Titers of ‘*Ca. Liberibacter asiaticus*’ in *Murraya paniculata* and *Murraya*-reared *Diaphorina citri* Are Much Lower than in *Citrus* and *Citrus*-reared Psyllids

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Abstract. Huanglongbing, one of the most devastating diseases of citrus, is associated with the bacterium ‘*Candidatus Liberibacter asiaticus*’ vectored by the Asian citrus psyllid, *Diaphorina citri*, in North America. *Murraya paniculata* is a common ornamental plant that is an alternate host of both the psyllid and bacterium. We tested *M. paniculata* and *Citrus sinensis* grown together in the same field for their titer of ‘*Ca. L. asiaticus*’. We found the bacterium in both *M. paniculata* and *C. sinensis*, but the titer was four orders of magnitude lower in *M. paniculata*. We also assayed *D. citri* from laboratory colonies reared on either ‘*Ca. L. asiaticus*’-infected *M. paniculata* or infected *Citrus* spp. Psyllids reared on infected *M. paniculata* also carried bacterial titers five orders of magnitude lower than psyllids reared on infected *Citrus* spp. These observations imply resistance to huanglongbing in *M. paniculata*.

Huanglongbing (HLB) is one of the most devastating diseases of citrus worldwide. It is associated with the fastidious bacteria ‘*Candidatus Liberibacter asiaticus*’ (CLAs), ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’. The bacteria are vectored by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (ACP), and the African citrus psyllid, *Trioza erytreae* (Del Guercio). The disease causes yellow shoots, a blotchy mottle appearance of the leaves, dieback, and the eventual death of a tree (Bové, 2006). HLB associated with CLAs vectored by ACP is jeopardizing the Florida citrus industry (Halbert et al., 2008) and threatens all citrus production in North America and the Caribbean basin.

Both CLAs and ACP have host plants within the family Rutaceae outside of the *Citrus*

genus (Deng et al., 2007b; Folimonova et al., 2009; Halbert and Manjunath, 2004; Peña et al., 2006). One plant species grown in Florida, *Murraya paniculata* (L.) Jack (a synonym for *M. exotica* L.; see USDA-ARS-NGRP, 2012), is commonly grown as a hedge and serves as a host of both ACP and CLAs (Damsteegt et al., 2010; Deng et al., 2007a; Halbert and Manjunath, 2004; Hung et al., 2000). The production and trade of *M. paniculata* in Florida has been regulated since 2008 as a result of concerns of *M. paniculata* being a reservoir of CLAs for commercial citrus (Clark, 2007). CLAs transmission between *Citrus sinensis* (L.) Osbeck var. ‘Madam Vinous’ and *M. paniculata* by ACP has been demonstrated (Damsteegt et al., 2010). In the field, *M. paniculata* is infrequently infected with CLAs, even in areas with high inoculum pressure (Walter et al., 2012). The importance of *M. paniculata* in citrus HLB epidemiology therefore deserves further exploration.

To help clarify the importance of *M. paniculata* relative to HLB epidemiology in commercial citrus in Florida, we investigated titers of CLAs in *M. paniculata* and *C. sinensis* plants growing in the same field and in ACP from colonies maintained on infected *M. paniculata* or infected *Citrus* spp. Because CLAs bacterial numbers in infected *M. paniculata* often are too low to be detected by 16S rDNA-based CLAs-specific quantitative polymerase chain reaction (Li et al., 2006), we compared the detected copy numbers of an internal repeat region of two CLAs prophage genes as a relative measure of CLAs abundance.

Materials and Methods

Prevalence of ‘Candidatus Liberibacter asiaticus’ in Murraya paniculata and Citrus

sinensis. We determined presence and titers of CLAs in 120 *M. paniculata* plants of unknown infection status interplanted with *Citrus sinensis* var. ‘Valencia’ at a USDA-ARS orchard near Fort Pierce, FL. High ACP pressure and CLAs inoculum existed in this orchard. The block of trees was planted in the spring of 2008. Half of the *M. paniculata* were sampled in Aug. 2010, and the others were sampled in Sept. 2010. Blotchy mottle-like leaf symptoms, similar to those observed in infected citrus, were noted on many of the *M. paniculata*, and symptomatic leaflets were preferentially sampled. Titers of CLAs in leaf samples from *Murraya* were compared with titers of CLAs in symptomatic leaf samples from 10 *C. sinensis* trees that had been identified during May 2010 as CLAs-infected and subsequently removed from the field. Infected *C. sinensis* from this field have CLAs titers that vary by less than 10^{1.5}-fold throughout the year with the lowest titers generally observed during the hottest months (D.G. Hall, personal observation). For both plant species, leaf tissue to yield 0.2 g of excised midvein was sampled from each plant.

DNA was extracted from the *M. paniculata* samples using the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA) for the samples collected in August and the NucleoSpin 96 Plant II Kit (Machery-Nagel, Bethlehem, PA) for the samples collected in September. Leaflet midribs were manually removed from the leaves with a sterile razor blade and midribs were finely chopped. Chopped midribs were placed in tubes containing slingshot pellets (August) or chrome steel beads (September) and homogenized by shaking in a Mini-Bead Beater (Biospec Products, Bartlesville, OK) for 5 min after lysis buffer and RNase were added. For the August samples, some of the samples were incubated for 60 min at 65 °C instead of 10 min following the recommendations of the manufacturer of the extraction kit to increase the yield of DNA. The September samples were incubated for 30 min at 60 °C. *C. sinensis* was extracted using a DNA precipitation method as described for the April plant samples in Walter et al. (2012). The amount of DNA for all the samples was quantified by spectrophotometry at 260 nm using a Nanodrop-1000 (Nanodrop Products, Wilmington, DE). Both kits had similar yields of DNA. However, the buffer-based method had apparently higher yield but more impurities as measured by the signal ratio of the 260- and 280-nm wavelengths that could inflate the estimated amount of DNA according to the spectrophotometric measurement. Samples were diluted so that 100 ng of plant DNA was loaded per polymerase chain reaction (PCR) to account for differences in extraction efficiency. As long as the same amount of DNA is tested, similar samples yield similar quantitative PCR results with all three extraction methods (A.J. Walter, personal observation).

Prevalence of ‘Candidatus Liberibacter asiaticus’ in Asian citrus psyllid. Because of the low prevalence of infection found in field-collected psyllids from *M. paniculata* (Walter et al., 2012), we sampled ACP from colonies

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maintained on infected *Citrus* spp. or infected *M. paniculata* under identical conditions in a growth chamber. All colonies were maintained at 25 °C, 70% relative humidity, 14:10 h light:dark, under 400 W high-pressure sodium and metal halide growth light bulbs (Metalarc M59 M400/U and Lumalux S51 LU400 bulbs;sylvania, Danvers, MA). The infection of *Citrus* plants originated from a field-collected shoot and was passed between plants by grafting or by placing an uninfected plant in a cage with an infected plant and an active ACP population. Colonies on *M. paniculata* were established in Dec. 2010 by placing several hundred adult ACP from an infected *Citrus* colony on uninfected *M. paniculata* plants for 7 d to transmit the pathogen, removing the adults, then adding a new population of uninfected adults to ensure that infected ACP taken from the colony originated from *M. paniculata*. All adult ACP sampled for this study had spent their entire life on the infected host plant. Samples of 21, 20, and 30 ACP were taken during Jan. 2011 from colonies reared in cages containing infected *Citrus jambhiri* Lush. (rough lemon), both infected *C. sinensis* and *Citrus paradisi* Macf. (grapefruit), or infected *M. paniculata*, respectively. An additional 24 adults were sampled from the *M. paniculata* colony in Feb. 2011. At the time of sampling, *Citrus* plants in the colonies had visual symptoms of HLB, and the *M. paniculata* were quantitative PCR-positive using the LJ900 primers (Morgan et al., 2012) with a maximum of 7678 *hyvI/hyvII* copies per reaction. Crude extracts of individual adults were made as described in Walter et al. (2012), and PCR was performed using 2 µL of crude extract per reaction.

Samples were assayed for the presence of CLAs using two quantitative PCR primer sets. We assayed for the presence of the internal repeat sequence of the *hyvI/hyvII* repeat prophage genes (Zhou et al., 2011) using the LJ900 primers (forward GCGGTTTAAACACAAAAGATGAATATC, reverse ATAAATCAATTGTCTAGTTTACGAC) as described by Morgan et al. (2012). This primer set is very sensitive to low-titer infections but is not ideal for absolute titer quantification because the primers target the nearly identical tandem repeats of two prophage genes with varying numbers of repeats in different CLAs isolates (Zhou et al., 2011). We use the number of detected repeats in the samples, as determined by standard curves built using negative samples from each extraction method and the pLJ153.1 plasmid described by Morgan et al. (2012), as a relative measure of the number of bacterial genomes. For the LJ900 primers, quantitative PCR was performed in a 15 µL reaction using PerfeCTa SYBR Green FastMix 2 × master mix (Quanta Biosciences, Inc., Gaithersburg, MD), a reaction concentration of 600 nM forward and 900 nM reverse primer, and nuclease-free water with a temperature program of 95 °C for 5 min, then 50 cycles of 95 °C for 3 s, followed by 62 °C for 30 s, then one cycle of 95 °C for 15 s, 62 °C for 1 min, and a gradual

ramp to 97 °C for 15 s. All samples that amplified on those primers were rerun in triplicate. A sample was not considered positive unless at least two of the three triplicate samples amplified and had the correct melt profile. Samples were also tested for the presence of the 16S rDNA of the CLAs genome using the HLBaspr primers (forward TCGAGCGCGTATGCAATACG, reverse GCGTTATCCCGTAGAAAAAGGTAG, probe AGACGGGTGAGTAACGCG with 6-FAM reporter dye and TAMRA quencher) developed by Li et al. (2006). This method is less sensitive than the LJ900 primers for low-titer samples. For the HLBaspr primers, quantitative PCR was performed in a 20 µL reaction using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), a reaction concentration of 0.4 mM of each primer, 500 nM probe, and nuclease-free water with a temperature program of 95 °C for 5 min then 50 cycles of 95 °C for 3 s followed by 60 °C for 30 s. Each sample was run only once on the HLBaspr primers. A detection threshold of 0.02 ΔRn was used for all samples. All PCR was performed on an ABI 7500 Fast system (Applied Biosystems).

We tested the normality of the *hyvI/hyvII* copy number using the Shapiro-Wilk statistic and compared the number of repeat copies detected in the plant or psyllid samples using the nonparametric Kruskal-Wallis test (Proc UNIVARIATE, Proc TTEST; SAS Institute, 2008).

Results

'*Candidatus Liberibacter asiaticus*' in plant material. Four of the 120 *M. paniculata* samples consistently amplified using the LJ900 primers, but the titer of bacteria in these plants was always low (Table 1). None of the *M. paniculata* samples that were negative using the LJ900 primers had detectable levels of CLAs 16S rDNA; one of the LJ900-positive *M. paniculata* samples amplified on the HLBaspr primers with a Cq value of 38.2. Yellowing symptoms had been observed on most of the sampled *M. paniculata* and did not appear to be associated with CLAs infection. All 10 of the *Citrus* samples had detectable CLAs 16S rDNA (Cq range, 26.9 to 30.6). The *hyvI/hyvII* copy numbers of the *C. sinensis* samples were non-normal ($W = 0.80, P = 0.0139$), so numbers of repeats were compared by the nonparametric Kruskal-Wallis test. The bacterial titer of *M. paniculata* was significantly lower than the titer found in *C. sinensis* (Murray range, 39 to 6.0×10^3 repeats per reaction; median, 811 repeats; *Citrus* range, 2.7×10^6 to 3.1×10^7 repeats per reaction; median, 6.8×10^6 repeats; $\chi^2 = 8.00, df = 1, P = 0.0047$) (Fig. 1). There was a 10^4 -fold difference in the median titer values of Murray and *Citrus* samples.

'*Candidatus Liberibacter asiaticus*' in Asian citrus psyllid. Although the titer of infected *M. paniculata* was very low, the plant may still be an inoculum reservoir for citrus if ACP from infected *M. paniculata* can transmit HLB. Therefore, we also compared the CLAs titer of ACP that completed their

development on infected *M. paniculata* and *Citrus* spp (Table 1). All 41 ACP sampled from the *Citrus* colonies yielded a quantitative PCR product using the LJ900 primers, and 40 yielded a 16S rDNA product (HLBaspr Cq range, 24.0 to 45.1). The number of *hyvI/hyvII* repeats detected in ACP from both *Citrus* hosts was non-normal (*C. jambhiri* $W = 0.56, P < 0.0001$; *C. sinensis/C. paradisi* $W = 0.75, P = 0.0002$), so results were compared with the Kruskal-Wallis test. There were no significant differences between the ACP from the different *Citrus* hosts based on detected repeats (*C. jambhiri* range, 67 to 6.0×10^6 repeats per reaction; median, 2.7×10^5 repeats; *C. sinensis/C. paradisi* range, 15 to 2.8×10^6 repeats per reaction; median, 1.9×10^5 repeats; $\chi^2 = 0.1742, df = 1, P = 0.6764$), so all ACP reared on infected *Citrus* were pooled for comparison with ACP reared on infected *M. paniculata*. Twenty-four of the 54 ACP taken from the Murray colony yielded a PCR product on the LJ900 primers, but only 16 yielded a product on the HLBaspr primers (HLBaspr Cq range, 28.4 to 45.8). The detected repeats from psyllids reared from *Citrus* sp. and *M. paniculata* was non-normal (Murray $W = 0.21, P < 0.0001$; *Citrus* $W = 0.59, P < 0.0001$). The CLAs titer was significantly lower for *D. citri* reared from *M. paniculata* than for *D. citri* reared from *Citrus* sp. (Murray range, 4 to 1.9×10^5 repeats per reaction; median, 28 repeats; *Citrus* range, 15 to 6.0×10^6 repeats per reaction, median, 2.6×10^6 repeats; $\chi^2 = 32.13, df = 1, P < 0.0001$) (Fig. 2). There was a 10^5 -fold difference in the median values of CLAs titers in ACP from colonies on Murray and *Citrus*.

Discussion

We have observed that the titer of CLAs in *M. paniculata* was four orders of magnitude lower than the titer observed in diseased *C. sinensis*. Our observations are similar to findings from a quarantine laboratory study where CLAs titers in *M. paniculata* inoculated with bacteria from *Citrus* were generally low and which subsequently declined or became undetectable over a period of 32 months (Damsteegt et al., 2010). Other reports support that titers of CLAs are low in field-collected *M. paniculata* (Deng et al., 2007a; Walter et al., 2012; Zhou et al., 2007). These results are valid even if the number of *hyvI/hyvII* repeats varies between CLAs in the two plant species as a result of the magnitude of the difference we describe. The lower titer found in *M. paniculata* relative to *Citrus* suggests that *M. paniculata* is resistant to CLAs. However, a large proportion of the CLAs present in infected citrus is non-viable (Folimonova and Achor, 2010; Trivedi et al., 2009), so it is possible that the results from *Citrus* are artificially inflated as a result of the sampling of dead bacteria.

More importantly, we found that the titer of CLAs in adult *D. citri* reared from infected *M. paniculata* was also four orders of magnitude lower than the CLAs titer in psyllids reared from infected *Citrus* based on differences in

Table 1. Date sampled, number tested, positive results by two quantitative polymerase chain reaction methods, and median copy number of *hyvI/hyvII* repeats from *Citrus sinensis* and *Murraya paniculata* growing in the same field and Asian citrus psyllid (ACP) cultured on infected *Citrus* spp. and *M. paniculata* under identical conditions.

Sample	Date tested	No.	Positive samples by HLBspr	Positive samples by <i>hyvI/hyvII</i>	Median copy number detected by <i>hyvI/hyvII</i>
<i>M. paniculata</i>	Aug. to Sept. 2010	120	0	4	811
<i>C. sinensis</i>	May 2010	10	10	10	6.8×10^6
ACP feeding on <i>M. paniculata</i>	Jan. to Feb. 2011	54	16	24	28
ACP feeding on <i>Citrus</i> spp.	Jan. 2011	41	40	41	2.6×10^6

Fig. 1. Titters (number of DNA copies) of 'Ca. Liberibacter asiaticus' (CLAs) in plant samples. Box plots showing 10th percentile (solid line on left, shaded box), first quartile (left side of shaded box, line inside shaded box), median (line in shaded box), third quartile (line inside shaded box, right side of shaded box), and 90th percentile (shaded box, solid line on right) for detected copy numbers per reaction of the *hyvI/hyvII* repeat sequence of CLAs in *Citrus sinensis* or *Murraya paniculata* grown in the same field. CLAs titers differed significantly between the plant species according to the Kruskal-Wallis test ($P < 0.05$).

Fig. 2. Titters (number of DNA copies) of 'Ca. Liberibacter asiaticus' (CLAs) in psyllid samples. Box plots showing 10th percentile (solid line on left, shaded box), first quartile (left side of shaded box, line inside shaded box), median (line in shaded box), third quartile (line inside shaded box, right side of shaded box), and 90th percentile (shaded box, solid line on right) for detected copy numbers per reaction of the *hyvI/hyvII* repeat sequence of CLAs in ACP reared from *Citrus* spp. or *Murraya paniculata* under identical conditions. CLAs titers in Asian citrus psyllid (ACP) from the plant species differed significantly according to the Kruskal-Wallis test ($P < 0.05$).

Cq values. The Cq values obtained from the colony-reared ACP in this study were similar to those observed in field-collected ACP from *M. paniculata* (Walter et al., 2012) and from *Citrus* spp. (D.G. Hall, unpublished data). If

ACP with low titers of CLAs are less likely to transmit the pathogen than ACP with high titers, then ACP originating from infected *M. paniculata* would be less likely to transmit CLAs than ACP originating from infected citrus.

It is not known whether the low CLAs titers that we observed in ACP from *M. paniculata* are the result of differences in CLAs load in the gut (for example, ACP feeding on infected *M. paniculata* may acquire less CLAs than when they feed on infected *Citrus*) or a consequence of biological differences in CLAs obtained from *Citrus* vs. *M. paniculata*. Although CLAs was reported to replicate in ACP (Hung et al., 2004; Inoue et al., 2009), the low titers of CLAs in ACP that acquired the bacterium from infected *M. paniculata* show that this replication does not overcome the titer differences resulting from a different amount of bacteria being acquired by ACP on the different hosts or possible differences in the rate of replication of CLAs acquired from different plants. ACP reared from infected *M. paniculata* have been shown to transmit detectable levels of CLAs to *Citrus* (Damsteegt et al., 2010). Whether the CLAs transmitted from *M. paniculata* to *Citrus* by ACP induce symptoms of HLB is currently under investigation.

CLAs occurred at much lower titers in both *Murraya paniculata* and ACP reared from infected *M. paniculata* than in both diseased *Citrus sinensis* and ACP reared from infected *Citrus* sp. Further work on *M. paniculata* may be useful on two fronts. First, we need to understand whether ACP that acquires a CLAs bacterium from *M. paniculata* is capable of transmitting CLAs that will cause typical HLB disease in *Citrus*. Second, *M. paniculata* may not support CLAs multiplication, which implies a potential resource for cloning resistance gene(s). The answers to both of these questions may yield new approaches for management of HLB in citrus.

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