Localization of *Candidatus Liberibacter asiaticus*, Associated with Citrus Huanglongbing Disease, in its Psyllid Vector using Fluorescence in situ Hybridization

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**Abstract**

The bacterium *Candidatus Liberibacter asiaticus* (Las) has been strongly associated with huanglongbing, or citrus greening, which is one of the most devastating citrus diseases worldwide. Las is transmitted by the Asian citrus psyllid (*ACP*) *Diaphorina citri* (Hemiptera: Psyllidae) in a persistent manner, but its interactions with the psyllid vector, particularly at the organ and cellular levels, are poorly understood. We have tested several fluorescence *in situ* hybridization (FISH) protocols and three nucleic acid probes for the localization of Las in haemolymph smears and dissected organs of ACP adults that fed on Las-infected plants in the field or laboratory and in sections from Las-infected citrus leaves. Las was detected by FISH and confocal laser scanning microscopy in the filter chamber, midgut, Malpighian tubules, haemolymph, salivary glands, ovaries and in muscle and fat tissues of ACP that acquired Las from infected plants, as well as in the phloem of infected citrus leaves. Las appeared as pleomorphic bodies or short thin rods that were much more dispersed and individually distinct in citrus leaf phloem and in ACP haemolymph, but more densely aggregated in cells of the salivary glands and other ACP organs and tissues. Our results provide the first *in situ* demonstration of Las infection in various psyllid organs and tissues and show the near-systemic infection of ACP by Las.

**Introduction**

Huanglongbing (HLB), or citrus greening, is one of the most destructive citrus diseases in many countries worldwide (da Graca 1991; Halbert and Manjunath 2004). Previously known primarily from Asia and Africa, HLB was identified in the Western Hemisphere in 2004 and was discovered in Florida in 2005 (Gottwald 2010). HLB disease agent infects all commercial citrus cultivars and causes substantial economic losses by promoting fruit drop, rendering fruit inedible and shortening the lifespan of infected trees (Miyakawa 1980; da Graca 1991; Bove 2006). Yield reduction can reach 30–100% depending on the proportion of the canopy affected and the age of trees during inoculation (Gottwald 2010). Currently, the three closely related, phloem-limited α-proteobacteria associated with this disease have been provisionally categorized as follows: *Candidatus Liberibacter asiaticus* (from Asia and the Americas), *Candidatus Liberibacter africanus* (from Africa) and *Candidatus Liberibacter americanus* (from Brazil) (Garnier et al. 2000; Bove 2006). The Asian citrus psyllid (*ACP*) *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is the principal insect vector of *Candidatus Liberibacter* spp. in Asia, Brazil and USA, whereas the African psyllid, *Trioza erytreae* (Del Guercio) (Triozidae), is the main vector in African countries, Mauritius and Réunion (Aubert 1987; Halbert and Manjunath 2004; Gottwald 2010).

Although Koch’s postulates remain to be completed, Tyler et al. (2009) provided evidence using phloem metagenomic DNA which strongly suggested that *Candidatus Liberibacter asiaticus* (Las) is the main, if not the only, phloem microbe present in plants with severe HLB symptoms in the USA. Las is transmitted by ACP in a persistent manner (Capoor et al. 1974; Xu et al. 1988; Hung et al. 2004). ACP developing on HLB-symptomatic trees can acquire the pathogen most efficiently during the nymphal stage, and emerging adults from these nymphs are infected and may immediately be able to inoculate the disease into healthy host plants (Capoor et al. 1974; Inoue et al. 2009; Pelz-Stelinski et al. 2010). Using quantitative polymerase chain reaction (qPCR), Inoue et al. (2009) reported that Las apparently multiplies in ACP 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, ACP nymphs and adults may remain inoculative throughout their life span (Xu et al. 1988; Hung et al. 2004). In most cases, low inoculation rates by single ACPs (1.3 – 12.2%) have been reported (Huang et al. 1985; Pelz-Stelinski et al. 2010). However, HLB often spreads quickly in a citrus planting, particularly if the planting is young (Xu et al. 1988; Gottwald 2010). To understand the epidemiology of this disease and hoping to develop novel ways to combat it, we need a better understanding of pathogen-vector interactions, particularly at the cellular and organ levels.

The alimentary canal and salivary glands are known as the most important barriers to the transmission of hemipteran-borne (Ammar 1994; Gray and Gildow 2003; Weintraub and Beanland 2006; Hogenhout et al. 2008). Because Las has been so far non-culturale and specific antibodies to it are not readily available, Las was only recently identified in various organs of its psyllid vectors using PCR (Ammar et al. 2011), with the exception of some electron microscopic observations of bacteria-like structures reported in the salivary glands and alimentary canal of ACP and T. erytreae that fed on HLB-symptomatic plants (Xu et al. 1988; Moll and Martin 1973). We detected Las in dissected alimentary canals, salivary glands and other organ tissues of ACP using qPCR (Ammar et al. 2011). The proportion of infected (PCR-positive) salivary gland was significantly lower than that of other organ tissues, but Las titre appeared to be much higher in both the salivary glands and alimentary canals compared with other tissues. However, in that investigation, only the salivary glands and alimentary canals were processed separately for PCR, whereas ‘other organs and tissues’ were processed together as a whole.

In the present work, several fluorescence in situ hybridization (FISH) protocols and three nucleic acid probes were tested and used with confocal laser scanning microscopy to study the distribution of Las in dissected organs of the psyllid vector. We localized Las in several organs and tissues of D. citri, and we report the near-systemic infection of this vector by Las bacterium. Additionally, we used FISH to localize Las in sections of HLB-symptomatic, Las-infected citrus leaves as positive controls.

Materials and Methods
Field-collected and laboratory-infected ACP adults and citrus leaves used
Live adults of ACP were collected from ‘Valencia’ orange trees [Citrus sinensis (L.) Osbeck] at the USDA citrus grove in Fort Pierce, in east-central Florida. A majority of these trees were naturally infected with Las, as evidenced by HLB disease symptoms and previous PCR tests (not included here). ACP adults were collected by aspiration into glass vials from trees showing symptoms of HLB on several occasions between 24 August 2009 and 11 May 2010. Additionally, we used adult ACP from a laboratory colony that has been maintained exclusively for several generations on HLB-symptomatic, Las-infected (PCR-positive) citrus plants (rough lemon, Citrus jambhiri Lush.). Healthy control ACP adults were taken from a laboratory colony established during the year 2000, which had been maintained on healthy orange jasmine trees [Murraya paniculata (L.) Jack] and, more recently, on healthy citrus trees (Citrus macrophylla Wester) in the greenhouse as described by Hall et al. (2007). No wild psyllids were introduced into this colony, and individuals from the colony were PCR-assayed every 3 months to ensure that the colony remained free of Las. Both healthy control and field- or laboratory-infected adults were starved (in glass vials) at room temperature for at least 3 h before dissection to help in clearing their gut contents. Subsequent to starvation, in some cases, ACP adults were kept in the refrigerator (at 4°C) for 1–2 days before dissection. However, all adults were still alive when dissected. FISH was also performed on sections of young mid-size leaves of HLB-symptomatic, Las-infected citrus plants, as a positive control (as confirmed by previous PCR tests), from our greenhouse that included Valencia orange, Ruby Red grapefruit (Citrus paradisi Macf.) and variegated lemon [Citrus limon (L.)]. Healthy control leaves from citrus plants were similarly processed for FISH.

Preparing citrus leaves and psyllid organ/tissues for FISH
Small pieces of leaves (5–6 mm wide and 5–6 mm long) that contained the midrib were cut from fresh citrus leaves using a clean razor blade and placed on a clean plastic Petri dish in a drop of 4% paraformaldehyde (for FISH protocols 1 and 2) or 70% ethanol (for FISH protocol 3). Cross sections were cut by hand as thinly as possible under a stereomicroscope (at 20×) and quickly transferred to a small sterile microfuge tube with one of the two fixatives described below.

For dissection of ACP adults, they were first immobilized by freezing at −20°C for 10–15 min and then dissected on a clean plastic Petri dish under a stereomicroscope (15–20×). Two fine forceps (Fontax no. 5; Electron Microscopy Sciences, Washington, PA, USA) and a new razor blade were cleaned with 70% ethanol and used for dissections. Further, to receive haemolymph drops from ACP, clean Tissue Tack microscope slides (Polysciences, Warrington, PA, USA) were set up by drawing three circles of liquid blocker (PAP Pen; Electron Microscopy Sciences) on each slide, and the haemolymph from each insect was collected on one of these circles as follows. Holding the tip of the abdomen with one forceps, using a clean razor blade a quick, clean cut was made between the thorax and abdomen. Immediately, the cut end of the abdomen was gently blotted and received inside one of the circles on the Tissue Tack slide. The haemolymph drops blotted on these slides were air-dried for a few minutes in a fume hood, then
30 μl of fixative was placed in each circle and a glass cover slip was placed on it, to minimize evaporation, for 2–3 h before further processing.

For dissection of the internal organs of ACP (Brittain 1923; Cicero et al. 2009; Ammar et al. 2011), both the cut abdomen and thorax–head parts were each placed in a drop of phosphate-buffered saline (PBS, pH 7.4; Polysciences). Using one forceps, a slight pressure was applied against the middle part of the cut abdomen, which pushed the filter chamber and midgut out and away from other tissues. Using two fine forceps, the cuticle was then gently torn away to expose the rest of the organs and tissues including the reproductive organs and fat tissues. However, all organs including the alimentary canal were left attached to the cuticular end of the abdomen for easier handling and to minimize loss or damage of these organs during various washing and processing steps for FISH. To expose the salivary glands, which are found at the anterior part of the thorax, the head–thorax part placed in a drop of PBS was held with one forceps, and the other forceps was used to remove the thoracic terga, wings, legs and most of the thoracic muscles. The salivary glands and compound ganglionic mass, which is an amalgamation of the thoracic and abdominal ganglia (Brittain 1923; Ammar et al. 2011), were left attached to the head, again to minimize the loss of these tiny organs during FISH processing. Dissected parts were quickly immersed in one of the two fixatives mentioned below placed in a small clean glass cavity dish (with a concave cavity ca. 20 mm wide and 6 mm deep).

**FISH probes and protocols tested**

The following three oligonucleotide probes, tagged with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), were tested for Las detection in sections of healthy and Las-infected citrus leaves and in dissected psyllid organs and haemolymph smears. Probe 1 (HLBas-AF488) is the Las-specific 16S rDNA qPCR primer described by Li et al. (2006), which is approved for regulatory Las detection in the USA. This probe was chosen to be highly specific to Las and to bind only the 16S rDNA and not the 16S rRNA. Probe 2 (USHRL-CL1r-AF488) is a 16S rDNA primer designed to be *C. Liberibacter* specific in plants and ACP, with a broader *C. Liberibacter* species range, and also to bind to the rRNA of which there are many more molecules per cell. This probe has been shown to amplify rDNA (in combination with another primer) from Las as well as *C. Liberibacter solanacearum* (McKenzie and Shatters 2009), which does not infect citrus and is not transmitted by ACP. Probe 3 (Clas-RDR-1) was designed to amplify a gene identified as ribonucleotide diphosphate reductase beta subunit that has four copies in the *C. Liberibacter asiaticus* psy62 genome (Duan et al. 2009) present in GenBank (NC_012985). This probe was shown to be *C. Liberibacter* specific

in psyllids and citrus tissues (data not shown) and would not bind to the RDR-1 mRNA. The probe designation and sequences are as follows:

(1) HLBas-AF488: (DNA) – Alexa 488 – TCG AGG GCG TAT GCA ATA CG (20 bases).
(2) USHRL-CL1r-AF488: (DNA)-Alexa 488 – TCC CTA TAA AGT ACC CAA CAT CTA GGT AAA (30 bases).
(3) CLas-RDR-1: (DNA)-Alexa 488 – TAT ATT GTT CAC ATG AGG GAG CAT TTA ACC (30 bases).

Probes were suspended in sterile DNA/RNA-free water and then diluted in hybridization buffer (HB) [20 mM Tris–HCl, pH 8.0; 0.9 m NaCl; 0.01% (w/v) sodium dodecyl sulphate and 30% (v/v) formamide] just before use. Dilutions of 50, 100 and 500 pmol/ml were used.

The protocols described here included modifications of FISH protocols adopted by previous workers to detect viruses or bacteria in whole-mount insect organs, including those by Ghanim et al. (2009), Fukatsu et al. (2007), Kikuchi et al. (2005, 2009) and Kono et al. (2008). In our work, all the FISH steps were carried out at room temperature. The hand-cut sections of plant leaves were processed in 0.3-ml microfuge tubes, whereas dissected insect organs were processed in the glass cavity dishes mentioned earlier. In both cases, various washing and other solutions were changed using drawn-out glass pipettes with minute tapered openings under a stereomicroscope (15–20×) so as to minimize loss or damage of plant sections or the tiny psyllid organs. Also, the Tissue Tack slides with haemolymph smears were always placed in a large Petri dish lined with moist filter paper to minimize evaporation of reagents between various steps. The following six protocols were tested on plant and insect material.

**Protocols 1a and 1b**

Fixation in 4% paraformaldehyde in PBS-T (PBS with 0.1% Triton X 100) for 3–4 h. This was followed by washing three times (20 min each) in HB, then hybridization (with probe in HB) for 3 h to overnight and washing (3 × 20 min) either in HB (protocol 1a) or TBS (Tris buffered saline (20 mM Tris–HCl [pH 7.4], 0.15 m NaCl) (protocol 1b).

**Protocols 2a and 2b**

Same as for Protocol 1, except that after fixation, specimens were washed in PBS-T (3 × 20 min), then permeabilized overnight in permeabilization buffer (PBS with 1% Triton X 100).

**Protocols 3a and 3b**

Fixation in Carnoy’s solution (ethanol:chloroform/acetic acid ratio, 6:3:1) for 3–4 h, followed by washing in HB (3 × 20 min), hybridization in probe plus HB (3 h to overnight) and then washing (3 × 20 min) in either HB (protocol 3a) or TBS (protocol 3b).
In all protocols, during hybridization and posthybridization washes, the specimens were kept in the dark and were only exposed to very little light as briefly as possible while changing solutions. Following each of these protocols, the specimens were stained for 5 min with 3 nM solution of the fluorescent nucleic acid stain propidium iodide (Invitrogen) that stained most of the tissues red, especially the nuclei, and thus helped in the identification of various insect and plant tissues against the green fluorescence of the Las probes used. This was followed by a final wash in PBS-T (3 × 20 min), before mounting the leaf sections and dissected organs on microscope slides with Fluoro-Gel (antifade mounting medium, Electron Microscopy Sciences, Hatfield, PA, USA). Haemolymph smears were similarly mounted and covered with glass cover slips. Mounted specimens were kept in the dark at 4°C until examination (within 1–4 days) using a confocal laser scanning microscope (Zeiss LSM 510) with 20× (dry), 40× or 63× (both oil immersion) objectives.

For each protocol (and probe), 2–5 FISH tests were conducted. In each test, dissected organs from 20–40 ACP adults that had access to Las-infected citrus plants in the field or laboratory and 10–20 healthy control adults were used, in addition to 10–20 leaf sections from healthy and Las-infected citrus plants. In each test, dissected organs from 20–40 ACP adults that had access to Las-infected citrus leaves and haemolymph smears (Figs 1a,b and 2g), whereas probe 1 with sections from Las-infected leaves and haemolymph smears (Figs 1a,b), probes 1 and 3 also produced positive results using protocol 3b with probes 1 or 2, green fluorescence indicating Las was detected normally as densely aggregated quasi-spherical or pleomorphic bodies. These were found in cells of the midgut and filter chamber (Fig. 1f), Malpighian tubules (Fig. 1h), salivary glands (Figs 2a,b), muscles (Fig. 2d) and in fat tissues surrounding the male and female reproductive systems (Figs 2e,f). Las was also detected, less frequently, in the female’s ovaries (Fig. 2e) but not in the male’s testes (Fig. 2f). In contrast to its aggregated localization in these organs and tissues, Las was detected as scattered pleomorphic bodies or short thin rods (similar to those in the phloem of infected leaves) in haemolymph smears from Las-infected ACP (Fig. 2g). In the salivary glands and other organs, Las appeared to be intracellular (mainly on the inside of the outer cell membranes) and intracytoplasmic (did not appear to invade the nuclei of infected cells) (Figs 1h and 2a,b). However, Las appeared to be extracellular in the haemolymph, that is not particularly associated with the haemocytes (indicated by red nuclei in Fig. 2g).

The proportions of infected (Las-positive) dissected organs of field-collected and laboratory-infected ACP adults using protocol 3b with probe 2 are shown in Table 1. In both field-collected and laboratory-infected adults, the proportion of infected salivary glands (8.3-28.6%) was considerably lower than that of infected alimentary canals (42.4-47.6%) or other organs (21.2-50.0%). The difference, in this regard, between the salivary gland and alimentary canal is significant in laboratory-infected psyllids ($\chi^2 = 5.30$, df = 1, P = 0.021) but not in field-infected ones ($\chi^2 = 1.06$, df = 1, P = 0.304). However, with pooled results from both populations (N = 33–79), the proportion of Las-infected salivary glands was significantly lower than that of both the alimentary canal ($\chi^2 = 4.82$, df = 1, P = 0.028) and other ACP organs ($\chi^2 = 4.29$, df = 1, P = 0.038).

Las was not detected by FISH, using any of the three probes mentioned previously, in any of the dissected organs (Figs 1g and 2c) or haemolymph smears (Fig. 2h) from healthy control ACP that had never been exposed, as nymphs or adults, to Las-infected plants.

Discussion
In a recent report, using qPCR, we detected Las in the salivary glands, alimentary canal and other organs’ issues of its psyllid vector D. citri/ACP (Ammar et al. 2011). In that investigation, ‘other organs’ issues’ represented the rest of the insect body (other than the head, salivary glands and alimentary canal) without specification. In the present work, we tested several FISH protocols and three nucleic acid probes to study the distribution of Las in psyllid organs by confocal laser scanning microscopy, and we provide the first in situ
demonstration that Las occurs in several organs and tissues of ACP. In addition to the salivary glands, Las was found to invade most of the vector’s organs and tissues including the haemolymph, filter chamber, midgut, fat and muscle tissues and ovary. As far as we know, this is also the first reported detection by FISH of Las in the phloem tissues of Las-infected citrus leaves. Furthermore, the FISH analysis clearly shows fluorescent staining of bacterial cells, indicating that nucleic acid detection is associated with the presence of intact bacteria in the observed tissues. Only one of six FISH protocols tested was successful in detecting Las in HLB-diseased citrus leaves and in dissected organs from ACP that acquired Las by feeding on Las-infected citrus plants either in the field or in laboratory. This successful protocol (using Carnoy’s fixation and posthybridization washing in TBS) contained a combination of steps modified from previous protocols used with whole-mount insect organs by Ghanim et al. (2009) and by Kikuchi et al. (2005, 2009). The specificity of the probes used is indicated by the fact that no specific fluorescence was observed in similarly treated sections of healthy citrus leaves (Fig. 1d) or dissected organs from healthy control ACP that were never exposed to infected plants (Figs 1g and 2c,h). Additionally, several qPCR tests performed in our laboratory on healthy and HLB-symptomatic citrus leaves, as well as on separate patches of ACP adults

Fig. 1 Confocal laser scanning micrographs of Las bacterium (green fluorescence) detected by FISH in the phloem tissue (ph) of Las-infected citrus leaves (a–c), as well as in the filter chamber (fc), midgut (mg) and Malpighian tubules (mt) of Las-infected psyllids (f, h). Panels d & e show sections from healthy control leaves, and panel g shows the alimentary canal of a healthy control psyllid. Specimens were tested with FISH probe 1 (a, b, d, e, f, g), probe 2 (h) or probe 3 (c), then stained with the nuclear stain propidium iodide (red) to differentiate various cells/tissues. Panels (d) and (e) show the same area, but in (e) differential interference contrast (DIC) was used to show boundaries of phloem cells (ph), which does not show clearly with normal laser scanning (in a–d). DIC was also used in panel (h), overlapped with the red and green channels, to show the boundaries of the Malpighian tubules. Abbreviations: fc, filter chamber; mg, midgut; mt, Malpighian tubules; n, cell nucleus; ph, phloem tissue; xy, xylem tissue
that fed on healthy or infected plants in the field or laboratory, confirmed the presence of Las in infected, but not in healthy control, citrus leaves and ACP organs or whole adults (Ammar et al. 2011; D. G. Hall and E.-D. Ammar, unpublished data).

The three nucleic acid probes tested detected Las in sections of infected citrus leaves and in haemolymph smears. One of these probes (No. 1) also detected Las in the alimentary canal but not in other ACP organs, and another one (No. 2) detected Las in both plant sections and in ACP organs and tissues. Probe No. 2 was designed to bind to both the 16S rDNA and the 16S rRNA. Because of the greater copy number of 16S rRNA molecules in a cell, compared with that of the rDNA gene, it is quite likely that the increased sensitivity of probe 2 is due to its ability to bind both the rDNA and rRNA. Additionally, the ability of probes to penetrate various organs/tissues may differ with the organ type and with the preparation method used, i.e. smears, whole mounts, or sections. Haemolymph smears from ACP and free-hand sections of citrus leaves are easier to penetrate, because Las appears to

Fig. 2 Confocal laser scanning micrographs of Las bacterium (green fluorescence) detected by FISH in dissected organs (a, b, d, e, f) or haemolymph smear (g) of Las-infected psyllids, compared with those from healthy control psyllids (c, h). Dissected organs (a–f) were tested with probe 2, whereas the haemolymph smears (g, h) were tested with probe 1; all were later stained with the nuclear stain propidium iodide (red) to differentiate various cell/tissues. Arrows in (a–c) indicate outer boundary of the salivary glands. Abbreviations: ft, fat tissue; he, haemolymph/haemocytes; mag, male accessory gland; mu, muscles; n, cell nucleus; ov, ovary; sg, salivary gland cells; ts, testis.
be extracellular in the haemolymph, and sectioning provides a more direct route for the penetration of probes and other reagents into cells and tissues that are normally bound by various cell membranes, cell walls (plant cells) or the basal lamina (insect organs). Also, the alimentary canal is probably easier to penetrate than other organs because it is composed mainly of one cell layer whereas most other insect organs are multilayered (Chapman 2003). Generally, however, whether in FISH or immunofluorescence labelling, whole-mount insect organs provide a more comprehensive picture of the distribution of various pathogens in insect organs and tissues without requiring the tedious and time-consuming serial sectioning (Ammar and Hogenhout 2005, 2008; Ghanim et al. 2009).

In the present work, the proportions of Las-injected organs of ACP using FISH (21–50%) were lower than those obtained by qPCR on dissected organs (45–81%) in our previous study (Ammar et al. 2011). This indicates that qPCR is more sensitive than FISH especially on whole dissected organs, which is to be expected because intact organs are presumably less permeable to various reagents than macerated organs used for PCR. The two techniques, however, are used for different purposes: PCR mainly for detection and FISH for in situ localization. In our work, FISH provided more detailed spatial localization of Las in ACP showing the near systemic infection of various organs and tissues of this psyllid vector. FISH results also indicated that Las is more densely aggregated in the salivary glands and other ACP organs than in HLB-infected citrus leaves, which is consistent with previous reports using qPCR that Las is highly dispersed and unevenly distributed in infected citrus plants (Gottwald 2010). Additionally, occasional detection of Las in ACP ovaries is consistent with previous reports on the occurrence of low-rate transovarial passage of Las from infected ACP mothers to their offspring (Pelz-Stelinski et al. 2010). However, detection of Las in eggs and newly hatched nymphs by qPCR in the latter study, or by FISH in the ovaries (in our study), does not necessarily mean that transovarial transmission can actually lead to Las inoculation into citrus plants by the offspring of infected mothers, which has not been experimentally proven so far.

The distribution of Las in the psyllid’s organs and tissues seems to be similar to that of propagative plant pathogens that are known to multiply in their hemipteran insect vectors, e.g. the leafhopper-borne, phloem-limited bacteria including several phytoplasmas, Spiroplasma kunkelii and Spiroplasma citri (Fletcher et al. 1998; Bové et al. 2003; Ammar and Hogenhout 2005, 2006), and the hemipteran-borne propagative plant viruses (Hogenhout et al. 2008). These propagative pathogens have a very wide, almost systemic, distribution in various organs and tissues of their vectors, which is markedly different from the more limited distribution of persistent, non-propagative plant pathogens in their vectors, that is mainly in the alimentary canal, salivary glands and haemolymph (Markham et al. 1988; Lett et al. 2002; Gray and Gill-dow 2003; Hogenhout et al. 2008; Ammar et al. 2009). Interestingly, to our knowledge, well-studied examples of circulative, non-propagative plant pathogens so far include only viral rather than bacterial pathogens. The near systemic distribution of Las in organs and tissues of ACP suggests that Las multiplies in its psyllid vector, which is also supported by our previous qPCR results showing the higher titre of Las in the salivary glands and alimentary canals compared with other tissues of ACP (Ammar et al. 2011). Additionally, other qPCR results on whole psyllids by Inoue et al. (2009) suggested the propagation of Las in ACP when acquired by nymphs. In our present study, Las appeared as pleiomorphic bodies or short thin rods in plant phloem cells and in the insect haemolymph, but mostly as quasi-spherical or pleiomorphic bodies in other insect organs. Electron microscopy images of sections in infected plants and psyllid vectors also show quasi-spherical, pleiomorphic and rod-shaped bacterial-like structures (Moll and Martin 1973; Xu et al. 1988; Hartung et al. 2010). Electron micrographs of a related bacterium, Candidatus Liberibacter solanacearum, associated with zebra chip disease of potatoes, also appear as pleiomorphic or rod shaped in plant phloem tissues (Secor et al. 2009). This dual morphology/poly morphism in Liberibacter bacteria is reminiscent of that in Spiroplasma spp., where generally, extracellular spiroplasma cells (in phloem sieve tubes and haemolymph) are more frequently helical, whereas spiroplasma cells that are located intracellularly are more often pleiomorphic, quasi-spherical or flask shaped (Özbek et al. 2003; Ammar and Hogenhout 2006).

Using two different methodologies (FISH and qPCR), our current and previous results (Ammar et al. 2011) show that the proportion of infected (Las-positive) salivary glands is significantly lower than that

### Table 1

Results of fluorescence in situ hybridization (FISH) tests on dissected organs from field- and laboratory-infected psyllids

<table>
<thead>
<tr>
<th>Field/laboratory population</th>
<th>Test. no.</th>
<th>AC</th>
<th>SG</th>
<th>OT</th>
</tr>
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<tbody>
<tr>
<td>Field</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>57</td>
<td>49</td>
<td>7/13</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2/13</td>
<td>2/11</td>
<td>1/20</td>
<td></td>
</tr>
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</tr>
<tr>
<td>Total</td>
<td>1435a</td>
<td>621a</td>
<td>28/56</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>2/10</td>
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</tr>
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<td>2</td>
<td>8/11</td>
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</tr>
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<td>33/79a</td>
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</tr>
</tbody>
</table>

1 Using FISH protocol 3b with probe 2 (at 500 pmol/ml) as described in the Methods section. In each of the five tests, 10–20 healthy control psyllids were dissected and similarly processed for FISH (none proved to be Las-positive).

2 AC, alimentary canal; SG, salivary glands; OT, other organs/tissues.

a,b In each row, proportions followed by different letters (a/b) are significantly different (χ² analysis).
of the alimentary canal or other ACP organs and tissues. In the present work, this trend was more pronounced in laboratory-infected than in field-collected psyllids, probably because the former had been reared exclusively on Las-infected plants and thus may have had longer access to infected plants during the nymphal stage. Taken together, these two studies suggest that the salivary glands may constitute a barrier to Las infection and/or transmission by ACP. This is consistent with previous reports in which the rate of HLB/Las transmission to plants by individual psyllids is normally much lower than the proportion of Las-infected (PCR-positive) psyllids. For instance, Pelz-Stelinski et al. (2010) recently reported that, whereas 40–60% of ACP that fed on Las-infected plants became PCR-positive, successful Las inoculation to citrus plants by individual D. citri ranged from 4 to 10%. In other well-studied systems of hemipteran-borne bacterial plant pathogens, e.g. mollicutes (Spiroplasma and phytoplasma), to be transmitted from diseased to healthy plants, the mollicutes need to circulate and multiply in the vector. This requires crossing the midgut epithelium, multiplying in the haemolymph or other organs and subsequently reaching the salivary glands. Only when the mollicutes have reached, and multiplied in, cells of the salivary glands can they be inoculated into a host plant, because they can be released with the insect’s saliva, which is known to be secreted into the plant during food uptake (Bove et al. 2003; Ammar and Hogenhout 2006). Specific interactions between pathogen surface proteins and receptors in membranes of the midgut, salivary glands or other vector tissues have been reported in other persistent propagative pathogens, e.g. plant viruses (Ullman et al. 2006; Hogenhout et al. 2008), Spiroplasma, phytoplasma and other bacteria (Fletcher et al. 1998; Yu et al. 2000; Bové et al. 2003; Weintraub and Beanland 2006; Killiny et al. 2006; Suzuki et al. 2006), and Plasmodium (Ghosh et al. 2001). These interactions are thought to be major determinants of the specificity with which such pathogens are transmitted only by certain species, races or developmental stages of their insect vectors (Ammar 1994; Bové et al. 2003; Weintraub and Beanland 2006). One of the suggested and innovative ways to combat such diseases is to find and block such receptors in the midgut, salivary glands or other tissues of the vectors (Ghosh et al. 2001; Hogenhout et al. 2008). We hope that our work on the distribution of Las in its vector’s organs and tissues will pave the way for continuing studies on HLB/Las vector interactions at the cellular and subcellular levels to further our understanding of such an economically important pathosystem.

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