Ultrastructural Changes and Putative Phage Particles Observed in Sweet Orange Leaves Infected with '*Candidatus Liberibacter asiaticus*'

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Abstract

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Huanglongbing (HLB), also known as citrus greening, is currently the most destructive citrus disease. Anatomical analyses of HLB-affected sweet orange were carried out by light and electron microscopy. As compared with healthy citrus, the phloem plasmodesmata were plugged with callose, and in some samples the phloem was collapsed. Chloroplast structures were deformed. Prophage sequences occupy a significant portion of the genome of '*Candidatus Liberibacter asiaticus*' and have been used to distinguish strains from Yunnan and Guangdong provinces in China and Florida. Interestingly, a large number of

possible putative phage particles were observed attached on the surface of '*Ca. L. asiaticus*' cells in plants inoculated with strain FJ3 from Fujian Province, China. Phage particles have been observed previously only in periwinkle plants artificially inoculated in Florida with '*Ca. L. asiaticus*' that carried the SC1-type prophage. PCR assays verified the presence of the SC1-type prophage sequences previously described from this bacterium in Florida in the FJ3 isolate. This is the first time that suspected phage particles have been observed in sweet orange trees infected with '*Ca. L. asiaticus*'.

Huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus worldwide. The disease is associated with three species of α -Proteobacteria, all non-culturable and phloem-limited: '*Candidatus Liberibacter asiaticus*,' '*Ca. L. africanus*,' and '*Ca. L. americanus*' (3). The causal agents have not been classically characterized because they have not been grown in pure culture, and so the name has provisional '*Candidatus*' status. '*Ca. L. asiaticus*' survives at high ambient temperatures while the other two species do not survive at ambient temperatures above 32°C (23). The pathogen is usually introduced into new areas by graft transmission with infected buds and is subsequently spread locally by either of two psyllid species, *Trioza erytreae* (Del Guercio) and *Diaphorina citri* (Kuwayama). Infected leaves develop typical symptoms of zinc deficiency with an uneven blotchy mottle, and newly emerged shoots may produce erect and uniformly yellow leaves. Recent work has shown that the root mass of sweet orange trees infected by '*Ca. L. asiaticus*' decreases substantially before any aboveground symptoms are observed (16). Infected trees produce fruit that are small and lopsided, with bitter juice and aborted seeds. However, all of these symptoms do not always occur at the same time on the same tree. Similar symptoms can be induced by the causal agent of citrus stubborn disease (CSD), *Spiroplasma citri*, which is a culturable, but wall-less prokaryote (2), and by either of two phytoplasmas (6,30) and *Citrus tristeza virus* (CTV). Management of the disease is difficult and costly, and currently no cure is available for infected trees. Growers work to slow the spread of the disease by consistent use of

pesticides to control the insect vector (29). Some growers also remove infected trees to reduce inoculum available to the citrus psyllid. However, '*Ca. L. asiaticus*' has infected nearly all commercial citrus in Florida. Growers seek to maintain fruit production by the use of proprietary nutritional programs (28,29), but the utility of this has been questioned (12).

'*Ca. L. asiaticus*' is surrounded by two double layered membranes as part of the gram-negative cell wall. The dimensions of the bacterium are not uniform and single cells have been seen to transition between rod and round cell shapes when viewed by transmission electron microscopy (TEM) (13). The round form of the cells ranges from 100 to 500 nm in diameter and the dimensions of the rod shaped bacteria are about 40 to 170 × 200 to 2,500 nm (13). '*Ca. L. asiaticus*' induces the collapse and proliferation of phloem cells (1). In mesophyll cells, chloroplasts become swollen and distorted and thylakoids break down. In infected leaves of the susceptible host, sweet orange, the middle lamella becomes significantly swollen surrounding sieve elements. Starch granules also accumulate in the leaf tissues, and crystal idioblasts are found (1,8). Infection by '*Ca. L. asiaticus*' leads to callose deposition in the sieve plates, with the effect that the size of sieve pores is reduced.

In dodder, *Cuscuta campestris*, '*Ca. L. asiaticus*' can reach high titers, but with an erratic distribution as also seen in citrus (13,20). Madagascar periwinkle, *Catharanthus roseus*, is also a very good non-rutaceous host plant in which the HLB bacteria can multiply actively in sieve tubes. Phage particles were observed by TEM in the phloem of '*Ca. L. asiaticus*'-infected periwinkles (31). These phage and prophage are designated as type SC1 and SC2 and FP1 and FP2 by different authors (31,32). Such phage particles have not been reported to date in sweet orange.

In this study, we explored ultrastructural changes in sweet orange leaves infected with '*Ca. L. asiaticus*' by light and transmission electron microscopy using new strains of '*Ca. L. asiaticus*' from Fujian Province, China. We observed bacterial cells in the phloem cells with associated damage to nearby tissues. In the course of this work we have also found what appear to be phage

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particles attached to the surface of bacteria inside infected sweet orange phloem cells.

Materials and Methods

Plant materials, grafting, and growing conditions. We collected samples from field trees with HLB symptoms located in Yanxi village, Changtai County of Zhangzhou City, Fujian Province, China. Infection by '*Ca. L. asiaticus*' was confirmed by PCR assays using the OI1/OIc2 primer set (15) and the pathogen was propagated by grafting infected buds on three 1-year-old 'Madame Vinous' sweet orange seedlings per strain. The inoculated and healthy plants kept as controls were maintained in a greenhouse at the Citrus Research Institute in Chongqing, China. Temperatures were maintained between 25 and 30°C and supplemental lighting was provided. The potting mix contained turf soil, sand, and rice husks (1:1:1). Chemical fertilizers with macronutrient content of 12:12:12 (NPK) were used every 7 to 10 days and rapeseed dregs were added to supply microelements.

Extraction of DNA and PCR assays. Three months after inoculation, sampling was initiated and DNA was extracted from leaf midrib tissue of inoculated plants following a CTAB-SLS protocol. Leaf midrib tissue (300 mg) was ground into a powder in liquid nitrogen and transferred to 1.5-ml tubes with 1 ml of extraction buffer (2.5% hexadecyltrimethylammonium bromide [CTAB]; 1 M 2-carboxyethyl phosphine-HCl, TCEP; 10% sodium lauryl sulfate [SLS]; 50 µg/ml proteinase K; pH = 8.0). The powdered tissue was mixed with the extraction buffer, incubated at 65°C for 30 to 40 min, and then centrifuged at 12,000 rpm (13,500 × g) for 2 min. The supernatant was transferred to a new 1.5-ml tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) solution was added, mixed, and incubated at 65°C for 5 min. The mixture was then centrifuged at 12,000 rpm (13,500 × g) for 3 min at room temperature. The supernatant was transferred to a new tube and an equal volume of chloroform/isoamyl alcohol (24:1) solution was added, mixed briefly, and centrifuged at 12,000 rpm (13,500 × g) for 2 min. The supernatant was then transferred to a new tube and 1/10 volume of 3 M sodium acetate, pH 5.2 and 9/10 volume of isopropanol was added to precipitate the DNA. The solution was incubated at 4°C for 30 min and then centrifuged at 8,000 rpm (2,200 × g) for 5 to 10 min. The supernatant was decanted, and the DNA pellet was washed three times with 75% ethanol and air dried. The DNA was dissolved in TE and stored at -20°C for use.

PCR to confirm infection of graft-inoculated trees with primers OI1/OIc2 was performed as described previously (15). PCR with primer pairs 766F/766R and 817F/817R, to detect SC1-type (22) and SC2-type phage, respectively, was carried out in 25-µl reaction volumes with MgCl₂ (2 mM), dNTP mix (120 µM), primers (200 nM each) with 1 U *Taq* polymerase. The PCR amplification program was 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 64°C (766F/R) or 56°C (817F/R) for 30 sec, and 72°C for 1 min, with a 10 min extension at 72°C after the final cycle. The primer pair 817F/817R was designed with Primer Premier software (Premier Biosoft, Palo Alto, CA) to amplify sequences from SC2 and FP2. The sequences of primers 817F/817R were 5'-AAG CCTCATAGTTAAAACCA-3' and 5'-ATAAGCCATTAGATGATT GGA-3'. Amplification products were separated by agarose gel electrophoresis.

Preparation of samples for light and transmission electron microscopy. After trees were confirmed to be infected by '*Ca. L. asiaticus*' by PCR, leaves from greenhouse-grown infected and healthy trees were collected for light microscopy and TEM. A standard fixation procedure was used. Leaf samples were cut into small pieces (1 × 1 mm) and fixed with 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer (PBS), pH 6.8, at 4°C for 4 h, then washed with PBS three times for 10 min each. Samples were post-fixed in 1% osmium tetroxide in PBS for 2 h at room temperature, followed by three washes with PBS. The specimens were then dehydrated by 10 min incubations with 1 ml of 30, 50, 70, 75, 80, 90, and 95% ethanol and finally with three washes with absolute ethanol. Specimens were then infiltrated by incubation with a mix-

ture of ethanol and Spurr's resin (2:1, 1:1, 1:2) for 12 h per step at room temperature, and finally incubated with pure Spurr's resin for 2 days (18). Semi-thin sections (0.5 to 1 µm) were stained with toluidine blue, and observed by light microscopy. Ultra-thin sections (80 to 100 nm) were mounted on 200-mesh formvar coated copper grids and stained with 2% uranyl acetate and 2% lead citrate. These thin sections were examined under a Hitachi H-7500 transmission electron microscope at Zhejiang University, China.

Results

'*Ca. L. asiaticus*'-infected buds were grafted onto sweet orange stems. Because '*Ca. L. asiaticus*' were unevenly distributed in infected trees, and the concentration was on average low, especially at the early stages of infection, it was difficult to observe '*Ca. L. asiaticus*' by TEM. PCR assays were used to confirm '*Ca. L. asiaticus*' in the trees after grafting. As expected, a single 1,160-bp band was present when DNA from inoculated trees was used as template. This product was not present when DNA from healthy trees was used as template, nor in the no template control reactions (Fig. 1A). '*Ca.*

Fig. 1. A, OI1/OIc2 specific amplicons (1,160 bp) in a 1.2% agarose gel, confirming the presence of '*Candidatus Liberibacter asiaticus*.' M, 100 bp ladder as size marker; FJ3, FJ7, and FJ16 denote isolates of '*Ca. L. asiaticus*' from Fujian; H, healthy; W, no template control. B, SC1-type phage DNA sequences (766 bp) amplified by primers 766F/766R. C, SC2-type phage DNA sequences (817 bp) amplified by 817F/817R.

L. asiaticus' was detected in leaf samples from new flushes of two trees, including tree FJ3 3 months after grafting and nine trees after 5 to 6 months or longer, including FJ7 and FJ16. Though the greenhouse-grown 'Madame Vinous' trees had some typical blotchy mottle symptom and were PCR-positive for the pathogen, the symptoms were much less severe than those observed on HLB trees in the field. The greenhouse-grown trees also lacked the typical yellow shoot symptom commonly seen in the field, where all the leaves on a new flush are uniformly yellow and erect. These samples were also tested with phage-related primers 766F/766R (SC1/FP1) (22) and 817F/817R (SC2/FP2). These assays detected prophage SC1 in samples FJ3 and FJ7 but not in sample FJ16, which had prophage SC2 but not prophage SC1 (Fig. 1B and C).

Leaf tissue from healthy and '*Ca. L. asiaticus*'-infected sweet orange were sectioned and stained for light microscopy. Comparison of the sections from the healthy and diseased leaf tissue showed that the phloem cells had divided in an irregular manner

and the tissue had become disorganized. In addition, starch granules were widespread in the pith and in the mesophyll of the diseased petiole section (Fig. 2A and B).

Leaf sections were also fixed and examined by TEM. This examination revealed that the chloroplasts of infected tissues had degenerated and the thylakoid membranes were completely disorganized. Osmiophilic granules were also observed as were numerous small and irregularly shaped starch granules. In contrast, chloroplasts from healthy tissue were well organized with well formed starch granules (Fig. 3A and B). Xylem tissues also contained crystal idioblasts, which most likely contained calcium oxalate crystals (Fig. 3C).

Many bacterial cells were observed in the sieve tube and companion cells and callose had accumulated in the plasmodesmata of sections prepared from strain FJ3 (Fig. 4A). Surfaces of these cells were not uniform as typically seen with '*Ca. L. asiaticus*' in tissue sections. Instead, surfaces of the bacterial cells were covered with particles. With higher magnification, many particles with the size and general appearance of phage had been bound to the bacterial surface of FJ3 (Fig. 4B and C). Phage particles have been observed previously only in periwinkle plants artificially inoculated in Florida with a '*Ca. L. asiaticus*' strain that carried the SC1-type prophage. Prophage sequences were obtained from FJ3 with primer 766F/R (Fig. 1B), consistent with an SC1-type prophage. The same phage particles were not observed from the healthy samples tested.

Discussion

'*Ca. L. asiaticus*' cells were seen within sieve elements and we observed callose accumulation in the plasmodesmata, consistent with previous reports (8,17), especially around plasmodesmata pore units (PPUs) before starch accumulated in the chloroplasts. This suggests that plugging of the PPUs and sieve pores by callose inhibits phloem transport, leading to the accumulation of starch in photosynthetic tissues (1,17). The accumulation of starch then causes photosynthesis to shut down and the chloroplasts to degrade. The accumulation of starch also leads to the stiffening and yellowing of leaves as was demonstrated with *Spiroplasma citri*, a bacterium with very similar pathogenesis (4). Osmiophilic granules were observed within chloroplasts. Such granules have been associated with plants suffering from some biotic or abiotic stress (26). Callose induction is part of a defense response controlled by distinct signaling pathways, which depends on both environmental conditions and pathogen-associated molecular patterns (PAMP) (26). It may also be formed in response to the physical deformation of the plant tissues that follows multiplication of '*Ca. L. asiaticus*' in host cells (8,27). Thus, there may be more than one trigger for callose induction in plants with symptoms of HLB.

Many crystal idioblasts were also found in diseased tissue. Similar cells contained deposits of calcium oxalate (10,11). The formation of idioblasts and the callose accumulation in our infected leaf samples were consistent with an active plant response to '*Ca. L. asiaticus*' and suggested imbalances in both calcium and carbohydrate metabolism.

The genome '*Ca. L. asiaticus*' was shown to be significantly reduced due to its lifestyle as an intracellular pathogen (7,14). However, two very similar prophage genomes, designated as SC1 and SC2, were integrated in the genome of strains Psy62 and UF506 from Florida (31). These prophage genomes accounted for approximately 1/15 of the entire '*Ca. L. asiaticus*' genome (7). *Liberibacter crescens*, from papaya in Puerto Rico, is the only member of the genus to have been grown successfully in culture. The genome of *L. crescens* was recently sequenced and annotated (19). Comparisons were carried out between the genomes of *L. crescens* and '*Ca. L. asiaticus*'. The genomes were largely syntenic, but the genome of *L. crescens* was slightly larger than that of '*Ca. L. asiaticus*'. Two putative prophages (LC1 and LC2) were also found in the *L. crescens* genome, as well as in the genome of '*Ca. L. solanacearum*', a pathogen of potatoes and tomatoes (21). The widespread presence of similar bacteriophage in different species of *Liberibacter* may provide insight into a virulence mechanism used

Fig. 2. Light micrographs of cross sections of midribs of 'Madame Vinous' sweet orange leaves. **A**, Cross sections of midrib of a healthy leaf. **B**, Cross section of a midrib of an HLB symptomatic leaf. Abbreviations: Ep, epidermis; Ph, phloem; Xy, xylem; Pi, pith. The white or light yellow particles in **B** are starch grains. Note the collapsed area of phloem tissue in **B** marked with yellow arrows. Black bars = 100 µm.

by '*Ca. L. asiaticus*.' Prophage are known to effect the evolution of bacteria, by disruption of the gene in which they integrate, the facilitation of homologous recombination, and the expression of new functions directly related to virulence, such as toxins, adhesions, etc. (9).

The two prophage genomes in '*Ca. L. asiaticus*,' designated SC1 (FP1) and SC2 (FP2), have been characterized (31,32). Two hyper-variable prophage genes with intragenic tandem repeats were used to demonstrate previously unknown diversity and plasticity of the

genome of '*Ca. L. asiaticus*' (32). A primer pair (766F/766R) based on the prophage terminase gene of '*Ca. L. asiaticus*' (CLIBASIA_05610) was used to assess the genetic variation in two '*Ca. L. asiaticus*' populations from geographically distinct provinces (Guangdong and Yunnan) in China (22). We have used this primer pair to detect the presence of a phage genome related to the SC1-type in samples FJ3 and FJ7 from Fujian Province. The SC1-type target sequence was found in samples from Yunnan Province much more frequently than in samples from Guangdong Province (22). The primer set 817F/817R was used to amplify fragments from prophage similar to the SC2-type from the Florida strains. Florida strain UF506 of '*Ca. L. asiaticus*' was shown to carry the SC1-type phage in the non-integrated, circular form in inoculated periwinkle (*Catharanthus roseus*) plants. Phage particles were also observed on '*Ca. L. asiaticus*' cells in periwinkle by TEM, and the presence of the phage genome was verified by Southern blotting (31). We have verified that an SC1-type prophage genome is present in our '*Ca. L. asiaticus*'-infected samples FJ3 and FJ7 from Fujian, China (Fig. 1B). Plants inoculated with strain FJ3 also had putative phage particles attached to the '*Ca. L. asiaticus*' cells within phloem cells of the host, and were among the first trees in the experiment to become PCR positive for '*Ca. L. asiaticus*'. The likely phage particles were present in FJ3-inoculated plants with high concentrations of '*Ca. L. asiaticus*' based on PCR, although the concentration of '*Ca. L. asiaticus*' observed in the cells with the phage particles was not nearly as high as has been observed for '*Ca. L. americanus*' or '*Ca. L. asiaticus*' in dodder (13).

The prophage of '*Ca. L. asiaticus*' have been shown to vary considerably in genome structure with different genotypes predominating in different locations as a result of two hypervariable regions

Fig. 3. Transmission electron micrographs of cross sections of '*Candidatus Liberibacter asiaticus*'-infected and healthy leaf tissue. **A**, Distorted structure of chloroplast in '*Ca. L. asiaticus*'-infected leaf. Note the collapsed thylakoids membranes, numerous small and disordered starch grains, and osmiophilic granules. **B**, A large starch granule in a chloroplast from a healthy leaf. **C**, Crystal idioblasts observed in xylem tissue. Abbreviations: CW, cell wall; St, starch grains; Chl, chloroplast; OG, osmiophilic granules; Mt, mitochondria; CI, crystal idioblast.

Fig. 4. Transmission electron micrographs of a vertical-section of '*Candidatus Liberibacter asiaticus*' strain FJ3-infected phloem tissue. **A**, Note the '*Ca. L. asiaticus*' cells and the heavy deposit of callose at the plasmodesmata (arrow). **B**, Higher magnification image of the area marked in **A**. **C**, Higher magnification image of the '*Ca. L. asiaticus*' cells. Abbreviations: Cc, companion cell; SE, sieve element; PI, plasmodesmata.

(32). The structure of these hypervariable regions has been shown to vary according to geographical origin of strains, and can distinguish strains both between China and Florida (5) and within China (22,24). We found two phage types in our samples from Fujian. Samples FJ3 and FJ7 had only SC1-type prophage, and sample FJ17 had only SC2-type prophage. None of our samples had both SC1- and SC2-type prophage sequences as found in strains of '*Ca. L. asiaticus*' from Florida (31). It is also worth noting that the structure of these hypervariable regions of tandem repeats is very similar to that of *pthA*, a gene that determines virulence of *Xanthomonas citri* on citrus (32). Differences in this hypervariable region may be reflected on the surface of '*Ca. L. asiaticus*' cells (32). In contrast to the hypervariable regions described above based on prophage, '*Ca. L. asiaticus*' strains also have stable variation in a series of variable number tandem repeats, with two genotypes present in Florida (25). Thus, there is ample variation in the '*Ca. L. asiaticus*' genome that could contribute to interaction of prophage with '*Ca. L. asiaticus*'.

The size of the putative phage particles attached to the cell surface in this study was similar to that observed previously in Florida (31), and they were present in samples from one (FJ3), but not another (FJ7), trees inoculated with strains of '*Ca. L. asiaticus*' that contained the SC1 genome, the genome previously identified in lytic phage in '*Ca. L. asiaticus*'-infected periwinkle. The lack of phage particles in samples inoculated with strain FJ7 may have simply been a result of the luck of sampling using very small specimens for electron microscopy. To our knowledge, this is the first time particles consistent with SC1-type phage have been observed in citrus. It will be interesting to learn the phage integration sites in the genome of the Fujian strain FJ3 used here and its copy number relative to the '*Ca. L. asiaticus*' genome.

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