



AN INTERDISCIPLINARY APPROACH TO COMBAT HLB

RESEARCH IN UC DAVIS' CONTAINED RESEARCH FACILITY

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This is an introductory article for an ongoing Citrograph report. In subsequent issues, progress on this time-course study will be reported. Readers will be informed of CRB-funded efforts to develop early, pre-symptomatic diagnostic methods for HLB.

An extensive background on the biology of disease development is provided that can be used as a reference by readers. In addition, each participating laboratory briefly describes the diagnostic method they have developed and introduces their research team.



BACKGROUND OF HUANGLONGBING

Huanglongbing (HLB), also known as Citrus Greening Disease, is the most serious disease threatening the citrus industry. The bacterium known as *Candidatus Liberibacter asiaticus* (or CLAs, for short) is the suspected causative agent for the disease, which can be transferred to plants through grafting infected material or via feeding by an insect known as the Asian citrus psyllid (ACP). Infection causes reduced fruit yield, poor fruit quality, fruit drop and early tree death. The time between initial CLAs infection, detection by nucleic acid-based methods (polymerase chain reaction [PCR]), and symptom manifestation, can take years to occur.

Early detection of CLAs in trees is the best prevention for future outbreaks in the state of California. This will enable growers and residents to make informed decisions for disease management and to act quickly to remove infected trees before the ACP vector

can spread the disease. Currently, PCR is the only technology approved for regulatory use for detection of infection. This technology relies on a minute amount of CLAs to be present in the sample. Since the bacteria are not uniformly distributed throughout the tree, early detection of CLAs by PCR is primarily a matter of luck in choosing the correct sample. However, just because PCR cannot detect the bacteria does not mean that CLAs is not present, and that ACP cannot feed on the infected tree, acquire, and then spread the bacteria. Because of the long latency period between infection and appearance of symptoms and the inability to detect the pathogen early, CLAs is able to spread unnoticed through urban areas and commercial groves. Though some citrus varieties appear to be more tolerant to the disease, all citrus cultivars and clones are susceptible. Fresh citrus production in California is particularly at risk.

HLB was first discovered in Florida in 2005. Just eight short years later, the entire state is under quarantine for the disease. During this period of time, more than \$4.5 billion in revenue, representing 30 – 40 percent of all citrus produced in the state, was reported lost to this pathogen, also resulting in more than 8,000 lost jobs.

In California, HLB has been confirmed in a single tree in an urban neighborhood of Los Angeles County in 2012. So far, adjacent trees have not tested positive for the presence of CLAs by PCR. At this time, it is unknown how far the bacteria may have spread from this one tree, since no early detection methods have been validated for this pathogen.

ACP insects are spreading from the south of California and have now been confirmed in Tulare County (about 45 miles outside of Fresno). Wherever the ACPs are found, the threat of CLAs infection, and thus HLB, is real. There is a desperate need to find early markers of infection to protect citrus growing areas, and more fully understand the pathogen, its insect vector and how it causes devastation to a tree. To address this, the Citrus Research Board,

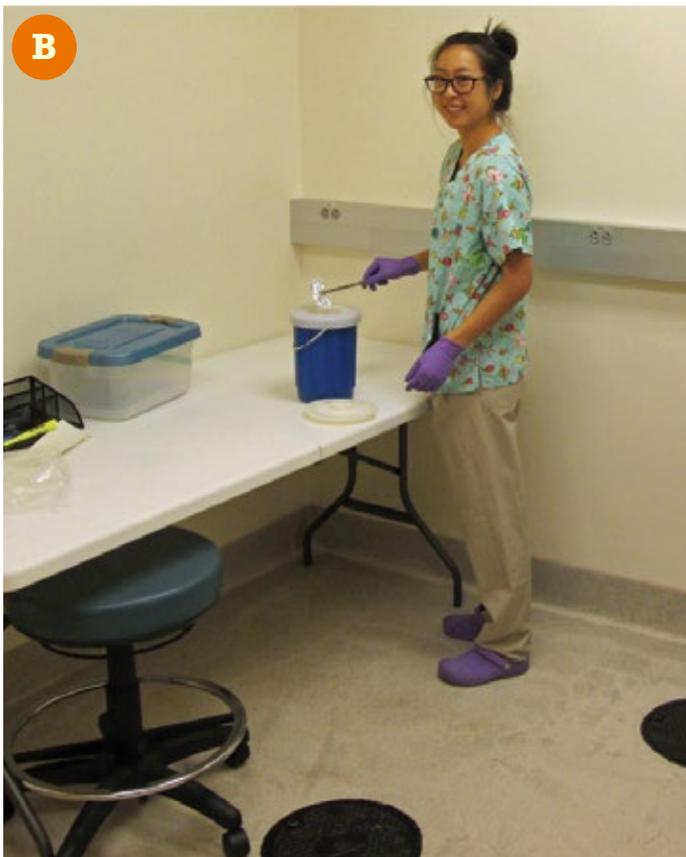


Figure 1. A. The Contained Research Facility (CRF) at the University of California, Davis. The CRF provides a tightly controlled environment to conduct experiments on plants. **B.** Graduate student Elizabeth Chin from the Slupsky lab preparing leaf samples in the CRF. **C.** CRB Board members Richard Bennett and Jeff Steen in the greenhouse during their visit to the facility. **D.** The experimental trees (Parent Washington orange, Tango mandarin and Lisbon lemon) for this CRB-funded project inside the greenhouse. Experimental trees are not infected with CLAs at this time.



together with researchers at UC Davis (Coaker, Davis, and Slupsky), UC Riverside (Ma and Jin), the California Institute of Technology Jet Propulsion Laboratory (Glenn Sellar), the Boyce Thompson Institute for Plant Research on the campus of Cornell University in New York (Cilia) and the University of Washington in Seattle (Bruce), have come together to tackle this important problem in the Contained Research Facility (CRF) at UC Davis.

RESEARCH ON HLB AT UC DAVIS CRF

The CRF at UC Davis has laboratory, greenhouse and growth chamber space that provides a highly controlled and contained research environment (Figure 1). Kris Godfrey and Tiffanie Simpson manage the facility. Elizabeth Foster is responsible for maintaining the plants, coordinating and helping with sample and data collection, as well as other research needs. Within the facility's greenhouse, the temperature and the amount of light and water the plant receives are regulated. These tightly controlled conditions are ideal for conducting experiments, because the variable

environmental factors and subsequent uncertainty in the results that come with sampling field trees are minimized or eliminated.

In the CRF, as many unknowns and variables as possible are removed so that information specific to the bacteria or tree response during the infection and disease development can be detected without confounding factors. Thus, changes to the plant over the course of CLAs infection from both graft and ACP inoculation will be studied. Three citrus varieties are being tested – Parent Washington navel orange, Tango mandarin and Lisbon lemon, all of which have different susceptibilities to HLB. Changes in gene, protein and metabolite regulation within the plants, beginning from the moment of inoculation until the development of HLB symptoms, will be examined. Comparison of the earliest time that CLAs infection can be detected using these methods will be compared with the gold-standard PCR technique (performed in the CRB diagnostic laboratory by Dr. Cynthia LeVesque, and John Morgan) to determine whether a more accurate early detection method may be found.

If successful, the data obtained will aid the researchers in applying for regulatory approval, the first step in making the method widely available. Furthermore, cooperation amongst the eight collaborators (whose projects are described below) will provide unprecedented information about the bacteria and disease, and potentially result in improved treatment methods.

Each member of the team brings a unique perspective to the study of the disease that, when combined, will provide a systems biology approach to understanding the pathogen and the insect, and the plant's response to them. Systems biology, as implied by its name, is interdisciplinary, combining multiple scientific fields to understand the complex interactions within a biological system, and emergent properties of the system that could not be revealed by studying any individual component in isolation. Our combined effort will provide a more complete understanding of the ACP/CLas/citrus system, and allow for development of early detection methods and a comprehensive management strategy to combat the disease.

PIECES OF A PUZZLE

The unique approach of each researcher in our interdisciplinary team has already provided a wealth of information, some of which has already been published. However, each of these approaches are just pieces of the larger systems biology puzzle (Figure 2) – Systems biology is the conglomeration of genomic, transcriptomic, proteomic and metabolomics information.

Studying the genome of citrus, the CLas pathogen or the ACP insect provides a blueprint of all the genes that these organisms can produce. Finding genes can help decipher all of the functions that an organism can perform, as the genome is the foundation upon which an organism is built.

However, not all genes are 'turned on' (also referred to as being 'expressed') and allowed to produce a functional product (RNA and/or protein); and if they were, one would not see differentiation between the root system and the trunk of the tree, the leaves or the fruit. Each of these parts of the tree contains the entire DNA of the organism, but not all of the genes encoded in the DNA are expressed in each tissue. The tree's DNA contains the essential information about how to carry out all of the tree's life processes and how to make all the tissue systems, such as the roots that absorb water and nutrients from the soil, the xylem and phloem cells that transport nutrients to all parts of the plant, and, of course, the leaves and fruit. This level of control of gene expression is through a process called epigenetics, where expression of certain genes can be either enhanced or repressed through the addition of methyl groups (a type of chemical group) to specific regions of DNA. Epigenetics determines how a leaf cell knows to be a leaf cell and not a root cell, even though all the cells in the tree have the same DNA. The epigenome can be altered through external influences, such as the amount of water and the presence/absence of micronutrients, soil bacteria or even pathogens like CLas.

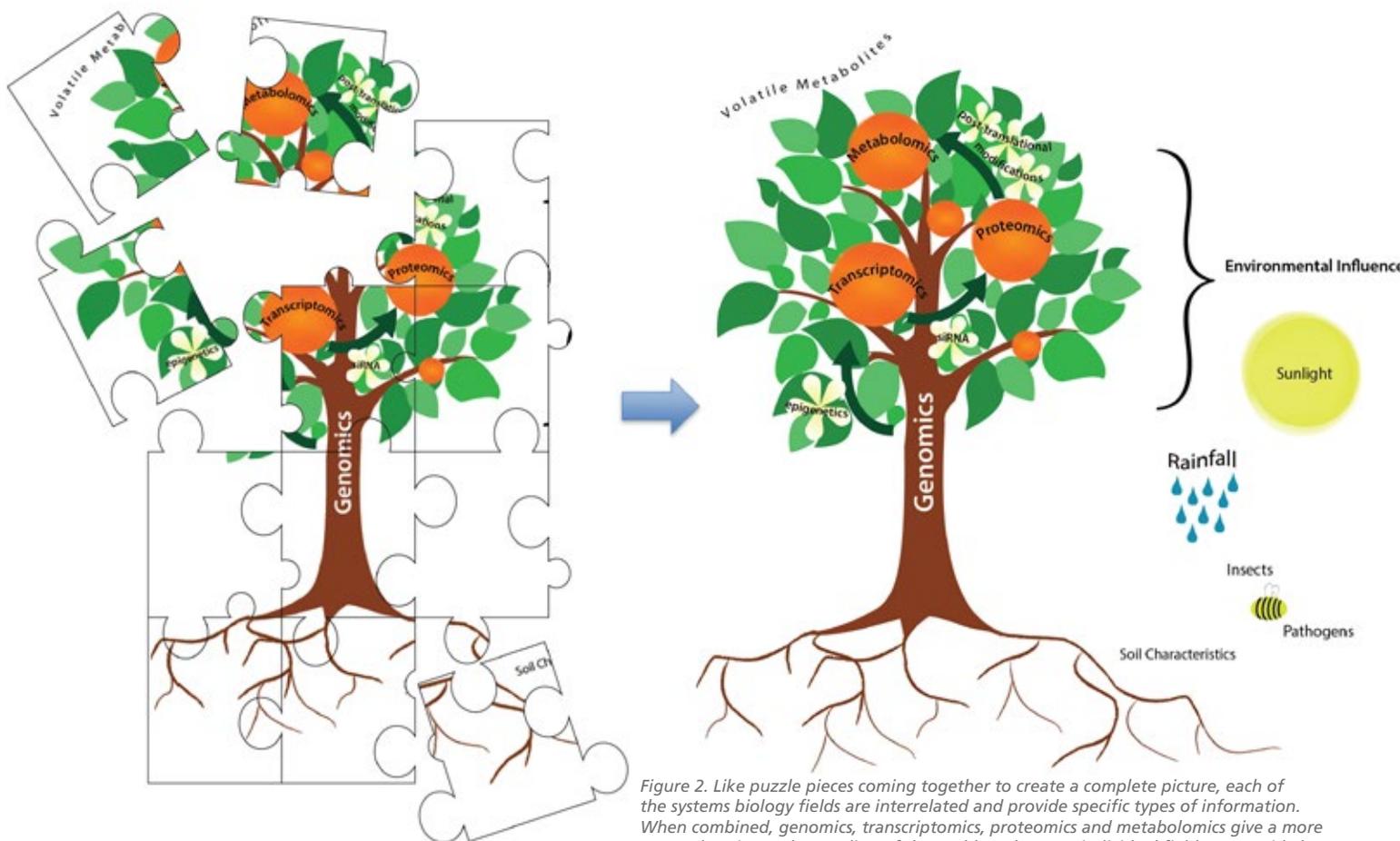


Figure 2. Like puzzle pieces coming together to create a complete picture, each of the systems biology fields are interrelated and provide specific types of information. When combined, genomics, transcriptomics, proteomics and metabolomics give a more comprehensive understanding of the problem than any individual field can provide by itself. A team of researchers from across the U.S. have come together to solve this puzzle for the most serious citrus disease that is now threatening fresh California citrus: HLB.

The first step of gene expression within DNA is a process called transcription. The DNA, or collection of genes, is 'read' and a transcript called messenger RNA (mRNA) is generated. Transcriptomics enables us to know what genes are being expressed and where. It can provide information about changes in the epigenome, and also provide information on the quantity of mRNA being produced from each gene.

Cues from the environment can cause changes in gene expression levels and thus mRNA production. For example, when the temperature rapidly decreases to close to freezing, specific genes will be turned on, and others will be turned off to help the tree cope with the change in the weather. Measuring which genes are turned on or off helps us understand the processes by which the tree is able to recover from exposure to cold, which genes are related to cold tolerance, and how the plant is regulating itself to deal with a change in weather.

After a gene has produced an mRNA molecule, the mRNA provides a template to make a certain protein through a process called **translation**. Proteins are essential for carrying out all functions inside a cell and between cells. They are involved in cell structure, cell signaling, metabolism (such as photosynthesis, respiration, energy transport, synthesis and breakdown of large molecules, etc.), defense against stress and pathogens such as CLAs, and even against insects such as the ACP. Proteins function as small molecular machines. Each protein has a highly specific shape that enables it to perform these important cellular processes. Understanding how proteins come together to form functional units is a critical part of proteomics studies.

Protein function can further be regulated through the addition of chemical groups (such as sugars and phosphate) to the protein that will either help or stop it from functioning. This process is called **post-translational modification**, and is essential in helping to define a protein's function or lack thereof. Regulation of protein expression can also occur at the gene level (through epigenetics, as described above), and at the mRNA level through pieces of RNA called small RNAs (sRNAs), which can be divided into two subclasses: micro RNA (miRNA) and small interfering RNA (siRNA). The latter are pieces of RNA that can bind to a specific mRNA transcript and prevent it from being translated into a protein. These sRNAs can be generated by the cell or by other organisms such as bacteria.

Metabolites are essential for the plant to grow and reproduce, as well as defend itself against pathogens, insects and other stresses. They are also responsible for providing sensory aspects of fruit such as taste and smell. These small molecules include things like amino acids (the building blocks of proteins), organic acids (such as citric acid, which is the main organic acid in orange fruit), sugars (such as sucrose, glucose and fructose), lipids (which form the cell membrane that surrounds the cell), various vitamins (such as ascorbic acid), and other molecules. They may be soluble or insoluble in water, and they may or may not be volatile. The sweet smell of citrus blossoms is an example of volatile chemicals produced through citrus metabolism to attract insects to help pol-

linate the tree. Metabolites are a reflection of the biological processes occurring in the tree. What happens at the metabolite end of things can be traced back to the proteome, the transcriptome, and the genome. If the amount of a metabolite changes, it tells us that there has been a change in the tree's activities.

All of these "-omic" pieces are interrelated, and the study of each contributes to our understanding of the others. Bringing each of these small, but significant, pieces of information together provides a holistic, molecular snapshot of the complete picture. Like puzzle pieces fitting together, genomics, transcriptomics, proteomics and metabolomics are related to one another. Together, they complete a picture that will enable us to create a robust early detection system, and allow development of strategies to combat HLB. Working with all the pieces is the only way to see the complete picture of what happens to a tree during CLAs infection.

GLOSSARY

Genomics

Study of the genetic make-up of an organism including DNA sequencing, mapping and gene function.

Epigenetics

Study of changes in gene function that are not the result of changes in the DNA sequence; determines whether a gene should be turned off or turned on.

Transcriptomics

Study of the how an organism's DNA sequence is transcribed into corresponding molecules of RNA.

Transcription and Translation

The process by which an organism's DNA sequence is converted from genes to RNA molecules to amino acids to proteins and other products.

Proteomics

Field of study that examines the structure and function of proteins in a cell.

Metabolomics

Study of metabolites, which are small chemicals or molecules used by proteins involved in plant metabolism.

AN INTERDISCIPLINARY TEAM

To create a more complete picture of how CLAs infection affects citrus, eight research groups and two infrastructure teams have come together from across the U.S. to work at the CRF. Each of the research groups is working on different pieces of the puzzle of how CLAs affects citrus:

Jin Lab

(UC Riverside, California) - sRNAs (transcriptomics)

Many sRNAs are differentially regulated by pathogen infection. The Jin lab is particularly interested in the sRNAs that are specifically induced or suppressed in CLas-positive plants. Their potential targets are likely involved in the tree responses against CLAs. The Jin lab will profile and compare sRNAs and mRNAs from HLB-tolerant and sensitive citrus plants using genome-wide RNA deep sequencing. They aim to identify a group of sRNAs and citrus genes that are specifically induced by CLas-infection, which could potentially be used as early diagnostic markers. Some of the sRNAs and genes are important for natural defense responses against CLAs, which will be ultimately used for citrus protection.

Ma Lab

(UC Riverside, California) - Antibody development (proteomics)

The Ma lab is developing antibody detection methods for robust diagnosis of HLB. Using the fully sequenced CLas genome, they predicted 27 CLas proteins that can be secreted outside the bacterial cell and presumably into the phloem of infected citrus. These secreted CLas proteins are called *effectors*. The Ma lab has further identified four CLas effectors that are highly expressed and, therefore, may be present in high abundance in CLas-infected trees. Using these effectors as antigens, the Ma lab has generated antibodies that can specifically bind to the target effectors and detect their presence with high efficiency. These antibodies may allow robust HLB diagnosis because, unlike the bacterium, the effectors are systemically dispersed in the infected trees through the central transport tissues of the plant and thus could be detected away from the infection site(s). CLas effector antibody-based detection may overcome the problems associated with the large variability in the distribution of the pathogen throughout the tree, and holds promise for bacterial detection in a simple, direct and highly specific manner.

Coaker Lab

(UC Davis, California) - Proteomics

The Coaker lab is using a proteomics-based approach to determine the expression pattern and citrus targets of the four CLas proteins that were identified by the Ma lab as being highly expressed in CLas-infected citrus trees. In other bacteria, effectors are required for pathogen virulence and can facilitate nutrient acquisition, insect feeding and suppression of defense responses. Her group will focus their efforts on investigating the expression patterns and citrus targets of these four unique CLas effectors. They hypothesize that these effectors are important for bacterial survival or HLB symptom development by targeting important citrus proteins to manipulate their host. They are also using mass spectrometry, a technique that enables protein identification, to isolate citrus proteins that are targeted by each CLas effector in different plant tissues. Successful completion of these experiments will facilitate HLB detection strategies, as well as provide a set of pathogen targets that can be manipulated to protect citrus against HLB.

Bruce Lab

(University of Washington, Washington) - Advanced Proteomics Technology Development

The Bruce lab is focused on development and application of advanced mass spectrometry technology for biological, biophysical and biochemical studies. This lab specializes in using technology called Fourier transform ion cyclotron resonance mass spectrometry for proteomics, biological, and biomedical applications. They will apply their cutting-edge Protein Interaction Reporter Technology and other technologies currently under development to gain insights into the protein interactions that regulate CLas transmission in collaboration with the Cilia lab.

Cilia Lab

(USDA - Agricultural Research Service, Boyce Thompson Institute for Plant Research, Cornell University, New York) - Vector-Host-Pathogen Proteomics

The Cilia lab is interested in understanding how proteins orchestrate the process of CLas transmission by ACP vectors and the spread of CLas inside of infected citrus trees. These processes involve changes in expression and protein interactions among ACP, citrus and CLas proteins. The Cilia lab is partnered with the Bruce lab to use advanced mass spectrometry technology developed in the Bruce lab for studying these complex processes. The Cilia lab will perform functional assays to understand which proteins and protein interactions are critical for CLas infection and transmission. Practical applications of the Cilia-Bruce collaboration include the development of early infection biomarkers for detection of HLB in citrus trees and the identification of novel molecular targets in the ACP for CLas transmission control.

Davis Lab

(UC Davis, California) - Volatile Metabolomics

The end products of some biochemical pathways are volatile organic compounds (VOCs) that radiate into the environment. The Davis lab measures these VOC metabolites in the canopy of citrus trees, and uses those "odors" to non-invasively diagnose a tree infected with a specific pathogen. In this CRF experiment, the Davis lab will use a suite of hardware and software approaches to monitor VOCs pre- and post-infection. First, they will use a mobile gas chromatograph differential mobility spectrometer (GC/DMS) to sample and analyze the VOCs emanating from the citrus leaves. This is a field-portable instrument that has previously been used to detect VOCs associated with citrus greening. Secondly, they will use an active sampling device to concentrate the VOCs onto a sorbent trap for later analysis on a mass spectrometer system for chemical identification. This experiment will help to determine when the VOC signature of a tree changes post-infection, and will provide valuable data regarding VOCs as early diagnostic markers for field detection of citrus greening.

Slupsky Lab (UC Davis, California) - Metabolomics

The Slupsky lab is measuring the metabolome of citrus infected with CLAs using a technique known as nuclear magnetic resonance (NMR). This technology is similar to that of an MRI (magnetic resonance imaging). NMR allows for each molecule's identity and concentration in a single sample to be determined at the same time. This group already has identified unique changes in citrus fruit that occur with infection. The changes have provided information showing that CLAs is able to "turn-off" the production of metabolites used for plant defense. These results partially explain why CLAs is so good at surviving in the tree and causing so much damage while evading plant defense. The Slupsky lab has begun to expand measurement from fruit to leaves and other tissues in citrus. Her work will provide valuable information on metabolic responses of the tree to infection, helping to understand why the pathogen is so difficult to detect early, and will be useful for validating early diagnostic metabolite markers of CLAs infection.

Sellar Lab (JPL, CalTech, Pasadena, California) - Optical Imaging

Inspection and detection are the first lines of defense against the spread of HLB. The interdisciplinary team at NASA's Jet Propulsion Laboratory (operated by the California Institute of Technology) is using optical data from NASA's "Airborne Visible and Infrared Imaging Spectrometer" ("AVIRIS") collected from an aircraft to produce maps of the distribution of citrus trees in urban areas to help guide inspection efforts.

Together, this interdisciplinary team is working to provide growers and the scientific community with as much information as possible about the disease itself, the bacteria that cause it, how the tree responds to infection and how it is transmitted. By putting together the pieces of this puzzle, our team will help to generate the 'big picture' so that we can develop early detection methods and a targeted approach to fully combat HLB. 🌍

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