

Outcome Driven Discussion: Model Systems for Studying HLB

OG3

Thanks to the many folks who provided information presented in this summary!

Rationale for Model Systems:

- Liberibacter inoculation produces HLB symptoms in citrus after 10-24 months.
- Model systems allow more rapid screening of potential therapeutic materials and transgenes through characterization of their expression, activity, and bio-availability
- Also may:
 - permit more efficient use of space- plants smaller than Citrus
 - Allow study in areas where HLB is not present
 - Have biological advantages, such as much greater depth of genomic data (i.e. Arabidopsis, Tobacco, Tomato.....)

CLsol / *B. cockerelli* Model Systems

- Appears to be almost exact parallel to HLB with phloem limited *Liberibacter* transmitted by psyllids
- Researchers working with tomato / CLsol, tobacco / CLsol, and potato / CLsol
- Symptoms apparent in just a few weeks
- Also some work on RNAi methods targeting *B. cockerelli* as a surrogate for *Diaphorina*

Table from Manjunath et al.

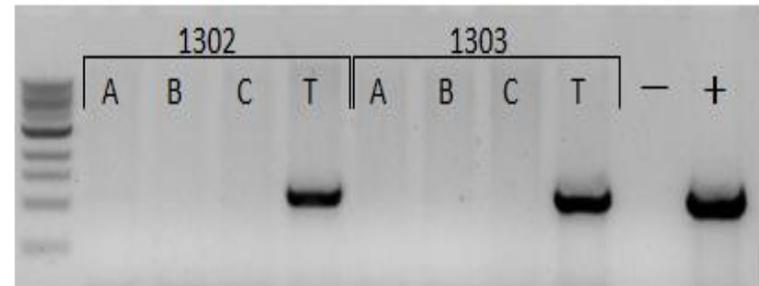
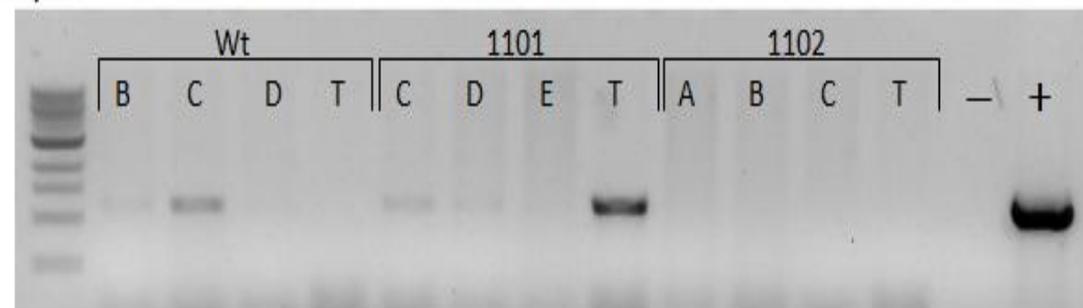
Characteristics	<i>Candidatus</i> L. asiaticus/Citrus	<i>Candidatus</i> L. psyllaourous/Tomato
Host range/hosts	Limited/Perennial	Wide host range/Annuals
Time required for disease symptoms to appear	Six months to two years	Two to three weeks
Rate of infection in psyllid vectors	Low level; ususally less than 15%	High level; nearly 100% infection under greenhouse conditions
Distribution in the host plant	Irregular.	Systemic infection with regular distribution
Status of host genome information		Genomes of several host plants has been sequenced (Tomato, <i>Nicotiana</i> , <i>Arabidopsis</i> , etc). Genome sequences of several members of Solanaceae are near completion.
Transgenic research capability	Transformation and evaluation of Citrus is a long process.	Transformation of Tomato is simple and evaluation can be done in months in
Ease of experimentation	Complicated due to irregular distribution of the pathogen; results can be inconclusive	Simpler methods of testing due to the uniformity of the pathogen in the plant system. Conclusive results may be obtained quickly.
Disease transmissibility	Graft and insect transmissible but at a low level.	Graft and insect transmissible in a very efficient manner.
Sources of Resistance	No good source of resistance	Resistant varieites, non-symptomatic hosts available

A tomato model system for studying citrus huanglongbing

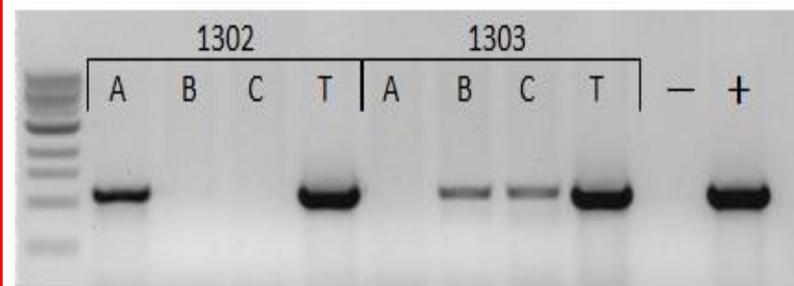
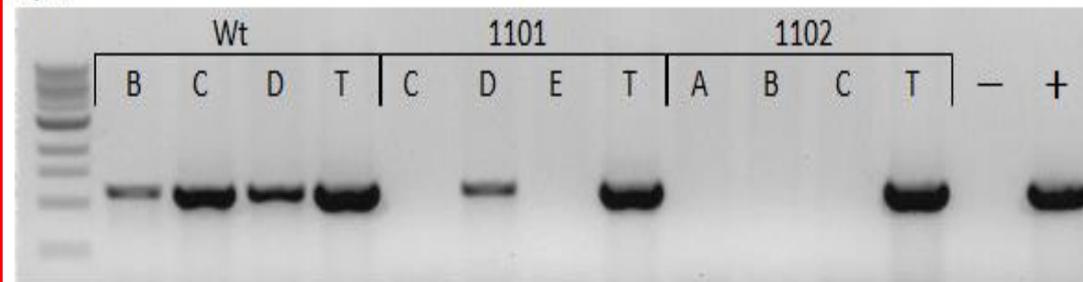
Keremane Manjunath, Chandrika Ramadugu, Greg Kund, John Trumble and Richard F Lee

- Host range studies using several members of Solanaceae with both graft and insect transmission of LPS revealed both resistant and susceptible varieties
- Nearly 100% of *Bactericera cockerelli* on tomato were found to carry the bacterium throughout the year , but colonies on pepper shows very low level of infection.
- Tomato plants can also be easily screened against antibiotics, chemicals and other agents under greenhouse conditions in a relatively short period

Day 15



Day 30



Mirkov- CLsol in a tobacco model system

- Wt is non transgenic
- Each cage has 3 tobacco plants of the same event and a Wt tomato plant (T) that is put in the cage 24 hours before the tobacco plants and infested with 20 hot psyllids.
- 1101 and 1102 are two different events for one of the defensins with no signal peptide.
- 1302 and 1303 are two different events co-expressing two defensins, and both have a signal peptide.
- PCR is at 15 and 30 days after tobacco plant are put in the cage.

Belknap & Munyaneza- Potato Model System

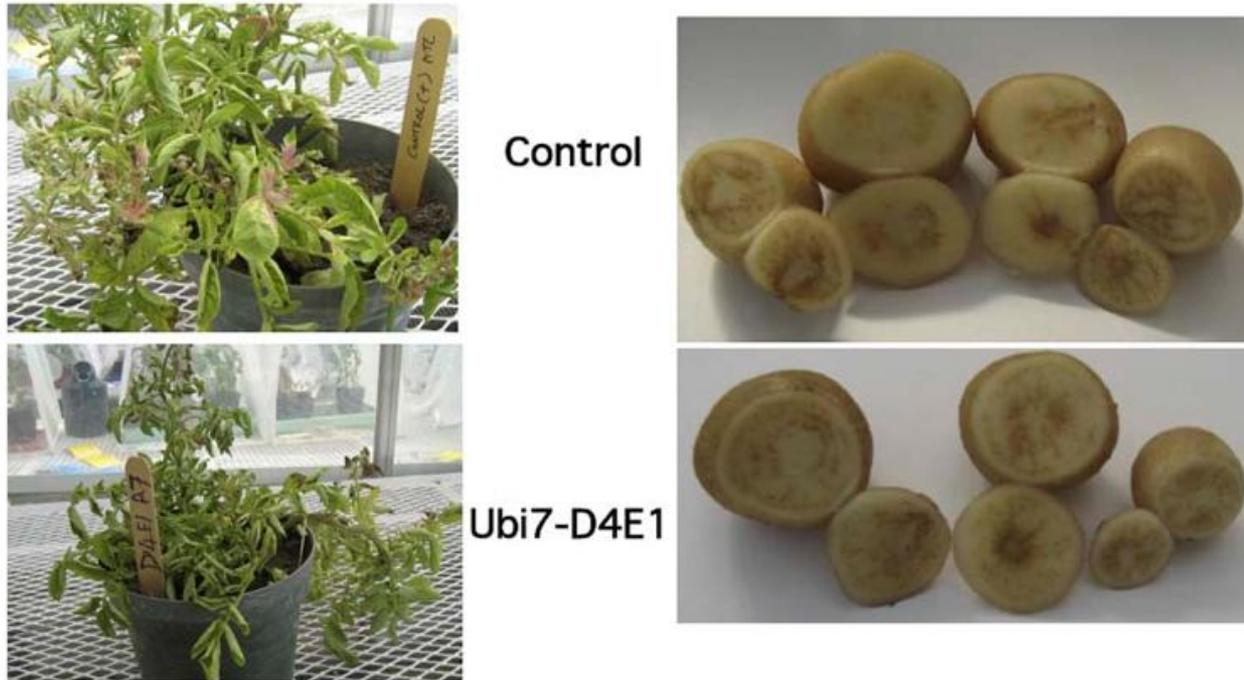
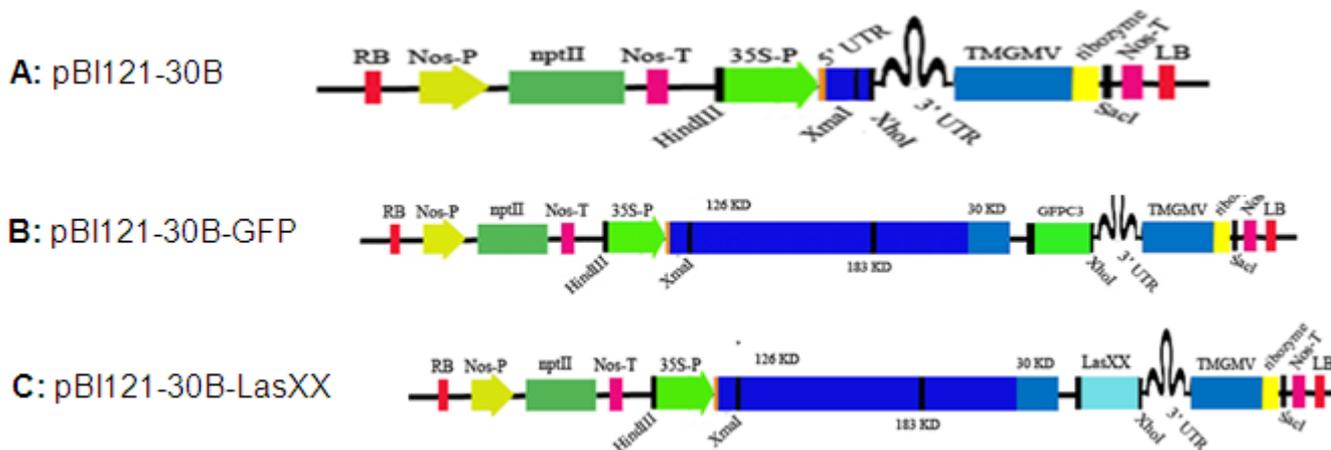


Figure 5. ZC symptoms in greenhouse infected potato (cv Atlantic) control and transgenic (Ubi7-D4E1) plants three weeks (plants) or two months (tubers) post-infection..

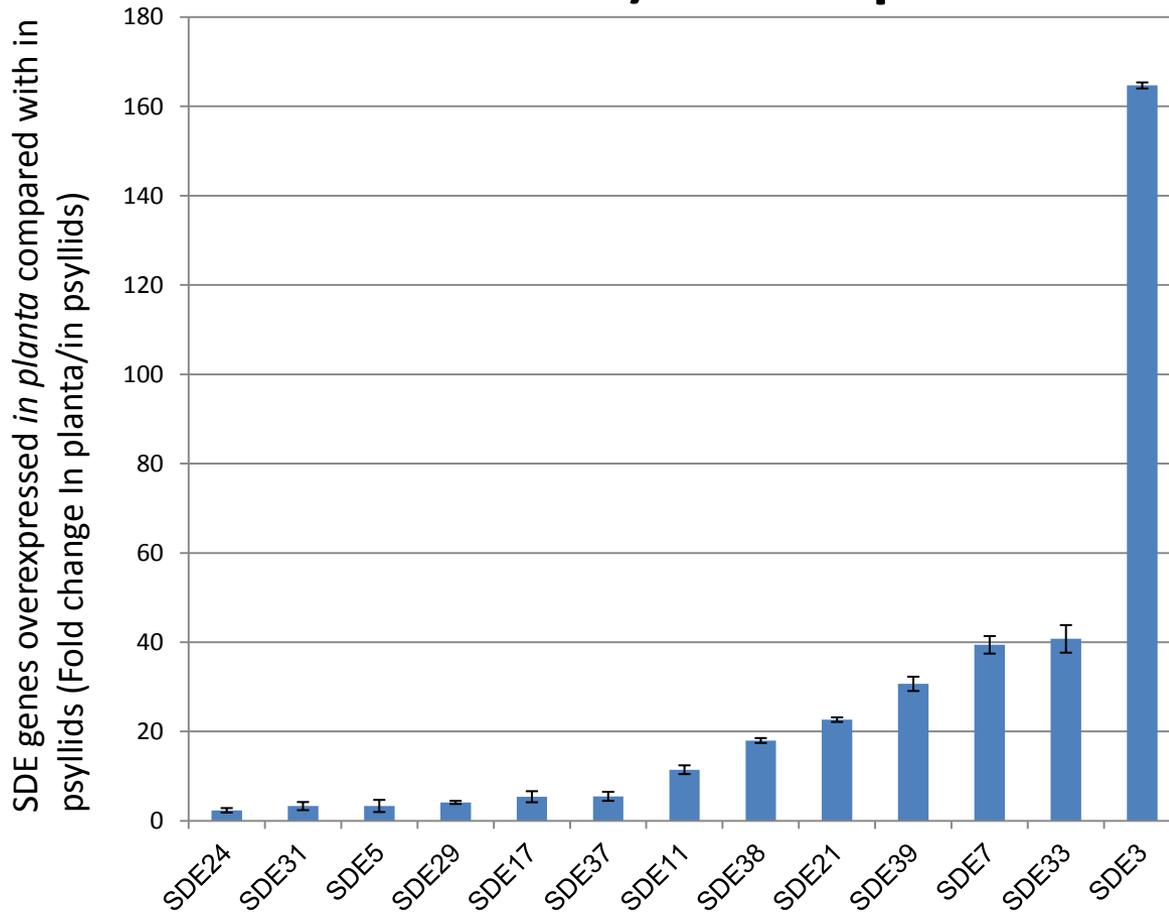
- Inserted transgene previously shown effective against *A. tumefaciens* and four citrus-derived GRPs (defensins), with no effect.
- Suspects level of challenge may be excessive, overcoming possible AMP benefit
- Repeating with psyllids at lower CLsol load

Using *N. benthamiana* in studying Sec dependent effectors (SDEs) from Clas- Nian Wang, William Dawson, Gene Albrigo, Kirsten Pelz- Stelinski:CREC-Lake Alfred

- Las is able to replicate and cause symptoms in tobacco (*N. tabacum* Xanthi) (Bové 2006)
- reasoned that the targets of the SDEs are likely to be conserved between citrus and tobacco
- transiently expressed the SDEs in *N. benthamiana* for preliminary screening using TMV expression vector
- model system speeds up the screening process to functionally characterize the SDEs of Las



Multiple SDE induced genes by Las are over-expressed *in planta* (*N. benthamiana*) compared within psyllids



Nian Wang,
William
Dawson,
Gene Albrigo,
Kirsten Pelz-
Stelinski
CREC-Lake
Alfred

Hong Lin & Chika Mwugo: Studying ZC as Primary Focus

- Four weeks after individual exposure to Lso + potato psyllid of 1-month old mini-tuber potato plants: Leaves and tubers +/-infection
- 2-DE and MALDI-TOF and LC-MS spectrometry analysis showed over 60 (leaves)and 50 (tubers)proteins differentially expressed
- Up-regulation of proteins involved in pathogen-response/defense, molecular chaperones, and energy production/general metabolism,
- ICP spectroscopy showed correlated with increase in K, Mn, Fe, and Cu in both potato leaves and tubers.

Hong Lin & Chika Mwugo: Studying ZC as Primary Focus

- Down-regulation of protease inhibitors in potato tubers and photosynthesis-related proteins in potato leaves, correlated with a reduction in Mg, Ca, and Zn concentrations in potato leaves.
- Novel insights into potential host-specific molecular and physiological responses to Lso infection
- Facilitates understanding the molecular process of host defense response and nutritional status of ZC plants.
- May also be relevant to HLB, some changes in gene expression similar and some not....

Arabidopsis/Psyllid Yellows as a Model System for Chemical Genomics of HLB

M. L. Roose and S. Patne, University of California, Riverside

- Arabidopsis can be infected with psyllid yellows (CLps) by potato psyllids raised on infected tomatoes
- Ct values in Arabidopsis shoots range from 24-28
- Tolerance variable, but no immune ecotypes found among 19 tested
- Infected plants have no visible symptoms, but seed germination is reduced
- Proposed to use in chemical genomics - test large numbers of chemicals to identify those that induce resistance/tolerance to vector or pathogen, then identify genes involved in this response.
- Chemical genomics screen is proving difficult because
 - plants grown in culture are not infected by psyllids
 - plants grown in soil require too much chemical

Arabidopsis as Model Systems – to Tomato.....

- only Arabidopsis in soil could be inoculated by infected psyllid, and only about 15% success rate
 - Transitioned to tomato as host
 - Using detached leaves of tomato (Ammar et al. system), get 80% infected in two weeks, with Ct<25

Oral Delivery of Double-Stranded RNAs and siRNAs Induces RNAi Effects in the Potato/Tomato Psyllid, *Bactericerca cockerelli*

Hada Wuriyangan, Cristina Rosa, Bryce W. Falk (PlosONE 2011)

- Used EST sequence information from *D. citri* to identify potential targets for RNA interference in *B. cockerelli*.
- Targeted ubiquitously expressed and gut-abundant mRNAs
- Applied double-stranded RNAs via injection and oral acquisition and siRNAs and were able to induce mortality in psyllids

Table 1. Mortality induced by injection of *BC-Actin* and GFP dsRNAs.

Injected dsRNA ¹	Total NO. of injected psyllids on day 0	No. and percentage of surviving individuals ³					
		Day 1 ²	Day 2	Day 3	Day 4	Day 5	Day 6
GFP	40	21 (100%)	19 (90%)	16 (76%)	16 (76%)	13 (62%)	12 (57%)
<i>BC-Actin</i>	40	24 (100%)	19 (79%)	10 (42%)	9 (38%)	2 (8%)	2 (8%)
GFP	40	26(100%)	26 (100%)	23 (88%)	22 (85%)	21 (81%)	11 (42%)
<i>BC-Actin</i>	40	22 (100%)	19 (86%)	10 (45%)	8 (36%)	4 (18%)	4 (18%)

1. Each individual was injected with 200 nL of 100 ng/μL dsRNA.

2. About half of the injected psyllids would die on day 1 after injection and this is most likely caused by the injection procedure.

3. The percentages of surviving individuals on day 2 - 6 were based on the NO. of surviving individuals on day 1 for each case.

Wuriyangan, Rosa, & Falk (PlosONE 2011)

- Knockdown of target mRNAs: oral acquisition primarily knockdown in the psyllid gut
- Concurrent with gene knockdown was the accumulation of target specific, 21 nucleotide siRNAs for an abundant mRNA for BC-Actin
- Results showed that RNAi can be a powerful tool for gene function studies in psyllids and may be tools for psyllid and plant disease control

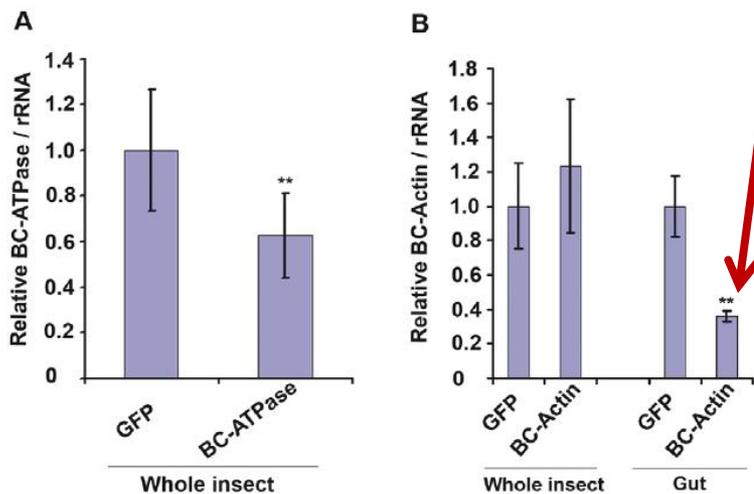


Figure 5. Knockdown of endogenous psyllid mRNAs by dsRNA feeding. The dsRNAs were

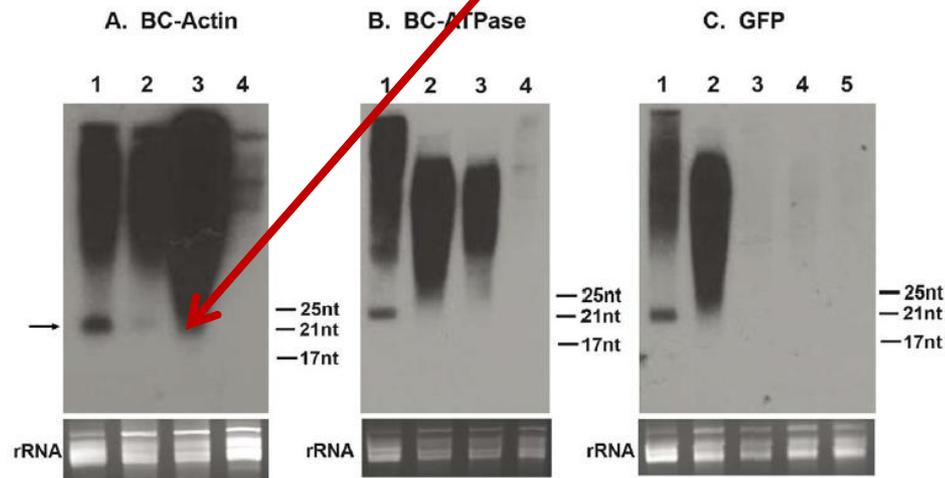
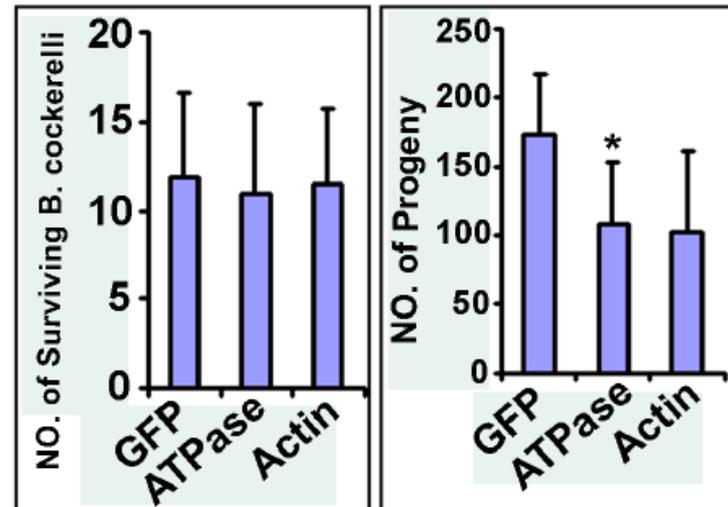


Figure 7. Northern blot detection of small RNA in *BC-Actin* dsRNA-fed psyllids. Teneral adult psyllids \

TMV Expression vector- Wurihanghan & Falk

- Expressed potato psyllid (BC, *B. cockerelli*) gene dsRNA in TMV, previously shown to suppress psyllid from exogenous dsRNA
- When nymphs were fed on tomatillo, tobacco or potato, leaf-disks expressing TMV-ATPase majority of trials showed reduced expression of ATPase
- Reduced psyllid fecundity resulted from TMV expression of BC-ATPase



Two Systems for Screening Chemicals to Therapeutically Suppress CLas- Duan lab

Periwinkle cutting

(Zhang et al, 2010 Phytopathology 100:239-245)

- Shorter incubation period (1-3 months)
- Easier to propagate the inoculum
- Requirement of special care, such as humidity and shade.
- Phytotoxicity needs to be tested against citrus again

Citrus graft-based

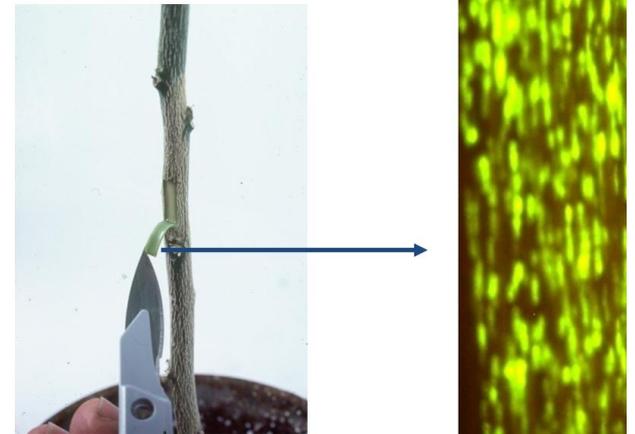
(Zhang et al., 2012 Phytopathology 102:567-574)

- Longer incubation time (3-6 month)
- Difficult to propagate large quantities of the inoculum
- Direct efficiency analysis of a treatment by testing both scion and root stock
- Direct assay on phytotoxicity and effectiveness in system of interest

CTV vector uses- Dawson lab

- Molecular tool for citrus biotechnology
- Test transgenes quickly before transformation
- Test transgenes that interfere with growth or development
- Test transgenes against pathogens
- Test transgenes for toxicity or repellency of insect vectors

CTV-
GFP



**CTV expressing
AMPs to control
Citrus Greening-
Dawson et al.**

**Have used to test
range of AMPs**

**Identified some
promising genes for
further study**

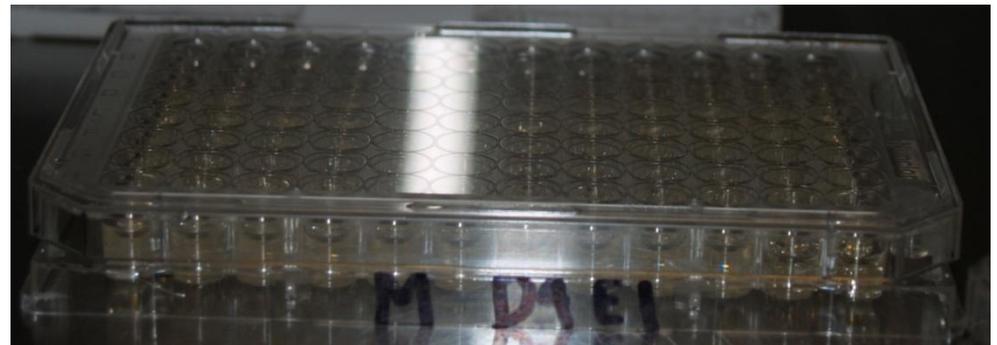
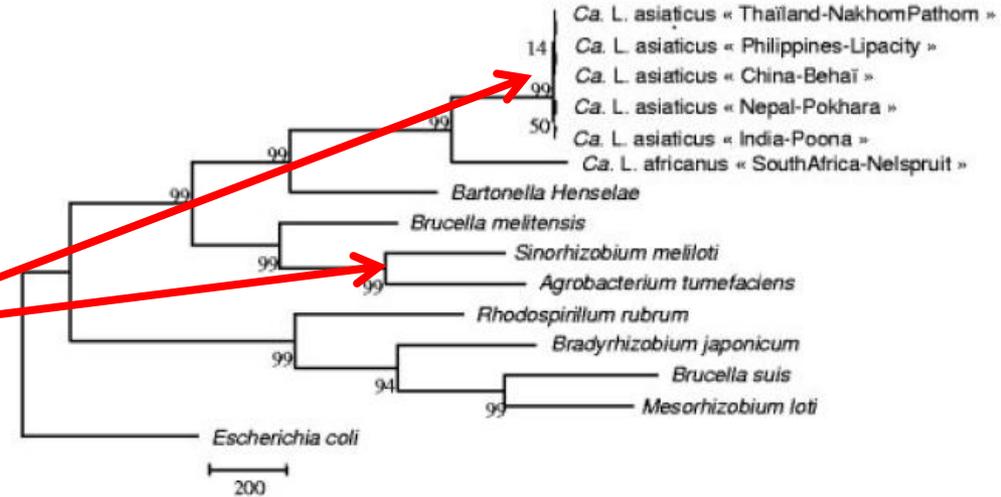


Use of ACP as a “Model” System

- Possibility of screening therapeutic materials against CLAs or specific step in pathosystem through artificial feeding to ACP, which would ultimately be implemented as transgenic product or applied exogenously in-planta
- Shatters indicates that artificial feeding of ACP on RNAi directed at blocking CLAs uptake shows significant differences from controls, but this appears to underestimate effectiveness achieved when delivered in-planta
- Reported that CLAs multiplies in the nymphs but not the adults (Inoue et al., 2009), so not useful for bacteriostatic materials
- Materials which may be degraded in the gut (perhaps scFvs or peptides?) may not be amenable to assessment in ACP for effectiveness in suppressing CLAs

In-Vitro AMP Screening- Stange et al.

- Since can't easily culture *Liberibacter*, used surrogates
- *Agrobacterium* and *Sinorhizobium* are related to *Liberibacter*, & easy to culture
- Also using *Xanthomonas citri*, causal agent of canker



	MIC (μ M)			Hemolytic Activity (%)
	At	Sm	Xcc	
AMP				
Tachyplesin I	0.3	0.3	0.3	3.0
SMAP-29	1	0.3	1	3.2
D4E1	1	0.3	1	3.6
D2A21	1	0.3	1	8.4
LL-37	1	1	1	5.1
Melittin	1	1	1	100.8
Cecropin A	3	3	10	1.1
Cecropin B	10	3	10	1.2
Indolicidin	10	3	3	2.0
Apidaecin IA	>30	1	>30	1.6
Drosocin	>30	3	>30	1.6
α -Purothionin	30	10	1	22.5
Pyrrhocoricin	>30	10	>30	1.9
Magainin I	>30	>30	>30	1.3
Magainin II	>30	>30	>30	1.5
Histatin-5	>30	>30	>30	1.8
Ib-4	>100	100	>100	
Cn-1	>100	>100	>100	
P4c	>100	>100	>100	

In-vitro assays: broad group of AMPs

- Quite repeatable across multiple runs
- Most were comparable in effectiveness across species, but some species x AMP interactions
- J. Jaynes used structure of most effective AMPs to derive new synthetics with potential for greater activity

Tests of Inoculativity as a “Model” System

- Ammar, Walter and Hall used an excised-leaf assay to speed up assessment of ACP transfer of CLAs into leaves from 3-12 months to 2-3 weeks
- Leaf stems were inserted into small tubes containing water and leaves were maintained in ventilated tubes with known infected ACP
- After 2 weeks of exposure to 5-10 ACP or 1 week followed by 1 week holding period an average of ~40% (0-62%) of leaves were CLas+ using LJ900 primers
- Variability reduced by larger numbers of leaves and ACP
- Relevant to just a few steps in the pathosystem, but could be useful in evaluating whether genotypes or treatments prevent feeding or transfer of CLas from ACP



CLas cultures as a “Model” System

- Vastly more efficient to screen therapeutic materials against CLas growing in-vitro
- Continue to be glimmers of light, but culturing remains intractable.....
- Perhaps the single most glaring “gap” in HLB research