Phloem Production in Huanglongbing-affected Citrus Trees

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Abstract. Citrus greening disease [Huanglongbing (HLB)] is the most significant and widespread threat to the citrus industry in recent history. A bacterium [*Candidatus Liberibacter asiaticus* (CLas)] vectored by the Asian citrus psyllid is the presumed causal agent of the disease, which results in the collapse of phloem tissue, leading to decreased productivity, chlorotic leaves, and bitter, misshapen fruit. Once infected, trees never fully recover and there currently is no cure, although foliar nutrient sprays and intensive irrigation appear to slow tree decline in some situations. Despite phloem necrosis in older tissue, new vegetative and reproductive growth occurs. Our current understanding of phloem collapse in citrus resulting from HLB is based on anatomical reports of trees in different stages of decline and does not explain the persistence of growth. Here, we present data that show new phloem cells are produced during the periodic flushes of vegetative growth and their subsequent collapse and plugging over a 6-month period. Cellular activity within the cambium and the ray parenchyma was diminished in HLB-affected petioles, suggesting an important link in the carbohydrate transport pathway is missing. Because of the short window of time during which the phloem appears healthy, the weeks immediately before and after the spring and summer flush are of critical importance for the management of citrus health.

Citrus HLB (synonym citrus greening) is a highly destructive, fast-spreading disease of citrus. Its presumed pathological agent, *Candidatus Liberibacter* spp., is a fastidious Gram-negative, obligate parasite, phloem-limited α -proteobacterium (Garnier and Bové, 1983; Jagoueix et al., 1994). Although not yet cultured to purity, recent attempts to do so have resulted in partial or mixed cultures of the organism (Davis et al., 2008; Sechler et al., 2009). Of the several species identified worldwide (Kim et al., 2009), CLas is the only species found in Florida (Wang and Trivedi, 2013). CLas is vectored by the phloem-feeding psyllid *Diaphorina citri* (Halbert and Manjunath, 2004) and transmitted into the phloem of citrus leaves during the feeding process. When transmitted into a citrus leaf by the psyllid, CLas unravels a cascade of physiological and metabolic changes that culminate in reduced vigor, diminished production, and ultimately tree death (Etxeberria et al., 2009).

Citrus trees affected by HLB exhibit a progressive degeneration of the phloem tissue (Achor et al., 2010; Schneider, 1968) that results in partial or total phloem collapse (Folimonova and Achor, 2010). At the anatomical level, the first observable indication of CLas infection is the conspicuous swelling of the middle lamella between and surrounding sieve elements (Folimonova and Achor,

2010). This swelling is followed by the deposition of callose plugs both in lateral pit fields (Koh et al., 2012) as well as in and around sieve plates (Folimonova and Achor, 2010; Koh et al., 2012). Although otherwise still functional at this early stage, the walls begin to collapse concurrently with increased deposition of callose and P-protein plugs at the sieve plates. The presence of callose plugs and phloem collapse is believed to hinder the transport of photoassimilates (nitrogenous and reduced carbon compounds) from photosynthetic source leaves to the remaining heterotrophic sink tissues (Schneider, 1968). As the flow of photoassimilates is progressively obstructed by phloem necrosis, starch granules begin to accumulate in sieve elements and all parenchyma cell types along the radial and longitudinal transport pathway, but especially in photosynthetically active cells (Etxeberria et al., 2009; Schneider,

1968). Callose plugging of the phloem and phloem degeneration are common symptoms of other phloem-specific diseases such as yellow vine disease of cucurbits (*Serratia marcescens*; Bruton et al., 2003) and phloem canker disease of walnut (*Erwinia rubifaciens*; Schaad and Wilson, 1970), whereas elevated starch levels have been reported in grapevines suffering from Bois Noir, a phloem-specific phytoplasma disease (Santi et al., 2013).

Despite the apparent total collapse of phloem elements in HLB-affected leaves and limbs, new vegetative growth continues to develop for several seasons. In fact, HLB-affected branches with evident HLB symptoms and seemingly obstructed phloem are capable of maintaining fruit production for some time (Ikpechukwu et al., 2011) (Fig. 1), although succeeding crops are progressively smaller and of lower quality. Furthermore, parenchyma cells in woody stems and bark tissue downstream from the leaf canopy (i.e., in the basipetal direction) also accumulate copious amounts of starch despite the evident dysfunctionality of the phloem tissue (Etxeberria et al., 2009). If photoassimilate transport were totally blocked at the leaf/petiole level, there would be insufficient carbohydrate flow to sustain the synthesis of starch in a basipetal direction and to support the growth of developing twigs, lower branches, and fruit.

A possible source for the carbohydrate accumulation in the bark and wood could be the result of temporary phloem transport in new foliage. In citrus trees, new growth emerges several times throughout the year with the most substantial flushes occurring in the spring and late summer (Reed and MacDougal, 1938; Syvertsen et al., 1981). Often, new leaves emerging from trees with a recent CLas infection appear healthy, although some leaves can be either severely deformed by psyllid feeding or eventually develop the typical blotchy mottling and yellowing patterns characteristic of the disease (Fig. 1). In trees known to have been infected with HLB for several years, new leaves do not grow to their typical size, are often upright in orientation, and remain a pale yellow color, never developing the dark green coloring of healthy leaves (Fig. 1). These observations suggest that, for a yet undetermined period of time, photoassimilate

Fig. 1. Production of healthy fruit by an Huanglongbing (HLB)-affected citrus tree. Subtending branch shows the yellow, upright leaves with blotchy mottle typical of symptomatic leaves.

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transport takes place in newly developed flush tissue and within the older supporting branches and trunk. At some point after leaf development, the phloem eventually collapses and becomes dysfunctional. Although previous studies have shown HLB symptoms at extreme stages of the disease, the goal of this study was to monitor the progression of phloem production over time in field-grown trees to determine anatomically how the trees are capable of sustaining new growth and then document the subsequent phloem collapse.

Materials and Methods

Plant material. Fully expanded and developed tissue from HLB-affected trees was collected based on their visual symptoms (i.e., symptomatic vs. asymptomatic) from 5-year-old ‘Valencia’ orange (*Citrus sinensis* L. Osbeck) trees grown at the Citrus Research and Education Center in Lake Alfred, FL, that had been previously determined by polymerase chain reaction (PCR) analysis to be infected with CLas. Trees were tested 3 months before sampling using the diagnostic facilities at the University of Florida’s Southwest Florida Research and Education Center using real-time PCR. Symptomatic tissue was characterized by blotchy leaf mottle, smaller and misshapen yellow leaves, and was confirmed with a starch test commonly used for HLB identification in the field (Etcheberria et al., 2008). We collected three stems, petioles, leaf midveins, and fruit peduncles from three different asymptomatic, symptomatic, and greenhouse trees on two separate dates, first in the late summer of 2011 and again in the late spring of 2013. Sampling in 2011 included tissue from both the spring and summer flush of that year, in which the spring and summer tissue was ≈6 and 2 months old, respectively. Sampling from 2013 included tissue from the late summer flush of 2012 and the spring flush of 2013, where the summer and spring flush were 6 and 2 months old, respectively. This spacing in sampling timing allowed us to capture the short- and long-term symptom development in HLB-affected tissue from both the spring and summer flush. All collected samples were qualitatively representative of the field or greenhouse populations.

Tissue preparation for light microscopy. Collected tissue was immediately placed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2. The tissue was kept at room temperature for 4 h and refrigerated overnight. Tissue slices were washed in the same buffer, postfixed for 4 h at room temperature in 2% osmium tetroxide in this buffer, dehydrated in an acetone series, and embedded in Spurr’s resin (Etcheberria et al., 2009). For light microscopy, 1-μm sections were cut with glass knives using an ultramicrotome, stained with methylene blue/azure A, and poststained in basic fuchsin. Light micrographs were taken on an Olympus Vanox compound microscope (Tokyo, Japan) with a digital camera.

FDA viability staining for healthy and HLB-infected ‘Valencia’ petioles. To determine the viability of cells within the vascular tissue of petioles we used fluorescein diacetate (FDA), a cell-permeant vital stain that, on hydrolysis by esterase activity, releases green fluorescence indicating cell viability and integrity (Ruzin, 1999). Eight transverse freehand sections from both healthy and HLB-infected ‘Valencia’ petioles were placed in labeled vials containing a 1:100 FDA solution. The vials were vacuum infiltrated for 15 s, capped with aluminum foil to minimize light exposure, and shaken for 40 min at 1000 rpm on an Eppendorf Thermomixer R (Eppendorf Inc., New York, NY). Each section was mounted on a slide and viewed with a 10× objective under a fluorescence microscope with a λ_{ex} = 488 nm, and a digital image was captured. Sample image brightness and contrast were adjusted equally for all samples using ImageJ (<<http://imagej.nih.gov/ij>>).

Results

The general anatomy of phloem tissue in healthy citrus trees consists of a ring of compact cells delimited by the distinctively larger xylem vessels to the interior and by thick-walled fibers along the outside (Figs. 2A and 3A). The newly produced metaphloem (Fig. 2A, black arrow) appears as a

layer of thin-walled cells between the cambium and the protophloem (Fig. 2A, white arrow). Differentiation of the metaphloem outwardly becomes evident with the maturation of sieve cells and companion cells toward the periphery. The phloem sieve elements and their companion cells are some of the smallest cells in the plant (Knoblauch and Oparka, 2012), have thin primary walls, and in petioles are often found in discrete bundles (Fig. 2A–B). Companion cells (Fig. 2B, black arrow) are easily distinguished from their associated sieve elements (Fig. 2B, white arrow) by their smaller size and denser cytoplasmic content. Large phloem parenchyma cells were invariably present scattered within the tissue and as part of the vascular rays and bordering the inner face of the tracheary ring (Figs. 2A–B and 3A, white arrow). Longitudinally, the sieve elements can be distinguished by their thin walls and oblique end walls (Fig. 3A, black arrow) as compared with the larger and more rectangular parenchyma cells (Fig. 3A, white arrow).

When affected by HLB, sieve elements initially become irregular with jagged boundaries (Fig. 2C). In the early stages of HLB, although phloem elements are not entirely collapsed, they showed a very dense cytoplasmic content, likely the callose and P-protein plugs associated with HLB (Figs. 2C and 3B, black arrow) (Achor et al., 2010). Plugging of phloem elements concludes with

Fig. 2. The progression of citrus greening symptoms in petioles of field-grown ‘Valencia’ trees. Representative transverse cross-sections through ‘Valencia’ petioles show that in healthy tissue (A) the phloem develops normally and at high magnification both sieve elements and companion cells are clearly visible (B, white and black arrows, respectively). Early Huanglongbing (HLB) symptoms (C) include deformed cambium cells (white arrow), collapsed phloem, and callose formation. Phloem parenchyma cells (gray arrow), however, retain normal shape as the surrounding sieve elements and companion cells collapse. As symptoms become more advanced (D), the cambium completely collapses (white arrow), callose plugs are common (black arrow), and the phloem cells collapse and are interspersed with misshapen parenchyma cells (gray arrow). Bars = 40 μm in A, C, E; 20 μm in B.

Fig. 3. Representative longitudinal light micrographs through the stems of (A) healthy greenhouse grown and (B) field-grown Huanglongbing (HLB)-affected ‘Valencia’ trees. The fibers (f), xylem (x), and parenchyma (white arrow) appear normal in both samples, but the phloem (black arrows) is severely collapsed in the HLB-affected tissue (B). Starch granule accumulation (gray arrow in B) is common in HLB-affected tissue. Bars = 40 μ m.

their eventual collapse as disease symptoms continue to develop (Fig. 2D). The only cells remaining largely unchanged in the phloem are the parenchyma cells (Fig. 2C, gray arrow), although some deformation occurs as the surrounding phloem cells collapse (Fig. 2D, gray arrow). Frequently, cambial tissue also appeared crushed and deformed in late-stage HLB (Fig. 2D, white arrow). The darker appearance of the phloem tissue in Figures 2D and 3B is the result of callose deposits, thickened cell walls, and from the collapse of the protophloem located at the cortex boundary of the tissue. Starch grains, occasionally seen in healthy leaves (Etteberria et al., 2009), became prominent in all parenchyma tissue of the petiole (Fig. 3B, gray arrow). Although cortex parenchyma appeared to have the highest concentration and larger

starch grains, xylem, phloem, and ray parenchyma cells also accumulated substantial amounts of starch (Fig. 3B).

In late summer, citrus trees exhibit a burst of vegetative growth termed “summer flush” (Fig. 4A–C). In young summer flush tissue developing on debilitated HLB-affected stems, both cambium and phloem appeared healthy in petioles (Fig. 4B) and stems (Fig. 4C). Phloem elements were free of callose, and cells appeared normal in shape and size (Fig. 4B–C). There was little or no evidence of HLB symptoms other than a few small starch grains in cortex parenchyma cells (Fig. 4B–C). In the older stem tissue (corresponding to spring flush), a wave of cambial activity was observed in petioles (Fig. 4E) as well as in mature stem tissue (Fig. 4F), although not always uniformly active around

the entire vascular cylinder. The newest ring of developing phloem appeared to be healthy for the first few cell layers in both petioles and stems (brackets in Figs. 4E and 4F, respectively). Starch accumulation in ray and xylem parenchyma was evident, and the protophloem was collapsed with significant plugging (presumably by callose; Kim et al., 2009; Koh et al., 2012) in all tissues investigated (Fig. 4E–F, gray arrow).

Phloem development in fruit peduncles differed from that of petioles and stems in that its production was continuous rather than in discrete bursts (Fig. 4D). From the cross-sections, it was evident that phloem in the peduncle eventually degrades (Fig. 4D, white arrow), but despite its condition, a significant amount of phloem and the cambium still appeared to be completely functional. It is noteworthy that in the older spring flush tissue, the cambium also appeared compressed (Fig. 4E–F) to a greater degree than in the younger tissue. In all cases, the newest rings of developing phloem appeared to be functional for the first few cell layers. As tissue from both sampling periods (spring and summer) aged, phloem collapsed.

Tissue collected in Spring 2013 from the previous summer (2012) and current spring (2013) flushes also showed signs of HLB (Fig. 5). The oldest tissue, the 6-month-old summer flush (Fig. 5A–B), showed the most advanced HLB symptoms in both stems and petioles (Figs. 5A and 5B, respectively), whereas the younger 2-month-old spring flush (Fig. 5C–D) showed fewer symptoms overall, and the petioles appeared healthy with only minor deformation occurring in the cambium (Fig. 5D). Petioles from the summer flush showed the most advanced HLB symptoms (Fig. 5B), where the older phloem was completely collapsed. HLB symptoms were variable in the midveins (data not shown) with the oldest tissue showing mild phloem collapse and the youngest tissue appearing healthy with little or no visible phloem degeneration, but overall symptoms in the midveins were similar to the other tissues. The youngest tissue from both the 2011 and 2013 samplings showed the least amount of HLB-related phloem degeneration: the phloem elements were free of callose and cells appeared normal in shape and size. It is noteworthy that despite the apparent damage to the cambium, xylem vessel development appeared to be largely unaffected by the disease.

FDA-stained healthy petioles showed living, metabolically active cells in the pith and throughout the ray parenchyma within the vascular tissue (Fig. 6A). Metabolically active cells were also visible in the cambial zone, characterized by a ring of bright emission points around the vascular cylinder (Fig. 6A, white arrow). The cambial zone also contains all active phloem and accompanying parenchyma cells. In direct contrast, HLB-affected petioles had few active cells in the ray parenchyma, and activity in the cambium was patchy with large sections of the cambium completely inactive (Fig. 6B). Some

Fig. 4. Representative transverse light micrographs through Huanglongbing (HLB)-affected 'Valencia' tissue. The branch diagram (A) shows the sampling locations on the stems, where the letters correspond to the following panels. Petiole and stem tissue (B and C, respectively) from the young summer flush from HLB-affected trees appeared healthy. Petiole and stem tissue from the same stems but developed during the spring flush (≈ 6 months old) showed advanced HLB symptoms in the oldest phloem, including obliterated phloem cells and misshapen parenchyma tissue (gray arrows D, E). The youngest phloem tissue from the spring flush, however, appeared to be healthy and functional for several cell layers (brackets in D, E). In the peduncle (F) the oldest phloem showed typical signs of HLB (gray arrow), but there was also new growth closest to the cambium (bracket) and more continuous growth and development. Bars = 40 μm .

autofluorescence from the xylem secondary walls was present in all samples, but the FDA signal from the healthy tissue was undoubtedly more intense than in the HLB-affected tissue.

Discussion

The cumulative effects of CLAs infection and HLB in citrus (Graham et al., 2013; Johnson et al., 2013), including the carbohydrate imbalances resulting from phloem collapse, have contributed to the reduction in crop yields in Florida and threaten the future of the entire industry worldwide. These imbalances are brought about in part by the reduction in root system (Johnson et al., 2013) and by the systemic breakdown of the phloem tissue, which often lead to secondary infections (Graham et al., 2013). Phloem collapse (Achor et al., 2010; Folimonova

and Achor, 2010; Schneider, 1968) results in the near total obstruction of phloem transport, hindering delivery of assimilates to developing sinks. However, despite the impaired phloem transport pathway, citrus trees affected by HLB continue to produce both vegetative and reproductive growth for several years, although of progressively lower quantity and quality as the disease wears on. The data presented in this communication suggest that in HLB-affected trees, production of vegetative and reproductive tissues is supported for a limited time by new phloem production during annual periodic flushes.

Our study indicates that a systemic wave of cambial activity can take place in stems, petioles, and midveins of fully expanded leaves and mature stems affected by HLB. In newly produced vegetative tissue, even after leaves had already fully expanded, phloem elements contain no signs of deteri-

oration in either stems or in leaves, a situation comparable to HLB-unaffected tissue. The phloem appears healthy and with conventional development of sieve elements and companion cells. The only indication of HLB symptoms early on is the presence of some starch in the parenchyma cells. As trees with HLB age, however, the disease symptoms appear to progress, either as a result of increased bacterial titer within the tissue or the prolonged disruption of the carbohydrate translocation pathway. Older HLB-affected phloem tissue that developed over the previous summer and throughout the winter shows the typical signs of degeneration (i.e., callose plugging, and collapse; Fig. 5). Under ordinary conditions, some natural senescence in the phloem is common in citrus, because leaves and stem tissue remain on the tree for multiple seasons (Schneider, 1952). In those cases, however, the magnitude of phloem disintegration and other symptoms typical of HLB are not present (starch accumulation, callose plugging, etc.).

In addition to the new vegetative tissue, fruit continues to develop from HLB-affected branches produced in spring. An earlier report suggested the possible symplastic movement of photoassimilates between the leaf photosynthetic tissue down the petiole through the abundant plasmodesmata connection in the petiole cortex and ray parenchyma (Etxeberria and Narciso, 2012). However, given the isolated nature of the vasculature that supplies the juice area in citrus fruits (Schneider, 1952), photoassimilates must enter the phloem elements at some point before the peduncle. When peduncles of developing fruit on HLB-affected trees were examined, phloem tissue appeared to be healthy in most areas, particularly those areas closest to the cambium (Fig. 4D). The size of healthy vascular tissue was always considerably larger than that of leaves and stems of the same age (Fig. 4E–F). Based on its dimensions alone from these static images, it appears that phloem in the peduncle is continuously produced throughout the growing season, or at least during fruit maturation, and not generated in waves as in other tissues (Fig. 4D). A notable observation was that healthy phloem was not always uniform around the vascular cylinder, a condition that can explain the misshapen, lopsided fruit common in trees affected by HLB. The partial collapse of the phloem on one side of the peduncle could limit cell expansion and development within the fruit such that one side develops at a different rate than the other. The fact that transport of photoassimilates from citrus leaves down to the fruit is restricted in its lateral distribution (i.e., minimal movement of photosynthate around the perimeter stem) supports our contention (Kock and Avigne, 1984). Therefore, the lack of lateral photoassimilate movement between sectors of the peduncle does not allow the system to compensate for phloem collapse, thereby resulting in uneven, misshapen fruit.

At this point, the implications of inactive ray cells are not clearly established; however,

Fig. 5. Transverse sections of Huanglongbing (HLB)-affected 'Valencia' trees collected in the spring of 2013. The previous summer (2012) flush (A–B) and spring (2013) flush tissue (B, D) show the effects of HLB after 6 and 2 months, respectively. Stem (A) and petiole (B) tissue from the summer flush (2012) showed new growth in the phloem despite advanced HLB symptoms. Stem (C) and petiole (D) tissue from the spring flush (2013) showed new growth but were less affected by HLB symptoms, and the petioles (D) appeared healthy with only minor HLB symptoms. Bar = 40 μ m.

Fig. 6. Transverse cross-sections through healthy (A) and Huanglongbing (HLB)-affected (B) citrus petioles stained with fluorescein diacetate (FDA) for cell viability. In healthy tissue (A), living cells were visible in the xylem ray parenchyma (x) and the phloem (white arrow). In HLB-affected tissue (B), viable cells were largely absent from the rays (green area in the xylem of B is the result of autofluorescence) and was patchy in the cambium (white arrow). Bars = 100 μ m.

these cells are known to be a critical link in the pathway of carbohydrate movement through plants, delivering carbohydrates from the phloem into the vascular parenchyma tissue (Tyree et al., 1999). Furthermore, the ray parenchyma are also known to play a significant role in drought recovery and are believed to help supply the vascular parenchyma with carbohydrates that are broken down into simple sugars as part of the vessel-refilling mechanism (Brodersen et al., 2010; Salleo et al., 2009; Schneider, 1968; Zwieniecki and Holbrook, 2009). Therefore, loss of the ray parenchyma could have important implications for drought recovery in field-grown citrus trees affected with HLB with a diminished root system (Johnson et al., 2013). The cause of cell death in this tissue is not known, but it could originate after the collapse of the phloem, and future work should be focused on the timing of these events. To our knowledge, this is the first documentation of decreased cellular activity in the ray parenchyma of citrus petioles affected by HLB.

The production of vegetative and reproductive tissues in HLB-affected citrus trees with highly compromised phloem systems suggests either an ancillary carbohydrate pathway or that much of the phloem transport takes place within a short window of time after each flush. With two important parts of the carbohydrate pathway (the phloem and ray parenchyma) becoming highly dysfunctional within a few months of development and with a lack of "wound" phloem (Jacobsen and Eschrich, 1990) production, the pathway of photoassimilate transport for new vegeta-

tive growth and fruit development remains unclear. We present evidence that waves of cambial activity concurrent with vegetative flushes are capable of temporarily alleviating phloem transport blockage caused by HLB-affected phloem tissue. Thus, the periods of time immediately before and after the spring and summer flush are likely the most critical from a management perspective.

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