Traditional vector control measures to reduce disease transmission

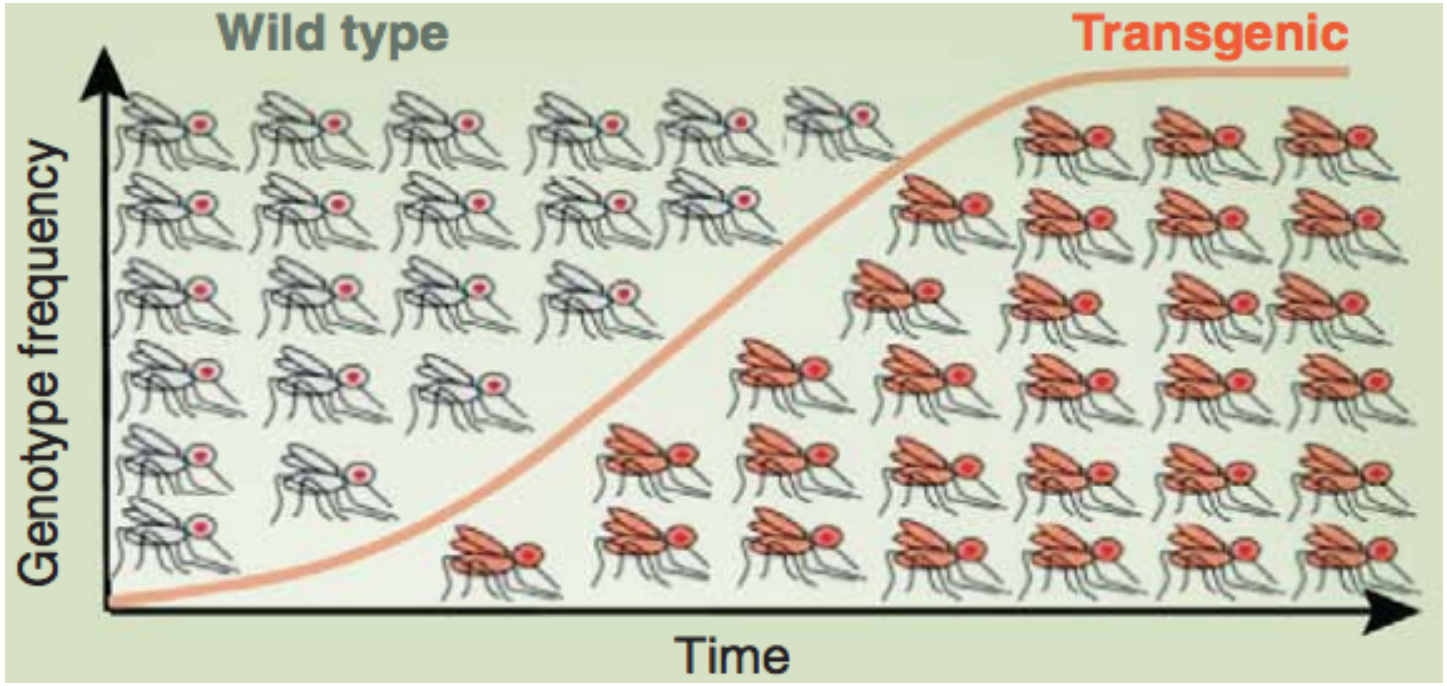
Insecticides

Biological control

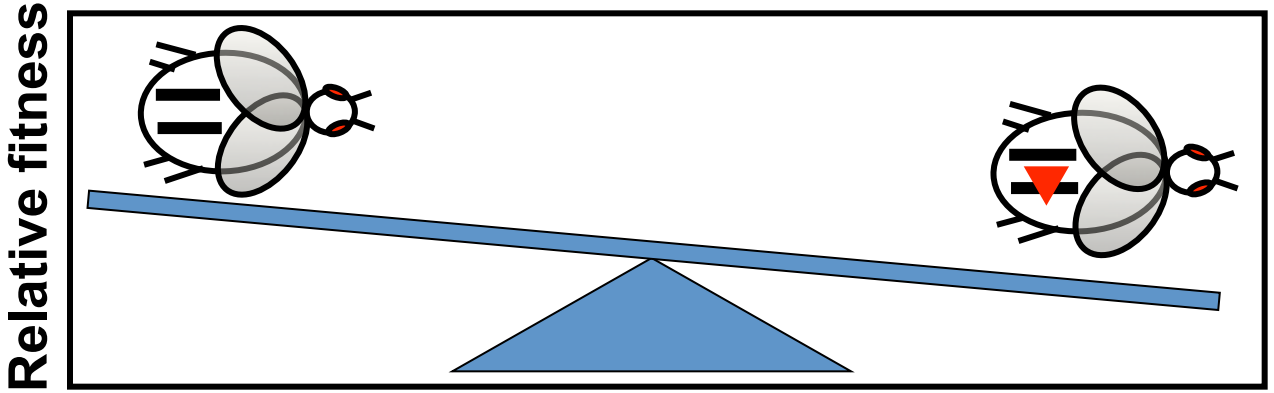
Traps

Trap crops Viruses

Population replacement to prevent remaining insects from transmitting disease

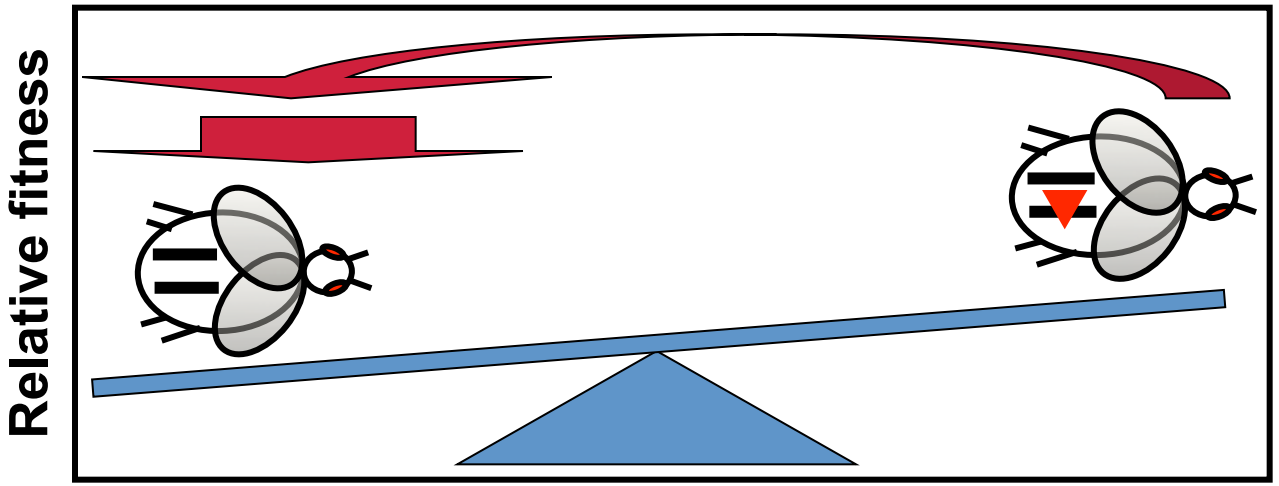


Problem: Genes that confer disease refractoriness are likely to result in a fitness cost to carriers

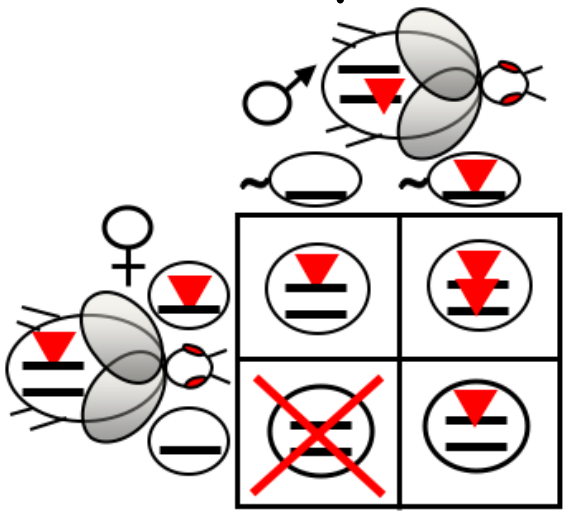


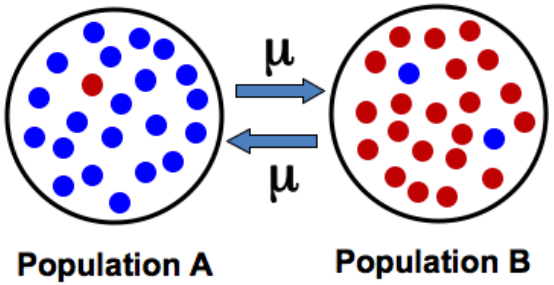
Solution: Increase the fitness cost associated with NOT carrying the gene of interest

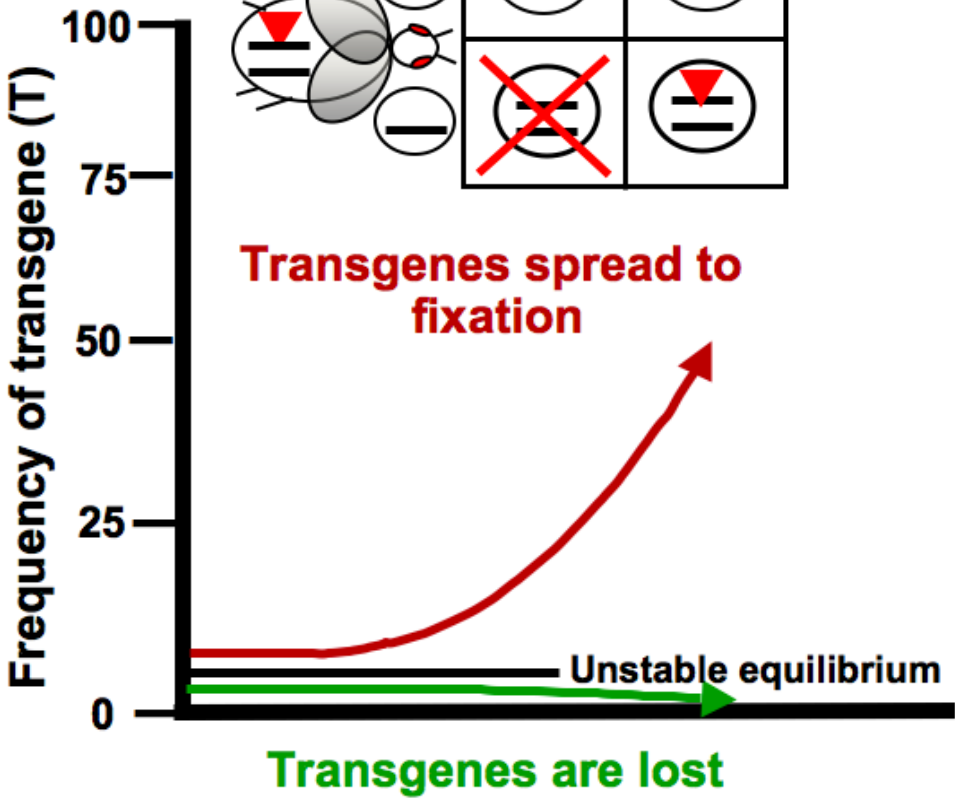
Gene drive

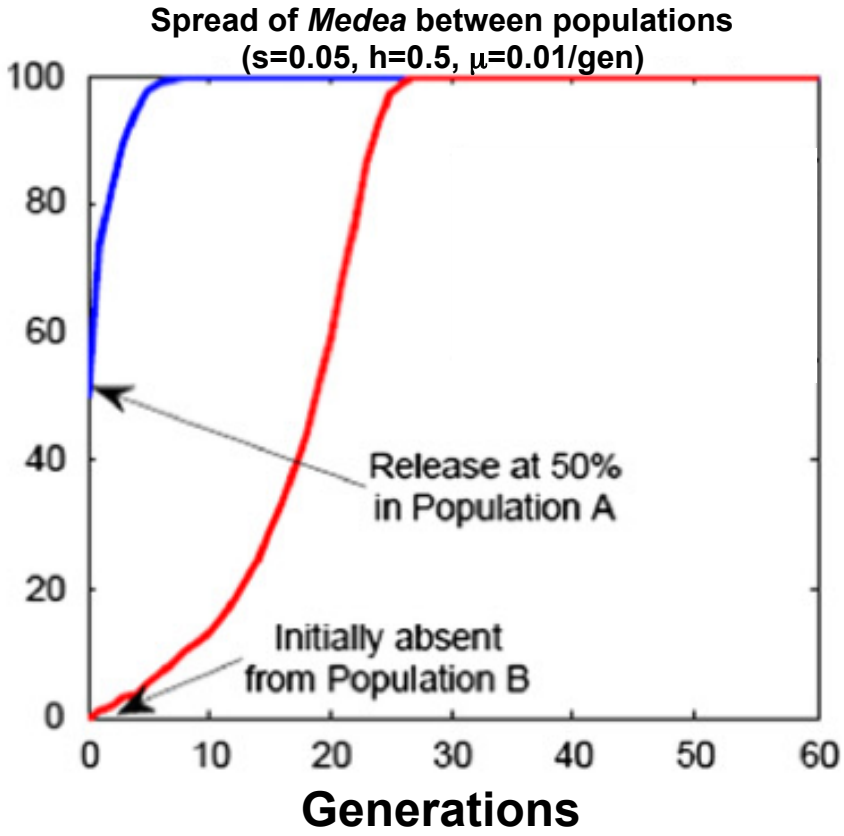


Medea, a low-threshold drive mechanism, is difficult to reverse, and spreads even when migration rates are low





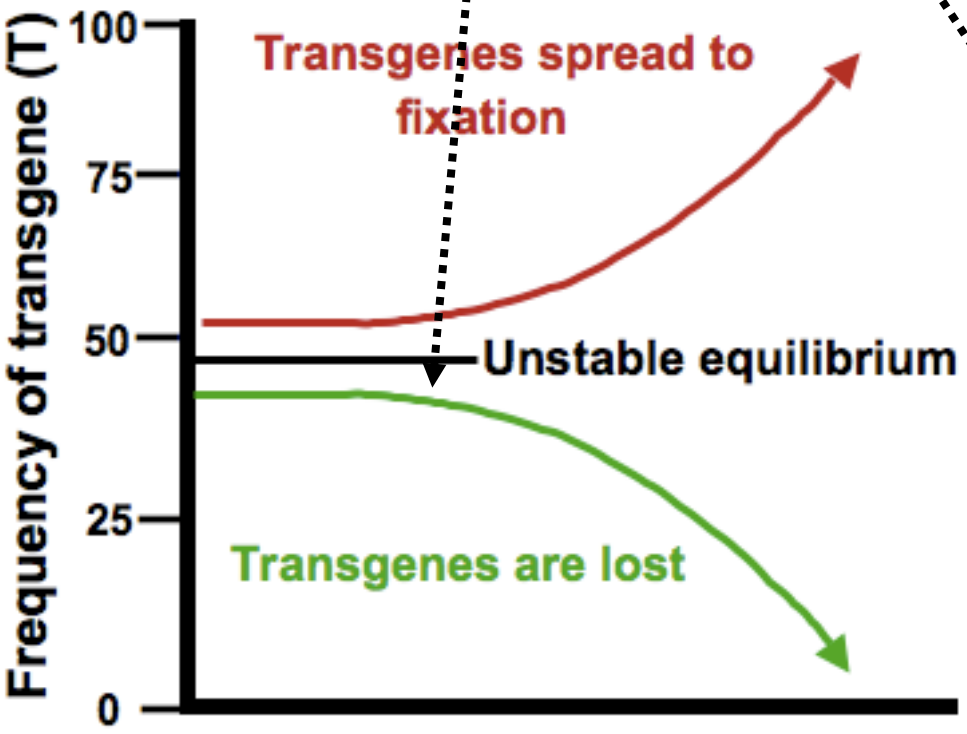


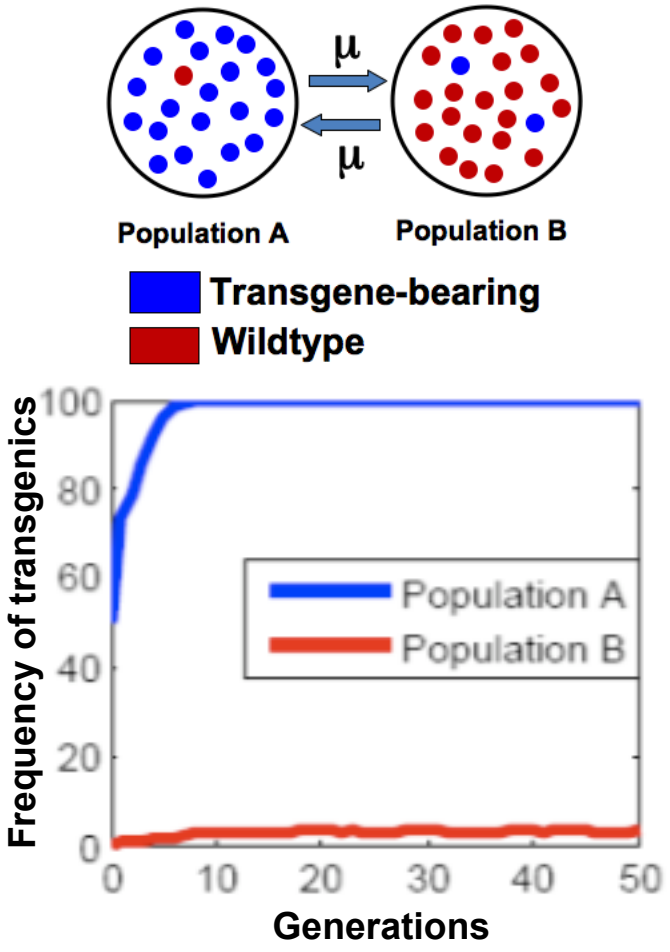


Ward et al. Evolution 65, 1149
Marshall and Hay, Journal of Theoretical Biol. 294, 153

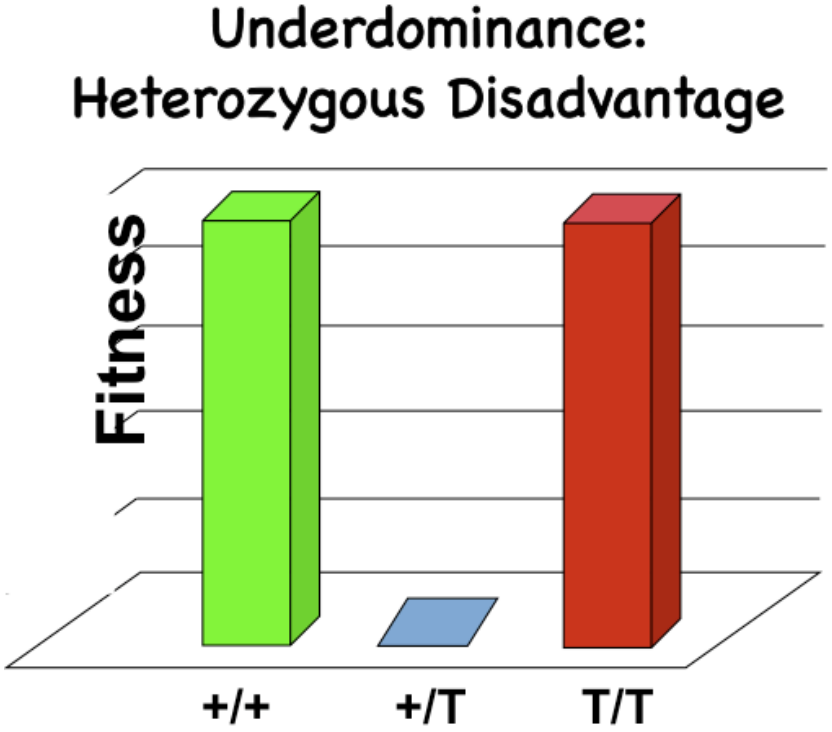
Many social and regulatory environments will require that spread only occur locally, and that it be reversible

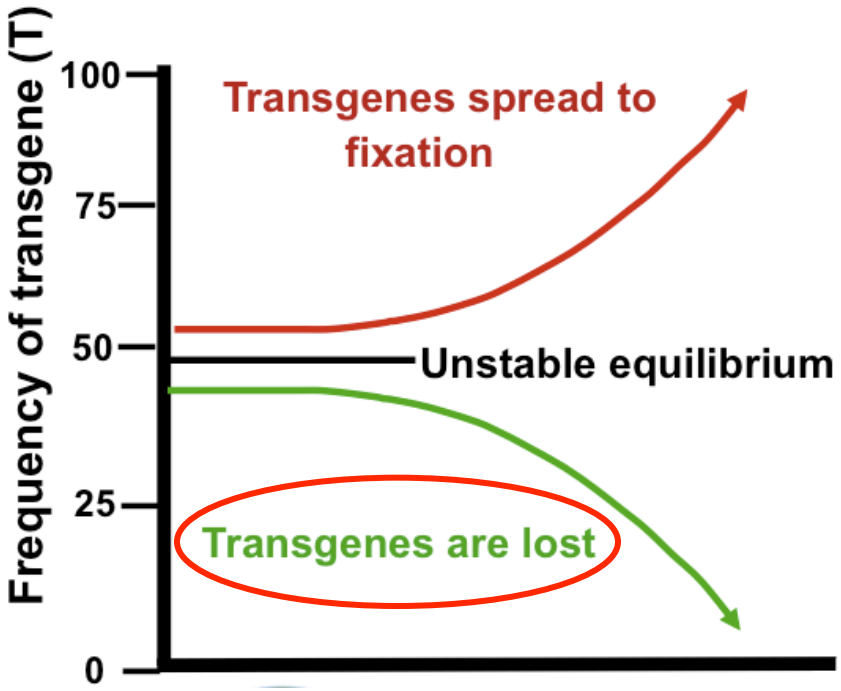
High threshold-dependent gene drive mechanisms bring about reversible and local population replacement



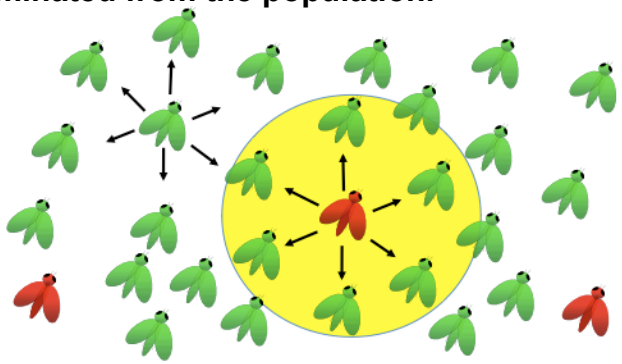


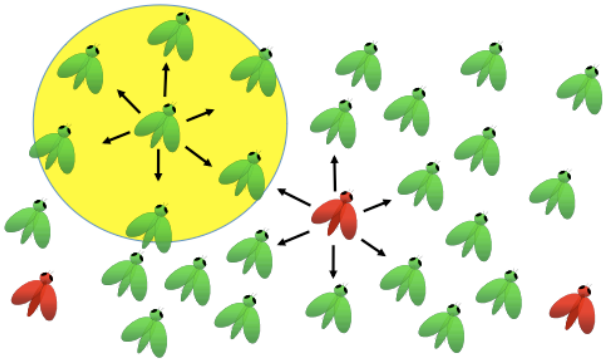
Underdominant systems show threshold-dependent, bi-stable behavior.





When wildtypes are common they mate mostly with each other, producing viable progeny carrying no transgenes.. Transgenics (T/T) mate mostly with wildtype, resulting in frequent loss of transgene-bearing chromosomes, and infrequent loss of wildtupe, non-transgene-bearing chromosomes, in unfit +/T heterozygotes. Transgenics are eliminated from the population.





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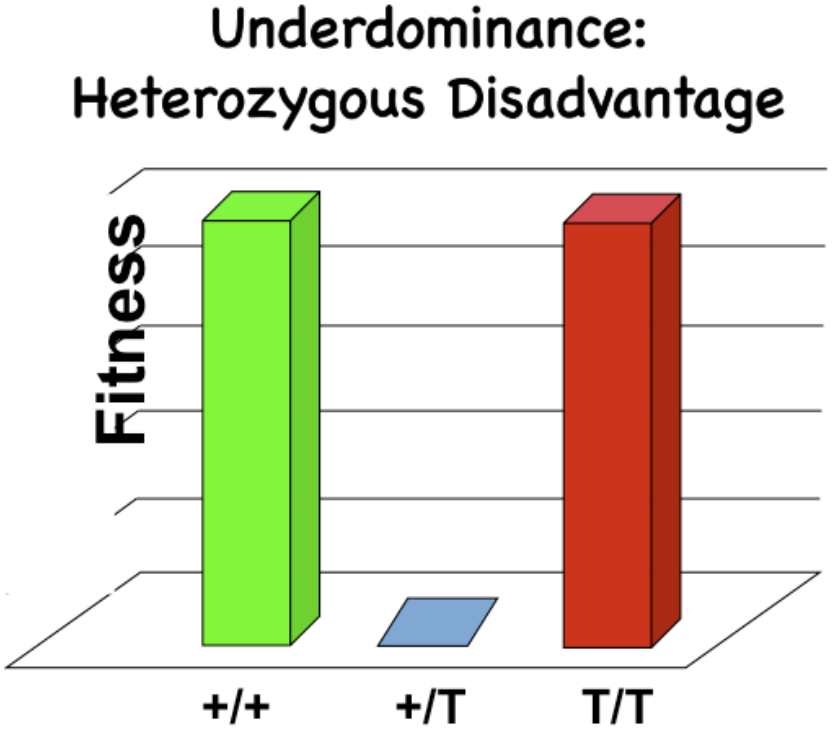
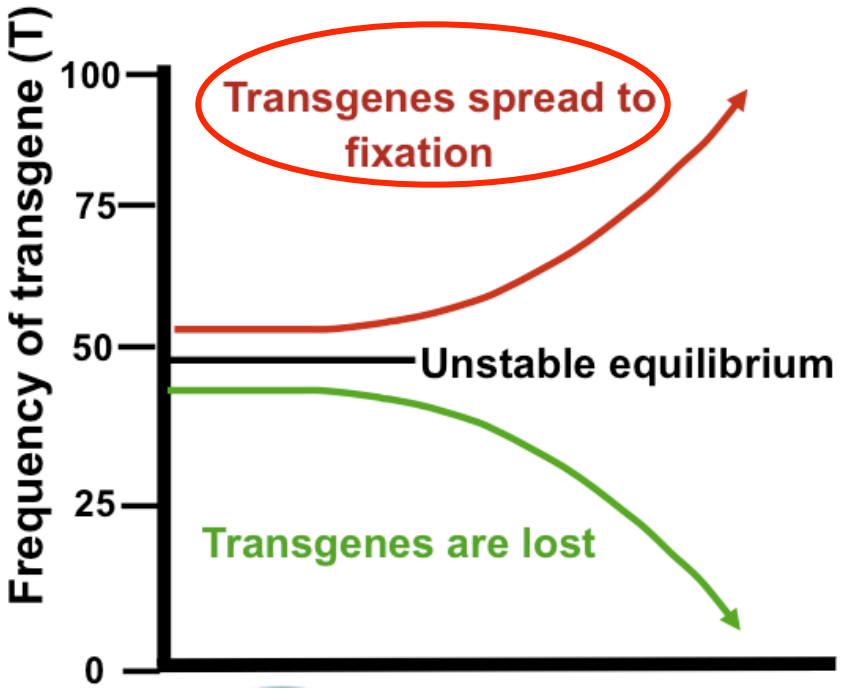
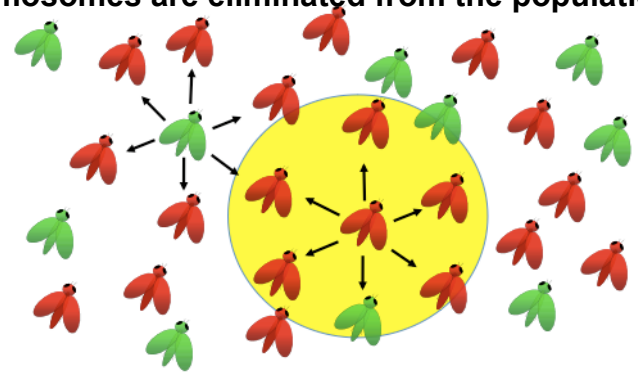
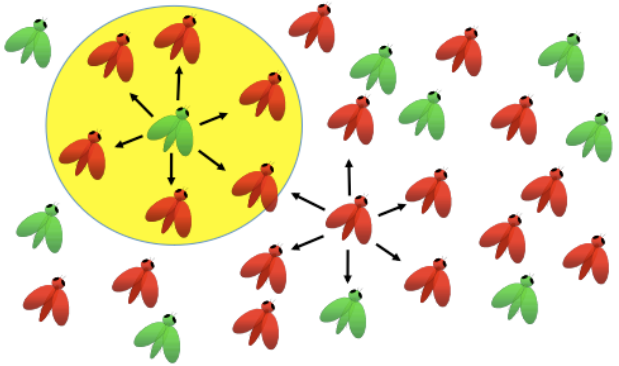


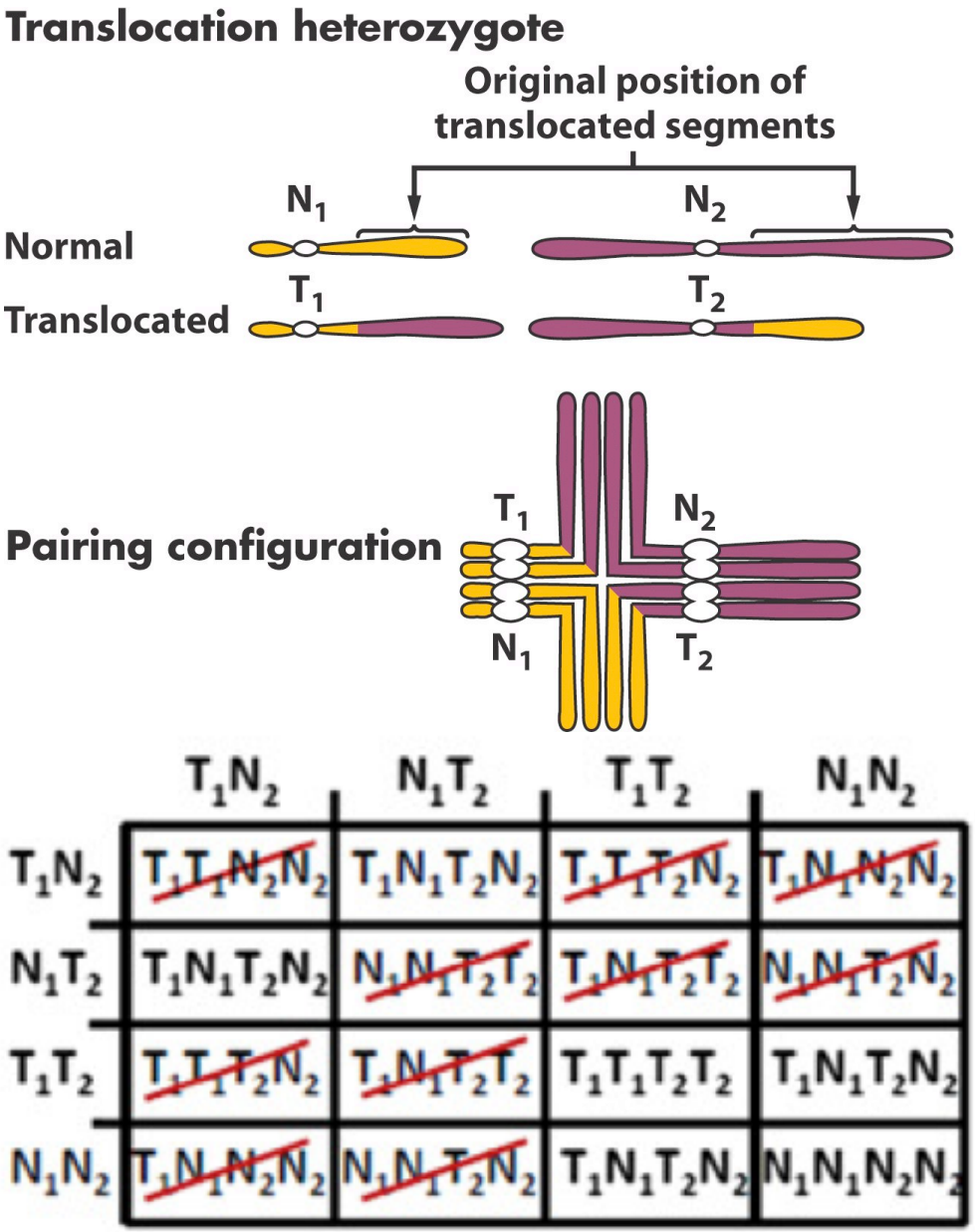


When transgenics (T/T) are common they mate mostly with each other, producing viable progeny carrying transgene-bearing chromosomes. Wildtypes (+/+) mate mostly with transgenics, resulting in frequent loss of wildtype chromosomes, and infrequent loss of transgene-bearing chromosomes, in unfit +/T heterozygotes. Wildtype chromosomes are eliminated from the population.

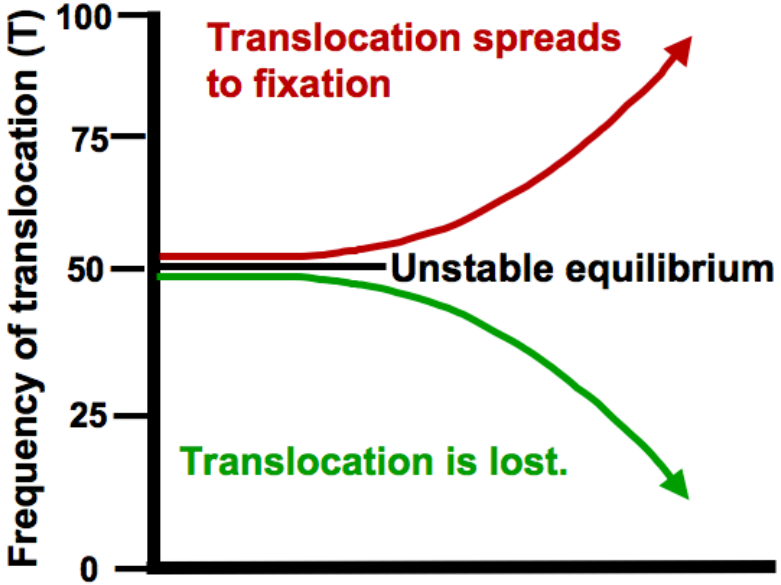


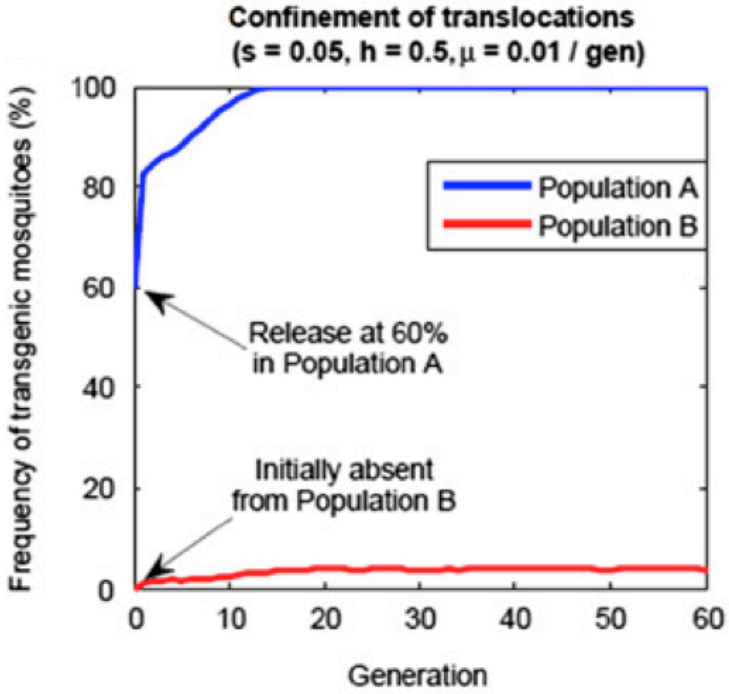


Reciprocal translocations constitute a very robust gene drive mechanism for reversible and local population replacement



	T_1N_2	N_1T_2	T_1T_2	N_1N_2
T_1N_2	~~$T_1T_1N_2N_2$~~	$T_1N_1T_2N_2$	~~$T_1T_1T_2N_2$~~	~~$T_1N_1N_2N_2$~~
N_1T_2	$T_1N_1T_2N_2$	~~$N_1N_1T_2T_2$~~	~~$T_1N_1T_2T_2$~~	~~$N_1N_1T_2N_2$~~
T_1T_2	~~$T_1T_1T_2N_2$~~	~~$T_1N_1T_2T_2$~~	$T_1T_1T_2T_2$	$T_1N_1T_2N_2$
N_1N_2	~~$T_1N_1N_2N_2$~~	~~$N_1N_1T_2N_2$~~	$T_1N_1T_2N_2$	$N_1N_1N_2N_2$





Useful characteristics of engineered translocations

1. Translocations are "natural", present in populations of all organisms

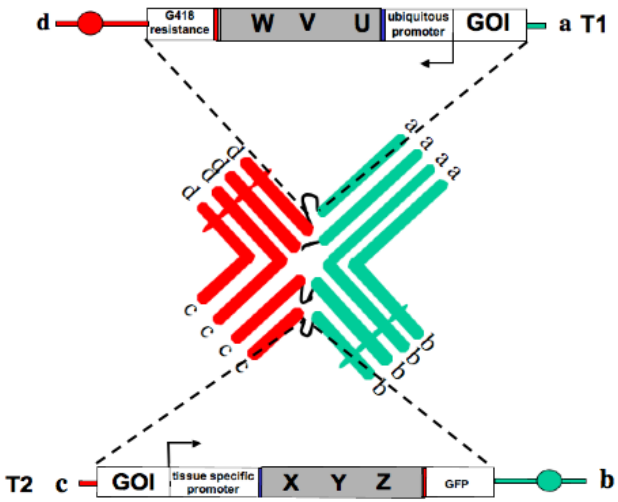
2. Translocations last (essentially) forever, and the GOI cannot recombine away when located at the breakpoint.

3. Translocation homozygotes carry two copies of each construct/chromosome, for a total of four GOIs

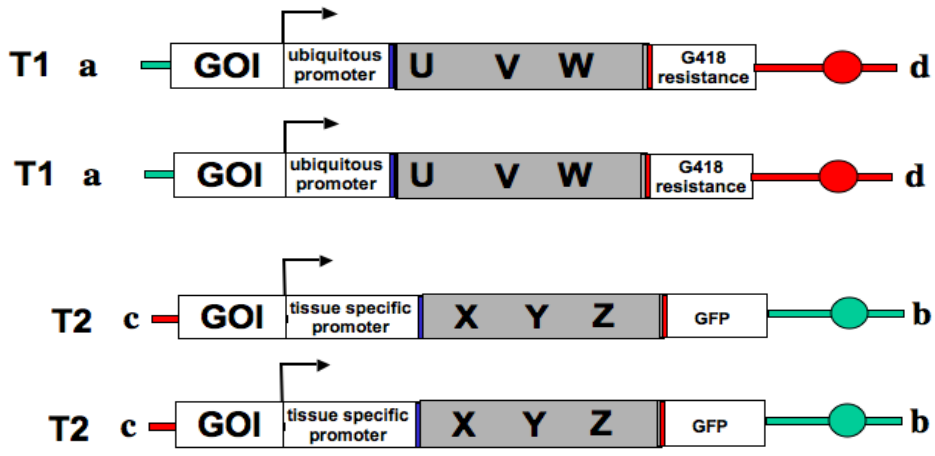
4. Local gene drive, and reversible through dilution

5. Can be created with limited knowledge about organism/ genome

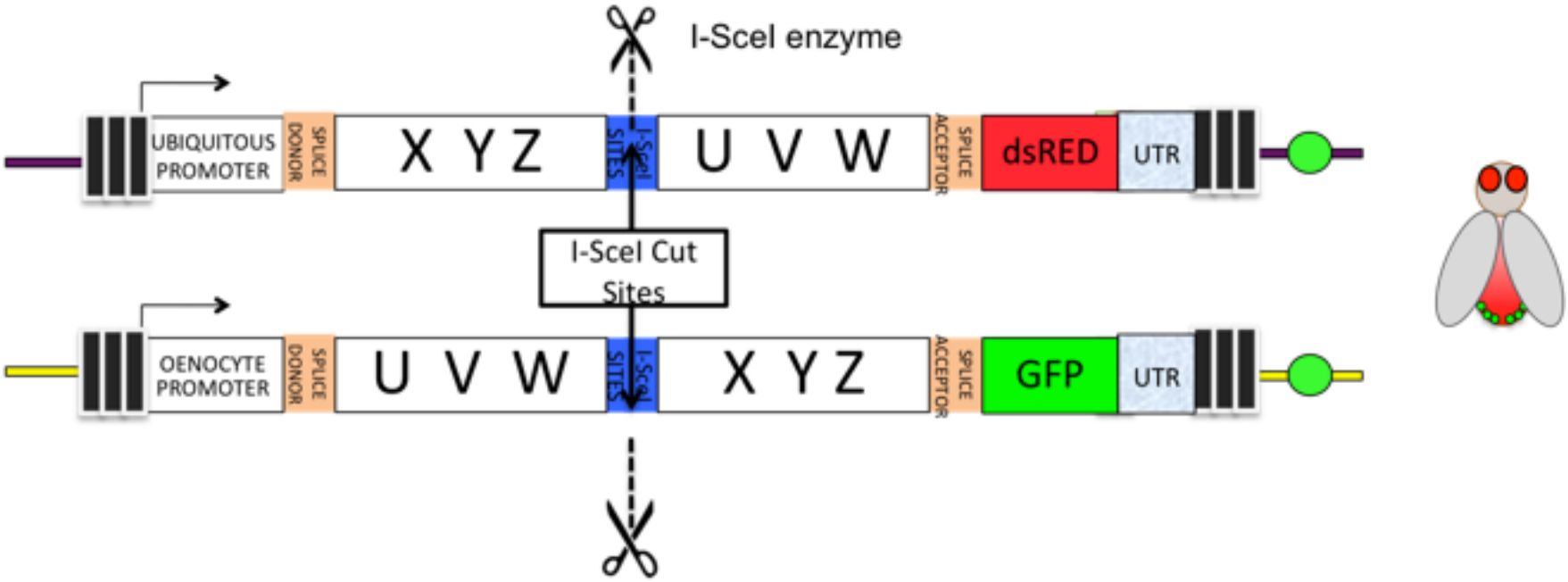
heterozygote



homozygote



Building a translocation: 1



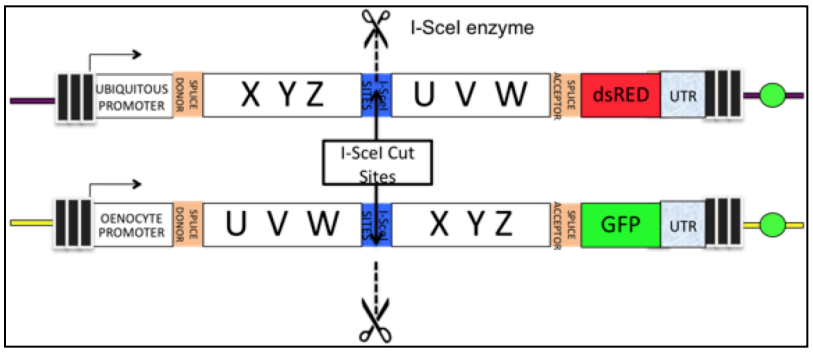
Generate stocks of each transgene-bearing chromosome.

Cross these to each other, and a third stock carrying heat shock driven I-Sce.

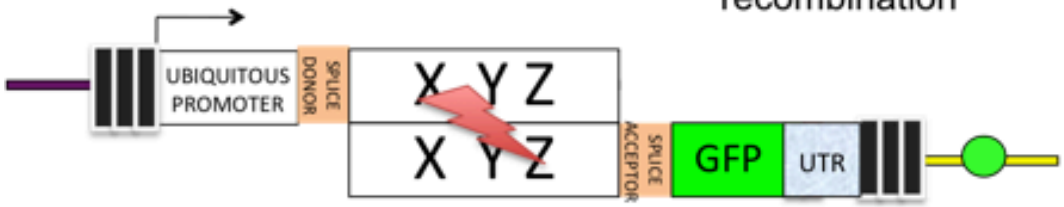
Heat shock progeny multiple times.

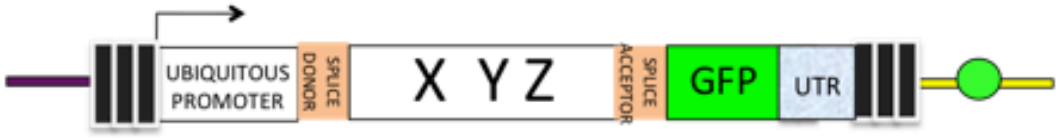
Outcross and look for recombinant chromosome-bearing progeny.

Building a translocation: 2

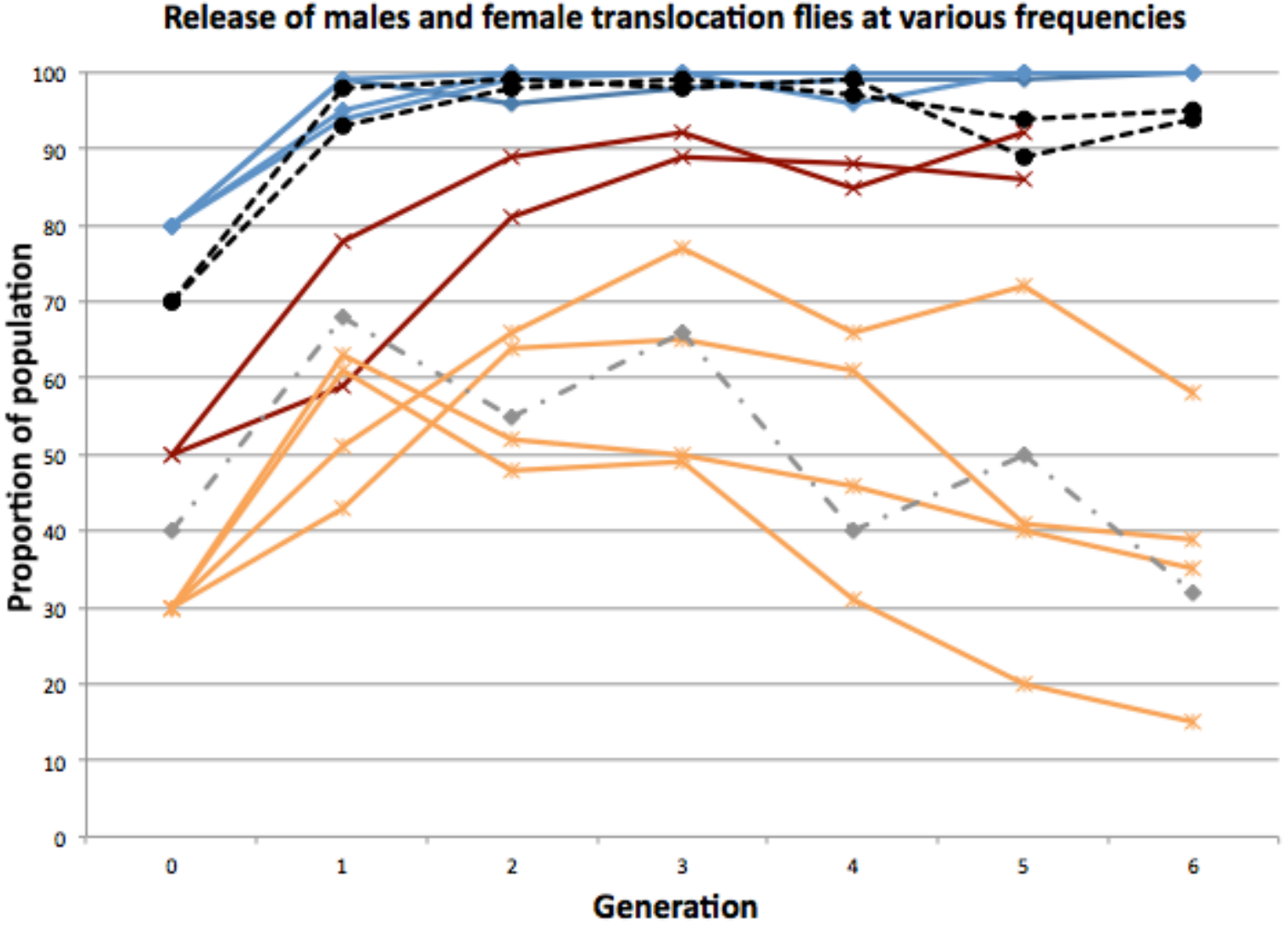


Repair via homologous recombination

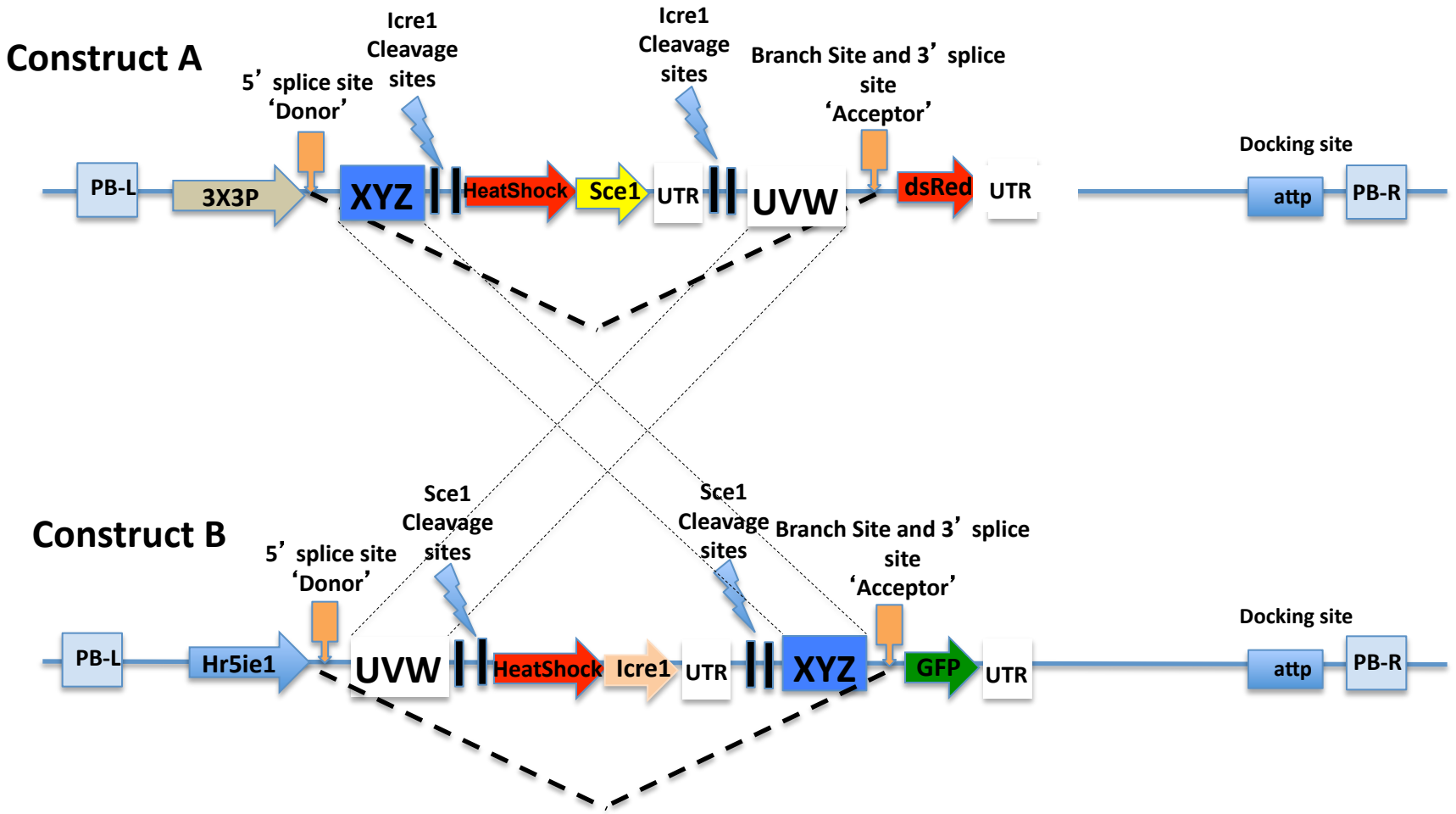




Translocation Drive

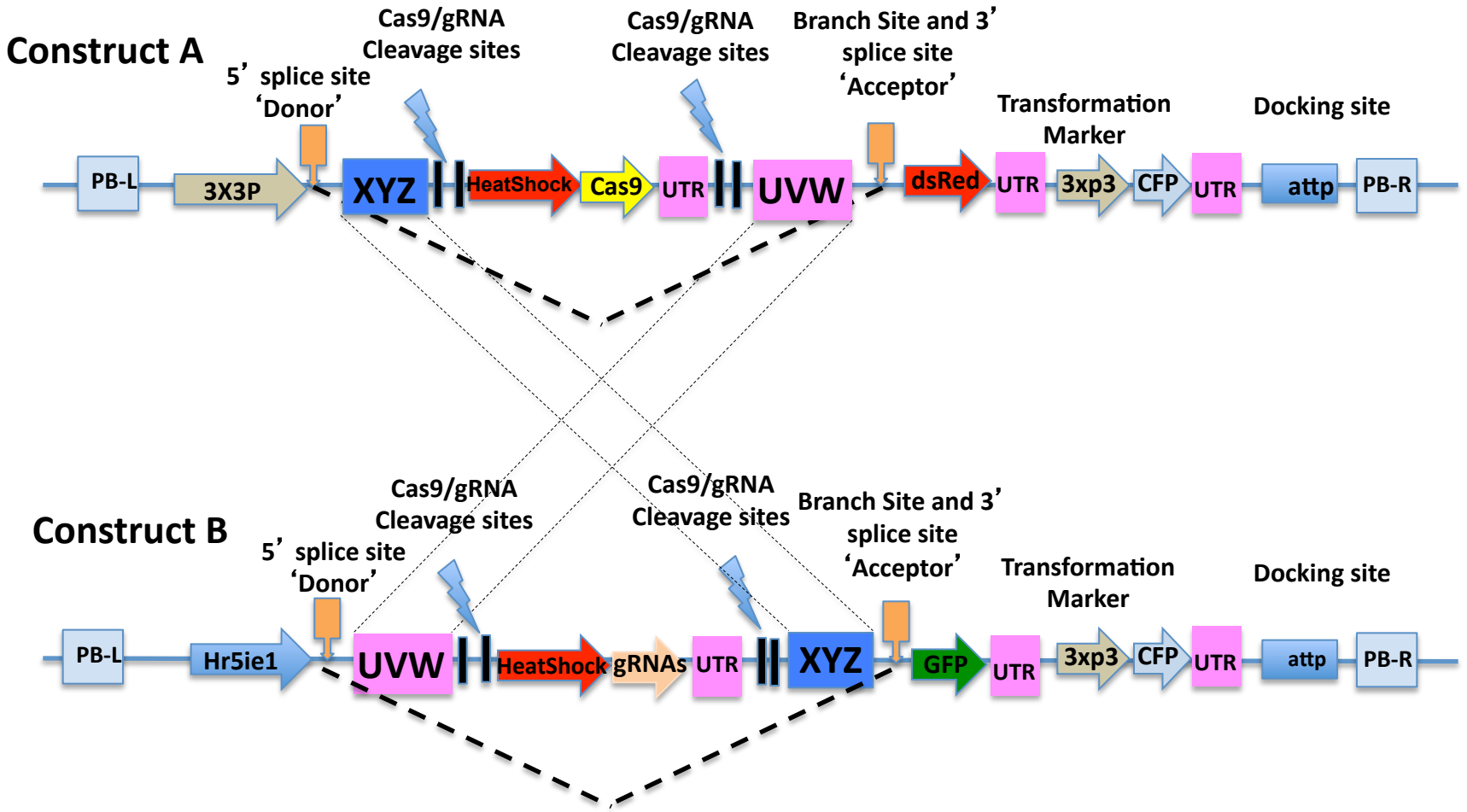


Generating a translocation through a simple cross between two stocks



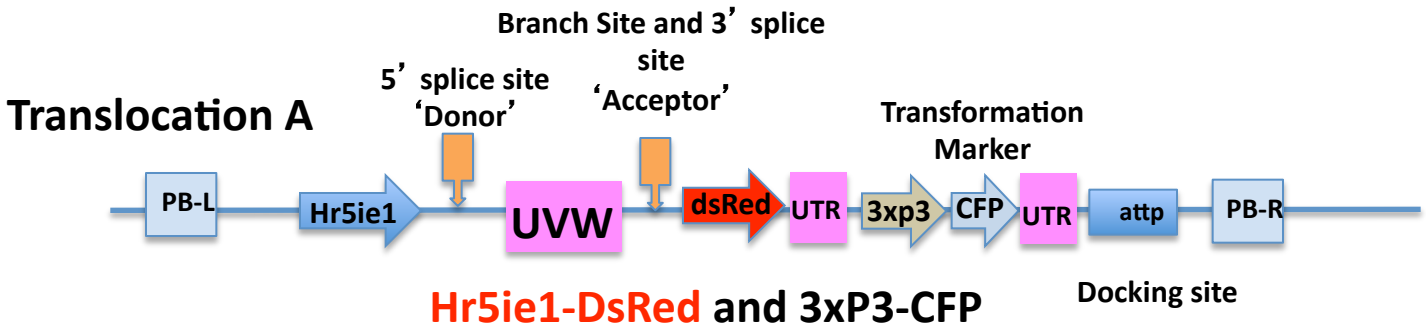
- Randomly integrate using piggybac to generate insertions on different chromosomes.
- Cross together stocks and heat shock to induce breaks.
- Screen progeny for translocation chromosomes. Test fitness.
- These plasmids/component genes should work in many species

Generating a translocation through a simple cross between two stocks

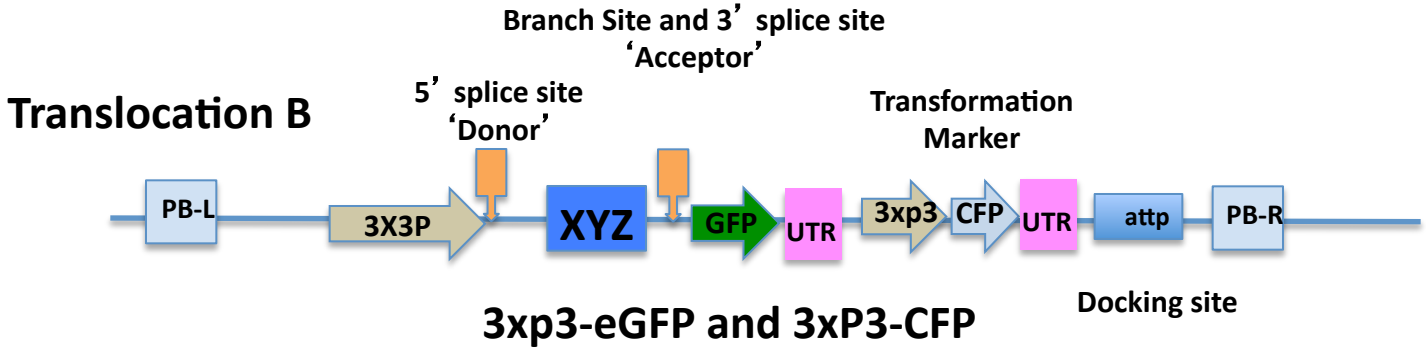


- Randomly integrate using piggybac to generate insertions on different chromosomes.
- Cross together stocks and heat shock to induce breaks.
- Screen progeny for translocation chromosomes. Test fitness.
- These plasmids/component genes should work in many species

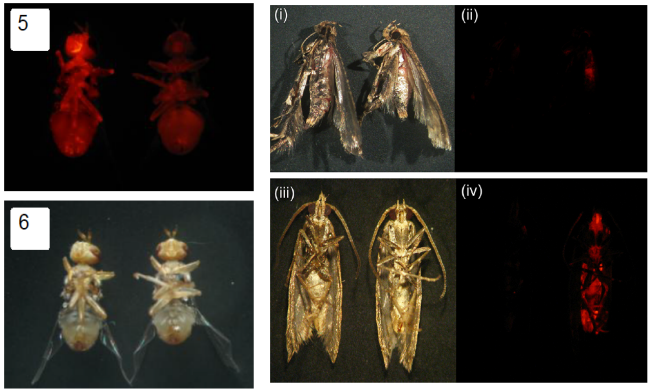
Translocation chromosomes generated through recombination



Hr5ie1-DsRed and 3xP3-CFP



3xp3-eGFP and 3xP3-CFP

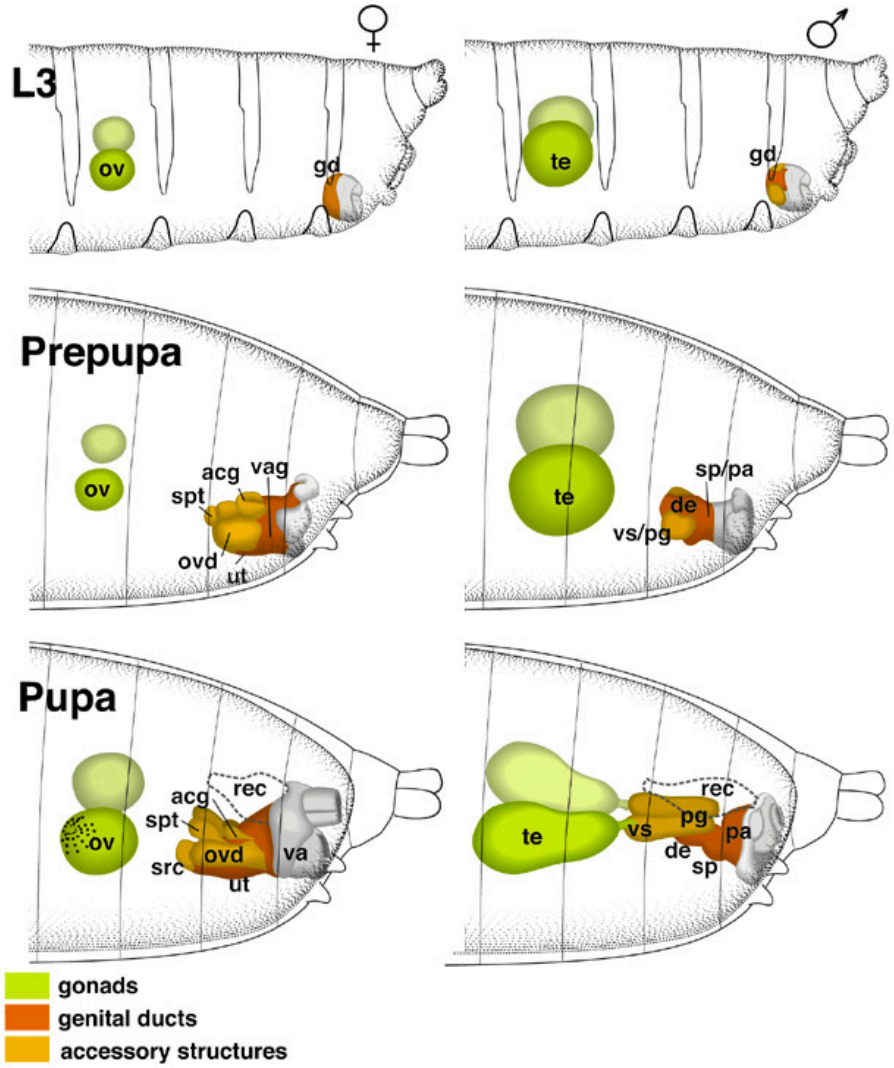


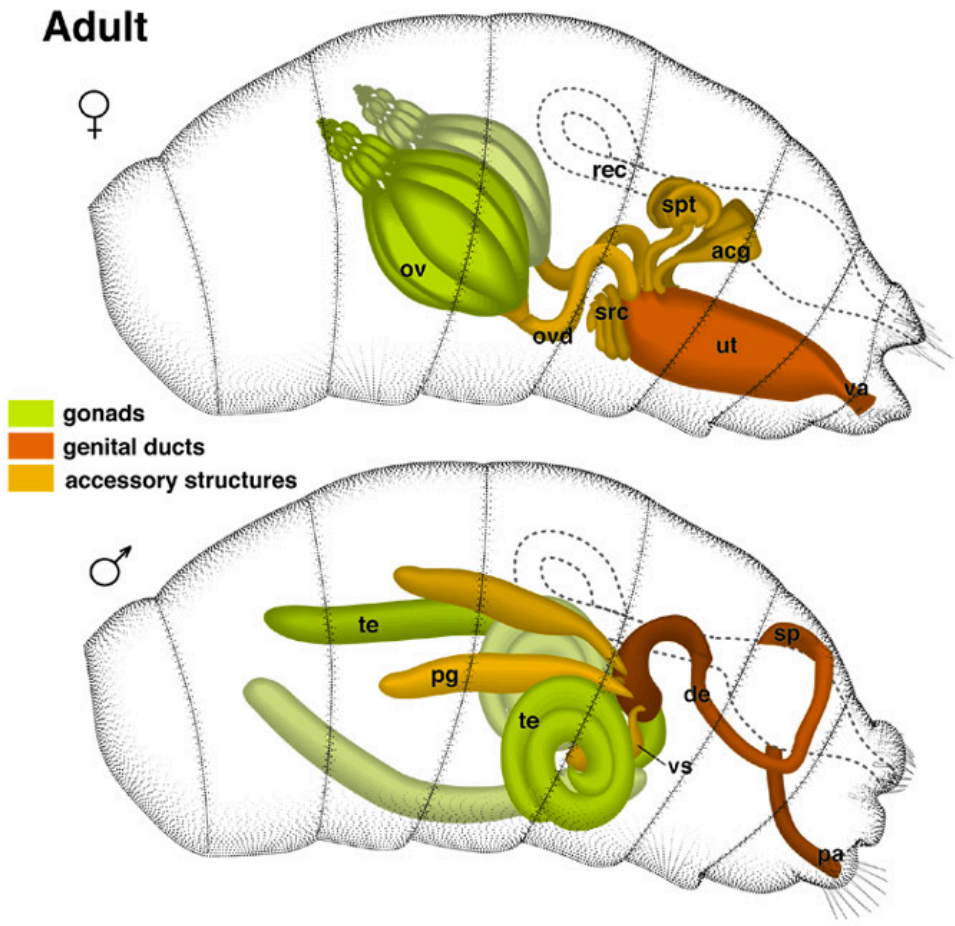
Hr5ie1-dsRed can be detected in the field

Oxitec

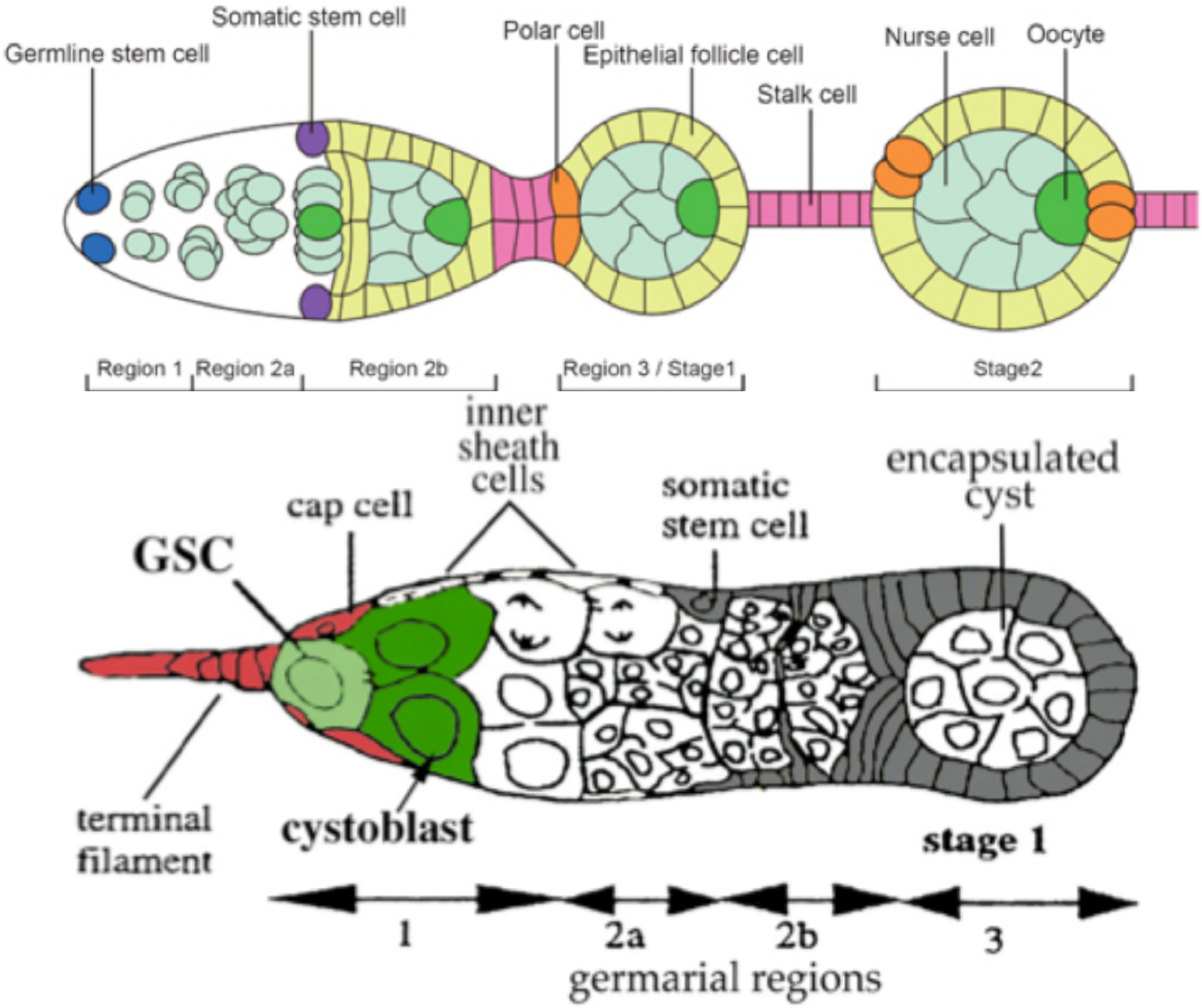
Olive Fly, Pink Bollworm, Diamondback moth, mosquito

Transgenesis through the adult; a fundamental difficulty in gaining access to the germline

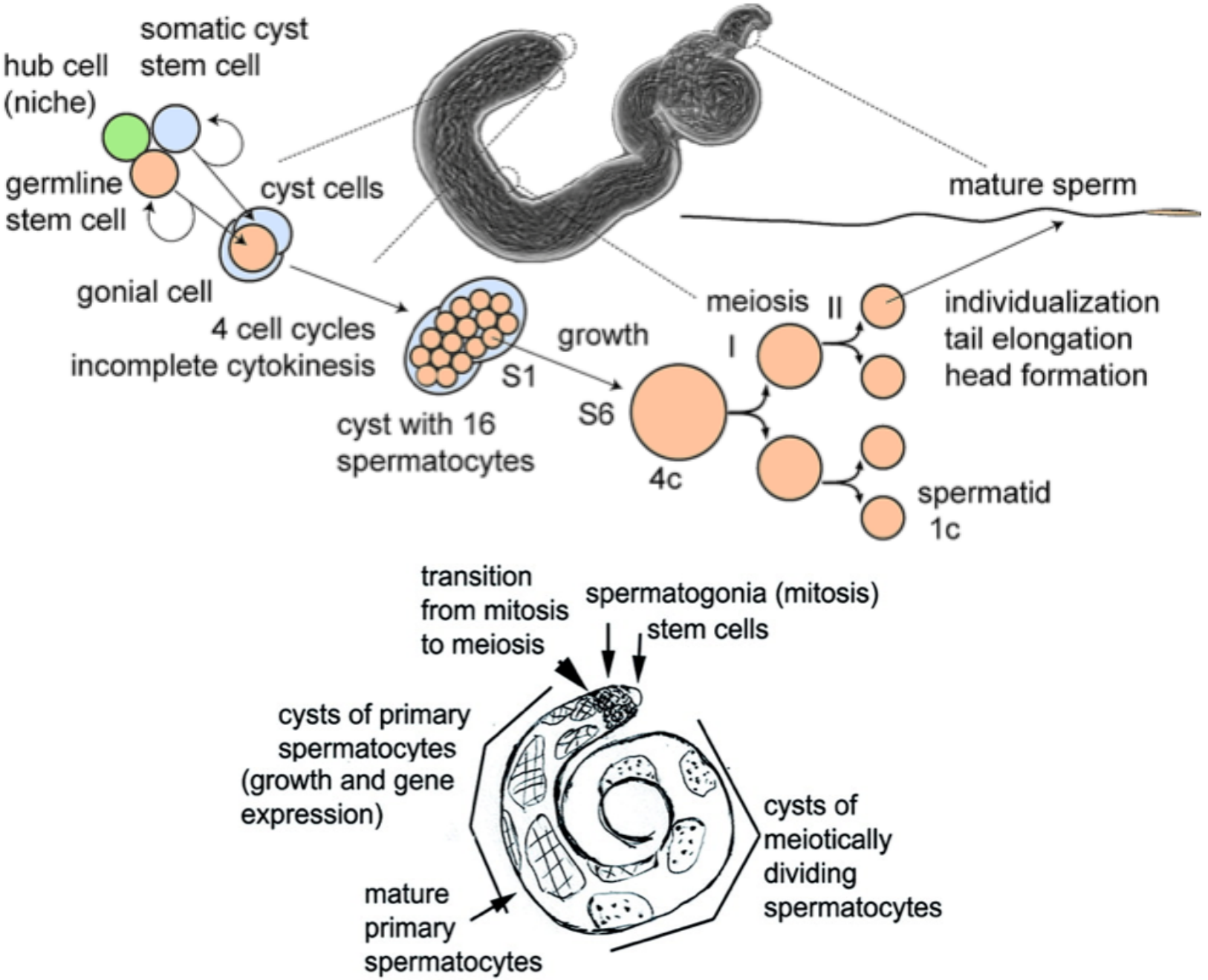




Ovary


Somatic stem cell
Germline stem cell
Polar cell
Epithelial follicle cell
Nurse cell
Oocyte
Stalk cell
Region 1
Region 2a
Region 2b
Region 3 / Stage1
Stage2
inner sheath cells
cap cell
somatic stem cell
encapsulated cyst
GSC
terminal filament
cystoblast
stage 1
1
2a
2b
3
germarial regions

Testis


somatic cyst
stem cell
hub cell
(niche)
germline
stem cell
cyst cells
gonial cell
4 cell cycles
incomplete cytokinesis
S1
cyst with 16
spermatocytes
growth
S6
4c
meiosis
I
II
individualization
tail elongation
head formation
mature sperm
spermatid
1c
transition
from mitosis
to meiosis
spermatogonia (mitosis)
stem cells
cysts of primary
spermatocytes
(growth and gene
expression)
mature
primary
spermatocytes
cysts of
meiotically
dividing
spermatocytes

Transgenesis

Reporter gene

Gfp, tdTomato

Promoter driving reporter

Baculovirus Hr5ie1

Ubiquitin

3x3p

Promoter driving transposase

Baculovirus Hr5ie1

Ubiquitin

Ribosomal protein

Delivery method

PEI

Jet prime PEI

Ca+, cell penetrating peptide nanoparticles

Pluronics

2 Baculovirus particles

1 with transposase

1 with transposon


Omar Akbari
Tobin Ivy
Marlene Biller
Anna Buchman

Small RNA deep sequencing and transcriptome profiles analysis to identify viruses in world populations of the Asian citrus psyllid, *Diaphorina citri*

Shahideh Nouri, Nida Salem, Thao Nguyen, Donald Coyle, Bryce W. Falk

Non-Plant based RNAi delivery

Can we achieve specific, systemic RNAi effects directly in hemipteran vectors without using plants to deliver the interfering RNAs?

Maybe we can use insect-infecting viruses

But where do we find them?

- Viruses are the most abundant microbes on the planet and many viruses are not pathogens and thus remain to be discovered.
- Next generation sequencing technology and bioinformatics tools offer powerful technique to discover novel viruses.
- If viruses can be identified, recovered and their genomes cloned as cDNAs to generate infectious viruses, then they can assessed for biological effects.

Our Rationale

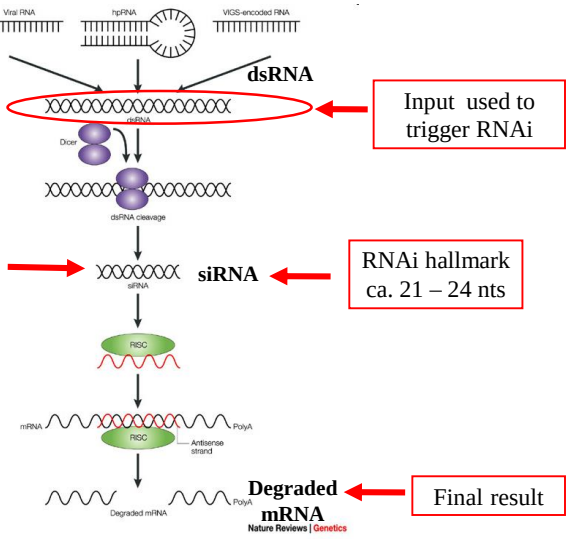
Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs

Qingfa Wu[a], Yingjun Luo[a], Rui Lu[a], Nelson Lau[b], Eric C. Lai[c], Wan-Xiang Li[a], and Shou-Wei Ding[a,1]

[a]Department of Plant Pathology and Microbiology, Institute for Integrative Genome Biology, University of California, Riverside, CA 92521; [b]Department of Biology, Brandeis University, Waltham, MA 02454; and [c]Department of Developmental Biology, Sloan–Kettering Institute, New York, NY 10065

http://www.acgov.org/cda/awm/agprograms/pestexclusion/sharpshooter.htm

Clone and sequence siRNA cDNAs, subtract the host sequences and analyze the remaining sequences for viruses.



JVI
Journals.ASM.org

Discovery and Evolution of Bunyavirids in Arctic Phantom Midges and Ancient Bunyavirid-Like Sequences in Insect Genomes

Matthew J. Ballinger,[a] Jeremy A. Bruenn,[a] John Hay,[b] Donna Czechowski,[b] Derek J. Taylor[a]
Department of Biological Sciences[a] and Department of Microbiology and Immunology,[b] State University of New York at Buffalo, Buffalo, New York, USA

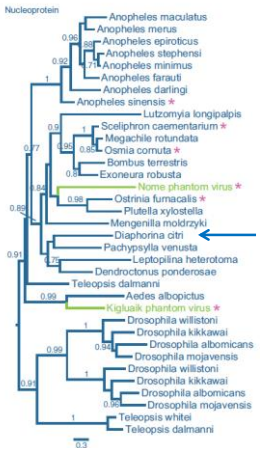


TABLE 3 Significant tBLASTn matches to phasmavirus genes in insect sequence databases

Host	Host common name	Host order	Gene(s) present	DNA[a]
Dendroctonus ponderosae	Mountain pine beetle	Coleoptera	G, N	Y
Chaoborus trivittatus	Phantom midge	Diptera	RdRp, G, N	N
Chaoborus cf. *flavicans*	Phantom midge	Diptera	RdRp, G, N	N
Drosophila spp.	Fruit fly	Diptera	N	Y
Aedes albopictus	Tiger mosquito	Diptera	G, N	N
Anopheles sinensis	Malaria mosquito	Diptera	RdRp, G, N	N
Anopheles epiroticus	Malaria mosquito	Diptera	G, N	Y
Anopheles minimus	Malaria mosquito	Diptera	G, N	Y
Anopheles stephensi	Malaria mosquito	Diptera	N	Y
Anopheles dirus	Malaria mosquito	Diptera	G	Y
Aedes aegypti	Yellow fever mosquito	Diptera	G	Y
Lutzomyia longipalpis	Sand fly	Diptera	G, N	Y
Teleopsis dalmanni	Stalk-eyed fly	Diptera	N	—
Teleopsis whitei	Stalk-eyed fly	Diptera	N	—
Culicoides sonorensis	Biting midge	Diptera	G	—
Rhodnius prolixus	Assassin bug	Hemiptera	G	Y
Pachypsylla venusta	Petiole gall psyllid	Hemiptera	RdRp, N	—
Diaphorina citri	Asian citrus psyllid	Hemiptera	N	Y

Even genome/transcriptome sequencing can find new viruses

Collected insects: US (FL, TX, HI, CA, Puerto Rico) and many foreign locations (Taiwan, China, Brazil and Pakistan).

Generated small RNA and transcriptome libraries

Deep sequencing of small RNAs and transcriptomes for identifying viruses associated with *Diaphorina citri*

Sequencing

Bioinformatics analysis

Small RNA (HiSeq)

RNA-seq (MiSeq)

RNA-seq (HiSeq)

Confirm virus presence by RT-PCR

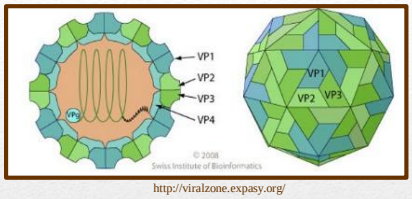
small RNA deep sequencing and transcriptome profiles analysis in world populations of *Diaphorina citri*

Name of Library	Number of reads	Avg. length	Number of reads after trim	Avg. length after trim	Number of reads after mapping to ACP transcriptome	Avg. length after map	Number of contigs
D. citri - China - 1	153,562,014	50.0	118,736,121	25.0	115,999,444	24.99	4,966
D. citri- Brazil - 1	195,655,932	50.0	101,869,794	25.5	98,131,266	25.45	6,932
D. citri – Florida - 1	210,667,442	50.0	190,207,640	26.3	184,436,525	26.29	11,972
D. citri - Taiwan	216,077,995	50.0	142,271,941	24.4	138,915,778	24.39	1,686
D. citri- Brazil - 3	In process	In process	In process	In process	In process	In process	In process
D. citri - China - 4	In process	In process	In process	In process	In process	In process	In process
D. citri - China - 5	In process	In process	In process	In process	In process	In process	In process
D. citri – Florida - 3	In process	In process	In process	In process	In process	In process	In process
D. citri – Hawaii	In process	In process	In process	In process	In process	In process	In process

Statistical information derived through bioinformatics analyses

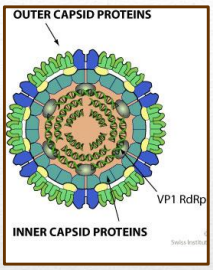
	Category	Family	Genus	Species	E-value	Population
Insect viruses	dsRNA viruses	*Reoviridae*	*Fijivirus*	*Nilaparvata lugens reovirus*	*0.0*	CH, TW, FL
	dsRNA viruses	*Reoviridae*	*None*	*Diaphorina citri reovirus*	*6.59e-132*	CH, TW, FL
	ssRNA viruses	*Iflaviridae*	*Iflavirus*	*Deformed wing virus*	*5.56e-21*	BR, CH, TW
	ssDNA viruses	*Parvoviridae*	*Iteradensovirus*	*Helicoverpa armigera Densovirus*	*4.21e-46*	BR, CH, TW, FL
	dsDNA viruses	*Polydnaviridae*	*Bracovirus*	*Cotesia congregate bracovirus*	*1.95e-95*	BR, CH, TW, FL
	dsDNA viruses	*Baculovirus*	*Alphabaculovirus*	*Autographa californica multiple nucleopolyhedrovirus*	*4.32e-90*	BR, CH, TW, FL
	ssRNA viruses	*Bunyaviridae*	*None*	*Kialuaik phantom virus*	*7.59e-37*	CH
	ssRNA virus	*None*	*None*	*Chronic bee paralysis virus*	*6.15e-04*	CH
Bacteriophage	dsDNA viruses	*Unclassified phages*	*None*	*Wolbachia endosymbiont of Culex quinquefasciatus WO prophage*	*2.1e-130*	BR, CH, TW, FL
Plant viruses	ssRNA viruses	*Luteoviridae*	*Polerovirus*	*Potato leafroll virus*	5.62e-46	CH, FL
	dsRNA viruses	*None*	*None*	*Gentian kobu-sho-associated virus*	2.74e-51	FL
	ssRNA viruses	*Virgaviridae*	*Tobamovirus*	*Cucumber green mottle mosaic virus*	5.65e-45	BR
	ssRNA viruses	*None*	*Umbravirus*	*Carrot mottle virus*	4.76e-53	BR
Animal viruses	ssRNA viruses	*Flaviviridae*	*Pestivirus*	*Bovine viral diarrhea virus 1*	1.49e-48	BR, CH, TW, FL
	dsDNA viruses	*Herpesviridae*	*Macavirus*	*Bovine herpes virus 6*	4.23e-100	BR, CH, TW, FL
	Retro-transcribing virus	*Retroviridae*	*Alpharetrovirus*	*Avian leukosis virus*	2.069e-68	BR, CH, TW, FL
Marine virus	dsDNA viruses	*Phycodnaviridae*	*None*	*Organic Lake phycodnavirus 1*	9.79e-30	BR, CH, TW, FL

Picorna-like virus (ssRNA)



http://viralzone.expasy.org/

Reovirus (dsRNA)



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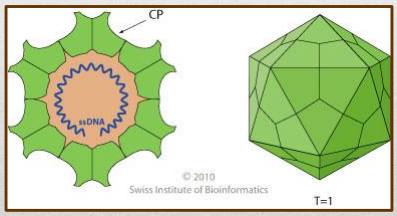
Densovirus (ssDNA)



http://viralzone.expasy.org/

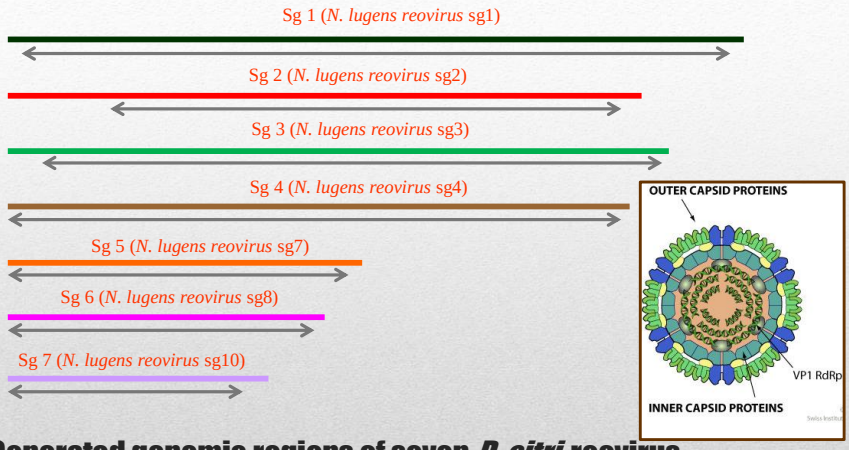
Newly discovered *D. citri* viruses

The highest BLAST hits in *D. citri* populations

Sample	Collector	NGS Platform			Identified Virus*		
		HiSeq small RNA	MiSeq RNA-seq	HiSeq RNA-seq	DCPLV	DcRV	DcDV
Taiwan 1	H.H.Y.	+	-	-	+	+	+
Taiwan 2	H.H.Y.	-	-	-	-	+	+
China 1	YC HW	+	+	-	+	+	+
China 2	YC HW	-	-	-	+	+	+
China 3	YC HW	-	-	-	+	+	+
China 4	YC	-	-	+	+	+	ND**
China 5	YC	-	-	+	+	-	ND
Brazil 1	TS	+	-	-	+	-	+
Brazil 2	DMG	-	-	-	+	-	+
Brazil 3	DMG	-	-	+	+	-	ND
Pakistan	AMK	-	-	-	-	-	+
Florida 1	WOD	+	+	-	-	+	+
Florida 2	WOD	-	-	-	-	+	+
Florida 3,4,5,6	KPS	-	-	(3)+	+/-	+	ND
Florida 7,8,9,10,11,12	KPS, BF, RP	-	-	-	-	+	ND
Puerto Rico 1,2,3		-	-	-	-	+	ND
California 1	DM	-	-	-	-	-	+
California 2	DM	-	-	-	-	-	+
California 3	DM	-	-	-	-	-	+
California 4	DM	-	-	-	-	-	+
Hawaii-Aiea	CH	-	-	+	-	+	+
Texas 1	AC	-	-	-	-	-	+
Texas 2	AC	-	-	-	-	-	+
CRF	KG	-	-	-	-	-	+

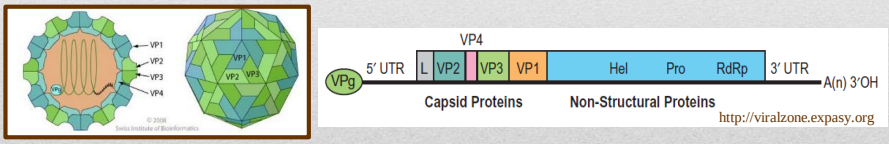
*Viruses confirmed by RT-PCR and Sanger sequencing
**Not Done.

D. citri RNA samples analyzed by NGS and RT-PCR for new viruses



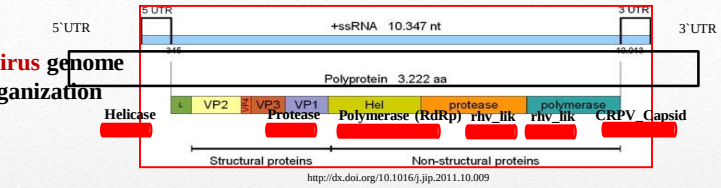
Generated genomic regions of seven *D. citri* reovirus genomic dsRNAs by bioinformatics analysis for *D. citri* reovirus strain China

- **Helicase:** 36-39% similarity (BLASTP) to *Deformed wing virus, Formica exsecta virus 2*
- **RdRp:** 35-38% similarity (BLASTP) to *Deformed wing virus, Nilaparvata lugens honeydew virus-3, Brevicoryne brassicae picorna-like virus*
- **CP:** 27-29% similarity (BLASTP) to *Deformed wing virus, Slow bee paralysis virus, Laodelphax striatellus picorna-like virus 2*

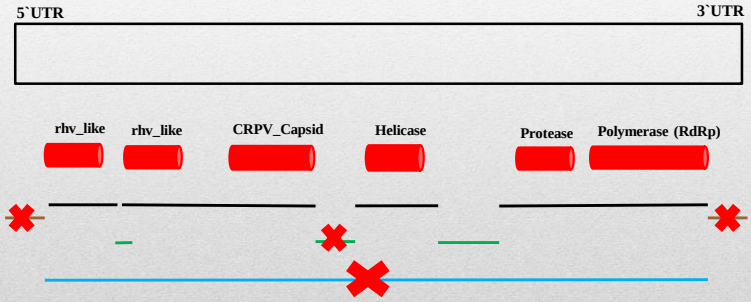


The highest BLAST Hits to iflaviruses

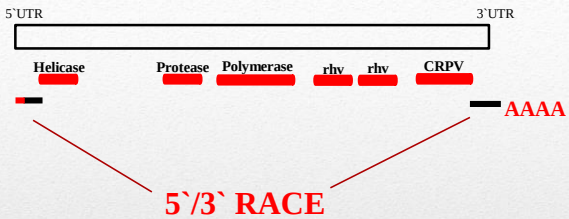
Iflavirus genome organization

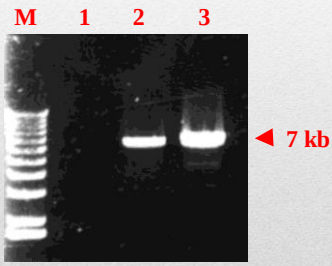


http://dx.doi.org/10.1016/j.jip.2011.10.009

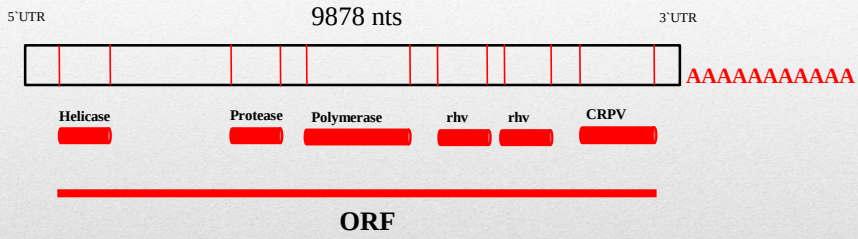


Generic genome organization and coding regions of the new picorna-like virus

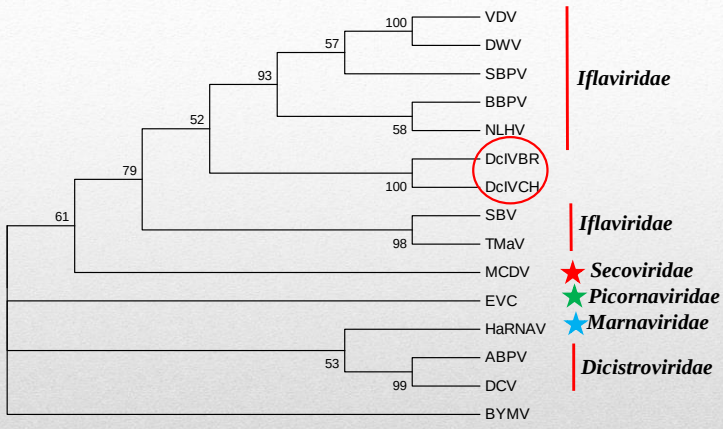




RT-PCR products from *D.ciri* RNAs amplified by using primers CP-reverse & Hel-forward. M) 1kb plus ladder; 1) *D. citri*-CRF; 2) *D.citri*-China; 3) *D. citri* Brazil



Diaphorina citri picorna-like virus genome organization

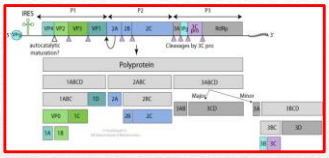


Phylogenetic tree constructed with the amino acid sequences of the RdRp by the NJ method.

DWV: *Deformed Wing Virus*; VDV: *Varroa destructor virus*; SBPV: *Slow bee paralysis virus*; BBPV: *Brevicoryne brassicae picorna-like virus*; NLHV: *Nilaparvata lugens honeydew virus*; SBV: *Sacbrood virus*; TMaV: *Tomato matilda virus*; ABPV: *Acute bee paralysis virus*; DCV: *Drosophila C virus*; MCDV: *Maize chlorotic dwarf virus*; EVC: *Enterovirus C* , HaRNAV: *Heterosigma akashiwo*

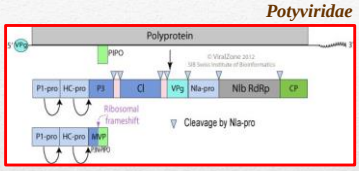
- *Picornaviridae*
  - *Aphthovirus*
  - *Enterovirus*
- *Calciviridae*
  - *Norovirus*
- *Potyviridae*
  - *Potyvirus*
- *Secoviridae*
  - *Comovirus*
  - *Fabavirus*
  - *Nepovirus*
  - *Torradovirus*
- *Dicistroviridae*
  - *Aaparavirus*
  - *Cripavirus*
- *Iflaviridae*
  - *Iflavirus*
- *Marnaviridae*
  - *Marnavirus*

Picornaviridae

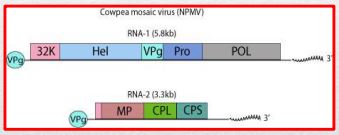


Calciviridae

Potyviridae

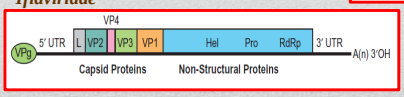


Secoviridae



Dicistroviridae

Iflaviridae

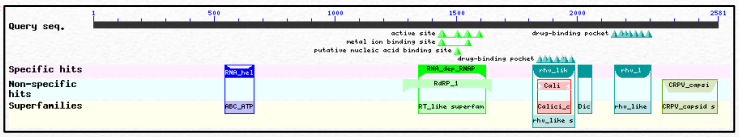


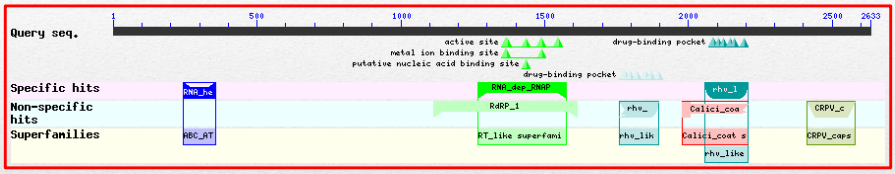
http://viralzone.expasy.org

Picornavirus-like Superfamily

Heterosigma akashiwo RNA virus

Found by metagnomic studies of phytoplankton





Name	Accession	Description	Interval	E-value
RNA_dep_RNAP	cd01699	RNA_dep_RNAP: RNA-dependent RNA polymerase (RdRp) is an essential protein encoded in the ...	1267-1576	2.22e-50
rhv_like	cd00205	Picornavirus capsid protein domain_like. Picornaviruses are non-enveloped plus-strand ssRNA ...	2058-2207	1.30e-19
RdRP_1	pfam00680	RNA dependent RNA polymerase;	1114-1614	1.13e-44
RNA_helicase	pfam00910	RNA helicase; This family includes RNA helicases thought to be involved in duplex unwinding ...	245-355	3.90e-14
rhv_like	cd00205	Picornavirus capsid protein domain_like. Picornaviruses are non-enveloped plus-strand ssRNA ...	1759-1896	3.08e-10
CRPV_capsid	pfam08762	CRPV capsid protein like; This is a family of capsid proteins found in positive stranded ssRNA ...	2412-2579	2.46e-08
Calici_coat	pfam00915	Calicivirus coat protein;	1977-2207	1.31e-05

BLAST search using Blastp

Summary

- **We have analyzed *D. citri* populations from 4 states, Puerto Rico, and 4 countries for *D. citri*-infecting viruses.**
- **3 viruses have been confirmed and we are focusing our efforts on a new virus, *Diaphorina citri picorna-like virus* (DCPLV).**
- **DPCLV does not appear to be common in U. S. *D. citri* populations.**
- **We have generated the complete nucleotide sequence of DCPLV (we think)**
- **We have obtained a USDA APHIS permit to perform biological studies with DCPLV within the UC Davis BSL3P Contained Research Facility.**

Future Directions

- **Generating full length cDNAs to DCPLV.**
- **Assess the infectivity and efficiency of the wild DCPLV (Infectious virus) in cultured psyllids.**
- **Engineer the DCPLV for delivering RNAs/proteins to *D.citri*. We will generate recombinant DCPLV (inserting the target insect mRNA sequences into virus) in transfected GWSS-Z15 or Sf9 cells. The recombinant virus will induce VIGS (Virus-induced gene silencing), and a negative phenotype.**
- **We are mining our data for additional potentially useful *D. citri* viruses.**

Diversity and ecology of *Wolbachia* in Florida Asian citrus psyllid populations

M. Hoffmann, C.W. Russell, M.R. Coy, and **K.S. Pelz-Stelinski**

University of Florida, Department of Nematology and Entomology, IFAS/CREC, Lake Alfred, FL

UF UNIVERSITY of FLORIDA IFAS

Citrus greening/Huanglongbing (HLB)

- Causal agent: *Candidatus* Liberibacter species
- HLB distribution in America: Continental USA, Caribbean, Central America, Mexico, Brazil
- HLB management: mainly insecticides & tree removal: $ 600 – 1000 per acre
- Economic impact of HLB on Florida citrus industry:

  Loss of $ 4.5 Billion between 2006 -2011 (~ 16% loss)(Hodges & Spreen 2012)

  Hodges & Spreen 2012: EDIS Publication #FE903, UF IFAS Extension Program, http://edis.ifas.ufl.edu/fe903

Middle Photo Source: EPPO https://www.eppo.int/QUARANTINE/bacteria/Liberobacter_africanum/LIBESP_images.htm

Citrus Greening Disease

The vector

- Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)
- Discovered in Florida June 1998
- Host range includes 25 genera of Rutaceae, including *Citrus*

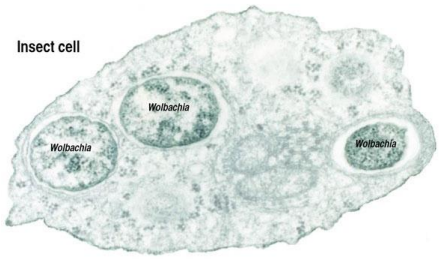
HLB management:

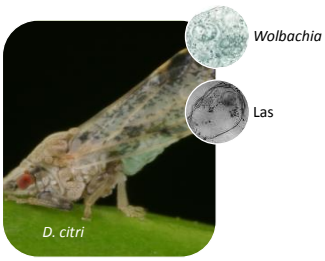
- Psyllid management (area-wide control)
- Management of infected plants, replanting

Endosymbionts:

- *Ca.* Profftella armatura
- *Ca.* Carsonella ruddii
- *Wolbachia pipientis*

Wolbachia

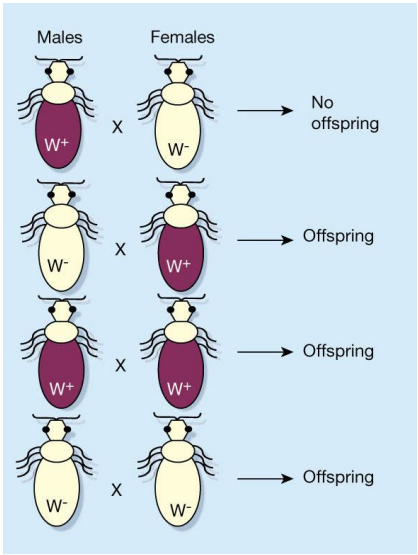




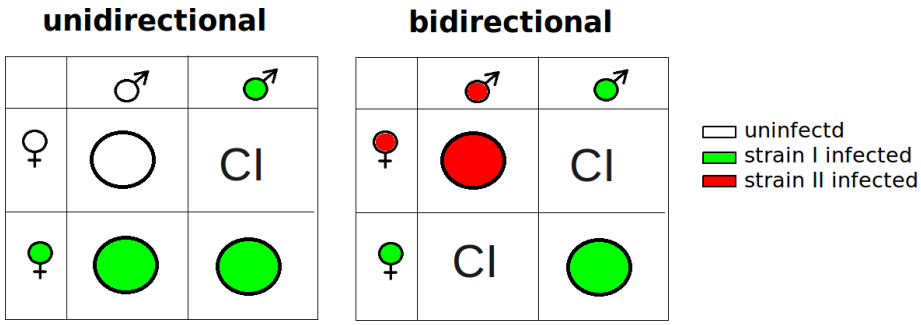
- A widespread intracellular bacterium, carried by an estimated 40% of insect spp.
- May interact with pathogens, effecting the probability of transmission (e.g. competitive exclusion, immune activation)
- Approach used in insect vectored human pathogen systems

Source: Genome Sequence of the Intracellular Bacterium Wolbachia. PLoS Biol 2/3/2004: e76. http://dx.doi.org/10.1371/journal.pbio.0020076

Cytoplasmic Incompatibility

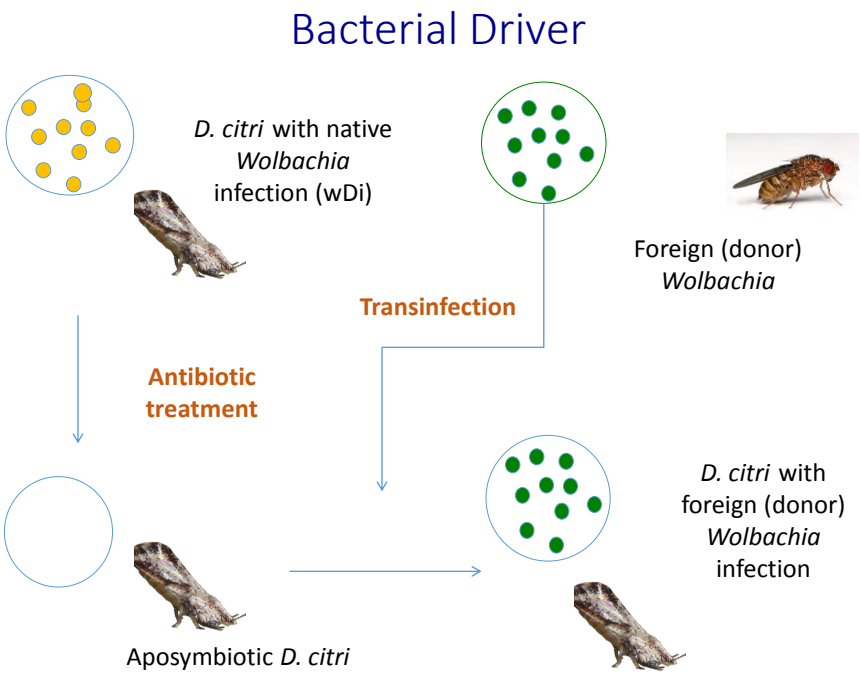


Cytoplasmic Incompatibility



Wolbachia for psyllid management: approach

- Identify geographic variation in *Wolbachia* infections among field populations
- Identify endemic *Wolbachia* types that reduce Las transmission/fitness
  - Minor strains good candidates for mass releases
- Develop breeding lines of *Wolbachia*-(co)infected and *Wolbachia*-free ACP: selective breeding and antibiotic treatment
- Establish stable somatic infections of non-native *Wolbachia* strains with infected insect cell cultures
  - Las transmission
  - fitness
  - cytoplasmic incompatibility (CI)



Objectives

1. Characterize diversity of *Wolbachia* in Florida *D. citri* populations by multilocus sequence typing (MLST)

- *D. citri Wolbachia* Sequence Types (ST) characterized by identification of accumulated nucleotide differences in five conserved genes
- Differences determined by comparing consensus sequences to MLST sequence database
- 5 MLTS genes: *coxA, hcpA, ftsZ, gatB, wsp*
- 10-33 individuals from 9 populations in Florida, 1 population from Hawaii

4

Objectives

2. Determine within-host densities of endosymbionts: *Wolbachia*, *Ca.* Proftella armatura and *Ca.* Carsonella ruddii

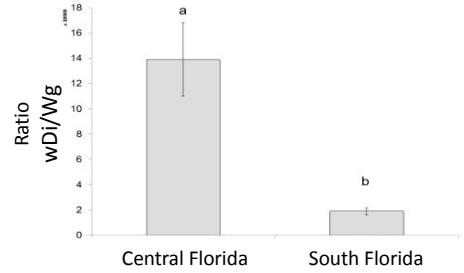
- Age (developmental stage)
- Geographic distribution of *D. citri*
- Relationship with *Wolbachia* sequence type

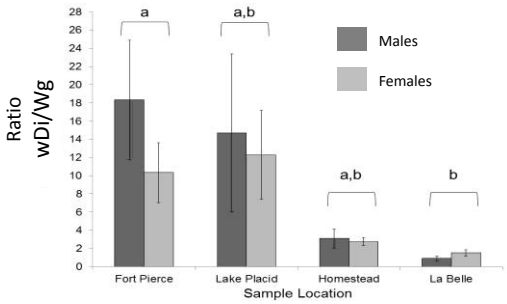
4

Geographic variation of *Wolbachia* infection density

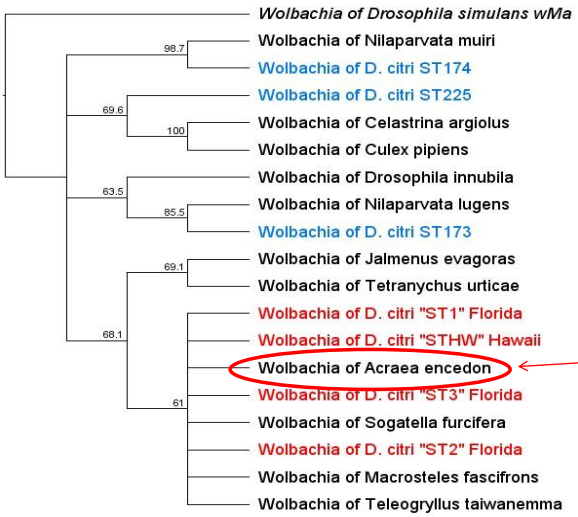
Wolbachia density may be associated with:

- Age
- Environmental conditions (temperature)
- Strain





Phylogenetic relationships of *Wolbachia* sequence types (STs) associated with Florida *D. citri*



Wolbachia of *D. citri*, this study

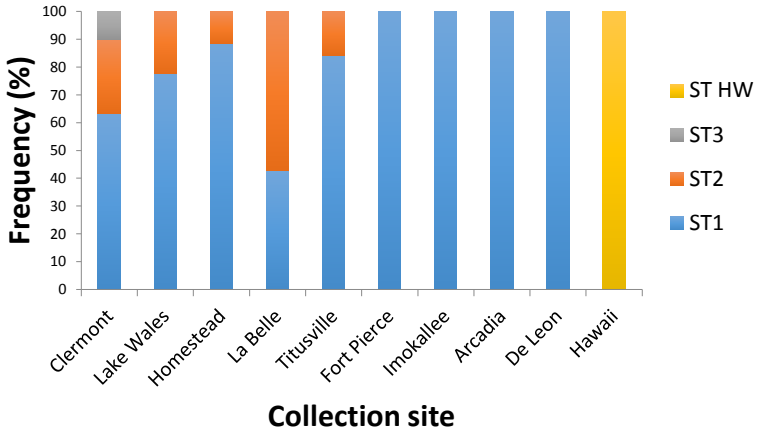
Wolbachia of *D. citri*, Brazil*

Male-killing *Wolbachia* strain (Lepidoptera)

*Guidolin & Cônsoli 2013: Microb Ecol. 65(2) 475-486.

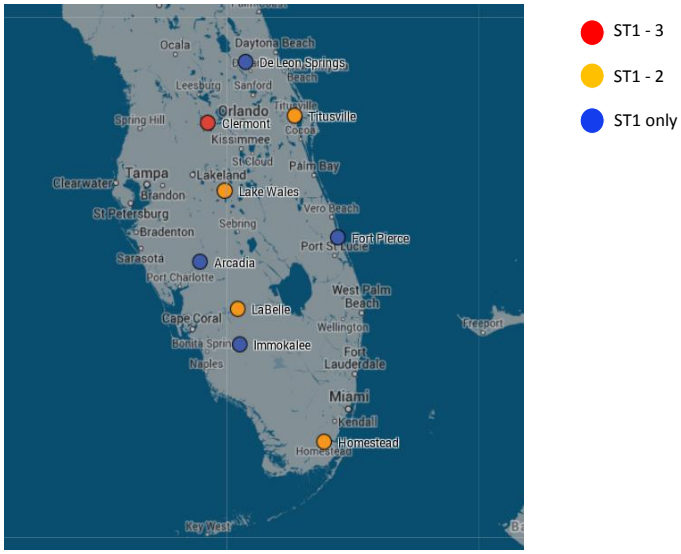
Neighbor-joining tree, cytochrome C oxidase gene, bootstrap values indicated at branches

Relative distribution of associated *Wolbachia* sequence types in *D. citri* populations



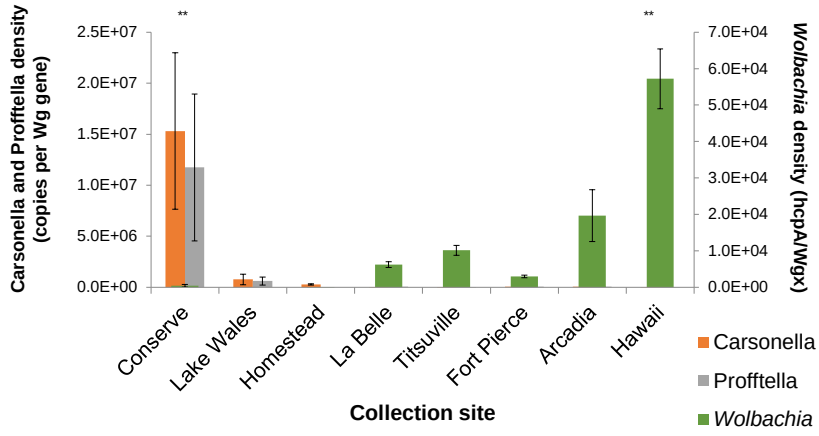
6

Occurrence of *Wolbachia* sequence types in Florida *D. citri* populations



6

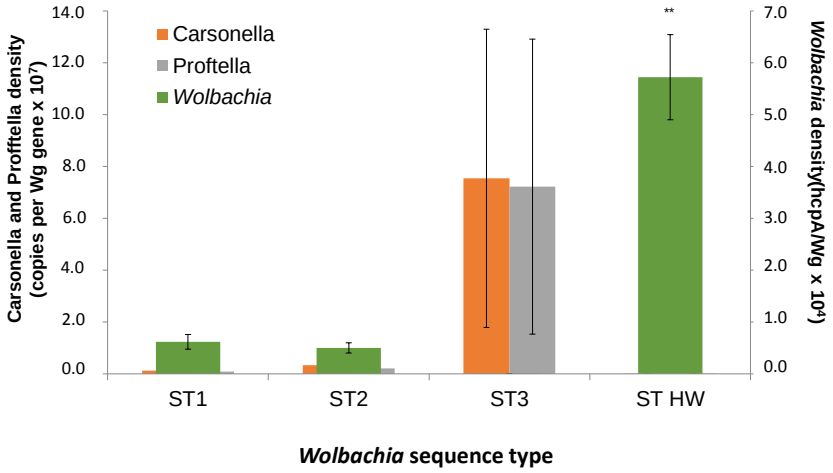
Within-host Density of *Diaphorina citri* Endosymbionts



Wolbachia densities and *Ca.* Profftella/Carsonella densities are inversely related

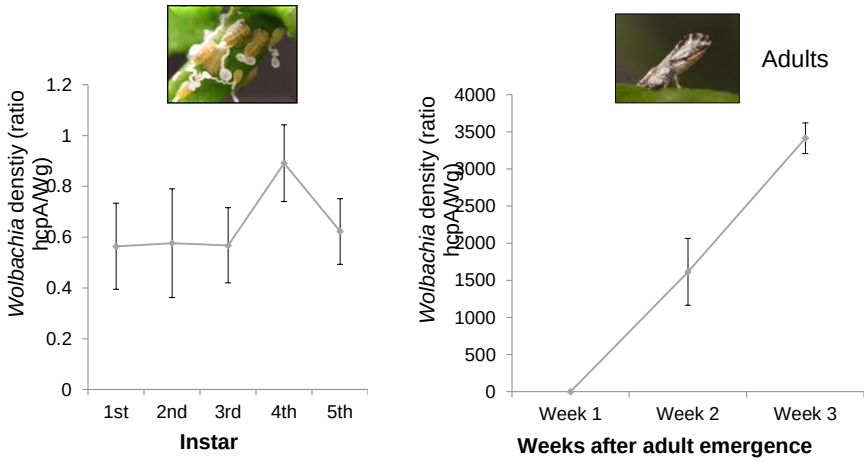
7

Inverse relationship between Carsonella/Profftella and *Wolbachia* ST3



8

Wolbachia titer increases with age



- *Wolbachia* density is low during larval development and increases following emergence

9

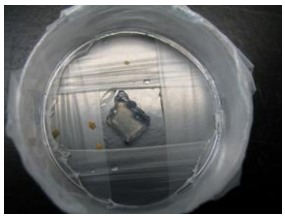
Conclusions

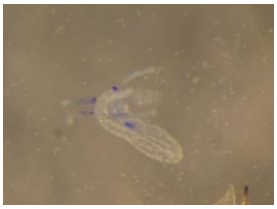
- Three sequence types of *Wolbachia* in *D. citri* populations in Florida
- One predominant sequence ST in all populations
- Minor ST a candidate for bacterial drive
- *Ca.* Carsonella ruddii / *Ca.* Profftella armatura and *Wolbachia* densities differ geographically
- Inverse relationship between *Wolbachia* and *D. citri* endosymbionts in adults
- Inverse relationship with endosymbionts associated with *Wolbachia* sequence type
- *Wolbachia* densities low during larval development and increase during adulthood

10

Ongoing Work

- Introduction of antibiotic cocktail in nymphal diet insufficient for removal of *Wolbachia*
- Currently targeting *Wolbachia* for silencing with RNAi
- Successful introduction of drosophila, mosquito *Wolbachia* strains using microinjection, nymph diet
- Evaluating efficiency of establishment in offspring, CI
- Confirmed establishment of isofemale line infected with ST3 (ILST3)
- Transmission efficiency by ILST3 line and transformed *D. citri*
- Competition among *Wol* strains/endosymbionts





Russell et al. 2015

Thank you!

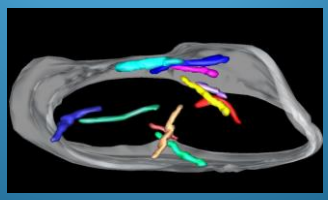
Funding:

Citrus Research and Development Foundation, Inc.

United States Department of Agriculture
National Institute of Food and Agriculture

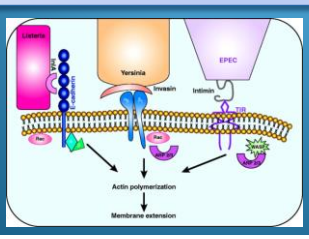
NuPsyllid: Effectors Mechanism 2

Robert Shatters (USDA, ARS)
El-Desouky Ammar (USDA, ARS)
John Hartung (USDA, ARS)
Marc Giulionatti (TPIMS)

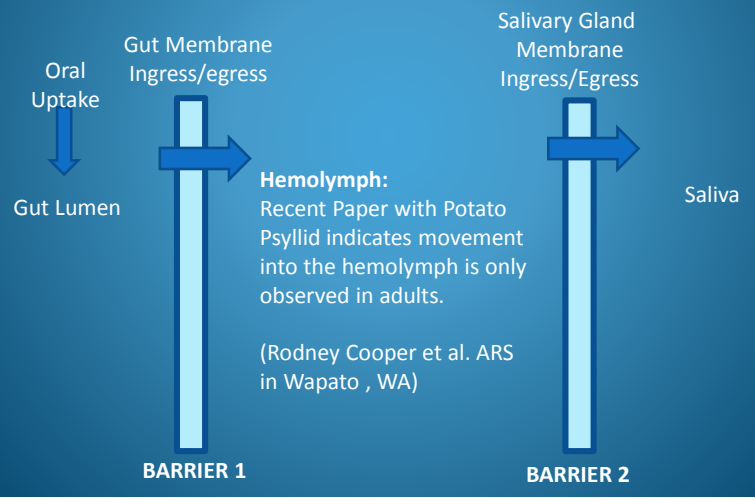


Effector Mechanism 2 Research Areas:

- Produce Single Chain Antibody Genes that target surface receptors on Liberibacter.
- Develop peptide library to identify peptides that bind gut membrane surfaces.
- Develop assay to screen for antibodies/peptides that inhibit Liberibacter-psyllid membrane interactions
- Test selected antibodies/peptides for inhibition of Liberibacter transmission



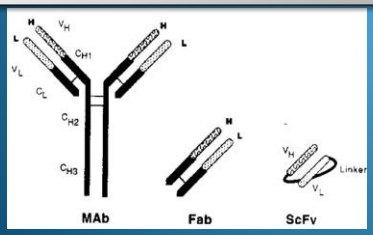
- Research indicates that adults are not efficient vectors
  - Are they blocked at acquisition or transmission



Effector Mechanism 2 Research Areas (John Hartung):

- Produce Single Chain Antibody Genes that target surface receptors on Liberibacter and on psyllid alimentary canal endothelium

- Library of scFv fragments with specificity for any target in 'Ca. Liberibacter asiaticus'.
- Genes encoding the scFv fragments
- Have isolated and verified scFv libraries highly enriched for scFv that bind to 8 different antigens representing surface exposed loops of proteins found embedded in outer membrane.
- 8 scFv clones that bind different surface antigens on the Clas.
  - IDed the targets through analysis of known Clas sequences
  - Expressed the surface antigens and "bio-panned" for cognate ScFVs



Effector Mechanism 2 Research Areas (John Hartung):

- **Produce Single Chain Antibody Genes that target surface receptors on Liberibacter and on psyllid alimentary canal endothelium**
  - **Currently have large volume prep of a very good polyclonal Ab to one surface antigen (for which an ScFv is also identified)**
  - **Currently have transgenic citrus expressing different ScFv's identified to bind different Clas surface antigens**
  - **Transgenic citrus expressing multiple scFv isolated by biopanning against InvA (invasin) and TolC (membrane efflux pump) were developed**
  - **Proposed to use transgenic plants in graft assay:**
    - Graft the transgenics onto high titer rootstocks, with appropriate controls.
    - CLas titer development will be monitored
    - Can also use leaf-caged ACP to assess ability of the ACP to pick up the CLas.
  - **Rabbit polyclonal antibodies against two of the Clas surface antigens have been developed. Outer membrane protein A and Kapsular polysaccharide polymerase (KpsA = Polysiallic acid polymerase PsaA).**

Effector Mechanism 2 Research Areas:

- **Develop peptide library to identify peptides that bind gut membrane surfaces.**
- Develop assay to screen for antibodies/peptides that inhibit Liberibacter-psyllid membrane interactions
- Test selected antibodies/peptides for inhibition of Liberibacter transmission

Positional Scanning Library

C'	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	N'	Amount (mg)
NH2	X	X	X	X											NH2	40.9
NH2	X	X	X	X											Biotin	40.3
NH2	**X**	**X**	**X**	**X**	**Gly**	**Gly**									**Biotin**	**41.5**
NH2	X	X	X	X	X										NH2	40.5
NH2	X	X	X	X	X										Biotin	40
NH2	X	X	X	X	X	Gly	Gly								Biotin	40.9
NH2	X	X	X	X	X	X									NH2	40.4
NH2	X	X	X	X	X	X									Biotin	40.7
NH2	X	X	X	X	X	X	Gly	Gly							Biotin	40.8
NH2	X	X	X	X	X	X	X								NH2	40.1
NH2	X	X	X	X	X	X	X								Biotin	41
NH2	X	X	X	X	X	X	X	Gly	Gly						Biotin	39.7
NH2	X	X	X	X	X	X	X	X							NH2	40.8
NH2	X	X	X	X	X	X	X	X							Biotin	40.7
NH2	X	X	X	X	X	X	X	X	Gly	Gly					Biotin	40.2
NH2	X	X	X	X	X	X	X	X	X						NH2	40.5
NH2	X	X	X	X	X	X	X	X	X						Biotin	40.2
NH2	X	X	X	X	X	X	X	X	X	Gly	Gly				Biotin	41
NH2	X	X	X	X	X	X	X	X	X	X					NH2	40.1
NH2	X	X	X	X	X	X	X	X	X	X					Biotin	40.8
NH2	X	X	X	X	X	X	X	X	X	X	Gly	Gly			Biotin	41.5

Screening Process Resulted in Detection of 8 Peptides that Bind the Psyllid Gut Epithelium

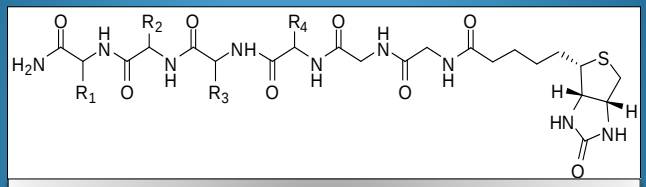
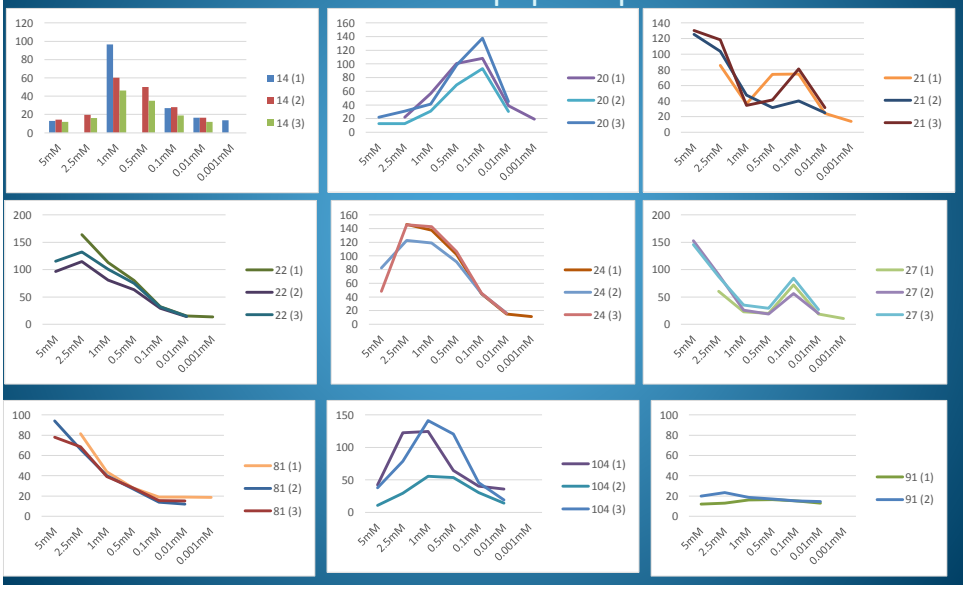


Figure 1. Structure of peptide library used in our screening assay to identify digestive tract binding peptides. The R represents the side group of any of the 20 amino acids. The four variable amino acids are separated from the biotin moiety by two glycine residues.

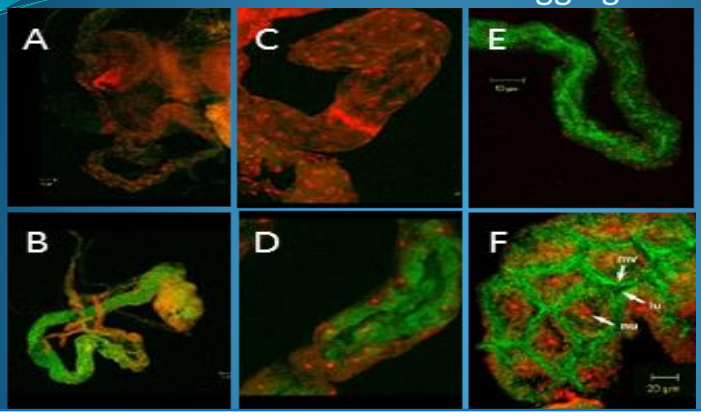
96-well plate assay for rapid screening

- **Psyllid gut membrane prep and binding protocol**
- **Preparation of gut membranes**
  - Adult psyllid guts are dissected placed in pH 6.5 buffer and stored at -20°C.
  - Homogenize, centrifuge, briefly sonicate, re-centrifuge.
  - Resuspend the pellet in 0.1 ml TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.0): 2 µl of gut-membrane preparation is equivalent to one gut.
- **Peptide binding to gut membranes**
  - Binding assays are carried out in filter bottom 96-well plates
  - Add the gut membranes (4 per well) and wash three times with 100 µl TBS, pH 7.0.
  - Add the peptides and wash.
  - Add Alexa Fluor 488, Wash 3 times.
  - Wells are ready to be viewed fluorometrically.

Concentration Effect on Gut Membrane Binding: All peptide show different binding kinetics related to concentration of peptide present



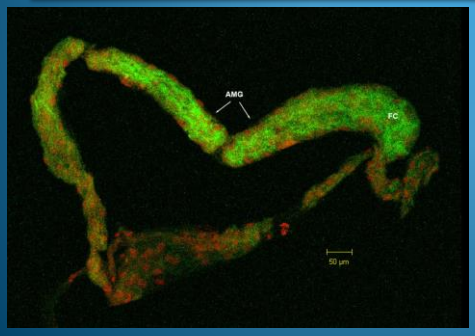
Gut-Binding Demonstrated for Specific Peptides by Fluorescent Tagging



Psyllid Digestive tract membrane binding of biotinylated peptides after feeding and clearing. ACP nymphs were fed on artificial diet for four days and transferred to diet alone to clear unbound peptide. Entire digestive tracts were dissected, fixed and stained for peptide using Alexafluor-488-streptavidin (green). Confocal microscopy of tissues backstained in (red) showing endothelial cells and nuclei. (A) Control of psyllids fed non-binding biotinylated peptide. (B) Psyllids fed mixture of all peptides in library. (C) increased magnification to show binding to lumen side of endothelium. (D) control for (C). (D and E) gut lumen binding if one of the 21 identified gut binding peptides showing intense binding in the brushborder membrane area.

Peptides Were Shown to Bind Nymph Gut Membranes As Well As Adult

- **ACP 4th instar nymphs that fed on peptide-diet (for 4 days) then cleared by feeding on excised healthy leaves (for 3 days).**
- **Confocal Results (10/16/14)**:

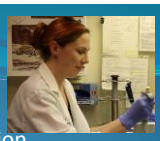


Binding Was observed in psyllids that remained as nymphs and psyllids that had emerged as adults

Effector Mechanism 2 Research Areas:

- Develop peptide library to identify peptides that bind bacterial and/or gut membrane surfaces.
- **Develop assay to screen for antibodies/peptides that inhibit Liberibacter-psyllid membrane interactions**
- Test selected antibodies/peptides for inhibition of Liberibacter transmission

Nymph Aquisition Bioassay



Peptide Loading

Adapted Kirsten Pelz-Stelinski's chambers

- 4th instar nymphs on diet chambers taken directly from colonies
- Kept on Diet for 3 days.

4th-5th instar

Peptide loading/Aquisition

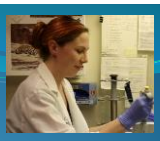
- Approximately 30 4th-5th instar nymphs transferred to single leaf (**healthy or infected**).
- Left on leaf until emergence. Transfer adults to healthy leaves.

Adults

Trasnsmission

- 10 adult psyllids feed on healthy leaf for 1-2 weeks.
- Psyllids are collected and tested for CLas.
- Leaves incubate in bags for another week and then are tested for CLas.

Nymph Aquisition Bioassay



Peptide Loading

4^{th}-5^{th} instar

Peptide loading/Aquisition

Adults or nymphs

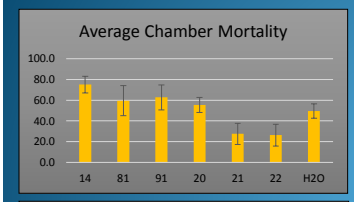
Monitor for Aquisition

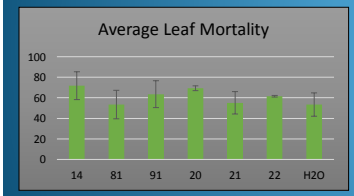
- 4^{th} instar nymphs on diet chambers taken directly from colonies
- Kept on Diet for 3 days.

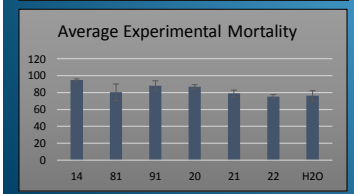
- Approximately 30 4^{th}-5^{th} instar nymphs transferred to single leaf (**healthy or infected**).
- Left on leaf until emergence. Transfer adults to healthy leaves.

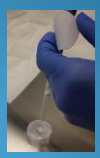
- Analyze Nymphs: Remove head/Thorax from gut and rest of body: PCR analysis of separate head thorax region and gut/body region.
- Looking for Movement into Head Region

Peptide 14 Consistently Induced Psyllid Mortality When Fed to 4^{th} Instar Nymphs









4 Days on Diet to monitor mortality

Consistent Problem: High Mortality in Diet only Controls. Note: We saw the same thing with RNAi feeding, but efficacy greatly improved when we were able to move to the whole plant assay (CTV expression system)

Overall Results With Bioassay

% Psyllids recovered	% Psyllids Clas +	Positives: Posterior/Abdomen Detection	Positives: Anterior/Head-Thorax Detection	% of Leaves Testing Positive for Clas (Transmission)
~10-20%	21%	67%	33%	~10%

- We do see acquisition and transmission.
- 33 to 50% of experiments fail due to high nymph mortality.

Summary

- Proposed to :
  - 1. ID and produce scFvs that bind bacterial surface antigens
  - 2. ID psyllid gut binding peptides.
  - 3. Develop bioassay for testing acquisition and transmission blocking characteristics of ScFVs and peptides

Summary

- To Date:
  - 1. **ID and Produce ScFV's that binding bacterial surface antigens (John Hartung)**
    - Specific Clas surface antigen binding ScFvs have been identified.
    - Stability in culture production has been problematic
    - However, transgenic citrus are now available expressing these (Collaborative work with John Hartung and Ed Stover).
    - These citrus are available for testing effect on Clas replication in the plant and acquisition/transmission analysis.

Summary

- Proposed to :
  - 2. ID psyllid gut binding peptides.
    - We have IDed 8 gut binding peptides and shown that they bind adult and nymph gut membrane preparations and intact adult and nymph epithelial layer.
    - Different binding kinetics and different fluorescent pattern of binding suggest at least some differences in interaction that may reflect different targets.
    - Low concentration binding is encouraging
    - At least one of these peptides shows reproducible toxicity to nymphal psyllids.

Summary

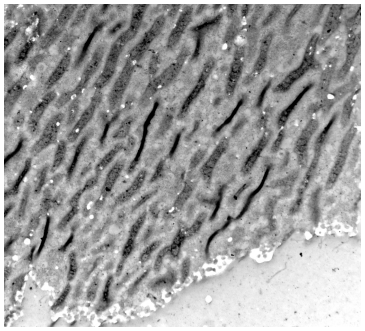
- Proposed to :
  - 3. Develop bioassay for testing acquisition transmission blocking characterisics of ScFVs and peptides.
    - Bioassay development has been the most challenging.
    - Kirsten Stelinski's chamber design has helped tremendously
    - High mortality continues to cause problems and requires us to do high number of replications.
    - We do have a working bioassay and we have shown we can monitor acquisition by looking for movement of Clas into the salivary glands (head/thorax) of psyllids
    - Currently testing peptides in this assay.

Next Steps:

- Test Transgenic citrus expression Clas surface antigen ScFV's?
- Need to finish competition studies with peptides:
  - Unbiotinylated vs biotinylated.
- Screen peptides in acquisition/transmission bioassays
- Psyllid toxicity of peptide 14?
  - Could be moved into transgenic citrus test? (nuclear or CTV?)

Outside the scope of the grant objectives-but advancement of findings

- Continue characterizing the peptide binding kinetics.
- Working collaboratively with Michelle Cilia on identifying the targets in the insect digestive tract that are binding the peptides.
- Determine if transgenic expression (nuclear or CTV) are viable alternative strategies for psyllid control.
- Screen of scFv transgenic citrus for effect on acquisition/transmission.

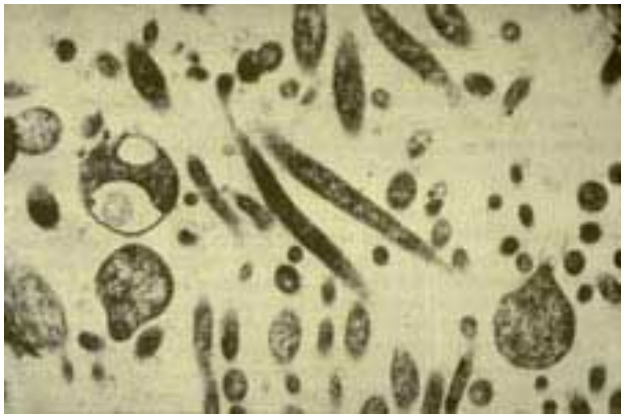


Psyllid transcripts with potential involvement in *Ca*. Liberibacter invasion and propagative transmission: Toward RNAi mediated abatement of citrus greening and zebra chip diseases

Judith K. Brown et al
School of Plant Sciences
University of Arizona
Tucson AZ USA

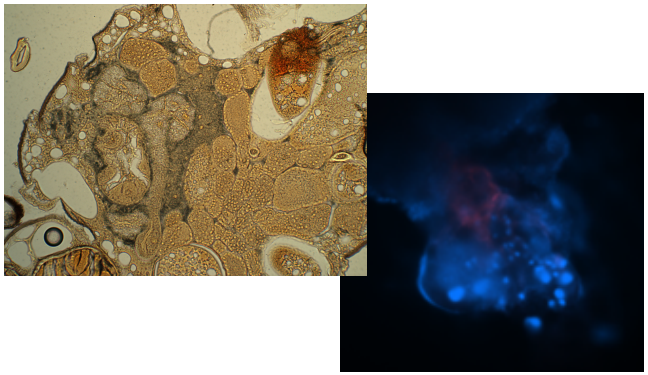
Multiple approaches:
(i) stalking the transmission pathway of the causal microbe of HLB…

an exotic microbe

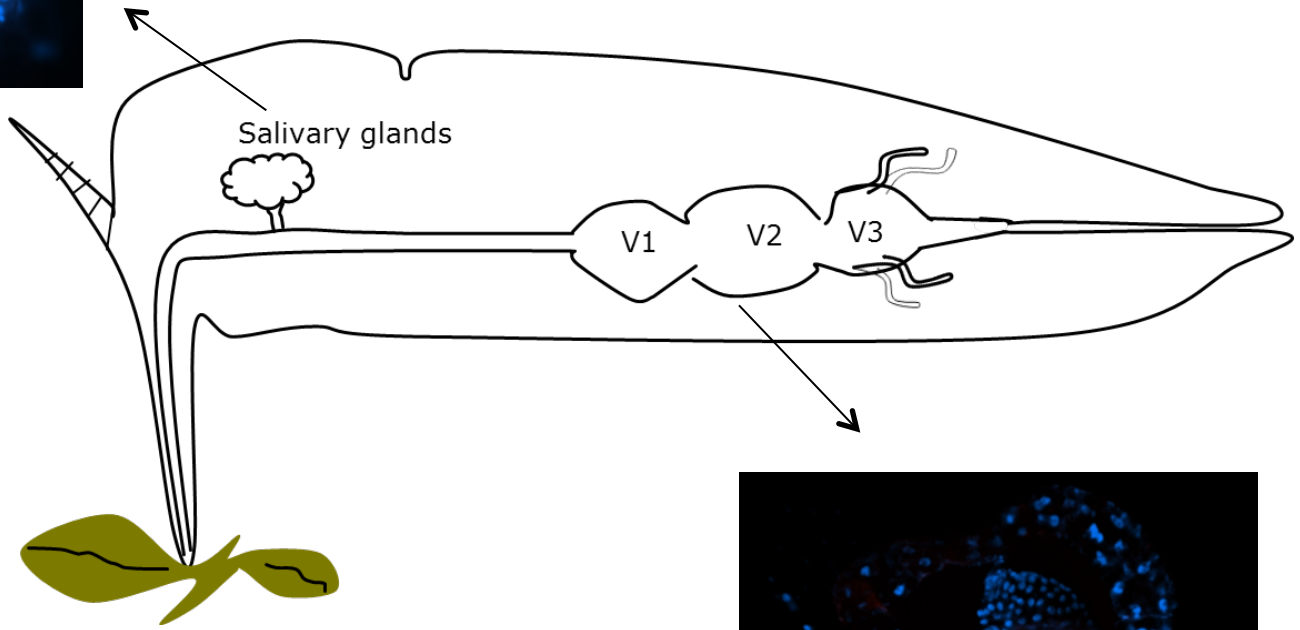


and an equally exotic psyllid vector

PoP anatomy and Lso Localization (FISH)



1. Fluorescent and gold-silver enhancement labeling in salivary glands

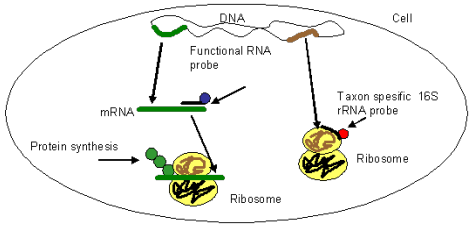


FISH

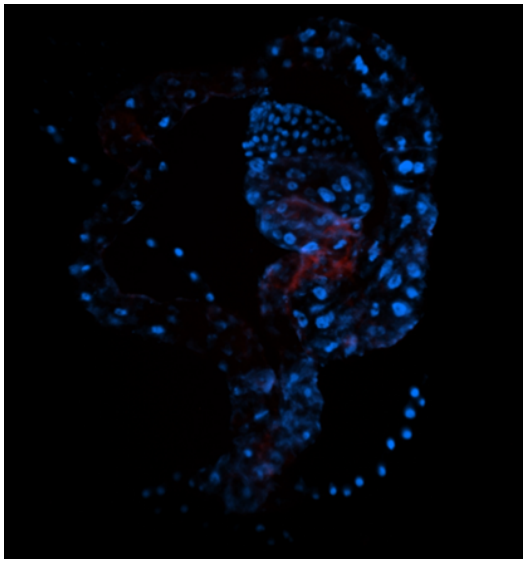
-Carnoy's fixative

-16S rRNA probe with Cy5 tag (red)

>20 pmol/ml overnight



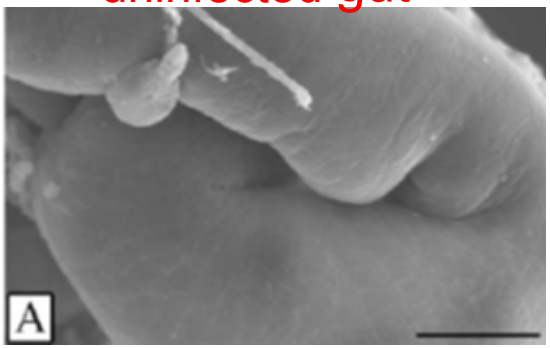
2. Fluorescent signal in gut (V1 section is shown, right)



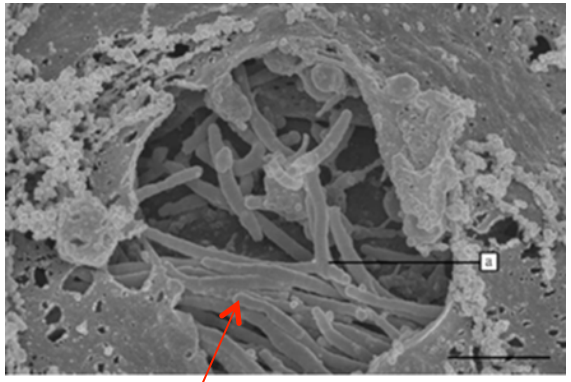
Cicero, Brown et al., unpublished

CLso localization in PoP (SEM)

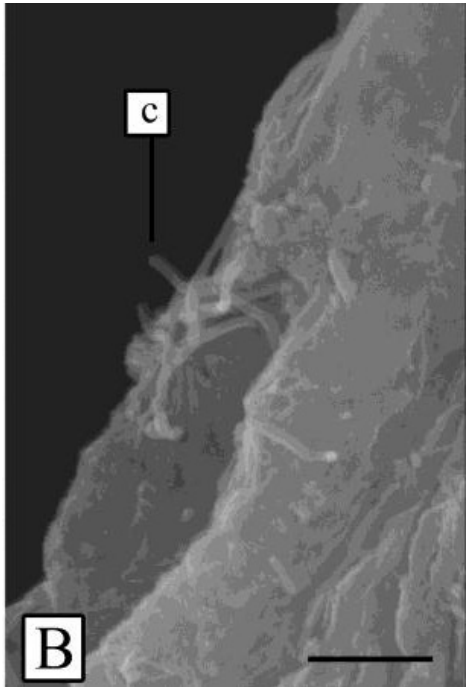
uninfected gut



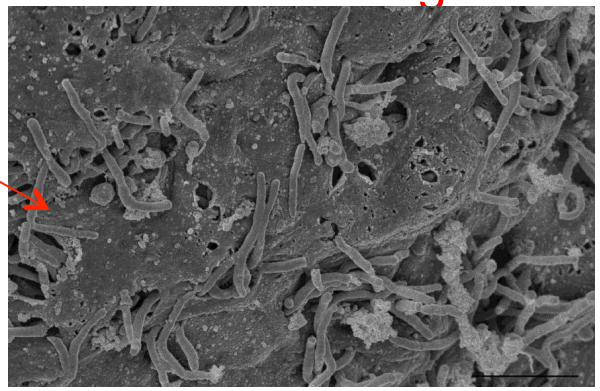
Liberibacter is seen in the esophagus and in the alimentary canal (gut)



infected esophagus



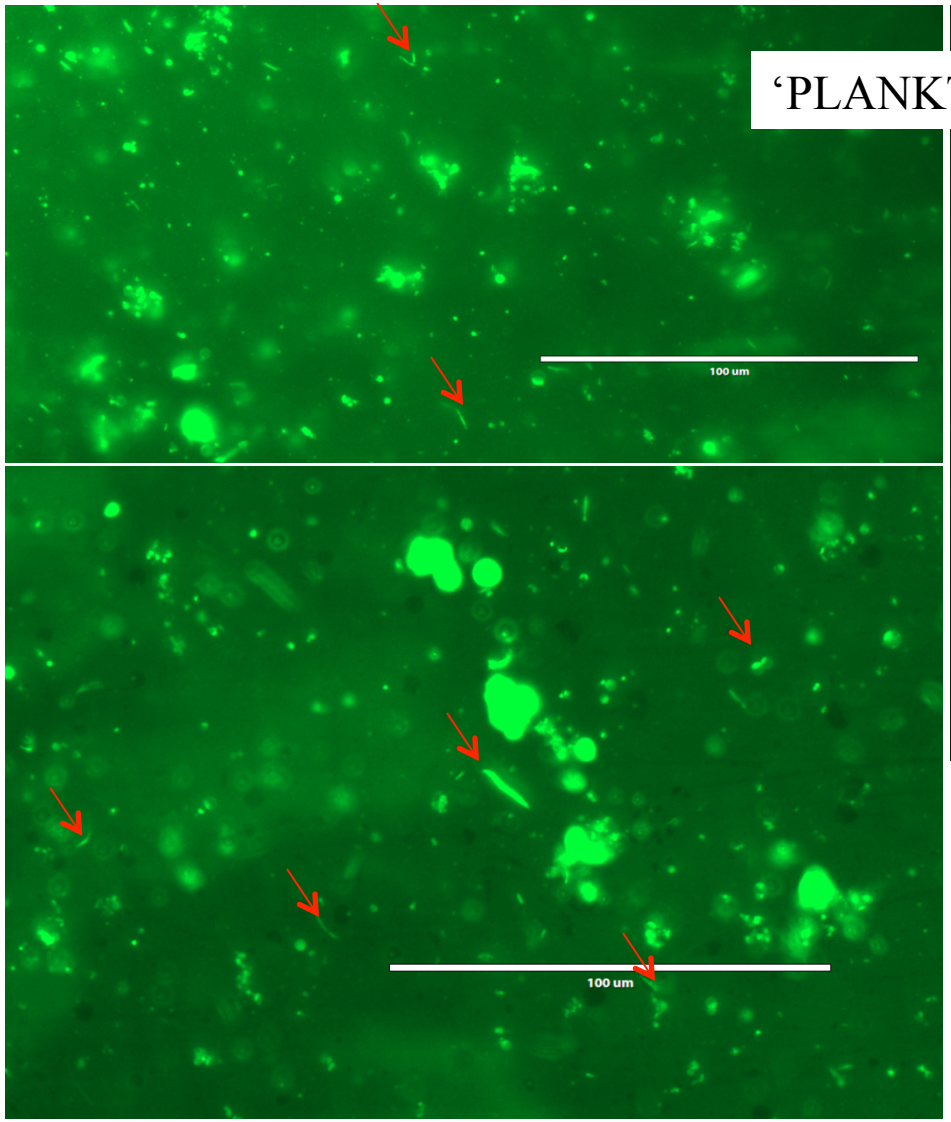
infected gut



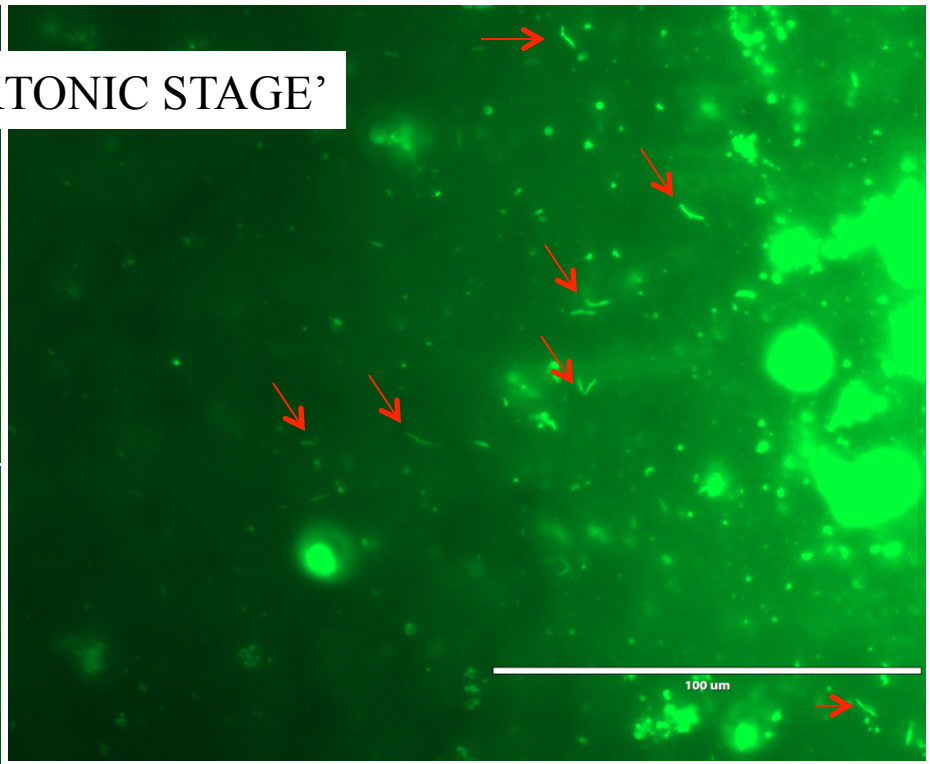
Lso bacteria

Cicero, Brown et al., submitted

Motile stage in SYTO13 stained extracts from infected psyllid gut



‘PLANKTONIC STAGE’



- **Presence of long rod shaped**
- **Liberibacter from infected gut**
- **Evidence of cells dividing?**

(M. Vyas, T. Fisher, J.K. Brown)

Mode of Transmission: *Circulative, propagative*
Pathway - entry via mouthparts, food canal, gut, blood, salivary glands/oral region

Virulence Factors
(some expressed in cell, some excreted, some membrane bound)
adherence/attachment (gut lumen)
biofilm formation formation/colonization (lumen)
quorum sensing (gut lumen)

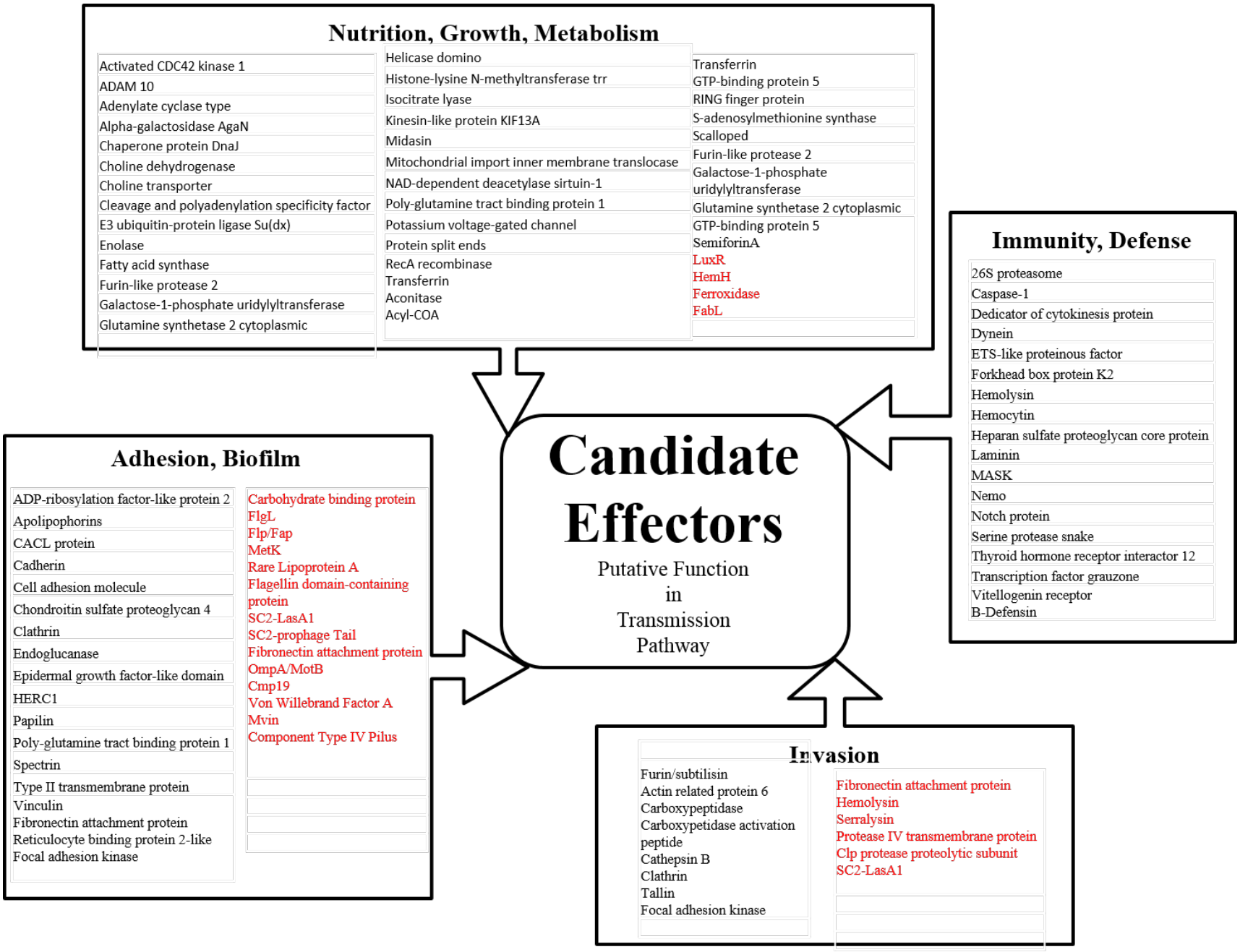
Immune response (psyllid) /immunosuppression/inhibition (Liberibacter)

Multiplication/nutrition (Fe+2, Ca+, energy ATPase)
invasion of epithelial lining /exit

Planktonic stage to establish new biofilms (external surface)/nutrition, multiplication or motile stage in blood to salivary glands / immune response/counter

Salivary gland invasion
multiplication/nutrition?

Summary


Nutrition, Growth, Metabolism
Activated CDC42 kinase 1
ADAM 10
Adenylate cyclase type
Alpha-galactosidase AgaN
Chaperone protein DnaJ
Choline dehydrogenase
Choline transporter
Cleavage and polyadenylation specificity factor
E3 ubiquitin-protein ligase Su(dx)
Enolase
Fatty acid synthase
Furin-like protease 2
Galactose-1-phosphate uridylyltransferase
Glutamine synthetase 2 cytoplasmic
Helicase domino
Histone-lysine N-methyltransferase trr
Isocitrate lyase
Kinesin-like protein KIF13A
Midasin
Mitochondrial import inner membrane translocase
NAD-dependent deacetylase sirtuin-1
Poly-glutamine tract binding protein 1
Potassium voltage-gated channel
Protein split ends
RecA recombinase
Transferrin
Aconitase
Acyl-COA
Transferrin
GTP-binding protein 5
RING finger protein
S-adenosylmethionine synthase
Scalloped
Furin-like protease 2
Galactose-1-phosphate uridylyltransferase
Glutamine synthetase 2 cytoplasmic
GTP-binding protein 5
SemiforinA
LuxR
HemH
Ferroxidase
FabL
Immunity, Defense
26S proteasome
Caspase-1
Dedicator of cytokinesis protein
Dynein
ETS-like proteinous factor
Forkhead box protein K2
Hemolysin
Hemocytin
Heparan sulfate proteoglycan core protein
Laminin
MASK
Nemo
Notch protein
Serine protease snake
Thyroid hormone receptor interactor 12
Transcription factor grauzone
Vitellogenin receptor
B-Defensin
Candidate Effectors
Putative Function in Transmission Pathway
Adhesion, Biofilm
ADP-ribosylation factor-like protein 2
Apolipophorins
CACL protein
Cadherin
Cell adhesion molecule
Chondroitin sulfate proteoglycan 4
Clathrin
Endoglucanase
Epidermal growth factor-like domain
HERC1
Papilin
Poly-glutamine tract binding protein 1
Spectrin
Type II transmembrane protein
Vinculin
Fibronectin attachment protein
Reticulocyte binding protein 2-like
Focal adhesion kinase
Carbohydrate binding protein
FlgL
Flp/Fap
MetK
Rare Lipoprotein A
Flagellin domain-containing protein
SC2-LasA1
SC2-prophage Tail
Fibronectin attachment protein
OmpA/MotB
Cmp19
Von Willebrand Factor A
Mvin
Component Type IV Pilus
Invasion
Furin/subtilisin
Actin related protein 6
Carboxypeptidase
Carboxypetidase activation peptide
Cathepsin B
Clathrin
Tallin
Focal adhesion kinase
Fibronectin attachment protein
Hemolysin
Serralysin
Protease IV transmembrane protein
Clp protease proteolytic subunit
SC2-LasA1

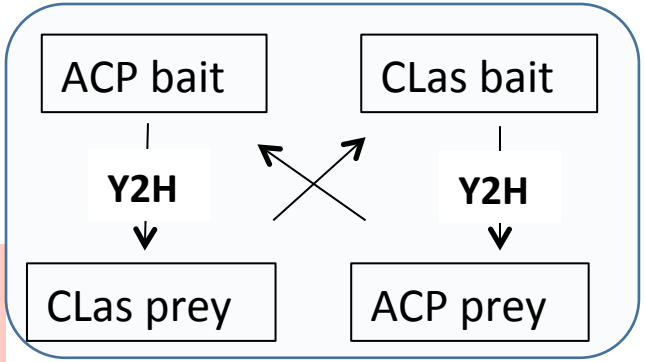
DECISION PIPELINE

Bioinformatics

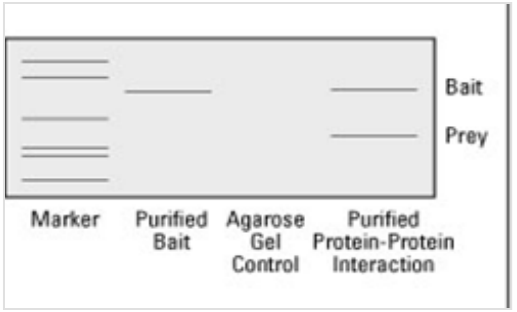
1. Literature searches
2. Psyllid transcriptomes
3. Databases (ex: NCBI)
4. Proteomics
5. Test for interactors

(4) Yeast-2-hybrid interactions - candidates

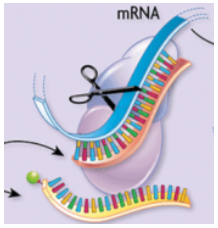
Test *in silico* candidates



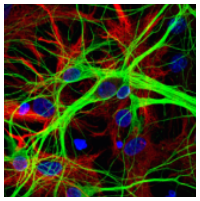
(5) Protein pull downs



RNA Interference (RNAi) in psyllids: knock downs



Transmission Interference without mortality: bioassay

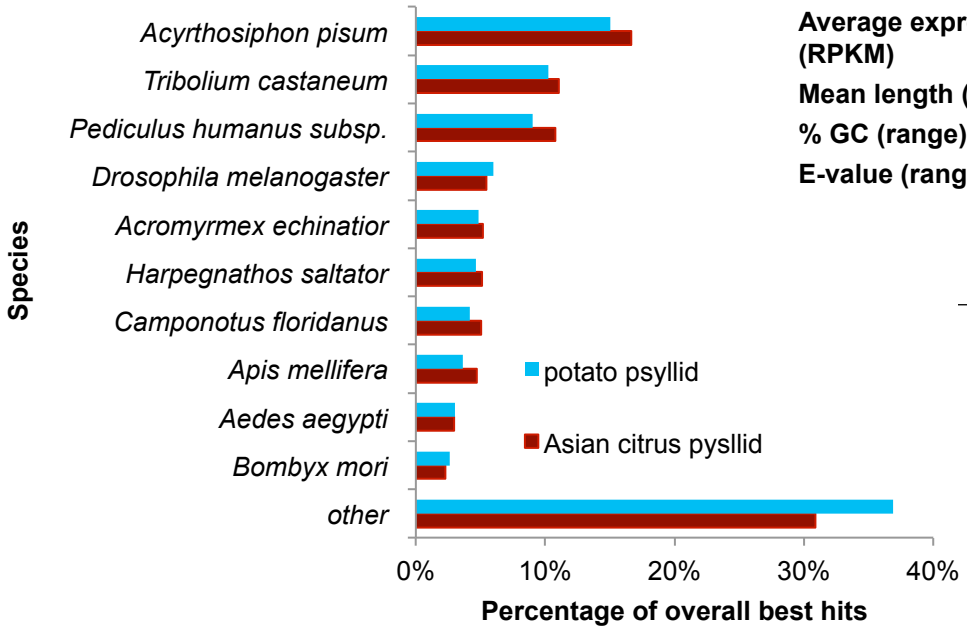


***(6) In vivo* detection of effectors, different FISH tags**

(2) The Transcriptomes

Annotated sequences (NCBI-Inv): 23,646 (51%)
Top hit: *D. citri*

		PoP	ACP
Sequencing (clean reads)	Wb	46,681,564	21,552,866
	WbL	53,240,863	46,865,913
	Ny	43,322,502	32,265,958
	NyL	55,836,522	28,947,167
	Total	199,081,451	129,631,904
Assembly	Total transcripts	82,224	45,976
	Average expression of transcripts (RPKM)	65	149
	Mean length (range) (bp)	651 (100- 27,405)	1,107 (150- 26,540)
	% GC (range)	40.7 (15.6- 82.4)	40.4 (10.4- 77.9)
Annotation	Total annotated transcripts (%)	16,496 (20%)	17,958 (39%)
	Average expression of transcripts (RPKM)	133	142
	Mean length (range) (bp)	1,754 (100- 27,405)	1,980 (150- 26,540)
	% GC (range)	45.3 (18.7- 75)	44.0 (12.3- 77.9)
	E-value (range)	2.5E-12 (0- 1.0E-10)	7.52E-13 (0- 1.0E-13)

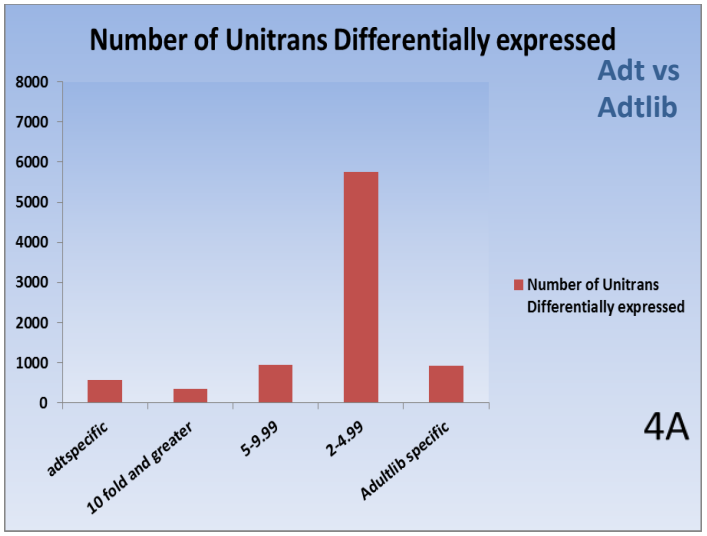


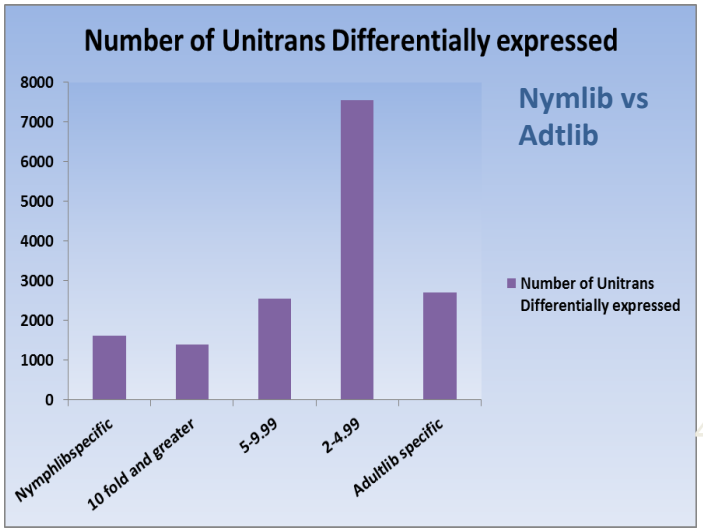
www.sohomoptera.org/ACPPoP
(Fisher et al., 2014; Vyas et al., *In Review*)

		PoP	ACP
Sequencing (clean reads)	Gt	13454866	44758331
	GtL	23929029	30395320
	Sg	241483758	244366344
	SgL	268312490	252770353
	Total	547180143	572290348
Assembly	Total transcripts	110,937	83,231
Annotation	Annotated (%)	20,976 (19%)	26,511 (32%)

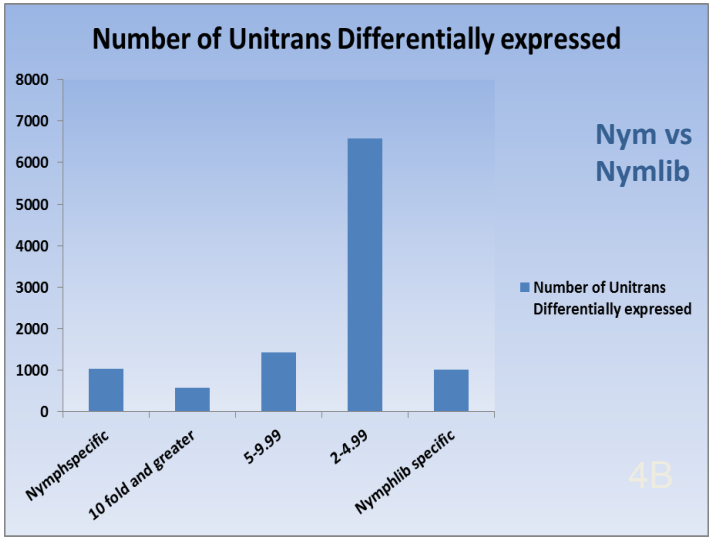
In silico predictions:

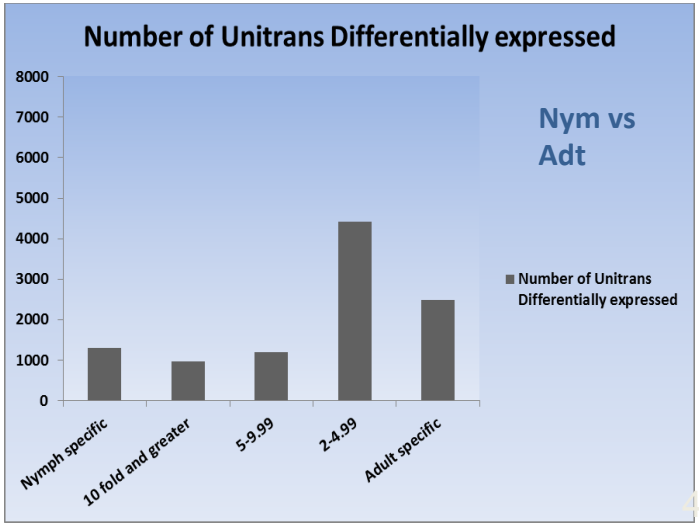
Asian citrus psyllid transcriptome: differential gene expression in infected/uninfected adult & nymph library comparisons; guts & salivary glands





Number of Unitrans

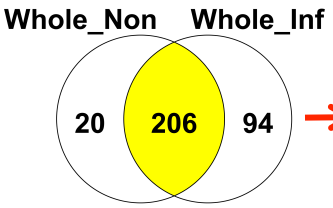


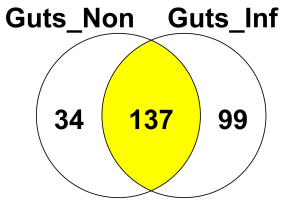


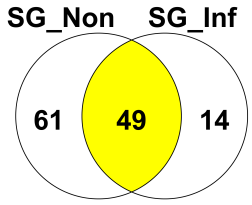
Infected vs Uninfected: Similar 2-5 fold increase abundant; some 5fold; some >10 fold

Results: LC-ESI-MS/MS

	Whole psyllid			Psyllid gut			Psyllid salivary gland		
	Total proteins	#Unique peptides	#Unique spectra	Total proteins	#Unique peptides	#Unique spectra	Total proteins	#Unique peptides	#Unique spectra
Uninfected	220	2382	2843	166	1445	1689	88	654	787
CLso-infected	288	3038	3652	225	2653	2653	57	633	722







Top 10 up-regulated

Transcript Id	Putative Function	Fold Change
BcGS_003899	Endocytosis	7.54
BcWN_12657	Nutrition	7.05
BcWN_03295	Endocytosis	6.98
BcWN_05858	Nutrition	4.10
BcGS_000280	Invasion/Adhesion	3.61
BcWN_08749	Endocytosis	3.52
BcGS_000339	Endocytosis	3.40
BcWN_05291	Stress	3.38
BcWN_07828	Endocytosis	3.29
BcGS_002146	Nutrition	3.28

Top 10 down-regulated

Transcript Id	Putative Function	Fold Change
BcWN_07348	Adhesion	7.8
DcWN_02041	Endocytosis	7.6
BcWN_15755	Unknown	7.2
BcWN_06552	Endocytosis	7.1
BcWN_07337	Nutrition	6.9
BcWN_23316	Stress	5.6
BcWN_81040	Nutrition	4.5
BcWN_13993	Nutrition	3.1
BcWN_07340	Unknown	2.9
DcWN_00198	Endocytosis	2.4

ex. Guts TCW transcriptomics – *in silico*

Biological Process GO levels 1-4 that contain significant number of DE transcripts

Invasion/Defense

Adhesion/Biofilm

GO Num	Level	Description	# Seq	GtGtL
GO:0008152	2	metabolic process	15055	0.0032
GO:0044710	3	single-organism metabolic process	14655	0.0042
GO:0044706	3	multi-multicellular organism process	99	0.04
GO:0044419	3	interspecies interaction between organisms	680	0.0071
GO:0044237	3	cellular metabolic process	13457	0.011
GO:0009058	3	biosynthetic process	8170	0.00074
GO:0044403	4	symbiosis, encompassing mutualism through parasitism	680	0.0071
GO:0006790	4	sulfur compound metabolic process	327	0.0057
GO:0044281	4	small molecule metabolic process	3887	3.80E-07
GO:0044711	4	single-organism biosynthetic process	1094	0.000086
GO:0009636	**4**	**response to toxin**	**341**	**0.038**
GO:0006979	4	response to oxidative stress	548	0.012
GO:0009612	4	response to mechanical stimulus	198	0.017
GO:0010035	4	response to inorganic substance	501	0.012
GO:0009629	4	response to gravity	66	0.028
GO:0072593	4	reactive oxygen species metabolic process	130	0.00049
GO:0015979	4	photosynthesis	72	0.0022
GO:0006793	4	phosphorus metabolic process	3332	0.043
GO:0055114	4	oxidation-reduction process	1015	0.00018
GO:0071704	4	organic substance metabolic process	14340	0.016
GO:0006091	4	generation of precursor metabolites and energy	729	0.024
GO:0071981	4	exit from diapause	53	0.015
GO:0017144	4	drug metabolic process	144	9.20E-10
GO:0051186	4	cofactor metabolic process	597	0.0013
GO:0044249	4	cellular biosynthetic process	7948	0.00066
GO:0016337	**4**	**cell-cell adhesion**	**681**	**0.0069**
GO:0071554	4	cell wall organization or biogenesis	82	0.000006

ex. SGs TCW transcriptomics

13 out of the 17 (76%) level 2 'Biological Process' GO categories contain a significant number of DE transcripts, compared to only 1 in gut comparisons

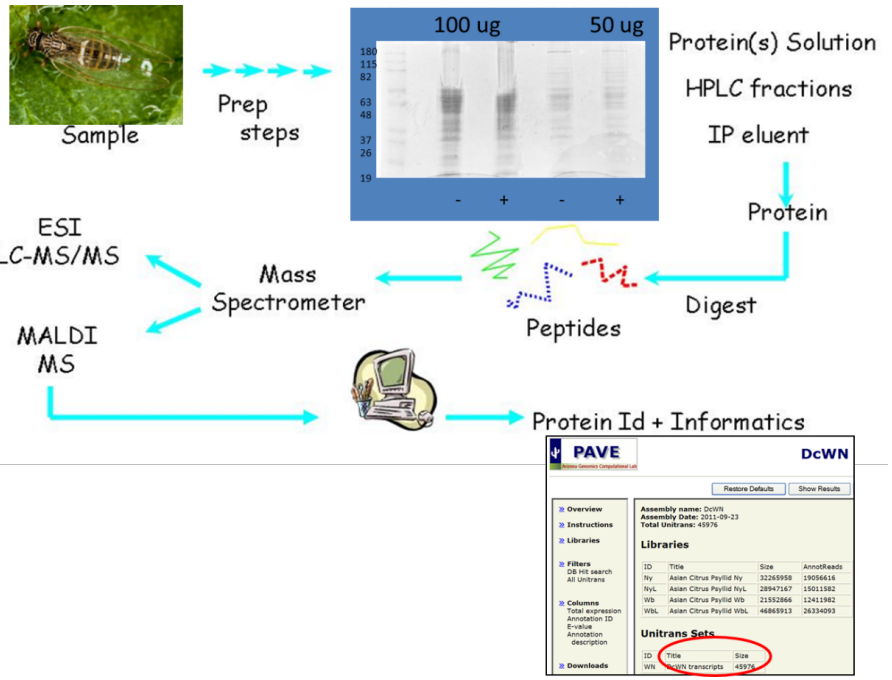
Nutrition

Immune/Defense

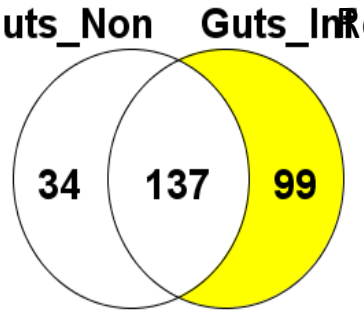
Invasion

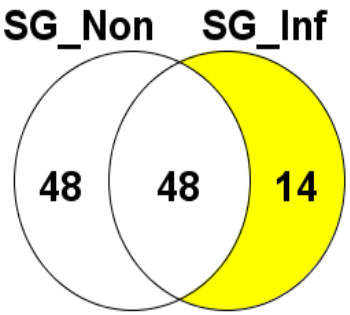
Go Num	Level	Description	# Seq	SgSgL
GO:0065007	2	biological regulation	11345	6.30E-24
GO:0009987	2	cellular process	18843	0.00003
GO:0016265	2	death	2023	0.019
GO:0032502	2	developmental process	10916	3.40E-38
GO:0040007	**2**	**growth**	**3602**	**0.013**
GO:0002376	**2**	**immune system process**	**1374**	**1.70E-03**
GO:0051179	**2**	**localization**	**7536**	**2.10E-26**
GO:0040011	2	locomotion	5016	0.0013
GO:0051704	2	multi-organism process	9034	2.40E-21
GO:0032501	2	multicellular organismal process	11227	2.00E-33
GO:0000003	2	reproduction	7409	4.30E-15
GO:0050896	2	response to stimulus	9327	8.5E-06
GO:0044699	2	single-organism process	12622	8.20E-24

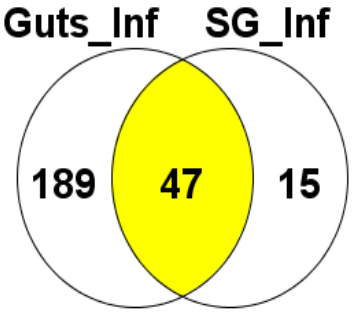
(3) Proteomics = protein ID



Results:





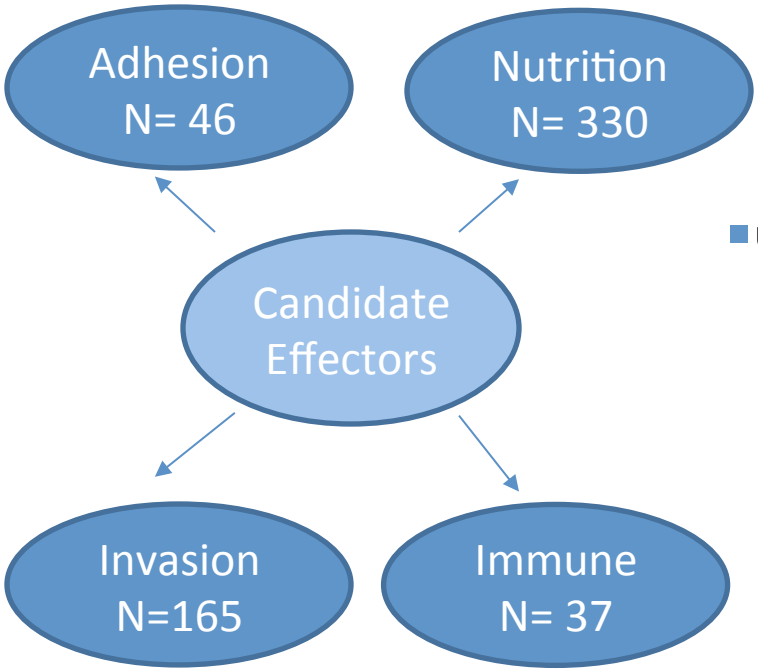


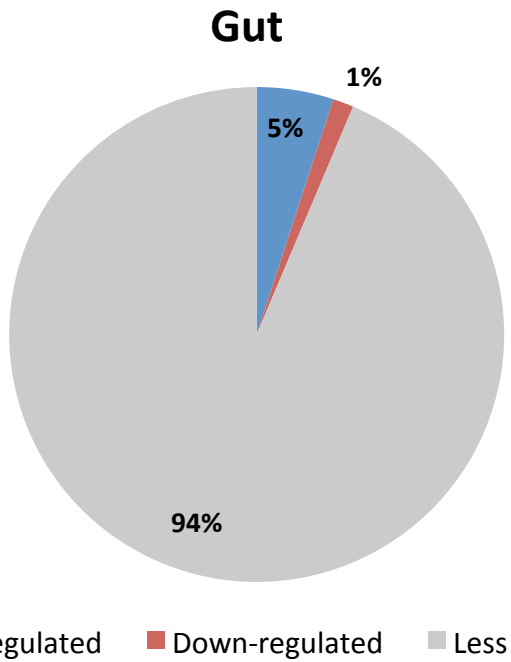
The majority of proteins found to be present in both the infected guts and salivary glands have putative functions associated with <u>invasion of host tissues</u>.

Results corroborated by *in silico* (TCW) comparative transcriptomics.

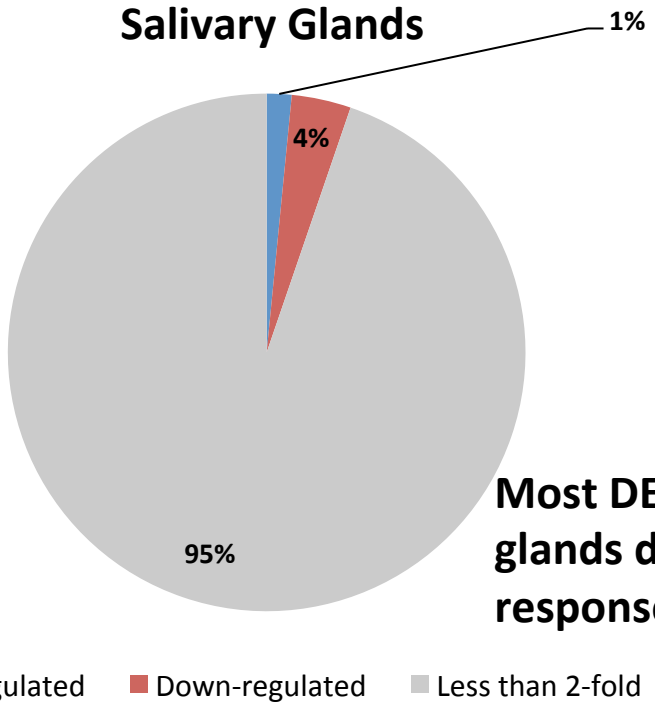
Collectively, these **two lines of evidence** are guiding the selection of potentially important effectors for systematic assessment in the decision pipeline.

Most transcripts in gut and salivary gland affected less than 2-fold by Liberibacter infection

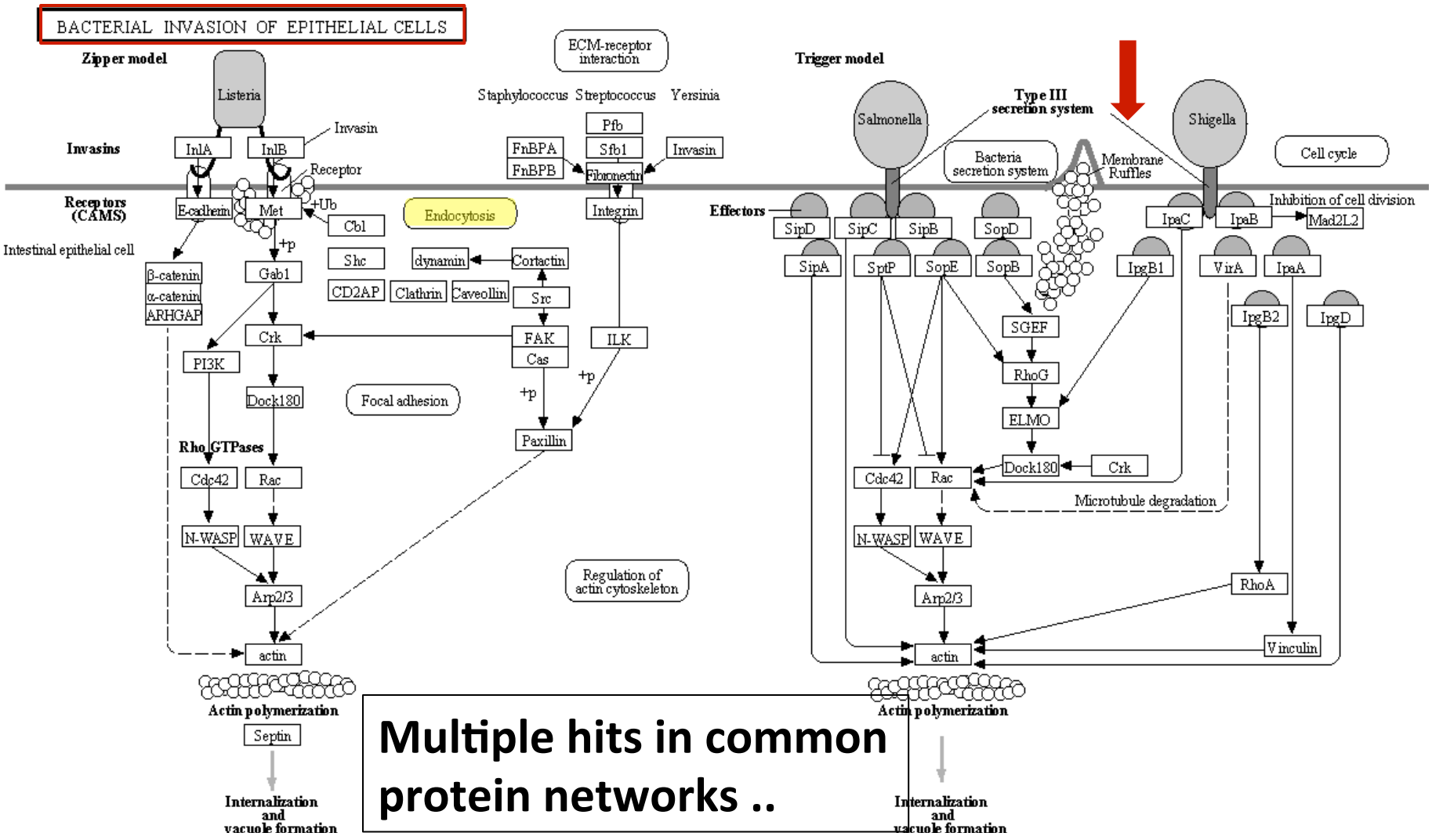




Most DE transcripts in gut up-regulated in response to CLas

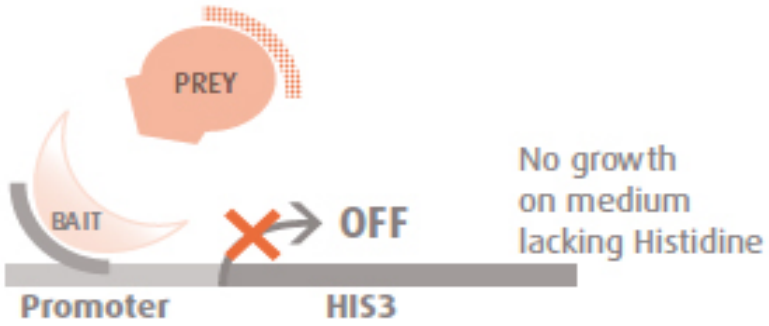


Most DE transcripts in salivary glands down-regulated in response to CLas

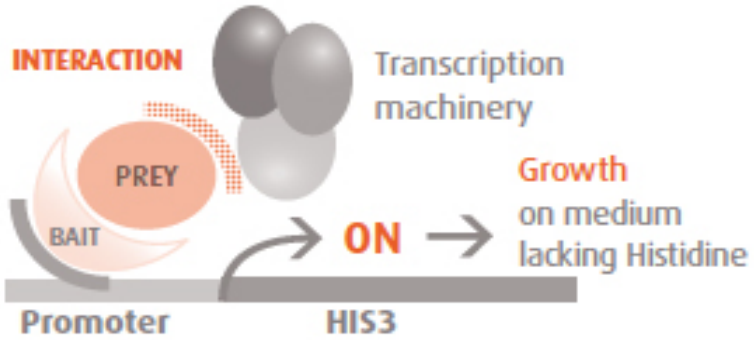


- Endo-exocyotic pathways, including phagocytosis, are known to be hijacked by pathogens to enable invasion of host tissues.
- Transcripts involved in these pathways are present in the transcriptome, and many are significantly expressed.

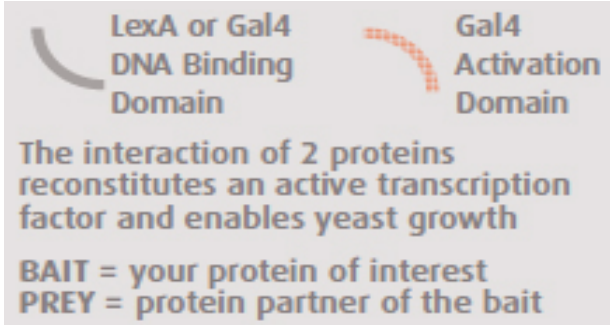
(4) Yeast two hybrid system



If the prey doesn't catch the bait, no transcription occurs.



If the bait protein interacts with the prey protein, the transcription factor's activator domain binds to the binding domain, and transcription occurs.



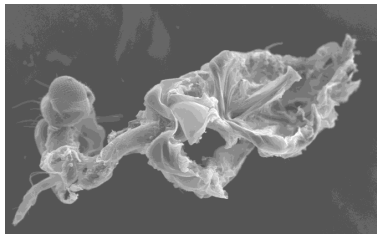
3 "Prey" libraries

uninfected-ACP

1. Gut

Cicero *et al.* 2009

2. Salivary glands



3. CLas

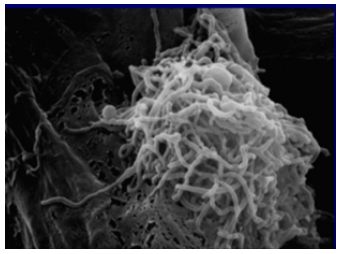


Photo courtesy of Mike Davis

Most Promising Results from CLas "Bait" mated against ACP "Prey" Libraries

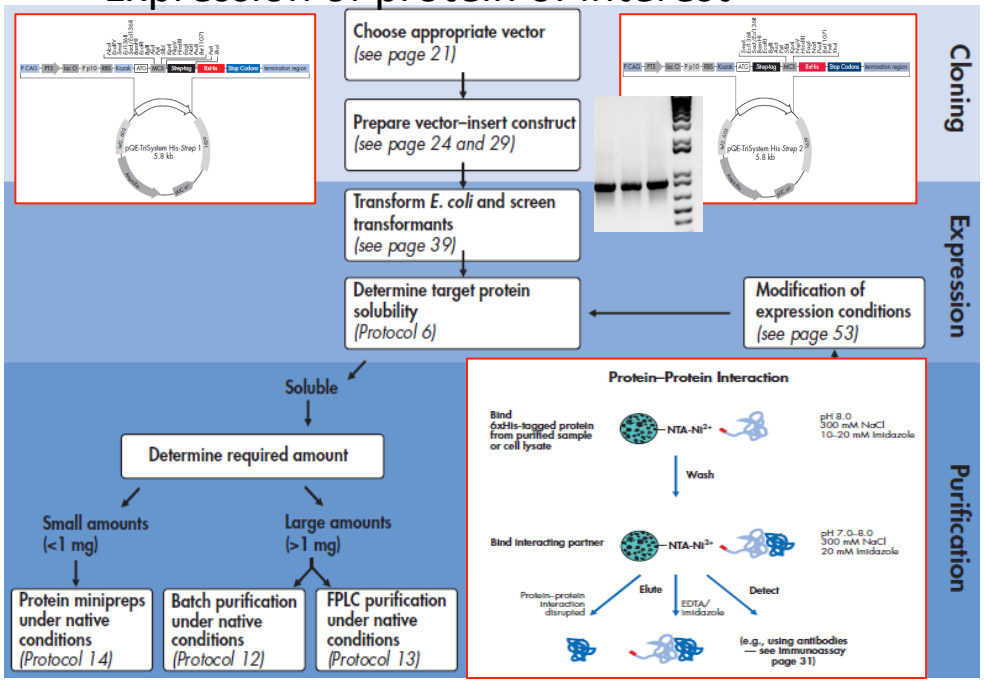
Mating No.	Bait (against ACP libraries)	Biologically Relevant Prey
1	Adhesion	2-Adhesion
2	Adhesion	1-Transport
3	Adhesion	1-Defense
4	Adhesion	1-Adhesion, 1-Defense, 2-Invasion , 1-Transport (Endocytosis)
6	Adhesion	1-Adhesion, 1-Defense, 1-Nutrition
7	Adhesion	1-Invasion
8	Invasion	1-Adhesion, 1-Nutrition
9	Invasion	1-Adhesion, 1-Invasion (Endocytosis)
10	Adhesion (Biofilm)	1-Defense, 1-Invasion
11	Adhesion	1-Invasion
12	Adhesion	1-Invasion
16	Invasion	1-Nutrition
17	Invasion	1-Invasion (Endocytosis)
18	Invasion	1-Invasion (Endocytosis)
19	Invasion	1-Invasion (Endocytosis)
20	Invasion	1-Invasion (Endocytosis)
21	Invasion	1-Invasion (Endocytosis)
22	Invasion	1-Invasion (Endocytosis)
23	Invasion	1-Invasion (Endocytosis)
24	Invasion	2-Adhesion, 1-Defense, 1-Invasion

Most Promising Results from ACP "Bait" mated against CLas"Prey" Libraries

Mating No.	Bait (against CLas library)	Biologically Relevant Prey
8	Adhesion	1-Adhesion (Biofilm), 1-Defense
9	Defense	1-Adhesion, 1-Defense, 1-Nutrition
11	Invasion (Endocytosis)	2-Adhesion, 1-Defense, 2-Invasion
15	Adhesion	1-Defense
17	Adhesion	1-Invasion
19	Adhesion	1-Defense
20	Invasion	1-Invasion
25	Invasion (Endocytosis)	1-Virulence

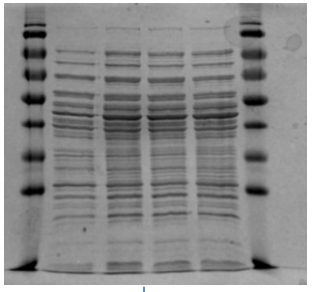
Protein expression and Co-Immunoprecipitation

Expression of protein of interest



Crude psyllid extract

- Twenty-five whole psyllids will be added to 200 microliters of pre-cooled buffer (*Strep*-tag®) with PIC.
- After sonication on ice, the homogenate is centrifuged for 20 min on max speed at 4°C.
- Supernatant contains soluble protein fractions and is mixed with protein of interest.

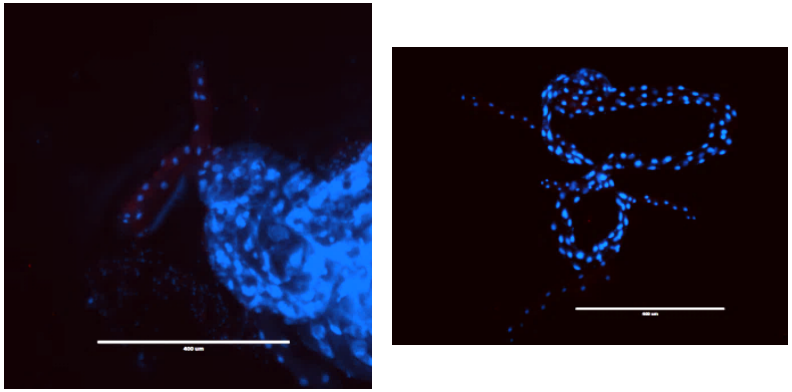


Identification of interacting components
LC/MS

Localization microscopy

Goal: Visualize gene expression patterns of effectors under normal conditions and after mRNA knockdown by RNAi

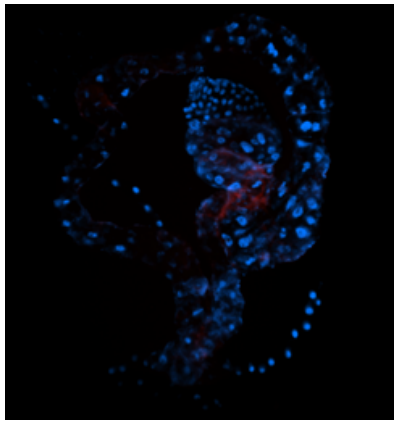
Results: Confirmed Carnoy's fixative and Cy5 fluorophore gives best results (no autofluorescence of non-infected tissues)



Antibody (Abcam)

*Anti-OMP antibody ab93127

*Designed for CLas, poor results may indicate non-specificity to CLso

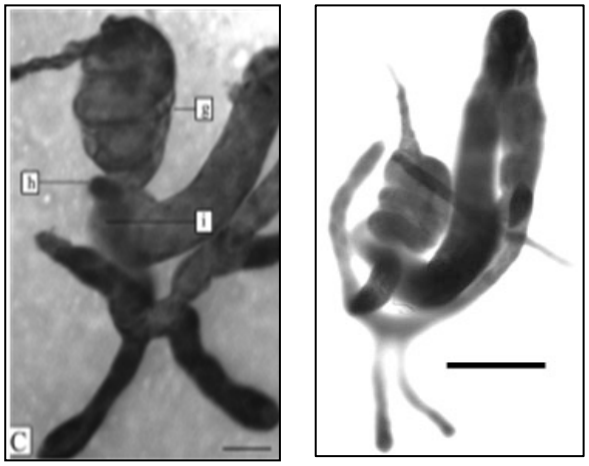


Oligonucleotide probe (Invitrogen)

CLso-specific 16rRNA

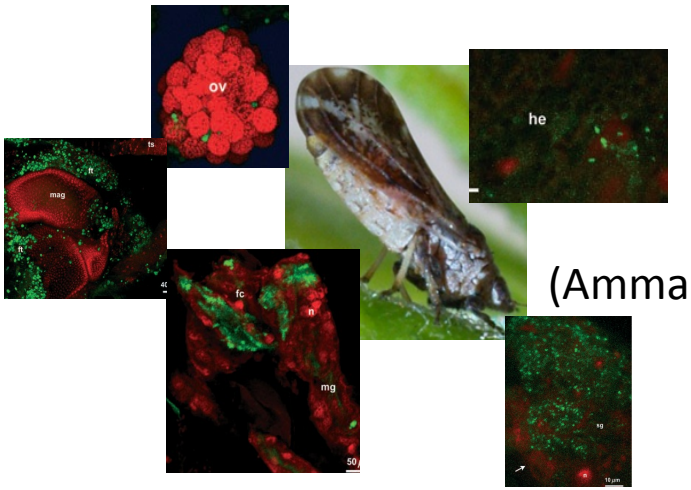
RNAi system

- ✓ **Alimentary canal and Liberibacter localization through circulative transmission pathway of ACP and PoP are very similar**



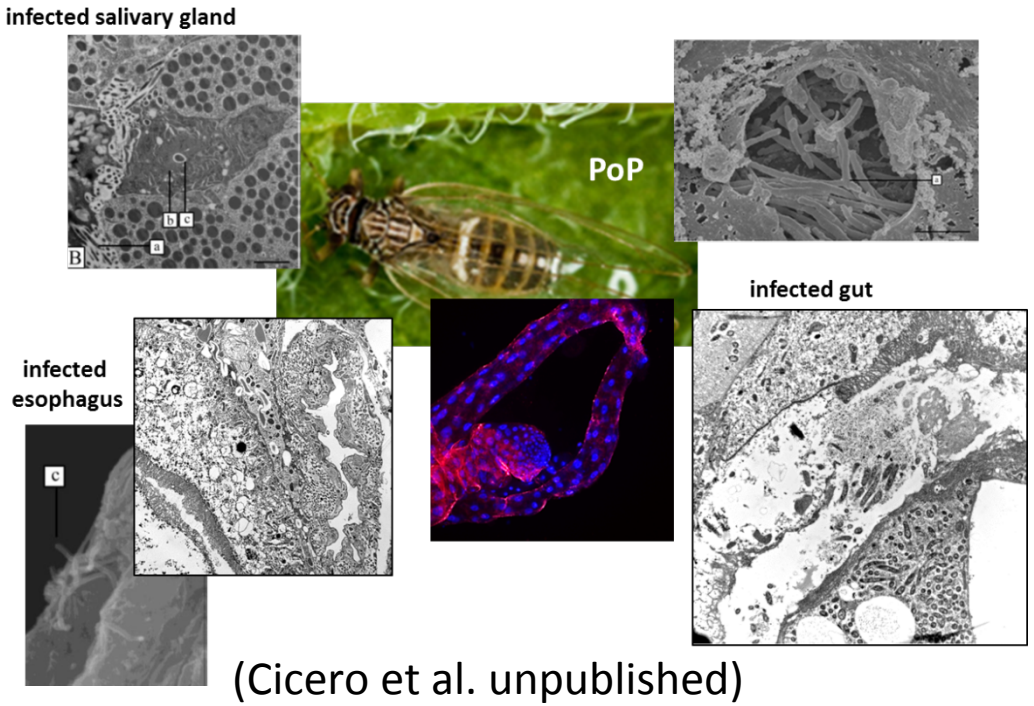
PoP ACP

(Cicero et al. 2009)



ACP

(Ammar et al. 2011)

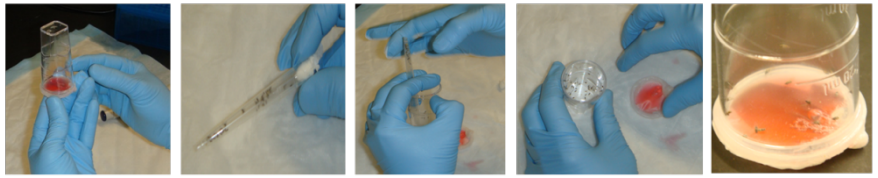


(Cicero et al. unpublished)

RNAi-strategy

Feeding assembly for oral-delivery of dsRNA

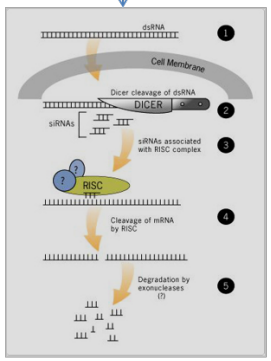
Feeding chamber ring | Stretching parafilm layer | Attaching to ring | Filling feeding solution | Solution layer



Feeding assembly ready | Psyllids transferred to feeding assembly | The experiment

dsRNA against target effector

300µl with 100 ng of dsRNA
3d feeding



* qPCR to measure gene knockdown

- After 3d feed
- Internal control psyllid 18S rDNA
- TaqMan probe technologies

Knockdown infected psyllids

5 psyllids per plant
14 d IAP

uninfected indicator plants

PCR for transmission efficiency
* conventional PCR with LibOA2/Ol2c primers

100% transmission control psyllids

~50% transmission control psyllids

5 psyllids per plant
21 d IAP

Infected psyllids

Infected source plant

4d AAP

Knockdown uninfected psyllids

RNAi results for uninfected psyllid for transmission bioassays

62% of genes tested (5 out of 8) reduced transmission in 'newly infected' (4d AAP) psyllids.

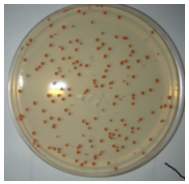
RNAi results for Clas-infected psyllid for transmission bioassays

25% of genes tested (3 out of 12) reduced transmission in psyllids born and reared on infected plants.

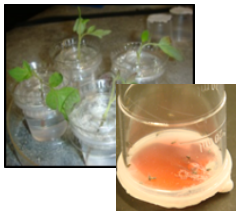
Summary

- **Transcriptomics-** More than 80,000 transcripts of which 32% (26,511) are annotated. Many showing significant differential expression in response to CLas. All assembled into a user-friendly platform to identify life stage- and tissue-specific candidate effectors important in Clas-ACP.

- **Proteomics-** More than psyllid 300 proteins identified using ACP transcripts. Proteins were isolated from whole body adults, midguts and salivary glands allowing for the identification of candidate effectors based on variation in abundance in response to CLas infection as well tissue-specificity.



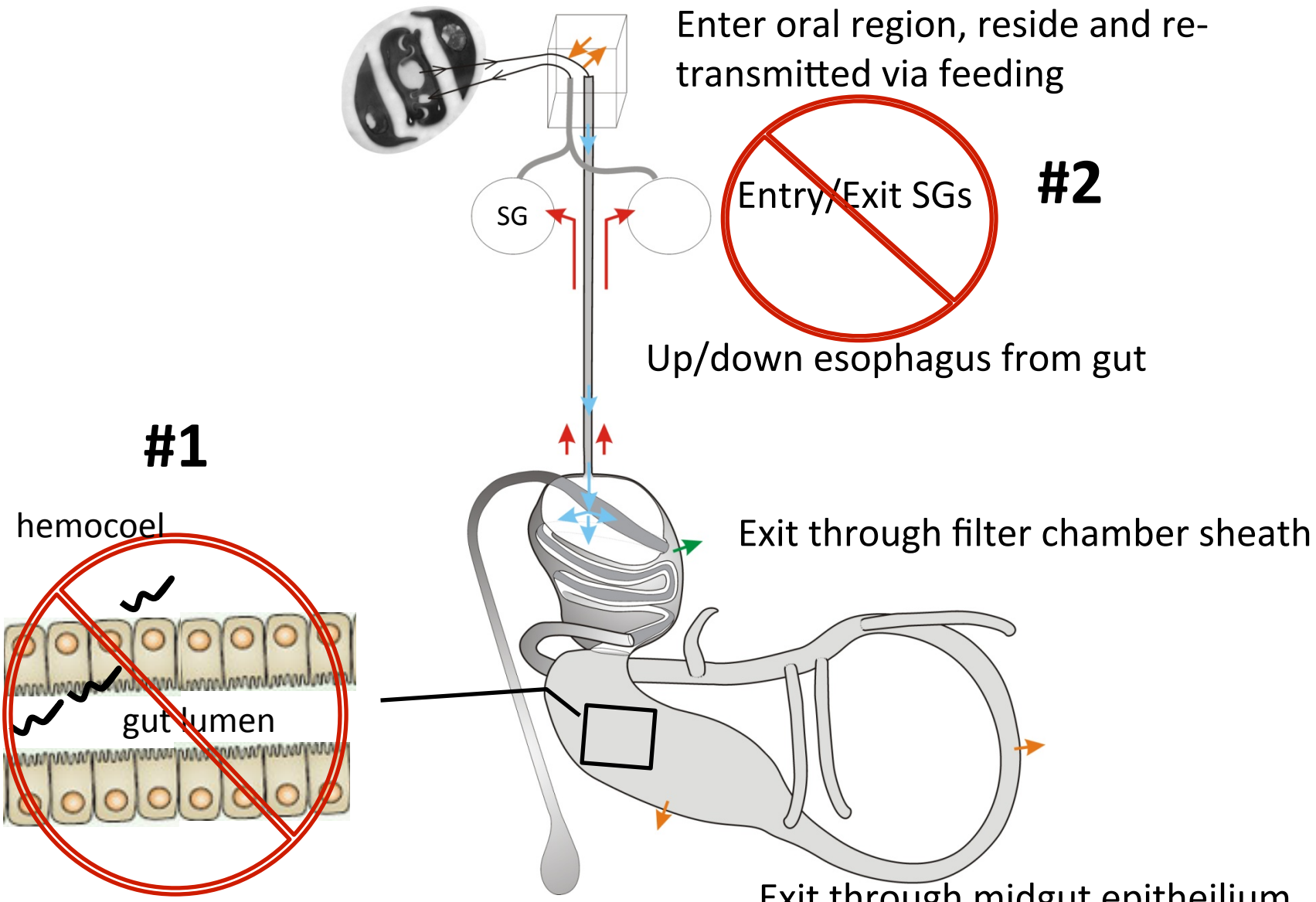
- **Yeast 2 Hybrid system:** the' interactions' of 22 genes (14 CLas genes) (8 ACP genes) reveal a model of pathogen invasion know in other bacterial pathosystems.



- **RNAi/Transmission Bioassay**: 17 interactors (genes/proteins) have been tested: transmission abatement ranges from 18-55%.
- Several are very promising as transgenic psyllid candidates:

Enolase (last step in glycolysis: 2-Phosphoglycerate to phosphoenolpyruvate) – muscles/ glycogen; Clathrin (coated vesicles/within cell transport); membrane ruffling effectors

Endocytosis: One target, Two Hits?



Acknowledgements

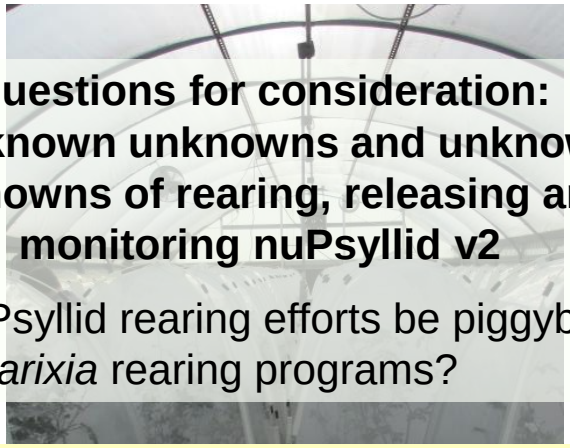
UA Brown Lab: Dr. Tonja Fisher, Tim Rast, M. Vyas
UA: Dr. Carol Soderlund & Dr. William Nelson (TCW db)
UA proteomics: Linda Breci

Dr. David Gang & Dr. Ruifeng He (WSU)

Support for aspects of this research was/is provided by

USDA-NIFA Nu-Psyllid CAPS project

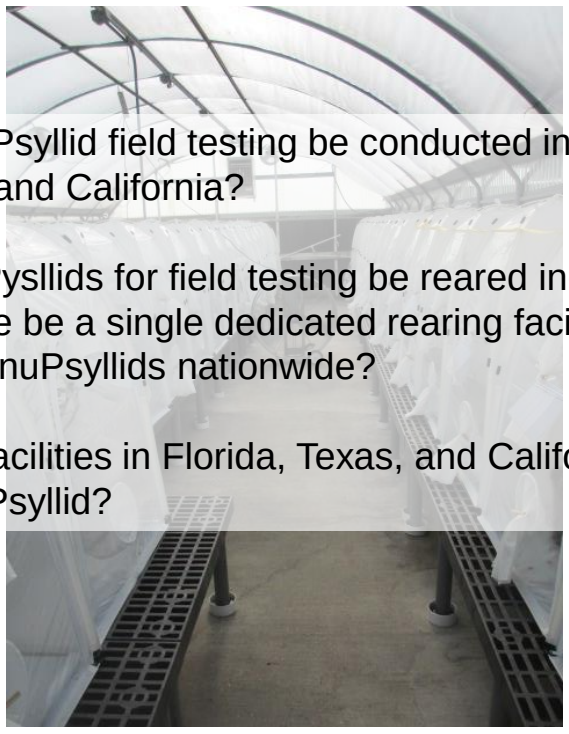
CRDF Citrus Research and Development Foundation, Inc.



Questions for consideration: The known unknowns and unknown unknowns of rearing, releasing and monitoring nuPsyllid v2

1. Will nuPsyllid rearing efforts be piggybacked on *Tamarixia* rearing programs?

Is the state-of-the art sufficient for producing WT ACP?
How nimble are we with respect to adapting rearing technology to a nuPsyllid with a certain level of fitness costs due to effector or driver mechanisms?
(*At least for initial field trials*)



2. Will nuPsyllid field testing be conducted in Florida, Texas, and California?

3. Will nuPsyllids for field testing be reared in-state or will there be a single dedicated rearing facility to provide nuPsyllids nationwide?

4. Which facilities in Florida, Texas, and California will rear nuPsyllid?

5. Can we ascertain whether there will be fitness cost(s) to nuPsyllids in terms of rearing them?

Can mass rearing of nuPsyllid be accomplished with current rearing techniques (i.e., plant-based) or will artificial diets be required to introduce the driver?

If artificial diet or some artificial diet phase is needed, how much research is needed to accomplish this?

Is this feasible considering the state-of-art and existing facilities?

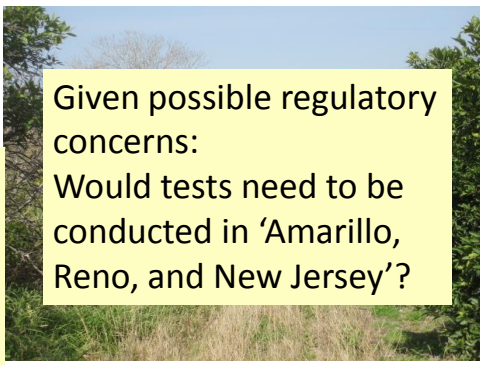
6. What does a nuPsyllid field test look like?

Type of planting array?
'All edge'? 'Blocks'? Size?

Designed for proof-of-concept success v. real world?

Conducted at a research farm or commercial site?

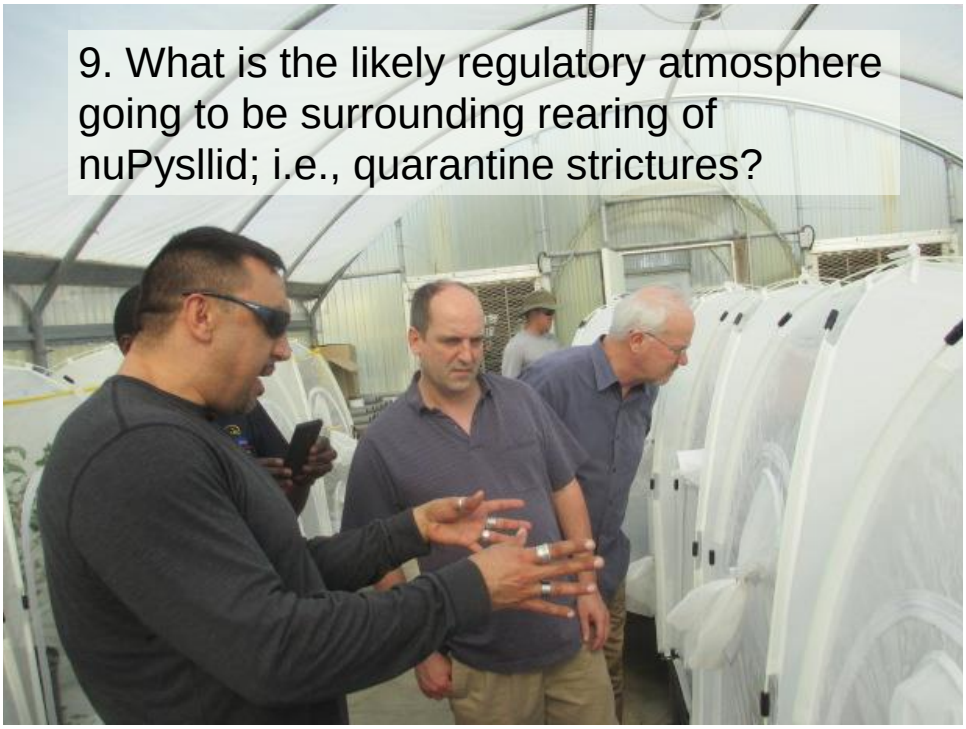
Is there a release model for us to follow? Oxitech?



Given possible regulatory concerns:
Would tests need to be conducted in 'Amarillo, Reno, and New Jersey'?

7. Can we estimate how many nuPsyllids would be needed to run a single field test?

Need to achieve 50% replacement of WT With nuPsyllid.
-Introduce following insecticide treatment
-Northern sites, introduce WT, then follow up with releases of nuPsyllid


9. What is the likely regulatory atmosphere going to be surrounding rearing of nuPysllid; i.e., quarantine strictures?


10. How will these questions be decided?

Plant transformation in the laboratory: Embryos are extracted from seeds and maintained on prepared culture media (unflavored gelatin fortified with essential nutrients). A bacterium, Agrobacterium*, is typically used to insert the target gene into the tender plant tissue. Marker genes for fluorescence or antibiotic activity are used to determine whether the gene was successfully inserted. Hundreds of transformation events are necessary to obtain only a handful of viable genetically modified seedlings.*

Photo credit: Maria Oliveira, Ph.D., USDA-ARS

GENETIC ENGINEERING TO PROTECT CITRUS FROM HLB

Carrie Teiken, Peggy Lemaux, Beth Grafton-Cardwell and Neil McRoberts

This past summer, the Citrus Research Board (CRB) and University of California Cooperative Extension hosted citrus grower seminars in Exeter, Riverside and Santa Paula, California. A range of topics was covered – including export challenges due to plant disease, strategies for dealing with water shortages, labor issues facing the California citrus industry, and the potential for using genetically engineered organisms to control the deadly citrus disease, huanglongbing (HLB).

A cure for HLB has not been identified, and all citrus varieties are susceptible to the disease. This is an issue of extreme importance for California citrus growers. Although to date only one HLB-infected tree has been identified in California, the Asian citrus psyllid (ACP), the insect that vectors the bacteria causing HLB, has spread throughout Southern California, is working its way up the coast and has been found in small numbers in the San Joaquin Valley, where 75 percent of commercial citrus is grown.

An important way to stop the spread of HLB is to stop the ACP; however, that is easier said than done. Natural enemies, such as parasites and predators, can reduce psyllid populations, but they do not eliminate the entire pest population; and so the disease continues to spread. Continuous broad-spectrum insecticide treatments can reduce psyllids to very low levels. However, even these treatments do not completely eliminate psyllids, and they are not economically and environmentally sustainable. Lastly, there are limited choices and problems with efficacy of insecticides for organic growers. Long-term solutions are needed, and these may include engineering a citrus tree that can withstand the pathogen and/or a psyllid that cannot transmit the disease. The industry is now faced with the decision as to whether or not an engineering solution should be employed to save California citrus.

ADDRESSING GE SOLUTIONS

Peggy Lemaux, Ph.D., spoke at the Santa Paula and Exeter grower seminars and addressed the topic of engineering citrus or ACP during her presentation, "Food fights in the marketplace: is there a path forward for citrus to address HLB disease." Genetically engineered (GE) crops (also called GMOs or genetically modified organisms) are already being grown commercially in the U.S. with crops like alfalfa, canola, corn, cotton, soybean, papaya and sugar beet; and GE acreages for most of these are above 90 percent.

Although widely grown, GE crops have not been widely accepted in California, leading to county-based bans on growth and propagation of such crops. In California and other states, there have been efforts to pass laws that would require labels on foods containing an engineered ingredient. Using the term "genetic modification" to describe these newly-engineered crops adds to the confusion, because classical breeding (which has long been used to alter the genetic information in crop varieties) also results in modification of the genetic material of the plant. GE crops are modified using some of the same mechanisms used during breeding to change traits of a crop, but the modifications are performed in the laboratory and then reintroduced into the plant.

Currently, genetic engineering for HLB resistance is focused on a number of approaches: GE citrus trees that are resistant to the bacterium, GE citrus trees that kill ACP when it feeds on the tree, and GE ACPs that are unable to vector the bacterium, '*Candidatus* liberibacter asiaticus' (CLas), that is closely associated with HLB. These technologies not only have the potential to save the citrus industry, but also will help growers reduce the number of pesticide applications used to control ACP, thereby reducing costs and increasing profits. Cutting back on insecticides will help growers maintain an integrated pest management program for all citrus pests and reduce pesticide resistance, secondary pest outbreaks and risks to the environment and workers.

However, GE organisms are often met with grower and general public apprehension. Concerns range from export issues (because some countries don't accept engineered crops), impacts on non-target organisms, movement of engineered genes to unintended crops and allergenicity caused by introduced genes. Yet, GE approaches will quite possibly be a component of the long-term solution for the HLB crisis.

CREATING A "nuPsyllid"

The federally-funded USDA National Institute of Food and Agriculture-Cooperative Agriculture Pest Survey's "nuPsyllid" project is a multiple research laboratory effort to engineer ACP and create a "nuPsyllid" that would replace the wild type ACP with a population that cannot transmit the HLB-associated bacterium HLB. The "nuPsyllid" non-vector then would be released into the ACP population, much like the release of *Tamarixia*, the parasitic wasp, and eliminate the wild ACP population.

Three methods currently are being studied to potentially modify the ACP. Bryce Falk, Ph.D., at the University of California Davis, is identifying naturally occurring ACP viruses. He then plans to genetically modify one of the psyllid viruses so that it will disrupt an essential function of the ACP, causing the psyllid to die or be unable to transmit CLas. Kirsten Pelz-Stelinski, Ph.D., at the University of Florida, is studying strains of *Wolbachia*, a bacterium that occurs naturally inside the body of many different types of insects. She plans to infect ACP with natural, foreign or altered *Wolbachia* to reduce the ACP's ability to transmit the bacteria. The third ACP modification is being investigated by Bruce Hay, Ph.D., at CalTech. Hay is working on creating a modified ACP that has a genetic element containing a toxin that kills the HLB-associated bacteria.

Several members working on the "nuPsyllid" project, including Neil McRoberts, Ph.D., University of California Davis Assistant Professor of Plant Pathology; Elizabeth Grafton-Cardwell, Ph.D., Director of the Lindcove Research and Extension Center and University of California Riverside IPM Specialist; and Carrie Teiken, University of California Davis Plant Pathology graduate student, are involved with investigating the socio-ecological consequences of engineering ACP. If a "nuPsyllid" engineering approach is successful, there likely will be reluctance to accept the altered psyllid, within both urban and grower communities, due to a variety of concerns. These concerns include the movement of introduced genes to other insects, consumer acceptance of oranges exposed to "nuPsyllid," potential damage to the crop by released psyllids and regulatory issues for organic citrus production. Therefore, the "nuPsyllid" Socio-economics and Modeling Team is evaluating how to effectively disseminate information on genetic engineering approaches to the citrus industry and provide them with an understanding of the potential long-term benefits and risks of the project.

SURVEYING THE INDUSTRY

The team began this evaluation task by individually surveying growers, pest control advisors and others who attended the March 2014 Citrus Showcase in Visalia, California, hosted by California Citrus Mutual with special presentations by the CRB. Attendees at the citrus grower seminars in Exeter and Santa Paula were given a similar survey, but were able to answer the questions with 'clickers' (handheld electronic transmitters). The clicker survey posed multiple-choice questions projected on a screen. Each participant then submitted their answers using the clicker, beaming a signal to the presentation computer, which collected the participants' answers and produced a chart that showed immediately how many participants chose each answer. The results of the survey were anonymous. A total of 259 responses were recorded: 46 at the Visalia Citrus Showcase, 42 in Santa Paula and 171 in Exeter.

Survey questions included information on citrus acreage grown or managed, age of participant, and their opinion on using genetic engineering to prevent HLB from spreading in California citrus. The survey also asked which type of engineering approach growers preferred: GE citrus trees that resist the disease, GE trees that kill the ACP when they feed, released GE ACPs that don't spread HLB, or none of the above. The last question asked growers to select what they believe is the biggest impediment to using GE approaches to manage ACP and HLB. Choices included grower acceptance, public acceptance, government approval or "I don't know."

KEY SURVEY FINDINGS:

• **What size are citrus farms?** The majority who were surveyed at the Visalia Showcase (63 percent) and the Santa Paula meeting (76 percent) farmed less than 100 acres of citrus. In Exeter, there were similar proportions of growers with less than 100 acres (39 percent) and those with more than 500 acres (37 percent). The remainder (24 percent) farmed between 100–500 acres **(Figure 1)**.

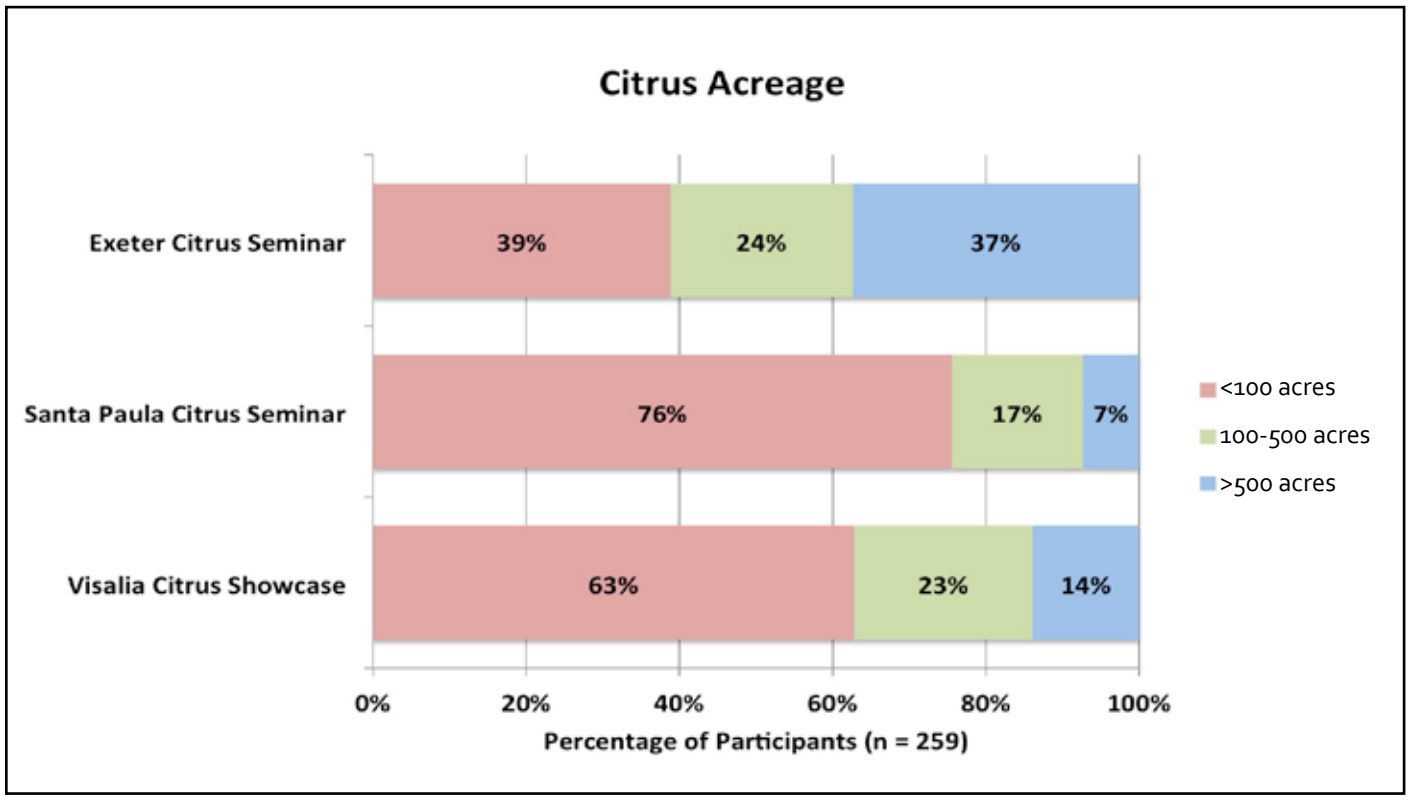


Figure 1: Number of acres of citrus grown.

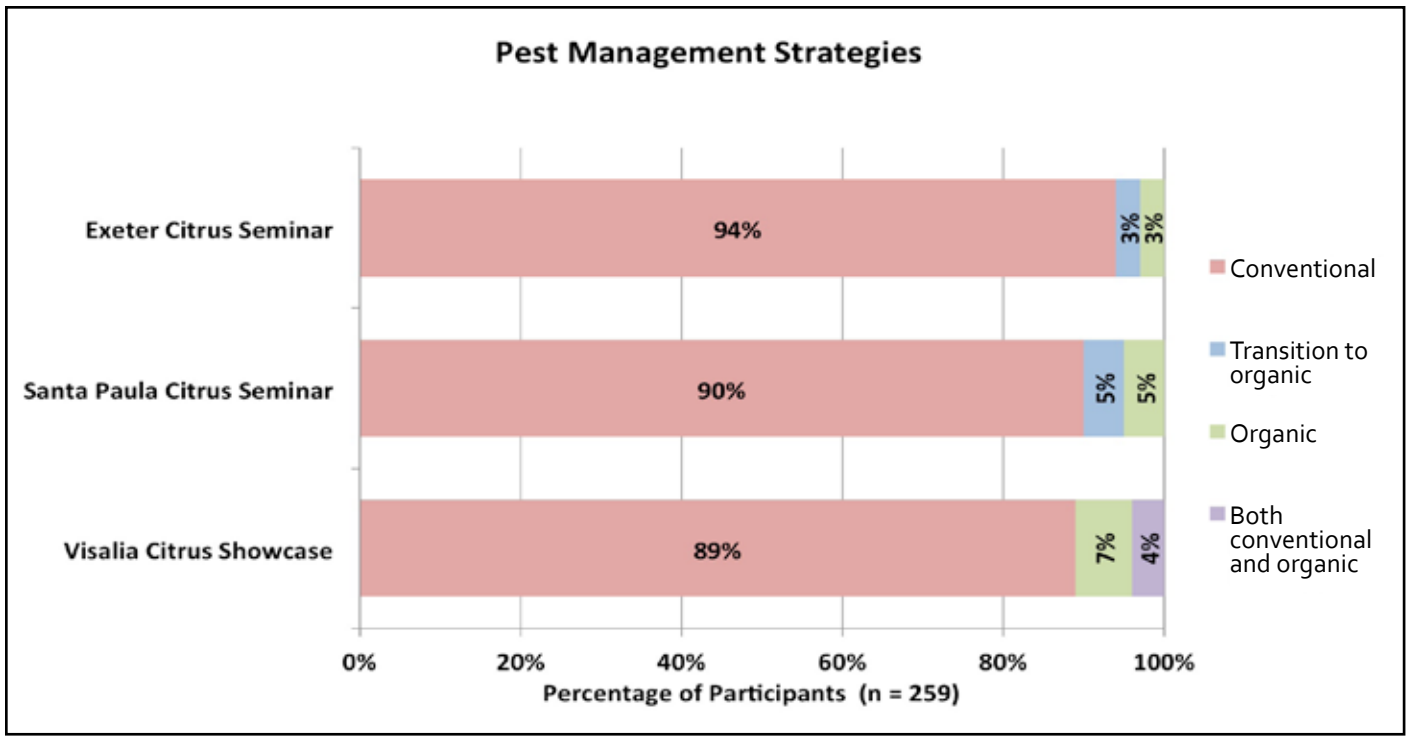


Figure 2: Proportion of growers practicing conventional, transitional and organic pest management strategies.

• **What types of citrus growers?** In all three locations, 89-94 percent of growers utilized a conventional pest management strategy of synthetic insecticides and herbicides; the remainder were organic growers or growers transitioning to organic **(Figure 2)**.

• **Thoughts on engineering?** Most of the survey participants were either strongly (65 percent) or cautiously (25 percent) in favor of a GE approach for controlling HLB. A low percentage (six percent) were indifferent or were completely against (six percent) GE approaches **(Figure 3)**.

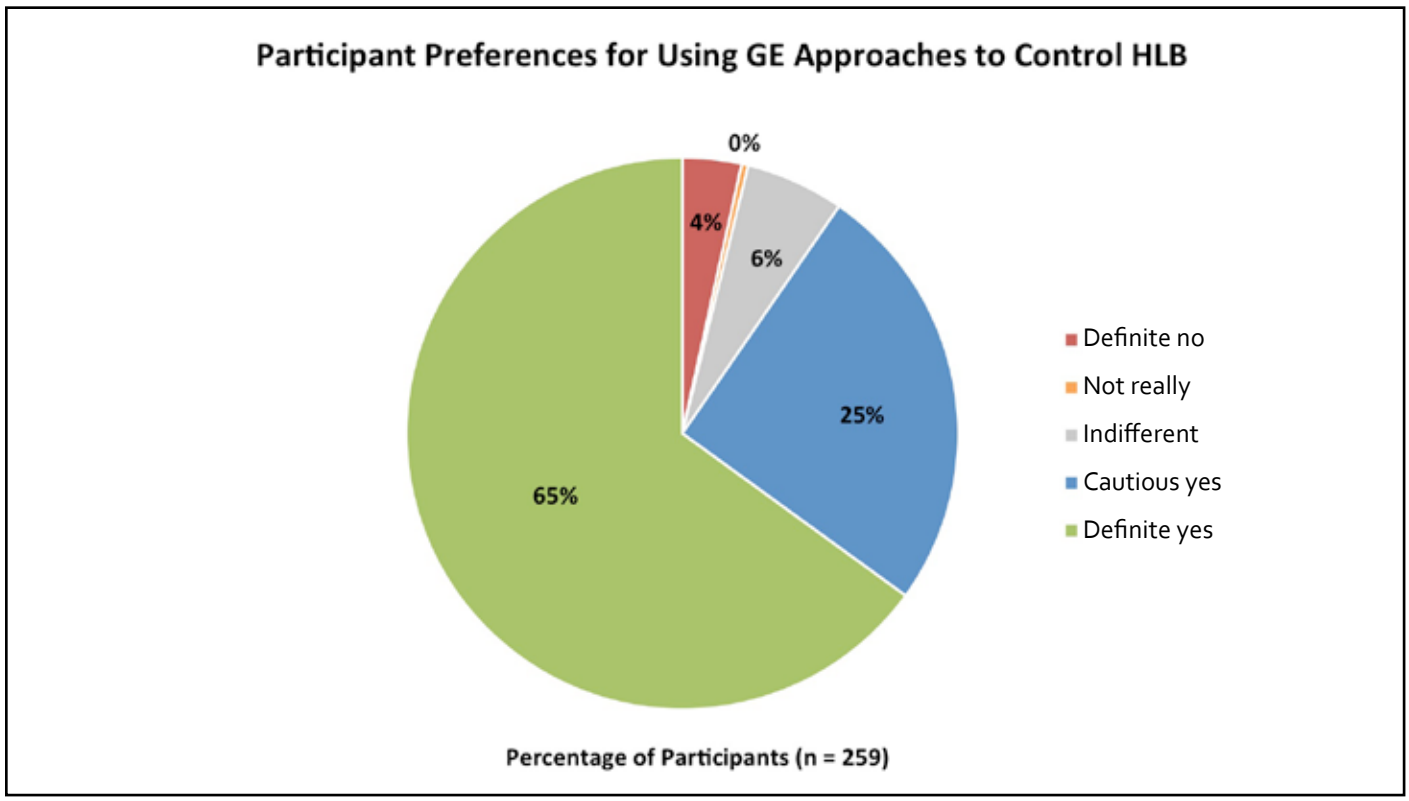


Figure 3: Participant preferences for using GE technology to prevent HLB from spreading in California.

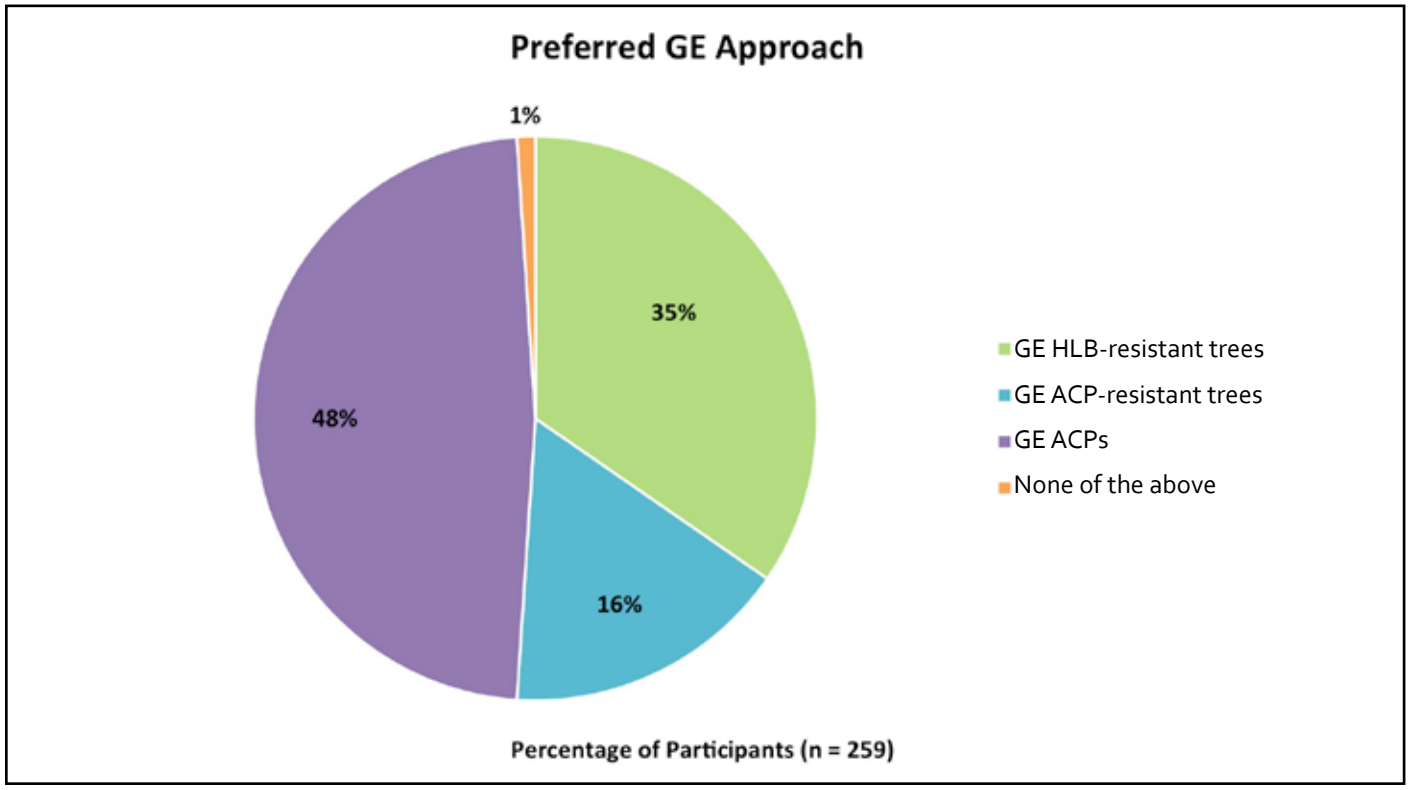


Figure 4: Preferred GE approach to control HLB.

• **What engineering approach?** Participants were evenly split between GE ACPs (48 percent) and GE trees (51 percent) as preferable for controlling HLB **(Figure 4)**.

Between the two techniques for GE trees, HLB-resistant trees were preferred (35 percent) over ACP-resistant trees (16 percent) **(Figure 4)**.

Growers with more than 500 acres of trees preferred GE trees (57 percent) to GE ACPs (43 percent); growers with fewer than 100 acres of trees preferred the opposite, GE ACPs (61 percent) over GE trees (39 percent) **(Figure 5)**.

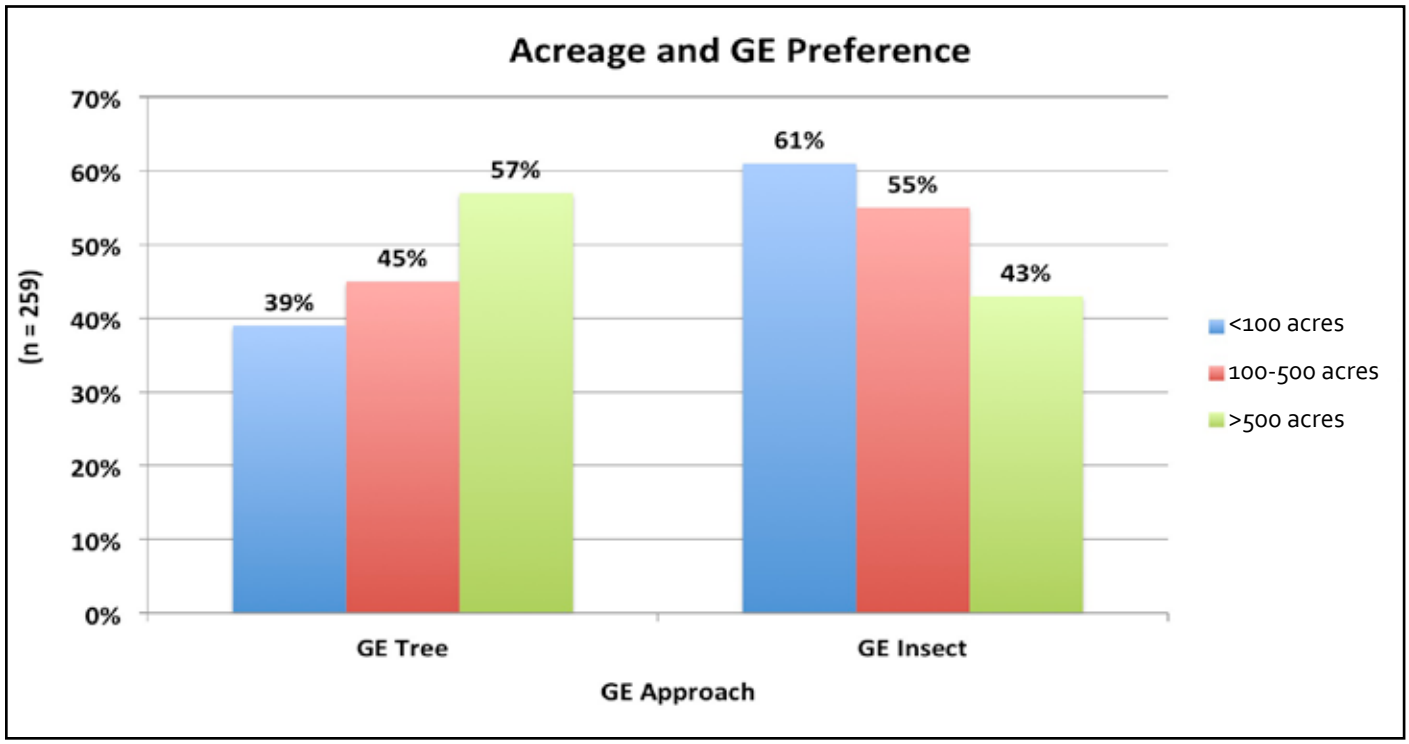


Figure 5: Grower acreage and preferred GE approach to control HLB.

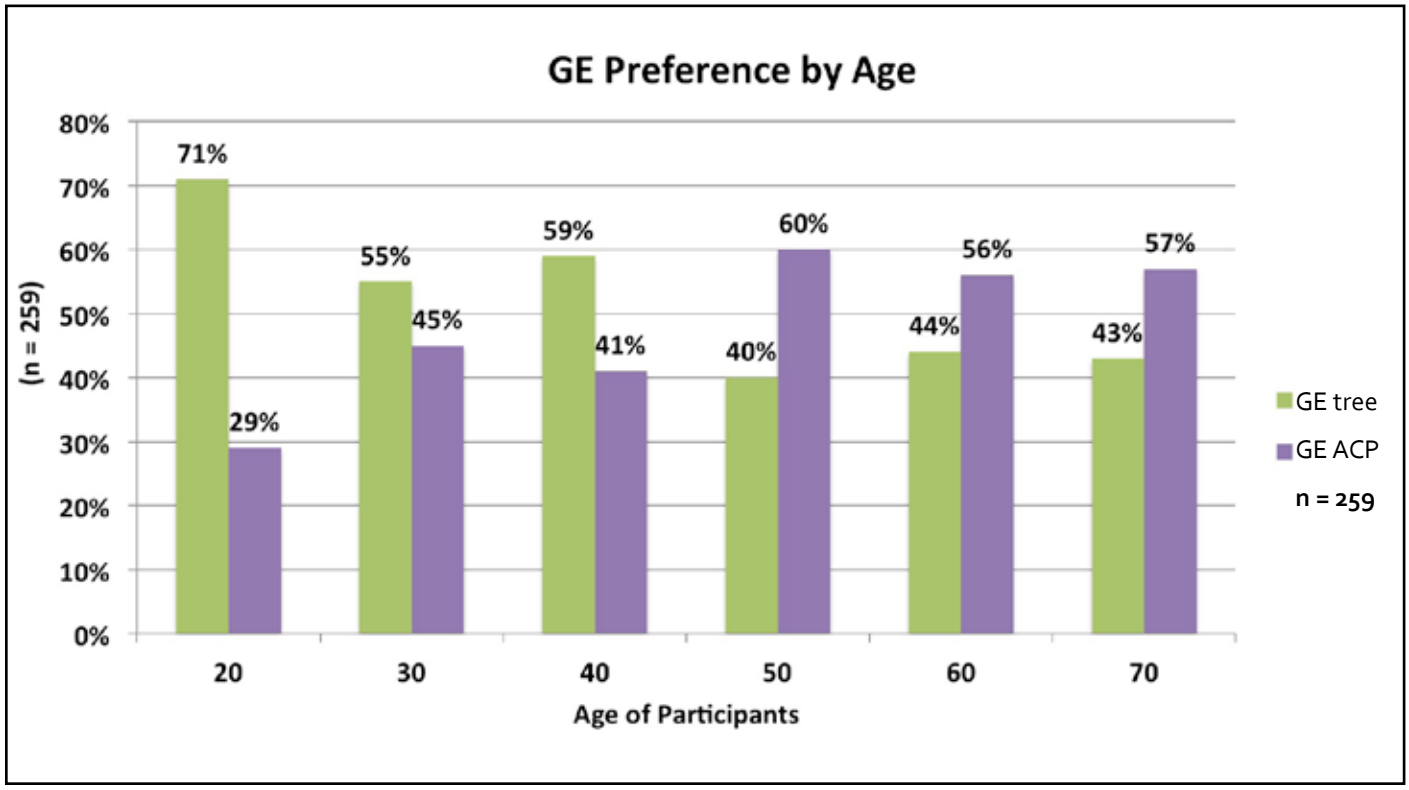


Figure 6: Age of survey participants and preferred GE approach to control HLB.

Participants aged 40 and younger had a stronger preference for GE trees (62 percent), while those over 50 preferred GE ACPs (58 percent) to control HLB **(Figure 6)**.

• **What would the impediment be?** Most attendees believed that public acceptance (56 percent) would be the biggest impediment to adoption of genetic engineering of either the tree or the psyllid, followed by government approval (33 percent). A small percentage thought grower acceptance would be an impediment (six percent), and some did not know (five percent) **(Figure 7)**.

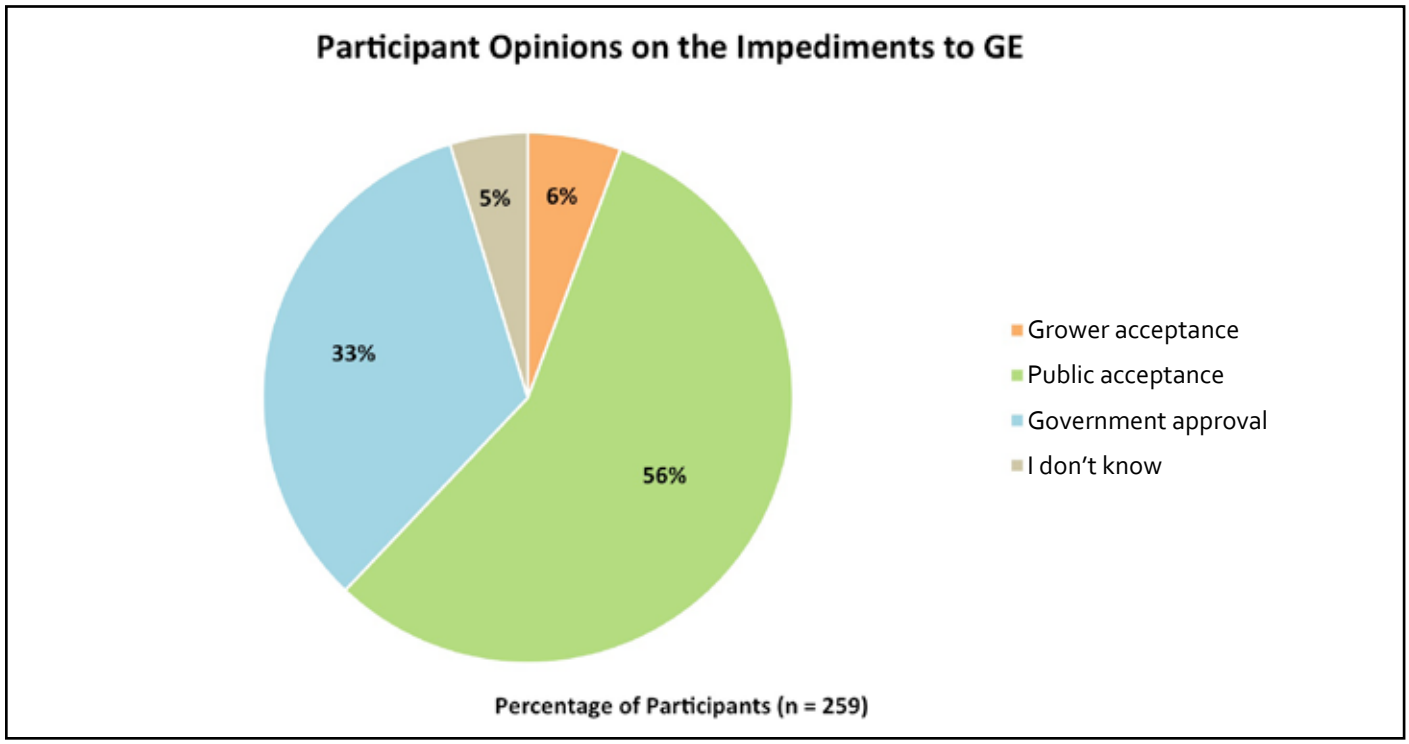


Figure 7: Participant opinions on the impediments to adoption of GE approaches to control HLB.

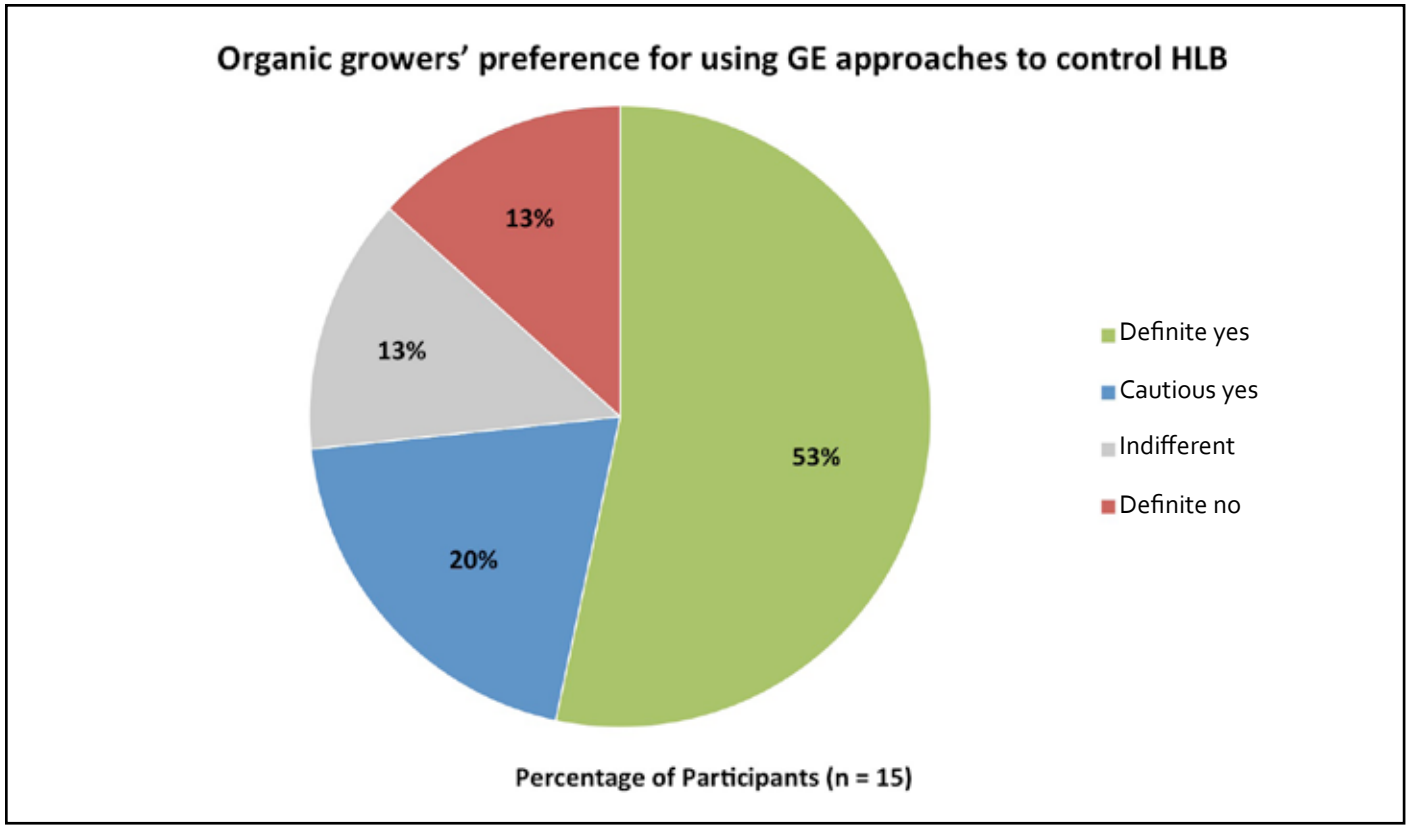


Figure 8: Organic growers' preferences for using GE technology to prevent HLB from spreading in California.

• **What did organic/transitional growers think?** While there were only 15 organic/transitional growers who participated in the survey, the majority either definitely or cautiously supported GE approaches to control HLB (73.3 percent). A small number completely rejected GE (13.3 percent), while some were indifferent (13.3 percent) **(Figure 8)**. Those who supported GE were split between GE trees (54 percent) and GE ACPs (46 percent).

Overall, the majority of those surveyed are in support of GE approaches to control HLB. Interestingly, preference for the GE approaches varied strongly based on age and acreage. When the Exeter audience was questioned about why they chose one GE approach over another, the older participants pointed out that they don't have time to replant citrus and reap the benefits of full production; and they, therefore, preferred modification of the psyllid. The younger participants felt that a GE tree would be a more permanent solution. Small growers preferred a transformed ACP solution, because replanting would have a negative impact on their income.

Although only 16 organic/transitional growers participated in the survey, the results showed most were in favor of using GE approaches to control HLB. One GE supporter at the Visalia meeting asked if GE would hinder one's status as an organic grower. We cannot answer that question at this point, because GE insects have not been released for agricultural purposes in the United States, and the regulatory process and consequences for the organic industry have not yet been determined.

IDENTIFYING POTENTIAL ISSUES

Many participants recognized that there are potential issues associated with GE technology that will need to be addressed. The majority felt that public acceptance would be the most difficult hurdle, followed by government approval. Only a few participants thought that growers would not support GE approaches, which was strongly substantiated by the grower survey responses. Several participants who completed the survey in Visalia also mentioned concerns about the safety of GE citrus for human consumption and its impact on the price of fruit. In Exeter, one participant was concerned that there would be fewer citrus varieties, and that the industry could lose some of the tastiest varieties since it takes time to engineer each variety and obtain regulatory approval to release into commercial production. Another expressed concern about having a monoculture of GE trees and the potential for the whole system to "crash and burn."

All GE technologies are under development at this time: transforming the plant itself to kill the bacterium or psyllid;

introducing a virus into the plant that carries an anti-bacterial gene; altering the psyllid itself so that it cannot transmit the bacteria; or introducing an organism into the psyllid to block its activity as a vector. If one or more of the GE approaches being researched is successful, one of the greatest challenges for the citrus industry will be to address the general public and regulatory concerns surrounding the technology. In all probability, both modified ACP and modified trees will be introduced along with other management tactics for a systems approach to addressing the devastating effects of HLB.

We are at the beginning of thinking about how to best deploy such technologies, and this is an on-going conversation in which the views of the industry are a crucial part. The recently announced investment in research to combat HLB by the federal government is likely to accelerate the pace at which new technologies are developed. The University of California extension and outreach team will be working hard to help with the education and implementation processes and we strongly encourage the active involvement of the grower community.

Carrie Teiken is a graduate student in the Department of Plant Pathology at the University of California Davis; Peggy Lemaux, Ph.D. is a cooperative extension specialist in plant and microbial biology at the University of California Berkeley; Beth Grafton-Cardwell, Ph.D. is the director of the Lindcove Research and Extension Center and a University of California Riverside integrated pest management specialist; and Neil McRoberts, Ph.D., is an assistant professor of plant pathology at the University of California Davis.