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# Movement of HLB Genetic Signal Within Citrus Trees: More Questions Than Answers

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**The systemic movement of *Candidatus* Liberibacter asiaticus (*C*Las) in citrus trees remains partially unresolved. Here, we report on additional experiments conducted in an effort to elucidate the mechanisms of HLB pathogenicity. Our results based on grafting and girdling experiments confirmed that *C*Las (or a genetic component) can spread within the xylem tissue. By blocking phloem transport and testing additional phloem-free tissues, we also confirmed that *C*Las genetic components detectable by qPCR analysis are capable analysis are capable of moving through plasmodesmata connections of living cells, but not by apoplastic pathways as evidenced by its absence in pollen, embryo, and nectar. The data in this communication support the presence of alternative pathways for the spread of HLB within citrus trees.**

In a previous report (Etxeberria et al., 2015), we presented evidence that challenged the prevalent belief that the causal agent of citrus huanglongbing (HLB), *Candidatus* Liberibacter asiaticus (*C*Las), moves exclusively in the phloem. For example, our data suggest that citrus phloem elements lack wall perforations (Schneider, 1952; Knoblauch and Oparka, 2012) sufficiently large to accommodate *C*Las movement in a lateral direction (Etxeberria et al., 2015). Furthermore, using fluorescent deoxy-glucose, we verified that phloem flow in citrus, as in other evergreen broadleaf species, is dominated by basipetal movement even during the development of new spring leaf flush (Epron et al., 2012). In separate experiments, we also demonstrated that the disease causing agent is capable of moving through the xylem and into the juice cells (Bai et al., 2013), despite the absence of vascular connectivity into the juice sacs (Koch and Avigne, 1990). Taken together, all available data are not adequate to explain the mechanisms of internal, systemic spread of HLB symptoms. Here, we present data resulting from additional experiments that help elucidate the mechanisms of HLB pathogenicity.

## Materials and Methods

**Plant material.** We used 'Valencia' (*Citrus sinensis* L. Osbeck) orange trees of different ages depending on the individual experiment. All experiments were conducted in greenhouses or groves located at the University of Florida, Citrus Research and Education Center in Lake Alfred, FL.

**Spiral girdling experiments.** Spiral girdles (one per tree) were performed on a lateral branch of 20 two-year-old 'Valencia' orange trees grown in a greenhouse to disrupt the most direct, axial pathway for phloem transport, thereby forcing phloem flow through lateral connections. The girdles were long enough to wrap around the circumference of the branch. The exposed area was covered with paraffin and any callus formation during the experiment was removed using a "Dremel" 4000 High Performance Rotary Tool (Robert Bosch Tool Corp., Chicago, IL, <https://www.dremel.com/en-us/Tools/Pages/ToolDetail.aspx?pid=4000>). All trees were bud-grafted with HLB+ material above the girdle and kept in the greenhouse under proper water and fertilization programs. Leaf samples were analyzed for HLB by qPCR every 3 months starting 3 months after grafting.

**Development of xylem connection between two trees.** To test the ability of *C*Las movement between trees through the xylem, we followed the grafting procedure detailed in Etxeberria et al. (2015). Briefly, two lateral branches from separate potted Valencia' trees were grafted together (Fig. 1A; Etxeberria et al., 2015). The graft tissue was allowed to heal and to develop new vascular connections for one year. Next, a complete phloem girdle was made on one branch below the graft union (Fig. 1B). Thus, the only intact pathway between the two trees was through the xylem. Then, HLB+ tissue was grafted below the girdle (Fig. 1C). Leaf samples from each plant were analyzed for HLB by PCR every 6 months, and wound callus tissue developing at the girdle was removed as above.

**Tissue collection for HLB analysis.** We collected samples of pollen, nectar, albedo, flavedo, flower buds, and unprocessed honey. Pollen was collected using pollen traps placed in three groves located around the Lake Alfred area during spring bloom 2016. At the same time, flowers from visible HLB-affected branches were excised and brought to the lab where nectar was collected using capillary tubes. Flower buds were collected at the same time and unheated honey was supplied by a local beekeeper.

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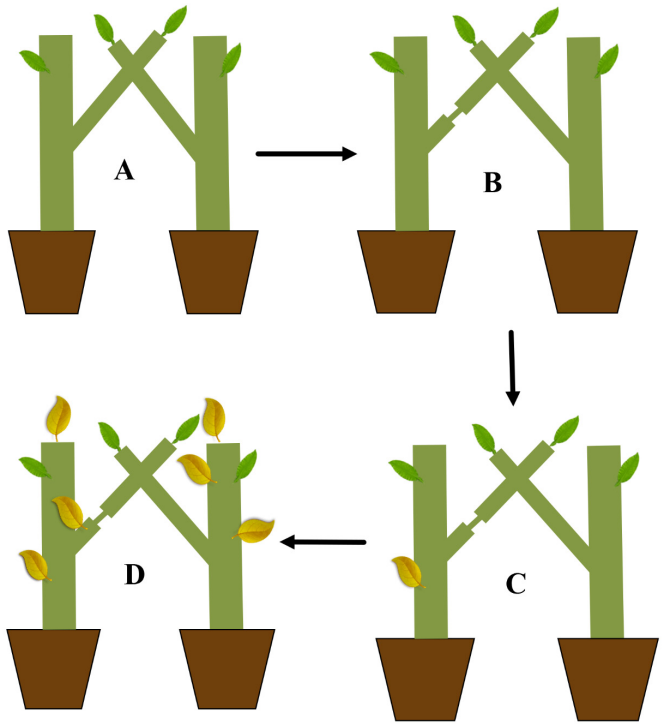


Fig. 1. Two separate potted *Citrus sinensis* 'Valencia' orange trees were grafted together by one branch (**A**), and the tissue allowed to heal and to develop new vascular tissue for one year. Afterwards, a complete girdle was made on one branch below the union area (**B**), and huanglongbing (HLB)$^+$ tissue grafted below the girdle (**C**). Leaf samples were analyzed for HLB by PCR every 6 months until HLB signal was detected by PCR (**D**).

**DNA isolation.** For isolation of '*Candidatus* Liberibacter' DNA, plant material was collected from leaf midribs. Approximately 100 mg (6 disks of 28 mm$^2$) of plant material was placed in an Ependorf tube, frozen in liquid nitrogen, and homogenized with one metal ball using a TissueLyser II (TL Qiagen, Valencia, CA). The pulverized tissue was extracted for DNA using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Yield and purity of DNA samples were estimated by measuring $OD_{260}$ and $OD_{260/280}$, respectively, with a NanoDrop Microplate Reader Spectrophotometer Epoch (Bioteck Instruments, Inc., Winooski, VT). The final concentration of DNA in all samples was diluted to 4 ng/$\mu$L for all samples. All samples were stored at –20 °C.

**qPCR.** Assays were performed on an Applied Biosystems 7500 PCR instrument (Life Technologies). The TaqMan Universal PCR Master Mix (Life Technologies) was used for the DNA assay. DNA samples were analyzed with degenerate genus-specific (*rpo*B) primer/probe sets. The final concentration of qPCR mix contained 1X TaqMan Universal PCR Master Mix (10 $\mu$L), forward and reverse primers 2 $\mu$L each (600 nM each), probe 1 $\mu$L (300 nM), 5 $\mu$L of 4 ng/$\mu$L DNA sample (20 ng total), and nuclease free water for a total of 20 $\mu$L. The qPCR assay consisted of 2 min incubation at 50 °C followed by 10 min incubation at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 1 min, respectively. Data were analyzed with the Applied Biosystems software (version 1.4.0). Ct values were calculated based on a previously determined standard curve according to Ananthakrishnan et al. (2013).

## Results and Discussion

The experiments conducted and described in this communication were intended to either confirm or complement existing data that would help define the mechanisms of *C*Las transport within a citrus tree. Figure 1 is a duplicate experiment to that described in Fig. 2 of Etxeberria et al. (2015). In the present experiment, 10 pairs of trees were grafted to each other by a side branch and allowed to heal for a year. After girdling below the graft area, leaving trees connected by xylem only, fresh HLB$^+$ material was grafted below the girdle. Of the original 10 tree pairs, two graft unions decayed and HLB transmission failed on a third tree. In six of the remaining seven pairs, HLB$^+$ leaves were detected above the girdle confirming that the HLB causing agent is capable of moving in the xylem tissue.

To retest for the possibility of lateral mobility of the HLB causing agent using a different experimental approach, we performed spiral girdles on lateral branches of 2-year-old trees before grafting HLB$^+$ tissue in a basipetal direction (Fig. 2). This experiment was conducted to sever direct phloem connection between the two sides of the spiral but still allow photoassimilates to move through symplastic pathways. In 18 of 20 trees, the HLB causing agent was able to traverse the girdled region

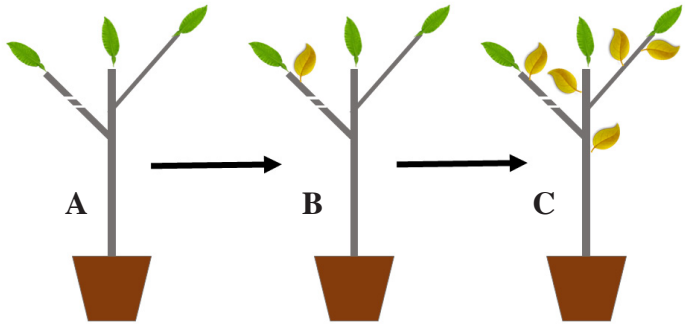


Fig. 2. Diagrammatic representation of spiral girdles where bark was excised in a spiral manner completely around a lateral branch of 2-year-old *Citrus sinensis* 'Valencia' trees (**A**). Huanglongbing (HLB)$^+$ bud material was grafted above the spiral (**B**) and leaf samples tested regularly. Tissues across the spiral girdle (**C**) became HLB$^+$ in 18 of 20 samples.

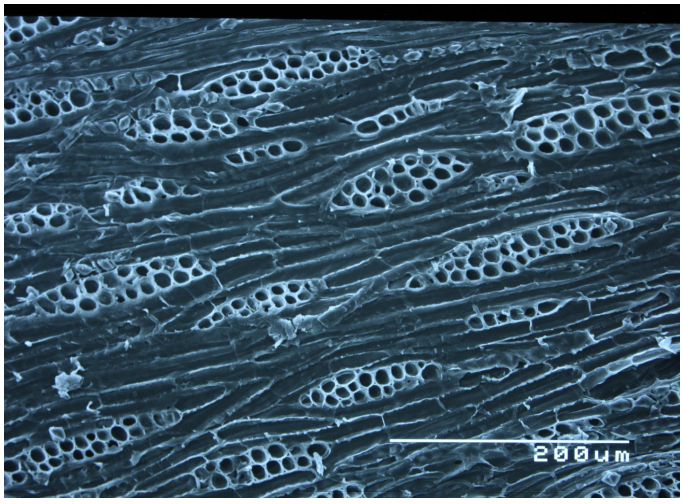


Fig. 3. Scanning electron micrograph of phloem tissue within the strip of bark of a *Citrus sinensis* 'Valencia' tree resulting from the spiral girdle. Bark was excised and phloem observed in a tangential.

in a basipetal direction, leading to symptom development in the remaining tree branches that then tested HLB+ by qPCR. These data can be interpreted in several ways. First, established phloem tubes bifurcate frequently enough to be able to circumvent the girdle area. This explanation, however, was not supported by microscopic observations of the phloem, where no bifurcations (Fig. 3) nor evidence of new phloem growth in a spiral pattern that might establish new connections along the bark spiral were observed. Second, *C*Las, or some constituent of *C*Las detectable with qPCR, is capable of moving through the xylem. Although xylem movement of *C*Las signal has been demonstrated (see above), in the present situation however, movement bypassing the spiral girdle would need to take place against the direction of the xylem current. Third, *C*Las detectable signal moves through the symplast of the xylem parenchyma.

The above data, plus observations of juice samples testing HLB+ by qPCR (Bai et al., 2013; Etxeberria et al., 2015), suggest that *C*Las, or at least part of *C*Las genome, is transported though non-vascular routes. To test this hypothesis, we analyzed a series of tissues with different forms of vascular or non-vascular connectivity by qPCR. We selected tissues containing vascular tissue (flower buds, leaves, seed coats), tissues with only symplastic connections (flavedo, albedo, juice cells), and those few tissues (or secretions) connected to the sporophyte exclusively through the apoplast (pollen grains, embryo and nectar). All sampled tissues were collected from HLB+ trees and from branches with evident visual symptoms. Table 1 shows the results of qPCR analysis demonstrating that all tissues containing either vascular tissue (xylem and phloem) or symplastic connections tested positive for *C*Las, whereas those symplastically isolated from the sporophytic body and connected only through the apoplast did not. These results imply that the genetic component that results in a positive qPCR signal is capable of moving not only through the vascular tissue, but through plasmodesmata as well, where movement of the *C*Las bacteria is physically impaired. The absence of *C*Las signal in apoplastically connected tissues indicated that either the *C*Las genetic material is not secreted or cannot cross the apoplastic space of ~5 nm (Etxeberria et al., 2016).

Figure 4 summarizes the data of Table 1 that supports the notion presented by Etxeberria et al. (2015) of an additional non-vascular pathway for the spread of *C*Las "genetic material" in citrus trees. This conclusion is based on our findings that genetic material is detected in the phloem-isolated but symplastically connected juice cells (Bai et al., 2013; Etxeberria et al., 2015) and in the albedo-flavedo (Table 1; Kim et al., 2009; Li et al., 2009) but not in symplastically excluded tissues such as pollen grains, nectar and embryo (Table 1; Graham et al., 2011; Hilf, 2011). *C*Las bacteria move in a vertical direction within the phloem elements but lateral transmission is impeded by the lack of physical connections (Kumar et al., 2014). Our results strongly suggest that because of the physical size exclusion barriers for *C*Las bacteria movement, some form of *C*Las genetic material moves between living cells through plasmodesmata-mediated symplastic connections, thereby leading to the qPCR+ values. It remains unclear which part of the *C*Las genome (fragments or the entire genome) is capable of moving through the symplast or whether these fragments of genetic material can engender disease symptoms. However, given that some of these tissues have tested qPCR+ using different primers, it is likely that the genetic component is of a significant size. This is presently under investigation.

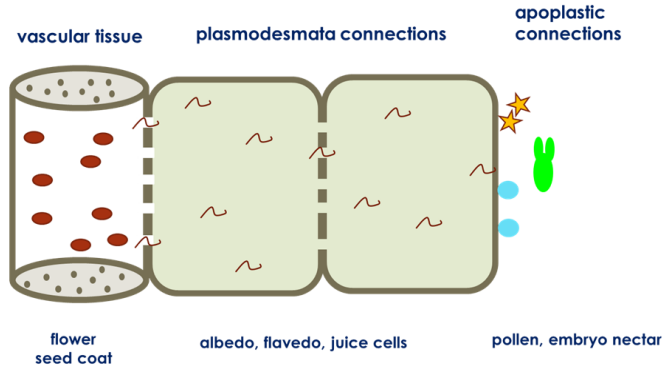


Fig. 4. Diagrammatic representation of plant tissues connected in various ways. *Candidatus* Liberibacter asiaticus (*C*Las) can be transported into organs containing vascular tissue through the phloem cells. Sieve plates separating sieve elements contain pores large enough to permit *C*Las passage from cell to cell and throughout the tree. In vascular isolated tissues such as juice cells, albedo and flavedo, exchange between cells is only possible through plasmodesmata connections (30–40 nm), not large enough to support *C*Las (2–5 $\mu$m) passage but allow crossing of genetic material. For apoplastic transport, materials have to be extruded by the cells into the apoplast then move through the cell wall matrix.

Table 1. List of tissues within the body of a citrus tree tested for huanglongbing (HLB) using qPCR. Tissues are grouped by whether they contain vascular tissue or are connected by the symplasm or apoplasm. Only tissues without cytoplasmic connections remain free of *C*Las genetic material.

| Vascular connection | Plasmodesmata connection | Apoplastic connection |
|---|---|---|
| Flower bud (+) | | |
| | | Pollen (–) |
| Fruit (+) | | |
| Albedo (+) | | |
| | Flavedo (+) | |
| | Juice (+) | |
| Seed (+) | | |
| Seed coat (+) | | |
| | | Embryo (–) |
| | | Nectar (–) |
| | | Honey (–) |

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