this tube. The caging tube is covered with a piece of fine-mesh screen cloth under the screw cap, with the flip-top part of the cap removed for ventilation. (B) Caging tubes in a tube-rack. (C) Psyllid adults feeding on the midrib (mr) of an excised citrus leaf; arrows indicate whitish honeydew excretions of the females (male honeydew droplets are colorless). (Online figure in color.)

25.7 ± 0.4°C) with 14 h of light per day. Asian citrus psyllid adults were caged in these tubes on the above-described leaves for an inoculation access period (IAP) of 1 or 2 wk as mentioned for each experiment (see Tables 1–3). The leaves were then washed (as described below) and either frozen immediately or after a 1-wk "holding" (postinoculation) period (HP), during which each leaf was placed in a sealed clear plastic bag with a slightly moistened paper towel to keep it from drying. During this HP, the bagged leaves were kept in the environmental chamber (at 25°C with 14 h of light per day) before being frozen and processed by qPCR by using either HLBaspr or LJ900 primers (Li et al. 2006, Morgan et al. 2012).

Experimental Treatments. We conducted six experiments on the excised leaf assay (Tables 1–3) to evaluate various factors affecting Las inoculation by Asian citrus psyllid, including psyllid density (1, 5, or 10 adults per leaf), IAP (1 or 2 wk), and HP (0 or 1 wk). In two of these experiments, we compared the inoculation of excised leaves to that of whole citrus seedlings by Asian citrus psyllid (see Table 4). To do this, we caged Las infected psyllids (one or five adults per seedling) on healthy excised leaves or seedlings of sweet orange (Ridge Pineapple) for 1 wk. The seedlings used were 15–23 cm in height, and the insects were caged on them in plastic tube cages. After inoculation by Asian citrus psyllid, the excised leaves were frozen after a 1-wk HP, whereas the seedlings were moved to an insect proof greenhouse in which the plants were regularly sprayed with pesticides to reduce the potential for an external inoculation of Las by any stray psyllids. These seedlings were sequentially sampled by qPCR by using HLBaspr primers at 5–6, 9–10, and 11–12 mo postinoculation (three pooled leaves per seedling per date). The temperature in the greenhouse

throughout the year ranged between 22.8 and 32.8°C. In each of the six experiments conducted, 30–50 healthy control psyllids were tested using 5–10 adults per leaf or seedling for 1–2 wk IAP. At the end of each experiment, the surviving psyllids and excised leaves they fed on also were tested by qPCR.

DNA Isolation From Citrus Leaves and Psyllids. All sample processing and DNA extraction were undertaken in a laminar flow hood. Excised citrus leaves on which Asian citrus psyllid adults were fed for an IAP of 1 or 2 wk were washed thoroughly in RNase-Away (Molecular BioProducts, Inc., San Diego, CA) and subsequently rinsed with DI water. This was done to remove Asian citrus psyllid honeydew excretions, eggs, or nymphs and any Las bacteria that may have been present on the leaf surface (e.g., possibly with or from anal excretions). Only the midrib of inoculated leaves was processed for qPCR. Each midrib was separated from the leaf blade and chopped into very small pieces with a new sterile razor blade. Each sample was then placed in an individual tube that was subsequently stored in a freezer at −80°C until further processing.

Total plant per Las DNA was extracted using the Nucleo-Spin Plant II kit (Macherey-Nagel, Bethlehem, PA). Chopped midrib samples were aseptically placed into sterile 2-ml impact resistant, screw-top tubes (USA Scientific, Ocala, FL) or into a single well of a 96-deep well plate (Macherey-Nagel).). The 2-ml screw top vials contained two 0.625-cm steel shots (Daisy Outdoor Products, Rogers, AR), whereas 96-deep well plate contained approximately 12 1.3-mm chrome steel beads per well (Biospec Products, Bartlesville, OK). To these wells, 400 μl of lysis buffer and 10 μl of RNase were added to the tubes/wells, and samples were subsequently homogenized by physical disruption by using a FastPrep 24 (MP Biomedicals, Solon, OH) for the 2-ml tubes, and a Mini Bead Beater 96 (Biospec Products) or Genogrinder (OPS Diagnostics, Lebanon, NJ) for the 96-deep well plates. Extraction was then carried out according to the manufacturer's protocols (Macherey-Nagel). After extraction, DNA was quantified by mass spectroscopy at 260 nm by using a Nanodrop 1000 (NanoDrop Products, Wilmington, DE), aliquoted, and diluted in nuclease-free water (QIAGEN, Valencia, CA) so that 100 ng of DNA was used in each qPCR reaction. Samples were stored at −20°C until used for PCR.

The inoculating psyllids used in the assay were collected at the end of each experiment and stored in 70% ethanol at 4°C until their DNA could be extracted. DNA was extracted from psyllids by using a crude extraction method derived from De Barro and Driver (1997). Each psyllid was placed in 150 μl of lysis buffer (5% 1 M KCl, 5% 1 M Tris at pH 8.4, 0.45% Tween 20, 0.45% NP-40, and 89.1% autoclaved DI water) in a 1.5-ml centrifuge tube (USA Scientific). Tubes were placed in a FastPrep-24 and homogenized for 30 s at 6 m/s. After homogenization, 100 μl of liquid was transferred to a clean 1.5-ml centrifuge tube and then placed in a water bath at 95°C for 5 min. Samples were immediately placed on ice for at least 10 min and then centrifuged at 14,000 g for 2 min. The supernatant was removed and stored in 0.5-ml PCR tubes (USA Scientific) at −20°C until analysis. In each qPCR reaction, 2 μl of crude psyllid extract was used.

qPCR. Extracted DNA from both leaves and psyllids was assayed for the presence of Las by using both the HLBaspr probe/primer set targeting the 16S DNA of LAS (5′→3′ sequences: forward TCGAGCGCGTATGCAATACG and reverse GCGTTATCCCGTAGAAAAAGGTAG, probe AGACGGGTGAGTAACGCG with 6-carboxyflourescein reporter dye on the 5′ end and TAMRA quencher on the 3′ end, Li et al. 2006) or the LJ900 primers targeting the *hyvI/hyvII* gene of a Las prophage (5′→3′ sequences: forward GCCGTTTTAACACAAAAGATGAATATC and reverse ATAAATCAATTTGTTCTAGTTTACGAC (Morgan et al. 2012). All primers and probes were manufactured by Integrated DNA Technologies, Inc. (Coralville, IA). The HLBaspr primers were run as a 20-μl reaction using Taqman Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.4 mM each of forward and reverse primer, and 500 nM of probe. The temperature program for the HLBaspr primers was 95°C for 5 min followed by 50 cycles of 95°C for 3 s followed by 60°C for 30 s. Samples were run in duplicate on the HLBaspr primers and rerun in cases when amplification occurred in only one of the two reactions. LJ900 primers were run in a 15-μl reaction using PerfeCTa SYBR Green FastMix Master Mix (Quanta Biosciences, Inc., Gaithersburg, MD), 600 nM forward primer, and 900 nM reverse primer. The temperature program for the LJ900 primers was 95°C for 5 min, followed by 50 cycles of 95°C for 3 s, followed by 62°C for 30 s, and then a disassociation cycle of 95°C for 15 s, 62°C for 1 min, and a gradual ramp to 97°C for 15 s. The presence or absence of amplification in LJ900 reactions was confirmed by examining the melt curve of the amplified product. Each sample was tested one time with the LJ900 primers, and positive samples were confirmed by running them in triplicate. Both sets of primers were run on 96-well skirted plates sealed with TemPlate film (USA Scientific). All qPCR plates contained positive controls and four to six wells of no template and negative controls. All qPCR reactions were run on an ABI 7500 Fast machine and analyzed with 7500 Software version 2.0.1 (Applied Biosystems).

Statistical Analysis. Initially, the proportion of Las-positive samples in the inoculated leaves or inoculating psyllids in each experiment was analyzed using chi-square test or Student's *t*-tests. However, because of the variability between experiments, the following more advanced methods were used to analyze the overall data from all six experiments (989 inoculated leaves in total).

Agreement between the two primer sets was assessed using Cohen's kappa coefficient that tests the hypothesis that two categorization methods agree no more than would be expected by chance given the frequency of the responses in each category. The effects of psyllid density (number of psyllids per leaf), IAP (time psyllids were confined to a leaf in days), and

Table 1. Results of inoculativity tests on individuals or groups of Asian citrus psyllid adults from a Las-infected colony by using excised citrus leaves for inoculation and qPCR with two Las primers

No. adults per leaf	Expt. no.	Tested psyllids			Inoculated leaves		
		No. tested	% Las positive		No. tested	% Las positive	
			HLBaspr primers	LJ900 primers		HLBaspr primers	LJ900 primers
10	1	32	75.00	93.75	10	40.00	60.00
	2	38	39.47	71.05	15	20.00	40.00
	Total	70	55.71	81.43	25	28.00	48.00
5	1	54	77.78	94.44	32	18.75	40.63
	2	25	40.00	64.00	20	10.00	10.00
	Total	79	65.82	84.81	52	15.38	28.85
1	1	22	81.82	95.45	45	4.40	11.11
	2	23	73.91	82.61	55	5.45	12.73
	Total	45	77.78	88.89	100	5.00	12.00

Asian citrus psyllid that had been reared on Las-infected citrus plants were fed on excised healthy sweet orange leaves for 1 wk, and the leaves processed for qPCR 1 wk postinoculation.

the HP (number of days between when psyllids were removed and the leaf was frozen for processing by PCR) on the probability of detecting Las transmission to excised leaves was analyzed by logistic regression. The full model contained all the first-order terms and their interactions (up to the three-way interaction between number of psyllids, IAP, and HP). The model was reduced by stepwise selection with $\alpha = 0.05$ (PROC LOGISTIC, SAS Institute 2008). Separate models were developed for the results of each primer set.

To determine whether the titer of bacteria found in individual psyllids at the end of the test affected the probability of detecting Las in inoculated leaves, we regressed the Cq (cycle threshold) value of the psyllids caged individually on leaves against both the probability that we detected Las in these leaves using logistic regression and the Cq value of each leaf where Las was detected using linear regression (PROC LOGISTIC and PROC REG, SAS Institute 2008). Psyllids that did not test positive for Las after the experiment was completed were excluded from the linear regression analysis. The effect of psyllid Cq was evaluated separately for the two primer sets.

As groups of 1, 5, or 10 insects per leaf were tested for inoculation, an estimate of the proportion of inoculative insects (if single insects were used per leaf) was calculated based on the properties of the binomial distribution by using the equation: $y = 1 - (1 - x)^{-(1/n)}$, where y is the proportion of infective insects in group or single tests, x is proportion of plants testing positive for the bacterium, and n is the number of insects per leaf (Madden et al. 2007).

For the comparison between inoculation of excised leaves versus whole seedlings, using HLBaspr primers in qPCR, the effect of experiment, number of Asian citrus psyllid per leaf, inoculated recipient (seedling or excised leaf), time elapsed postinoculation, and the two-way interactions involving number of Asian citrus psyllid on the probability of Las detection were tested via logistic regression (Proc Logistic, SAS Institute 2008). The excised leaves were assigned a value of 0 mo after inoculation, and the seedlings were tested for Las infection at 5, 9, and 12 mo postinoculation.

Results

Effects of Primer Sets, Psyllid Density, IAP, and HP. Results of the six experiments conducted on Asian citrus psyllid inoculation of excised citrus leaves by using 1, 5, or 10 Asian citrus psyllid adults per leaf from the infected colony, with 1- or 2-wk IAP and 0–1 wk HP are shown in Tables 1–3. In experiments 1 and 2 (Table 1), percentage of Las-positive adults was significantly higher using LJ900 primers (88.9%) than using HLBaspr primers (55.7%) ($\chi^2 = 19.71$, df = 1, $P < 0.0001$). The Cq values were normally lower (indicating higher titer of Las) with LJ900 than with HLBaspr primers. The average Cq value for Las-positive psyllids was 27.024 ± 0.596 by using HLBaspr primers and 23.868 ± 0.957 by using LJ900 primers ($t = 2.79$, df = 117, $P = 0.006$). The percentages of Las-positive samples in the inoculated leaves were also significantly higher using LJ900 than HLBaspr primers ($\chi^2 = 7.34$, df = 1, $P = 0.007$). These percentages, using 1, 5, or 10 adults per leaf were 5–12%, 15.4–28.9%, and 28–48%, respectively (Table 1). Later experiments showed similar trends (Tables 2 and 3). None of the healthy control psyllids or leaves in any of these experiments proved Las positive by either primer sets (data not shown).

In all six experiments, 10.62% of the inoculated leaves ($N = 989$) were Las positive by using both primer sets (HLBaspr and LJ900), whereas 76.54% of these leaves were Las negative by both primer sets. The HLBaspr primers were negative and the LJ900 primers were positive in 9.40% of the leaves tested, and the HLBaspr primers were positive and the LJ900 primers were negative for 3.24% of the leaves tested. Cohen's kappa coefficient test strongly rejected the hypothesis that the results of the two primer sets were random ($\kappa = 0.558$, $P < 0.0001$), showing that in general the two methods agreed on whether a sample was infected or not, although more positives were detected using the LJ900 primers.

Using qPCR results from the HLBaspr primer set, the selected logistic regression model showed significant effects for psyllid density (number of adults per leaf), IAP, HP, the interaction between psyllid

Table 2. Results of 1- and 2-wk inoculativity tests on Asian citrus psyllid adults from a Las-infected colony by using excised citrus leaves for inoculation and qPCR with HLBaspr primers

No. adults per leaf	Expt. no.	Tested psyllids		Inoculated leaves			
				1-wk IAP		2-wk IAP	
		No tested	% Las-positive	No tested	% Las-positive	No tested	% Las-positive
5	3	184	54.35	41	14.63	37	18.92
	4	293	55.29	40	47.50	60	61.67
	Total	477	54.93	81	30.86	97	45.36
1	3	75	30.67	75	4.00	74	2.70
	4	92	18.48	80	5.00		
	Total	167	22.16	155	4.52	74	2.70

Asian citrus psyllid that had been reared on Las-infected citrus plants were fed on excised healthy sweet orange leaves for 1 or 2 wk, and the leaves processed for qPCR immediately or 1 wk post-inoculation.

density and IAP, and the interaction between psyllid density and HP (Table 4). According to our selected model, increasing the psyllid density, (between 1 and 10 adults per leaf) or the inoculation access period (between 1 and 2 wk) increased the probability of detecting Las transmission into inoculated leaves. Also, increasing the HP (between 0 and 1 wk postinoculation) increased the probability of Las detection in inoculated leaves for single psyllids but not for groups (5 or 10 psyllids per leaf). The effect of increasing the HP was less pronounced for longer IAPs.

With qPCR results using the LJ900 primer set, the selected logistic regression model showed significant effects for the HP, and the interaction between psyllid density and IAP (Table 4). Longer IAP combined with higher psyllid density per leaf increased the probability of detecting Las transmission into inoculated leaves.

Correlation Between Psyllid Cq values and Detection of Las in Inoculated Leaves. From all the psyllids tested individually (one adult per leaf) that survived the 1 or 2 wk inoculativity tests, and proved to be Las positive by qPCR, our data set included 216 inoculations with single psyllids according to the HLBaspr primers (34.8% of Las-positive psyllids) and 295 inoculations using the LJ900 primers (47.5% of Las-positive psyllids). Using the HLBaspr primers, the Cq value of psyllids caged individually on excised leaves negatively affected the probability of Las detection in inoculated leaves (df = 1, parameter estimate = -0.2835, SE = 0.0684, Wald $\chi^2 = 17.2084$, $P < 0.0001$). Understandably, psyllids with a lower titer of Las (higher Cq values) were less likely to inoculate the leaf (Fig. 2A). Our data set included Asian citrus psyllid with HLBaspr Cq values between 21.9 and 40.98; the highest Asian citrus psyllid Cq where we observed inoculation was 30.85. Using the HLBaspr primers, there was a significant positive relationship between the Cq value of the psyllid and the Cq of the inoculated leaf (df = 1, $F = 6.11$, $P = 0.0220$, parameter estimate = 0.49, SE = 0.20) (Fig. 2B). Similarly, based on the LJ900 primers, the Cq of psyllids was negatively related to the probability that Las transmission was detected (df = 1, parameter estimate = -0.1831, SE = 0.0377, Wald $\chi^2 = 23.5809$, $P < 0.0001$) (Fig. 2C) and positively related to the Cq value of inoculated leaves (df = 1, $F = 9.69$, $P = 0.0038$, parameter estimate = 0.48, SE = 0.16) (Fig. 2D). Asian citrus psyllid Cq values for the LJ900 primer set were between 13.54 and 37.08; the highest Asian citrus psyllid Cq on the LJ900 primers where transmission has been observed was 30.02.

Table 3. Results of 1- and 2-wk inoculativity tests on Las-infected Asian citrus psyllid adults by using excised citrus leaves with 0- or 1-wk holding (postinoculation) period

No. adults per leaf	Expt. no.[a]	Inoculated leaves											
		1-wk IAP						2-wk IAP					
		No holding period			1-wk holding period			No holding period			1-wk holding period		
		No tested	% Las +		No tested	% Las +		No tested	% Las +		No tested	% Las +	
			HLBaspr primers	LJ900 primers		HLBaspr primers	LJ900 primers		HLBaspr primers	LJ900 primers		HLBaspr primers	LJ900 primers
5	5	16	18.75	75.00	17	11.76	47.06	5	0.00	20.00	9	11.11	44.44
	6	20	0.00	5.00	20	20.00	35.00	10	30.00	30.00	8	0.00	0.00
	Total	36	8.33	36.11	37	16.22	40.54	15	20.00	26.67	17	5.88	23.53
1	5	49	2.04	10.20	40	0.00	20.00	21	0.00	33.33	16	6.25	12.50
	6	50	2.00	10.00	49	0.00	4.08	25	0.00	8.00	27	0.00	14.81
	Total	99	2.02	10.10	89	0.00	11.24	46	0.00	19.57	43	2.33	13.95

Asian citrus psyllids that had been reared on Las-infected citrus plants were fed on excised sweet orange leaves for 1 or 2 wk, and then the leaves were processed for qPCR immediately or after 1 wk holding (postinoculation) period.

[a] In experiment 5, adults were fed on three consecutive groups of leaves in this order: 1 wk, 2 wk, and 1 wk; but in experiment 6, psyllids were fed in a different order: 2 wk, 1 wk, and 1 wk; this order was done to minimize the effect of feeding order on the results.

Table 4. Logistic regression model to describe the effects of the experimental factors on detecting Las in leaves inoculated by the Asian citrus psyllid by using two primer sets

Primer	Parameter	df	Estimate	SE	Wald χ^2	P
HLBaspr	Intercept	1	−2.5197	0.7676	10.7747	0.0010
	No. of Asian citrus psyllid per leaf	1	−0.2790	0.1419	3.8648	0.0493
	IAP	1	−0.0836	0.0819	1.0417	0.3074
	Holding (postinoculation) period	1	0.1696	0.0835	4.1294	0.0421
	No. of Asian citrus psyllid × IAP	1	0.0812	0.0167	23.5499	<0.0001
	No. of Asian citrus psyllid × holding period	1	−0.0215	0.0078	7.6945	0.0055
LJ900	Intercept	1	−1.7630	0.5213	11.4383	0.0007
	No. of Asian citrus psyllid per leaf	1	0.0228	0.1143	0.0399	0.8417
	IAP	1	−0.0445	0.0513	0.7519	0.3859
	Holding (postinoculation) period	1	−0.0683	0.0230	8.8164	0.0030
	No. of Asian citrus psyllid × IAP	1	0.0453	0.0123	13.5136	0.0002

The full model contained the first order terms for number of psyllids per leaf, inoculation access period, holding (postinoculation) period, all two- and three-way interactions among the first order terms. The model was reduced by stepwise selection with $\alpha = 0.05$ (PROC LOGISTIC, SAS Institute 2008).

Estimation of the Proportion of Inoculative Psyllids From Group or Individual Tests. Using data obtained from experiments 1–6 (Tables 1–3), we estimated the proportion of inoculative psyllids from group or individual tests by using the binomial distribution equation mentioned under Materials and Methods. The estimated proportion of inoculative psyllids in our study using 1, 5, or 10 adults per leaf were 10.65, 7.55, or 3.56% respectively, by using the HLBasper primers, and 9.93, 9.97, or 7.99%, respectively, by using the more sensitive LJ900 primers.

Intermittent Las Inoculation by Asian Citrus Psyllid. In three experiments using excised leaves, two consecutive tests were conducted using 1, 5, or 10 adults per leaf, with 1 or 2 wk IAP (Supp Table 1 [online only]). Percentage of leaves that proved Las positive in both tests varied according to the number of psyllids tested per leaf. Using the more sensitive

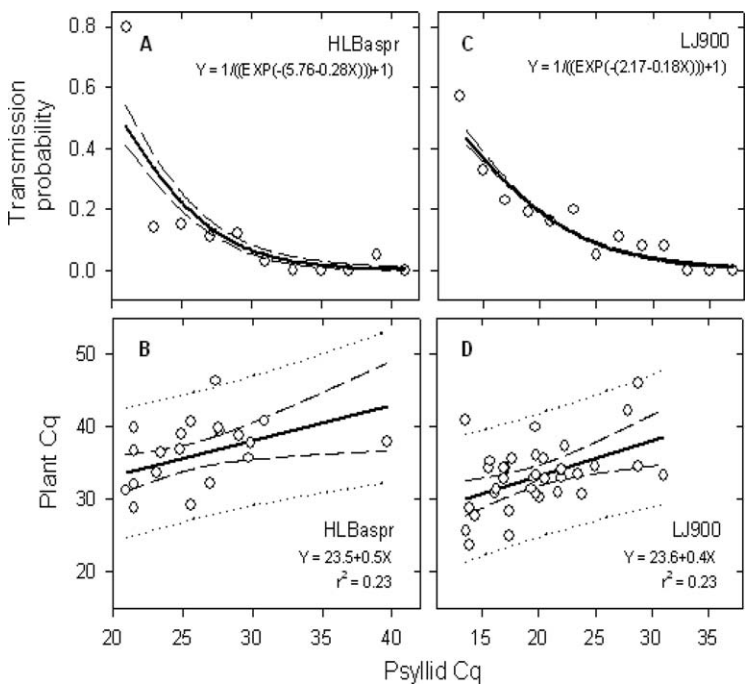

Fig. 2. Observed probability of Las detection (open circles in A and C) and Cq values obtained from infected leaves (open circles in B and D) following inoculation by single psyllids, grouped here according to Cq values (21–23, 23–25, etc.); predicted relationships (solid lines) with 95% confidence intervals (dashed lines) and prediction intervals (dotted lines) for: (A) logistic regression of the effect of Asian citrus psyllid Cq on the probability of detecting Las transmission by using the HLBaspr probe/primer set, (B) linear regression of the effect of Asian citrus psyllid Cq on excised leaf Cq by using the HLBaspr probe/primer set, (C) logistic regression of the effect of psyllid Cq on the probability of detecting Las transmission by using the LJ900 probe/primer set, and (D) linear regression of the effect of psyllid Cq on excised leaf Cq by using the LJ900 probe/primer set. All regressions were significant at $P < 0.05$. Logistic regressions were performed using the results of individual trials although the probability detected in groups is plotted on the graph for ease of interpretation.

Table 5. PCR results, using HLBaspr primers, of excised leaves and whole seedlings inoculated for 1 wk, consecutively, by the same individuals or groups of Asian citrus psyllid from a Las-infected colony

No. Asian citrus psyllid per leaf or seedling	Expt. no.	Inoculated excised leaves[a]		Inoculated seedlings[b]					
				5–6 mo		9–10 mo		11–12 mo	
		No. tested	%	No. tested	%	No. tested	%	No. tested	%
5	2	20	10.0	20	0.0	20	0.0	20	0.0
	4	40	47.5	40	30.0	39	2.6	39	7.7
	Total	60	35.0	60	20.0	59	1.7	59	5.8
1	2	55	5.5	54	3.7	41	14.6	30	0.0
	4	80	5.0	100	3.0	99	1.0	98	3.1
	Total	135	5.2	154	3.2	140	5.0	128	2.3

[a] Leaves were processed for qPCR 1 wk after inoculation.
[b] Seedlings were placed in the greenhouse and sampled for processing by qPCR 5–12 mo after inoculation (three pooled leaves per seedling/date).

LJ900 primers, 4/6 (66.7%) of leaves with 10 adults per leaf tested positive in both tests, compared with 4/11 (36.4%) of leaves with five adults per leaf, and only 1/14 (7.1%) of leaves with one adult per leaf. This suggests that Las inoculation by infected Asian citrus psyllid adults occurred intermittently within the tested period.

Inoculation of Excised Leaves Versus Whole Seedlings. In two experiments (Table 5), both excised leaves and young healthy seedlings of sweet orange were consecutively inoculated for 1 wk each by the same psyllids or groups (one or five adults per leaf or seedling). In both experiments, the order of inoculation was reversed for half of the tested psyllids, with leaves or seedlings tested first, to minimize any possible feeding order effect. Inoculated leaves were frozen and tested by qPCR 1 wk postinoculation, whereas leaf samples from inoculated seedlings were frozen and tested by qPCR 5–12 mo postinoculation.

Logistic regression analysis of the results showed significant effects due to Asian citrus psyllid density (one or five adults per leaf), experiment, HP, Asian citrus psyllid density-experiment interaction, and Asian citrus psyllid density-time postinoculation interaction. However, no significant effect was found between the inoculated recipients (excised leaves versus seedlings), or Asian citrus psyllid density-recipient interaction (Supp Table 2 [online only]).

Discussion

HLB, or citrus greening disease, is the most destructive citrus pathosystem worldwide (Bové 2006, Gottwald 2010). The three Liberibacter pathogens strongly associated with this disease in different parts of the world are transmitted either by Asian citrus psyllid or by the African citrus psyllid *T. erytreae* (Halbert and Manjunath 2004, Bové 2006). Two of the major difficulties in studying the epidemiology and vector interactions of HLB in various populations or regions are as follows: 1) although a high proportion (up to 100%) of the psyllids that feed on HLB-diseased plants may become infected with Liberibacter (as evidenced by PCR tests), a much smaller proportion of these insects (<10%) can normally inoculate the bacterium to healthy citrus plants; and 2) plants that are inoculated by infected Asian citrus psyllid only show HLB symptoms or become PCR positive for Las or other Liberibacter spp. usually 3–12 mo after inoculation with infected psyllids (Inoue et al. 2009, Bonani et al. 2010, Gottwald 2010, Pelz-Stelinski et al. 2010). In our study, we demonstrated that excised citrus leaves can be successfully used to assay the rate of inoculativity in Asian citrus psyllid adults and that such assays can take only 2–3 wk rather than 3–12 mo as with whole seedling. Our results using the more commonly used, but less sensitive, HLBaspr primers indicated that the percentage of Las-positive Asian citrus psyllid used in various experiments ranged from 22 to 81%. Yet, when single Asian citrus psyllid were tested per leaf, only 2–6% of the Asian citrus psyllid transmitted the pathogen, a finding that is largely comparable to results obtained by previous studies using whole citrus seedlings for Las inoculation by single Asian citrus psyllid adults (e.g., 5% by Pelz-Stelinski et al. 2010). Using LJ900 primers, however, higher rates of inoculation by single psyllids were indicated (10–20%). This excised-leaf assay method therefore can be much more efficient when using more sensitive Las primers. LJ900 primers have been shown previously to reduce the relative detectable Las threshold by ≈3–9 qPCR cycles compared with conventional 16S rDNA-based qPCR methods, and thus LJ900 primers detected Las from otherwise nondetectable levels in plant and insect samples (Morgan et al. 2012).

Logistic regression (Table 4) showed that greater proportions of Las-positive leaves were positively correlated with higher densities of inoculating Asian citrus psyllid adults per leaf (5–10 adults per leaf, compared with single psyllids), longer IAPs (2 wk rather than 1 wk), and 1-wk IAP followed by a 1-wk HP. Thus, our study suggests that the best conditions for this assay would be either using single psyllids per leaf for 2-wk IAP, or 1-wk IAP followed by 1-wk HP. Alternatively, one can use 5–10 insects per leaf for 1-wk IAP with or without a HP. Using 5–10 Asian citrus psyllid per leaf saves more time, labor, and research material, especially when the rate of Las inoculativity in Asian citrus psyllid is <10%. In addition, with group tests, the binomial equation can be used to estimate the proportion of inoculative

psyllids (Madden et al. 2007), making group testing much more efficient than single-insect testing. However, in our study, this equation tended to underestimate the proportion of inoculative psyllids when larger groups were tested (between 5–10 adults per leaf), especially with the less sensitive (HLBaspr) primers. Thus, using five adults per leaf may give a better estimate of inoculative Asian citrus psyllid individuals than using 10 adults per leaf.

Regardless of the primer set, our results indicated that a positive correlation exists between Las titer (as evidenced by Cq values) in Asian citrus psyllid adults tested singly and the probability of Las being transmitted to a leaf (Fig. 2A and C), which is logical and can be very useful in epidemiological studies. Because it is much easier and quicker to identify infected psyllids by PCR than to test their inoculativity by allowing them to feed on healthy plants, this positive correlation can be used to estimate the rate or proportion of Las-inoculative psyllids from that of PCR-positive psyllids in an Asian citrus psyllid population. Our results also suggest that the lower rate of Las transmission by Asian citrus psyllid compared with the proportion of Las-positive psyllids, as reported here and in previous investigations (Inoue et al. 2009, Pelz-Stelinski et al. 2010), is due to a low innate rate of Las inoculation by Asian citrus psyllid. This low innate rate may be largely related to a salivary gland "exit/escape" barrier thought to exist in Asian citrus psyllid (Ammar et al. 2011a,b) but could be influenced by insect age, feeding behavior, and acquisition access period (Inoue et al. 2009, Bonani et al. 2010, Pelz-stelinski et al. 2010). Transmission barriers associated with salivary glands have been reported with other insect-borne plant and animal pathogens, including viruses and bacteria (Hardy 1988, Ammar 1994, Hogenhout et al. 2008, Ammar et al. 2009). Possible mechanisms that may account for a salivary gland exit/escape barrier are that a lower pathogen titer is produced in the salivary glands or secreted during the feeding process to infect the host (Hardy 1988). The first factor does not seem to be the case with Asian citrus psyllid because Las titer in infected salivary glands was higher than that in most other tissues (Ammar et al. 2011a). Another possibility is that the pathogen may be inactivated by salivary secretions of the vector. The possible role(s) of chemical and physiological conditions of the salivary secretions in enhancing or obstructing pathogen transmission by insect vectors remains largely uninvestigated (Ammar 1994), but inhibitory effects of the salivary secretions of aphids on some aphid-nontransmissible viruses have been reported previously (reviewed by Nishi 1969).

Las has been reported to replicate in Asian citrus psyllid when acquired by nymphs but not when acquired by adults (Inoue et al. 2009). It is possible that the lower rate of Las replication in adults may be responsible for the intermittent transmission of Las by Asian citrus psyllid in two consecutive tests (Supp Table 1 [online only]). Such intermittent transmission by vectors has been reported earlier for Las and HLB (Capoor et al. 1974, Inoue et al. 2009) and for other insect-vectored plant pathogens (Ammar 1994, Ammar et al. 2009). It also can be related to a salivary gland exit barrier that may include the width of the salivary canal in the psyllids maxillary stylets. In Asian citrus psyllid adults, the diameter of this canal has been reported to be 0.3–0.5 μm (Garzo et al. 2012), whereas the diameter of Liberibacter cells in phloem tissues has been reported to be 0.33–0.66 μm (Hartung et al. 2010). This suggests that Asian citrus psyllid salivary canal may not be wide enough for the larger Liberibacter cells to go through and could thereby limit the rate of inoculation or contribute to intermittent transmission of Las.

That the Las bacteria detected by qPCR in excised leaves had been inoculated by psyllids during the IAP into the phloem tissue rather than other leaf tissues and thus can move systemically in the plant to potentially cause disease, is indicated by the following: 1) no significant differences were found in the percentage of Las-infected samples between inoculated excised leaves and whole seedlings when the latter were tested by qPCR several months postinoculation; 2) only the midribs were processed and tested by qPCR in both the excised leaves and leaf samples from the whole plants inoculated by Asian citrus psyllid, thereby increasing the chances of sampling mainly vascular tissue (phloem and xylem) compared with other leaf tissues; and 3) logistic regression analysis of qPCR results by using the two primer sets showed significant increase in the probability of Las detection in inoculated leaves for single psyllids after a 1-wk HP, which suggested that the inoculated bacteria may have replicated in the excised leaves during this period.

In experiments where we tested inoculation of excised leaves or whole seedlings by the single psyllids or groups, the probability of detecting Las in these seedlings decreased with time between 5 and 12 mo postinoculation. The decrease in Las detection in inoculated seedlings with time may have occurred for three possible reasons: 1) as the seedlings grow older (larger), we may be less likely to select infected leaves as Las is unevenly distributed through the plant (Gottwald et al. 2007, Folimonova et al. 2009, Gottwald 2010), 2) physiological processes in the plant may be negatively affecting Las populations initially inoculated by the psyllids, or 3) higher temperatures or other environmental conditions in the greenhouse may have negative effects on Las infection of inoculated seedlings (Folimonova et al. 2009). Although Lopes et al. (2009) indicated that Las in Brazil is heat tolerant, compared with *Ca.* Liberibacter americanus, it is possible that Florida isolate(s) of Las may be different in this regard than those in Brazil. Regardless, these results suggest that an excised leaf assay may give a more realistic picture of Las inoculation that actually takes place than whole seedling tests, despite what happens to the inoculated bacteria in planta after inoculation. If the lower detection efficiency in seedlings that we report here is due to processes in planta and not purely the effects of growth or environmental conditions, then examining the magnitude of the dif-

ferences in Las detection in excised leaves versus plants kept for several months may be useful as a Las resistance screening test.

The excised-leaf assay method, reported here with Asian citrus psyllid and Las, can probably be used with other psyllid vectors and other Liberibacter agents of HLB. Merits of using excised leaves to assay the inoculativity of psyllids with Las or other HLB agents, compared with using citrus seedlings, include the following: 1) much faster time to get the results (2–3 wk compared with 3–12 mo), which may be crucial in epidemiological and vector relation studies; and 2) reducing cost of research material (e.g., plants) and greenhouse space that can be important in larger studies or when space is limited. In addition, using this method can avoid or limit possible factors that may affect the pathogenicity or detection of Las in whole plants placed in the greenhouse for several months to a year, including the effect of variable or higher temperature or possible contamination by Las or HLB from any stray psyllids in the greenhouse. It is known that the latency of Las or HLB infection in host plants (postinoculation) is highly variable and is apparently greatly affected by tree age, horticultural health, and other factors (Gottwald 2010).

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