U.S. Horticultural
Research Laboratory
Ft. Pierce, Florida

Breeding Citrus for HLB Resistance

Kim Bowman

Greg McCollum

Randy Niedz

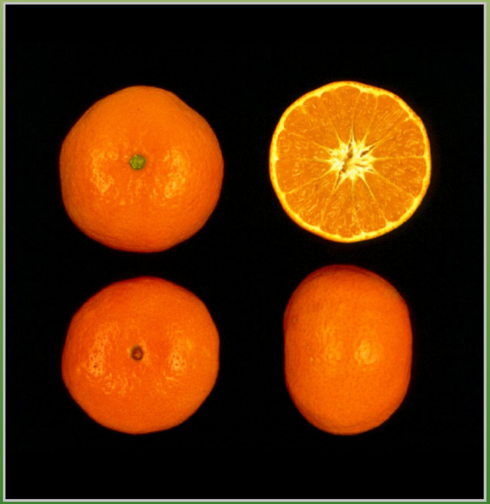
Robert Shatters

Ed Stover

Ute Albrecht
Lesley Benyon
Randy Driggers
Mizuri Hert
Ric Stange
Janice Zale

Principle Collaborators:

YongPing Duan
David Hall
Bob Shatters
Richard Lee
William Belknap
Abhaya Dandekar
Jesse Jaynes
Fred Gmitter
Dennis Gray
Mikeal Roose



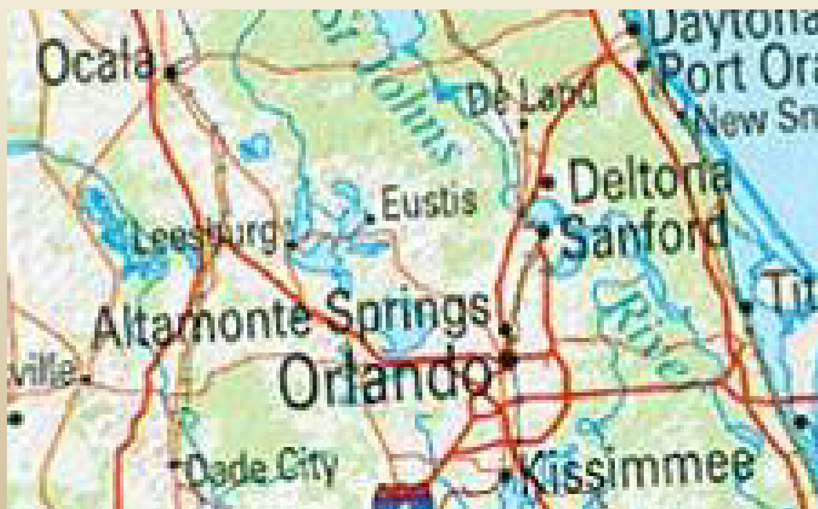
ARS Citrus Improvement

The oldest citrus breeding program in the world

W. T. Swingle

H.J. Webber

Sub-Tropical Laboratory
Eustis, Fla.
Swingle and Webber
1893-1897



>75% of US citrus industry has rootstock and/or scion from USDA program…. mainly rootstocks!

Rootstocks from USDA

- Carrizo/Troyer most important rootstock in CA and 2nd most important in FL
- Swingle is most widely used rootstock in FL
- US-852 released in 1999
- US-812 released in 2001
- US-802 released in 2007
- US-897 released in 2007
- US-942 for release in 2010

Hamlin sweet orange

Osceola County

Trees 16 years old

**Dr. Kim Bowman,
rootstock breeder geneticist**

US-802 rootstock

US-897

USDA Citrus Scion Releases

Year	Release	Pedigree
1930	Minneola Orlando	Duncan x Dancy
1959	Robinson Osceola Lee	Clementine x Orlando
1963	Page	Clementine x Minneola
1964	Nova	Clementine x Orlando
1979	Sunburst	Robinson x Osceola
1987	Fallglo	(Clementine x Orlando) x Temple
1987	Flame	Nucellar sport of 'Ruby Red'
1987	Midsweet	Nucellar sport of 'Homosassa'
1989	Ambersweet	(Clementine x Orlando) x midseason orange
2009	US Seedless Pineapple	Irradiated Pineapple
2009	US Early Pride	Irradiated Fallglo

Newest releases made by Dr. Greg McCollum

Scion Improvement Objectives

- Outstanding fruit quality
  - Flavor and appearance
  - Easy peeling
  - Flesh texture
  - Seedless
- Range of harvest time
- Resistance to pests
- Productivity
- Postharvest performance
- HLB and Canker Resistance

Creation of new scions
Sexual Hybridization

Controlled pollinations to combine parents with desirable traits

Fruit harvested
Seed extracted

Seedlings grown
For field planting

Evaluation of seedlings
1st Test

Propagation of promising selections

Replicated field trials
2nd Test

Mutation (Irradiation) Breeding

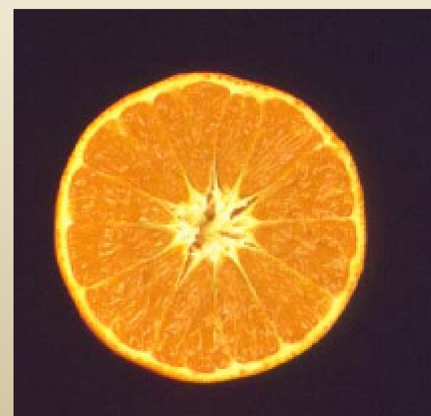
Used to develop seedless versions of high quality, but seedy selections

Budwood of promising scion

Irradiate

Irradiated budwood grafted onto liners

Field testing



‘Seedless Kishu’

Huanglongbing, AKA Citrus Greening

- First identified in Florida in 2005 (Brazil 2004) now found in all FL citrus producing counties
- Estimated that 2-8% of FL citrus trees are infected, but some groves no longer productive
- Associated with a bacterium, *Candidatus* Liberibacter asiaticus, vectored by the Asian Citrus Psyllid (in TX, AZ, CA), phloem limited
- Within a few years of infection, many citrus trees become weak, have poor quality fruit, with lots of fruit drop, and trees may die or become useless

Photos Bove, 2006

Citrus Bacterial Canker

- Found in Florida in 1912 & eradicated by 1933; again 1986 & eradicated by 1994; again 1995……
- Caused by bacterium, *Xanthomonas citri pv. citri*, which is spread on people, equipment, wind-driven rain. Grapefruit are especially susceptible, but also sweet orange. Spread widely by 3 hurricanes 2004.
- Infection makes fruit unattractive, greatly increases preharvest drop, causes leaf lesions / defoliation / reduced productivity, and interferes with exports

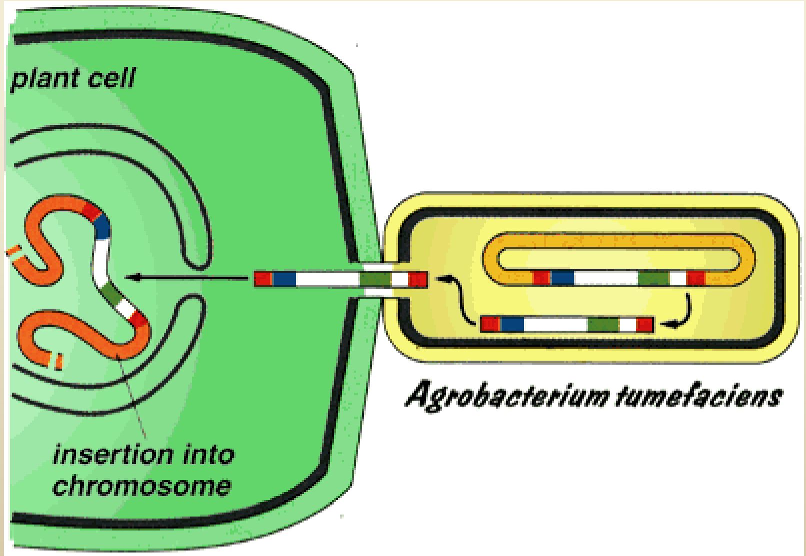
Photo Gottwald et al., 2002

Focus on Developing HLB-and ACP Resistant Citrus

- **HLB likely the single greatest threat to citrus**
- **No strong HLB resistance has been identified in cultivated Citrus scion varieties**
- **Transgenics appear to be best medium term solution for strong HLB resistance**
- **Goal: add genes to reduce survival, growth, and/or virulence of HLB vector, genes to deter psyllid vector, possibly suppress host disease response**
- **With little known about host /pathogen interaction, antimicrobial peptides have been a major focus- may also confer resistance to canker and CVC**
- **Other types of genes also being explored……….**

Using nature's genetic engineer: *Agrobacterium tumefaciens*

A. tumefaciens causes crown gall disease in many plant species



By removing At genes for growth regulators and replacing with:

1) promoter
2) gene of interest etc.
3) gene for selectable marker (antibiotic)

can express genes when and where you want, without gall formation.

Image: www.plantsci.cam.ac.uk/.../GFP/plantrans.html

Citrus Transformation: Major Focus for HLB and Canker Resistance

Transformation

Selection

Regeneration

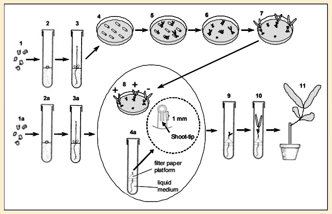
Micro-grafting

Grow out

Evaluation

Slide from Kim Bowman

Transgenic Project: Parallel Tracks



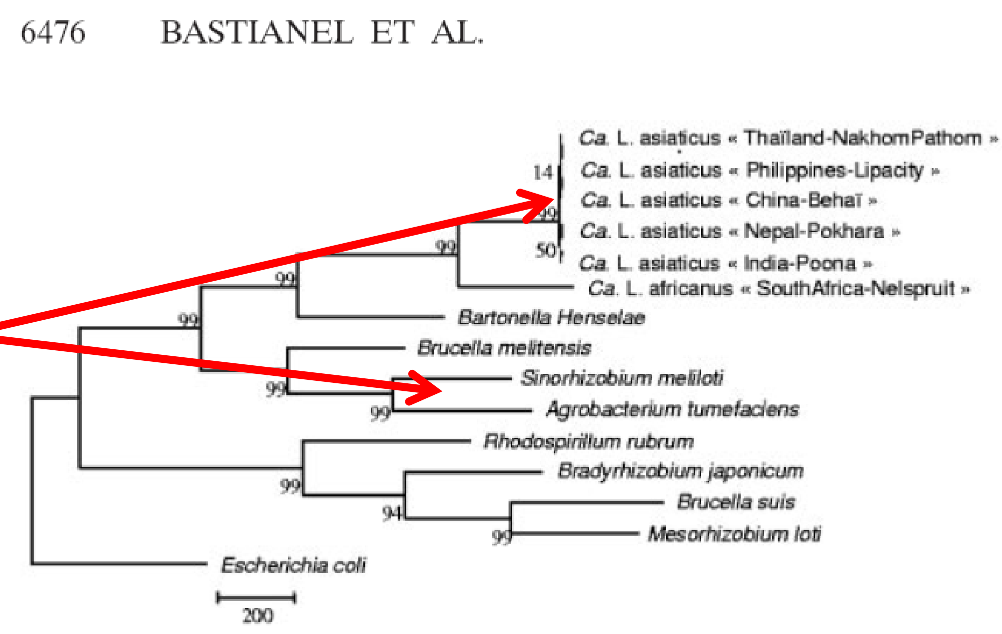
- Fastest track- possible “home run” using best available technology on rootstocks, sweet orange and grapefruit- high throughput.
  - Goal is earliest possible resistant variety in field
  - Emphasizing components which are deregulated in crop plants
- Experiments to overcome transformation limitations including mature tissue transformation
- Identifying new targets for transgenes
- Exploring other promoters, constructs, etc. first with easily transformed rootstock types

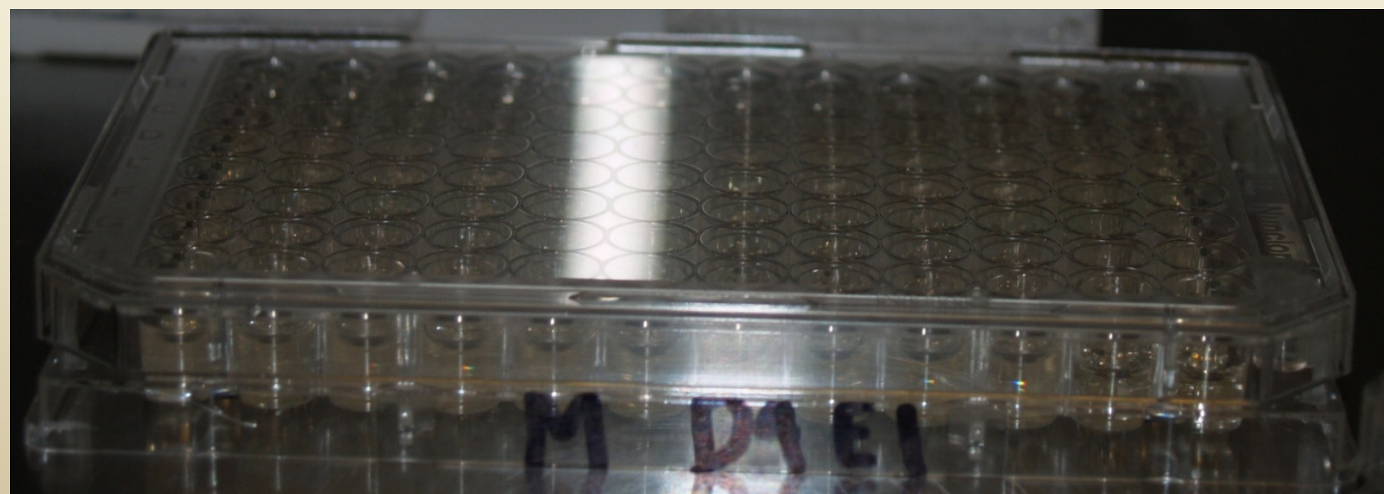
Antimicrobial Peptides

- Broadly active against groups of micro-organisms
- Initial active defense to combat infection in multicellular organisms
- Most are very small molecules, MAY move systemically
- Results in microbial death or prevents growth

In –Vitro AMP Screening

- *Agrobacterium* and *Sinorhizobium* are related to *Liberibacter*
- Also using *Xanthomonas citri*
- Best AMPs, including D4E1 are effective in 1 μM range





AMP transgenics being tested with CLas infected psyllids

Harnessing nature's genetic engineer: *Agrobacterium*

Gene constructs:

1) **promoter - express genes when and where you want**
2) **gene of interest etc.**
3) **gene for selectable marker (antibiotic)**

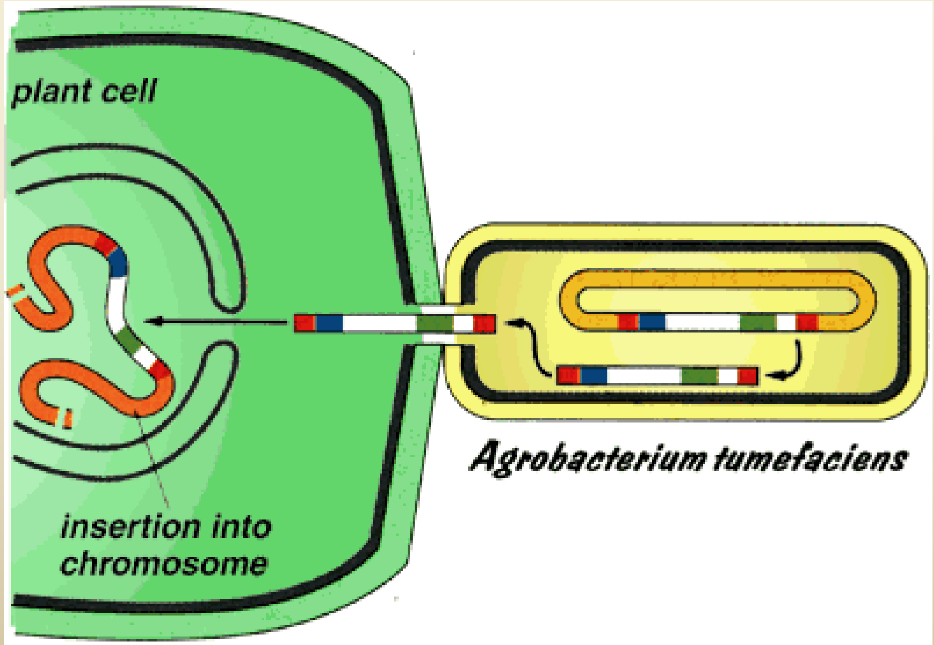
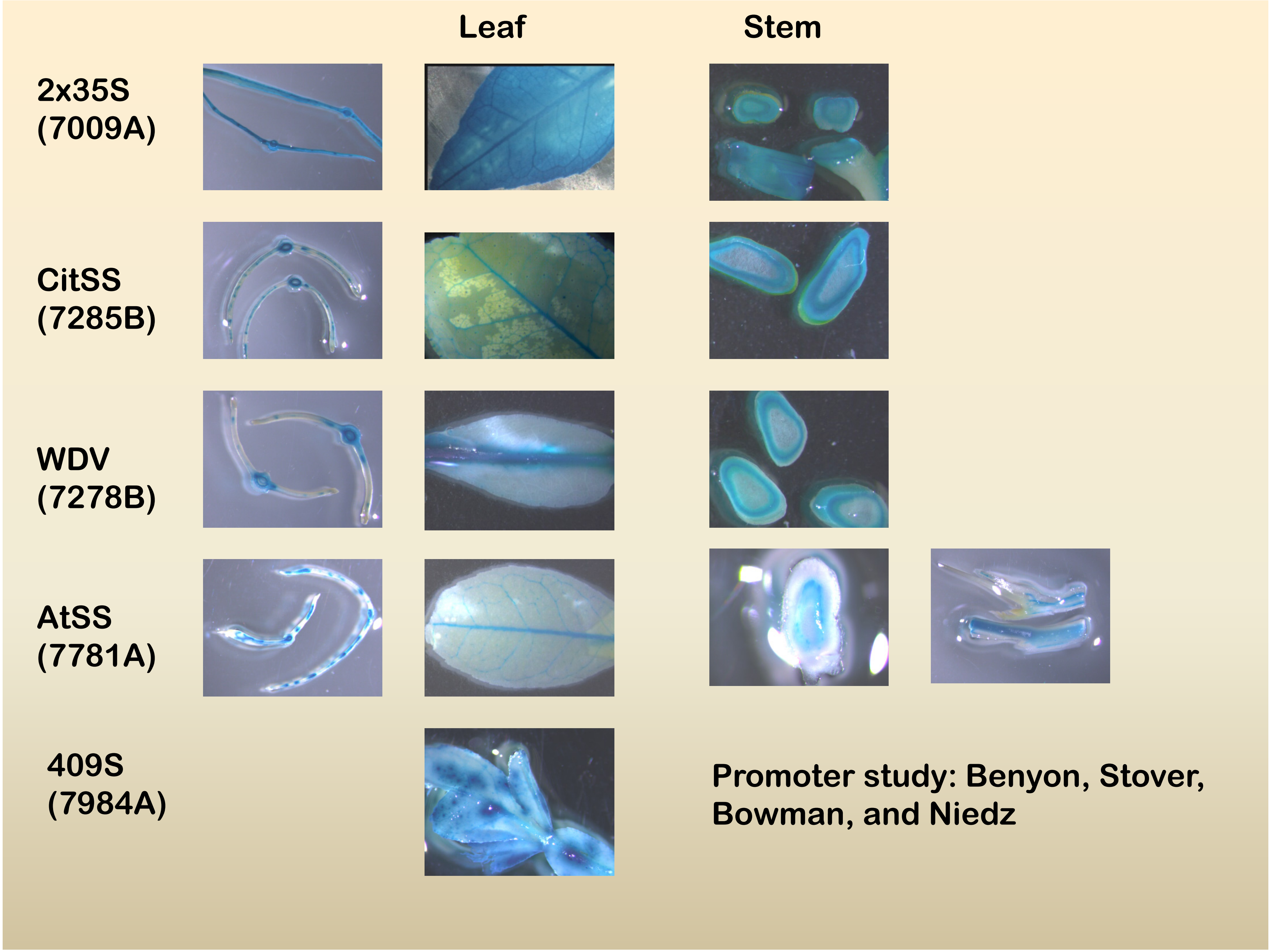


Image: www.plantsci.cam.ac.uk/.../GFP/plantrans.html

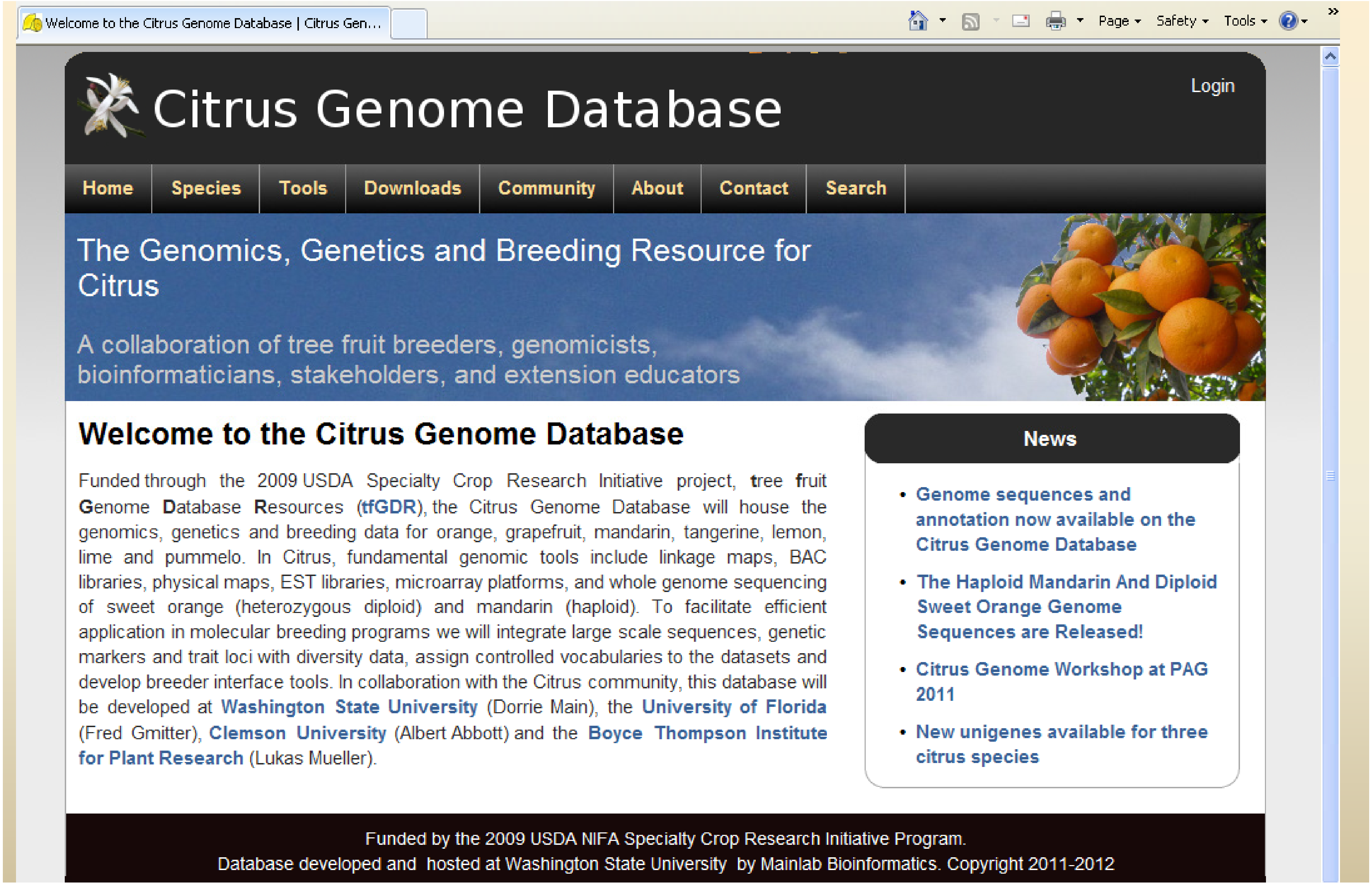


Leaf
Stem
2x35S
(7009A)
CitSS
(7285B)
WDV
(7278B)
AtSS
(7781A)
409S
(7984A)
Promoter study: Benyon, Stover,
Bowman, and Niedz

Other Transgenes for HLB Resistance

- Transmembrane protein from Liberibacter is a target identified from the *Liberibacter asiaticus* genome (working with Duan group at USHRL)
- Phloem-specific protein induced during HLB infection (Bowman data USHRL)
- Working with ARS scientists in California to develop constructs so that ALL inserted genes are from Citrus!

Screening genome of Citrus and Poncirus for construct components: defensins, promoters, tDNA analogues etc

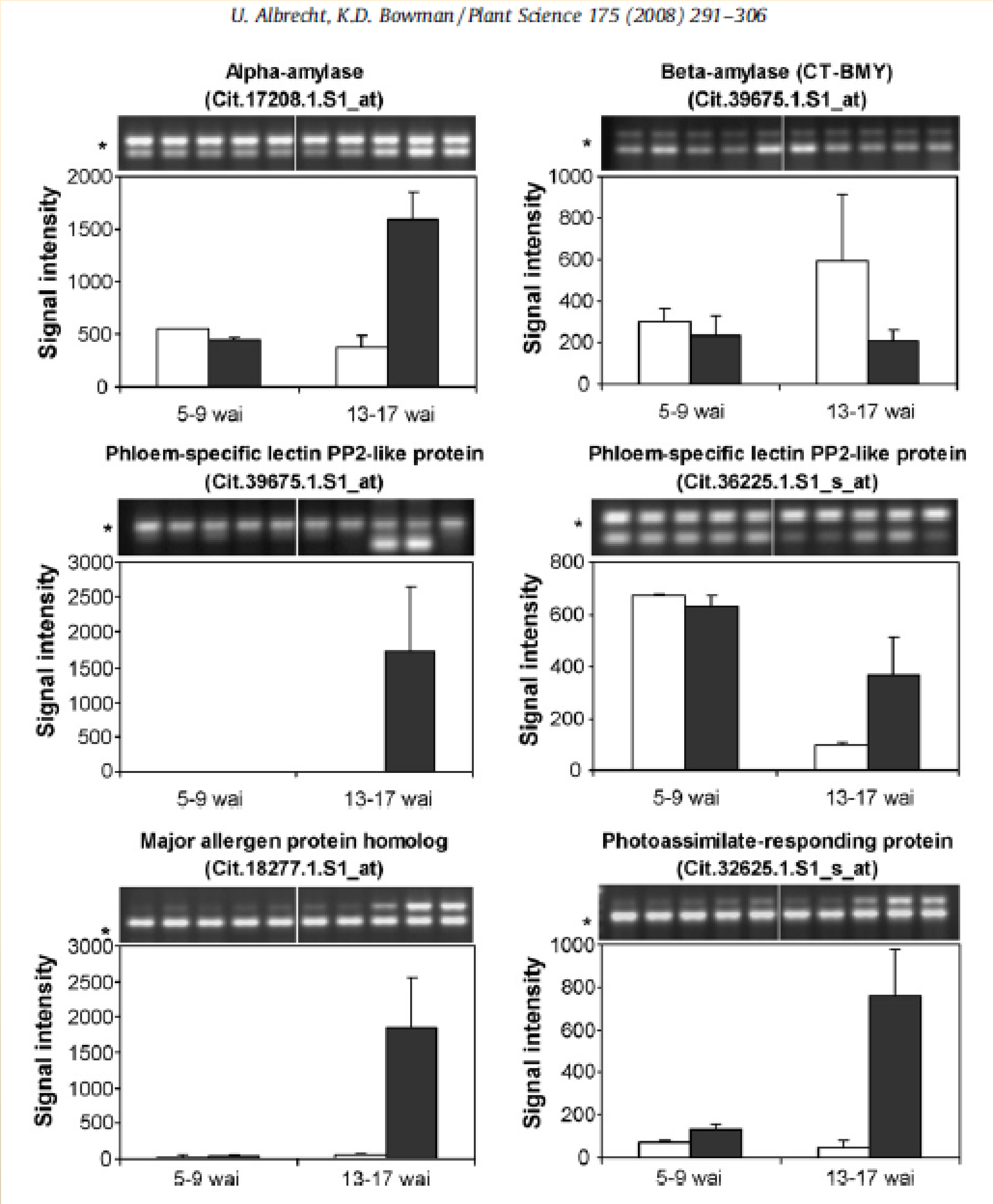


Gene expression differences in HLB-infected vs. healthy sweet orange.

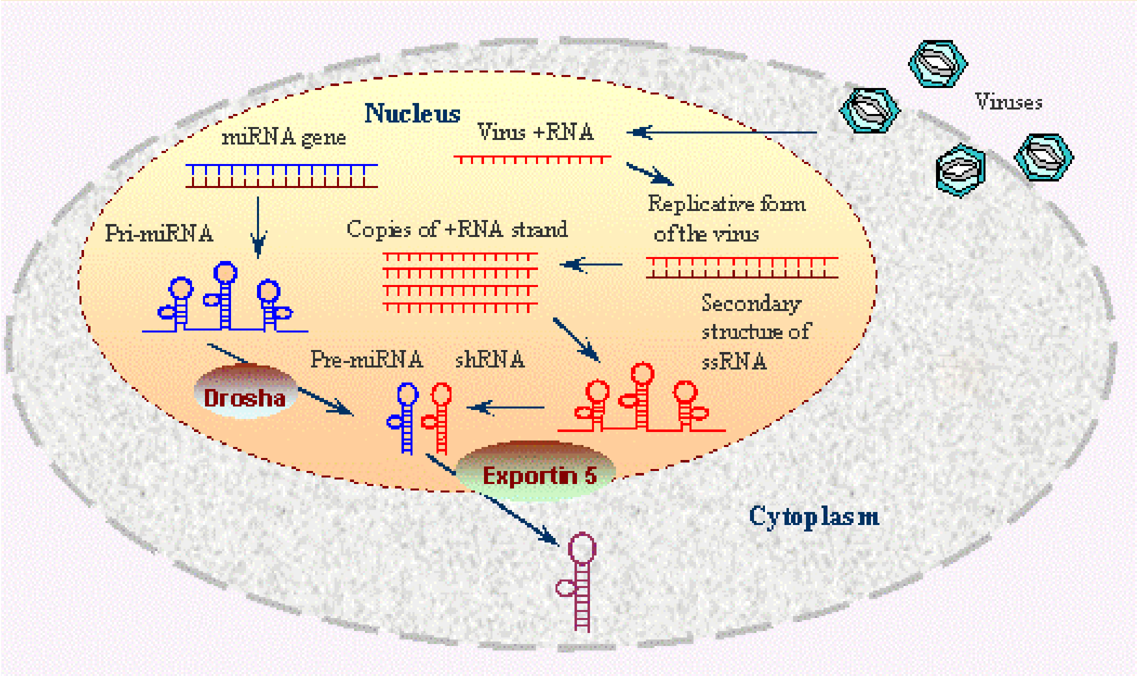
U of FL has shown PP-2 and callose comprise phloem plugs in HLB+

Albrecht and Bowman show PP-2 is upregulated in HLB+ Cleo not US-897

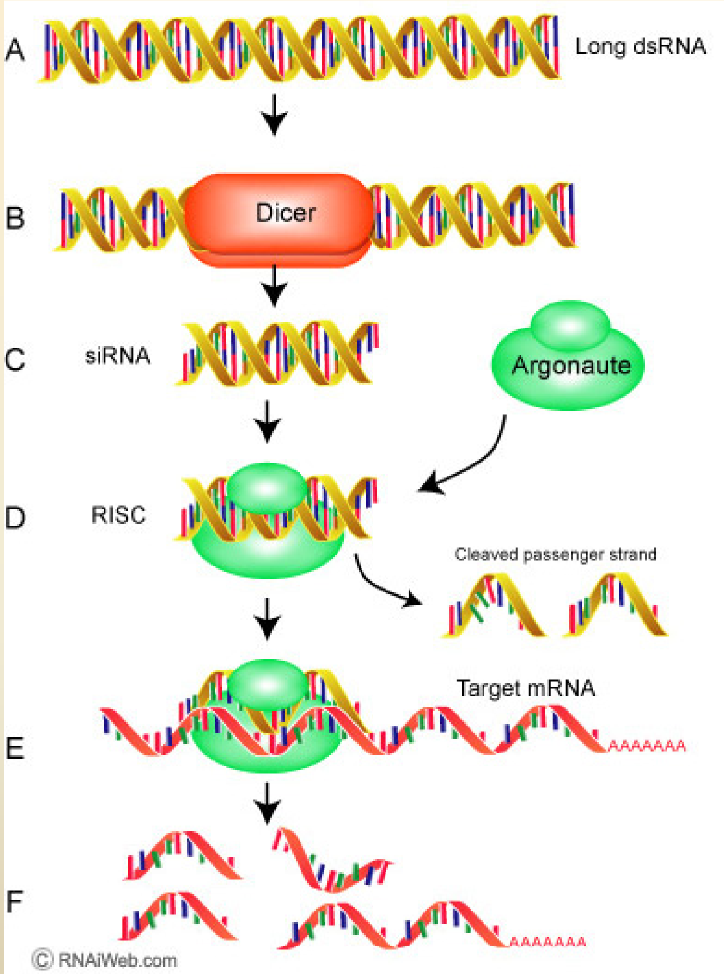
We are testing hairpin constructs of PP-2 for RNAi strategy



RNAi- harnessing a tool that is used against viruses



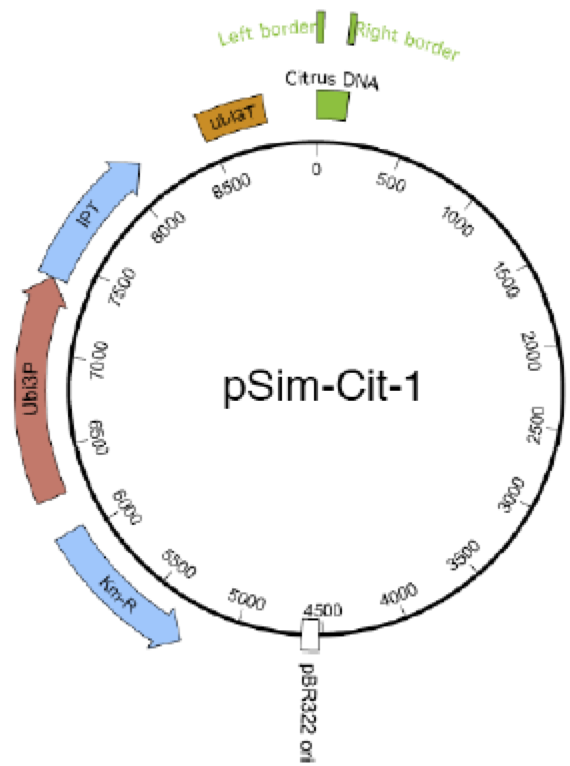
ncbi.nlm.nih.gov



rnaiweb.com

Myb genes as selectable marker, possible bacteriostatic agent?- Dennis Gray UF is coPI

Citrus Intragenic Binary Vector



Right Border

CACTACAACTCAATATATCCTGTACGAGATATCACAG
AAAGACANCAANATATATCCTGTCA (Consensus)

Left Border

GGGGCAGGATATATCCAAATGGAAAGACTAATCTGTCAGAAGGAAAAAGAAGAAGGACCTGTGTCAG
NNGGCAGGATATATNNNNNTGTAAA (Consensus)

Simplot

Caius Rommens and Kathy Swords

Bill Belknap and Randy Niedz

Resistance to HLB in Citrus gene pool?

- Folimonova et al. (Dawson group) tested 30 genotypes for HLB response
- Symptoms varied greatly, with sweet orange in the most sensitive, highest titer group
- *Poncirus trifoliata* and *Citrus latipes* displayed the greatest resistance in symptoms and low/no titer

HLB-resistance from distant citrus and relatives

Lee, Stover, Hall, Keremane, Halburth

- 85 accessions: planted June 2009
- Seed from Riverside repository
- 8 plants each randomized in Ft Pierce
- In this experiment, *Poncirus* was slowest to develop CLas (Lee et al.) and most resistant to ACP colonization in citrus gene pool (Westbrook et al.).
- Trifoliate hybrids variable and intermediate

Using trifoliate genes for HLB resistance

- U of Florida (Fred Gmitter), UC Riverside (Roose) and USHRL (Stover) are collaborating on a trial of diverse citranges and other trifoliate orange hybrids, to identify genes associated with HLB-resistance
- When mapped and identified, can use genes in intragenics and as a marker in conventional breeding

U2

Diapositiva 29

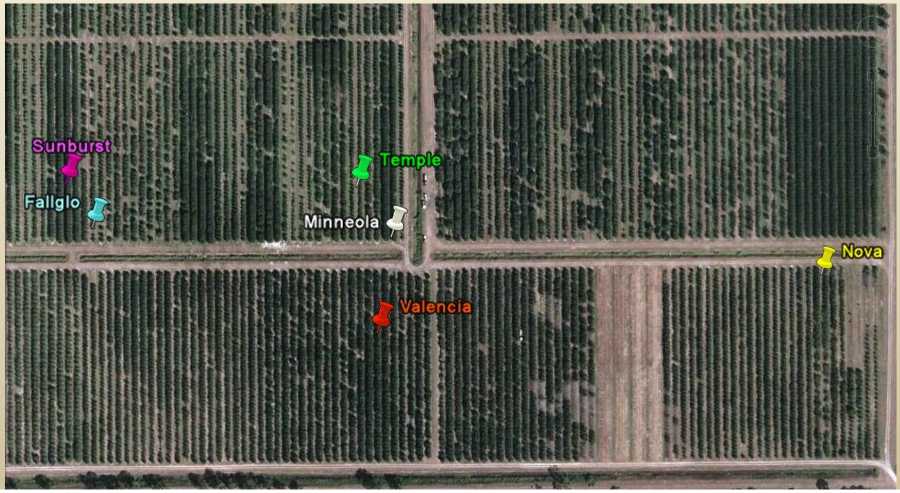
U2 REd text: Confusing
USHRL; 02/06/2010

Carrizo transformed with D35S:: Citrus FT

Gloria Moore- UF Horticulture

Evaluating varieties for HLB-tolerance

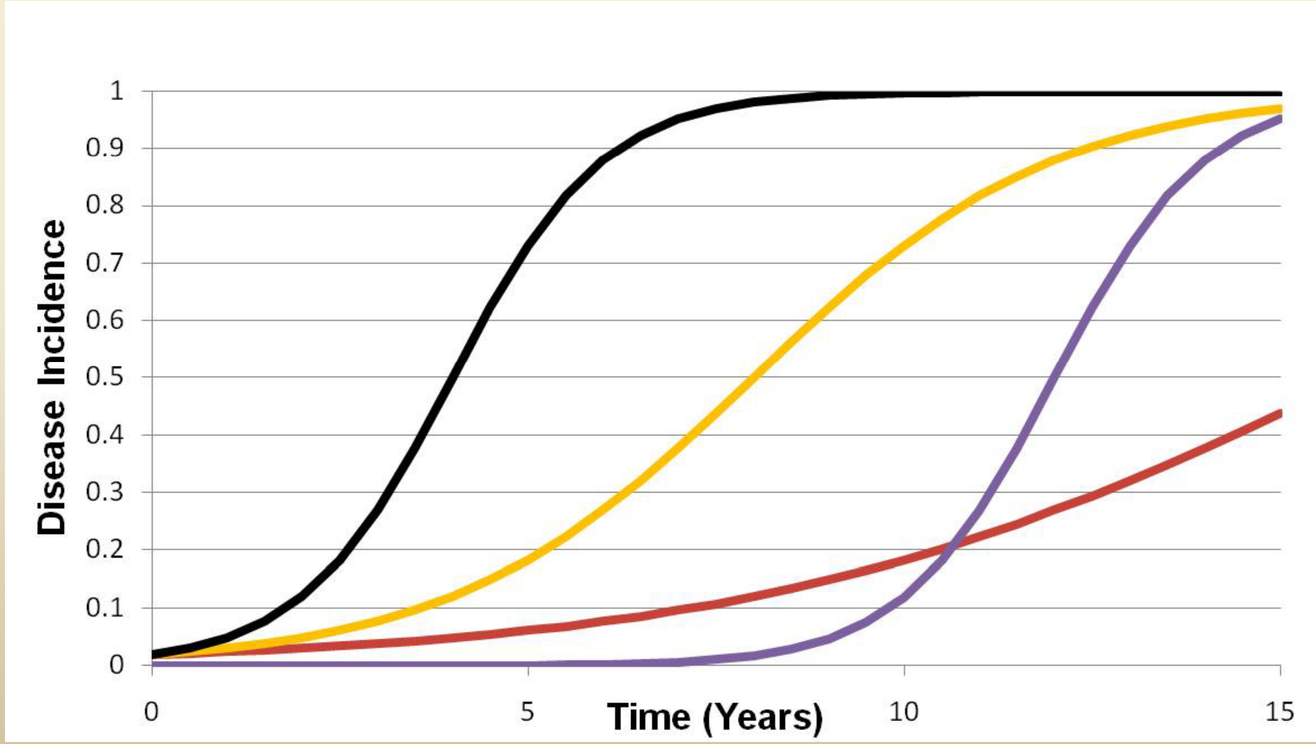
- Appears that citrus varieties vary widely in their rate of developing HLB
- Identified 8 groves in the Indian River area reporting presence of HLB and multiple specialty cultivars
- Avoided reported edge effects and bias, randomly sampled 20 trees /cultivar /grove
- Ran q-PCR using 16S CLas rDNA “Wenbin” primers (APHIS standard); 760 trees were sampled



HLB bacterium level for citrus cultivars in Indian River area groves: April 2010.

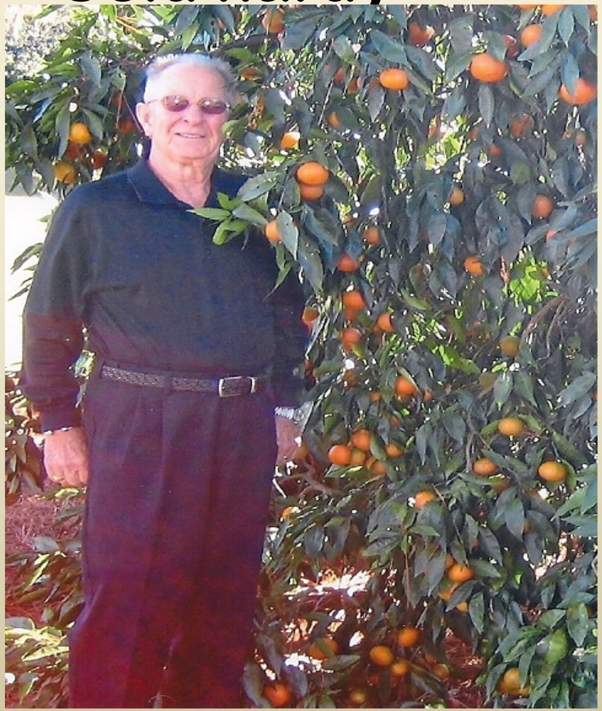
	Mean # HLB bacteria genome/sample*	% trees Ct<36
Minneola	304	43%
Murcott	168	44%
Sweet orange	236	31%
Grapefruit	40	20%
Temple	9	15%
Fallglo	13	18%
Sunburst	107	13%

- In time immune trees will be found… in the meantime
- If resistance or tolerance is confirmed, how may this benefit citrus industries?
- How much delay in symptom development is needed to be useful?



USDA 1-37-12

- (King x Chang Sha) x Satsuma
- Mid Season (Dec)
- Easy peeling
- 20 seeds / fruit
- Good bearer
- Cold hardy



- Jack Hearn, USDA geneticist, retired
  Created USDA 1-37-12
- 12 year old USDA 1-37-12 tree
  South Georgia 12/2009
- Tolerated temps as low as 18 °F

USDA 1-77-19

Irradiated selection of 5-75-8 (Pearl tangelo x Duncan grapefruit)

- Mature in Sept.
- Seedless
- Low acid
- Non-bitter

USDA 1-105-106

- Complex hybrid: (Clementine x Orlando) x sweet orange
- Replicated trials have been conducted
- Fruit matures in December
- Good bearer
- Fruit have deep orange color, produces high quality juice
- Mandarin background could mean less canker

FF-1-75-113 The parentage of this selection is Ambersweet X 1-30-52 (Wilking X Valencia) so it's makeup is half orange. It has the most orange-like juice of any hybrid selection we have. Most tasters say it tastes and smells exactly like really good orange juice. The fruit grow in clusters on the seedling tree and have a somewhat tear drop shape. Juice quality is extremely high with 14.5 brix, 1 percent acid, and 37.7 color when it ripens around December 1st. This will be entered into a replicated trial in the summer of 2011.

FF-1-63-77   1-37-12 X Sunburst   This selection is ¼ Satsuma and went through this year's 20 degree Fahrenheit cold spell with hardly a wrinkled leaf. It has good color internal and external, peels easily but isn't so loose as to be likely to cause problems with shipping. The segments separate cleanly with little leaking. Seed count is high on the seedling tree but may drop in a planting without cross pollination---- if not we will irradiate the budwood. The fruit plugs slightly more than the sunburst parent. Propagated for a replicated trial in early 2011.

Ftp- 6-16-172 Low Acid Pummelo X 5-75-8 This selection has a small amount of grapefruit- like bitterness but very low acid. The taste is very mild. Fruit on the seedling tree ripen very early and would probably pass grapefruit standards in September in Fort Pierce. It has a deep red internal appearance that rivals the deepest color found in grapefruit such as Star Ruby. After irradiation to remove the seeds, this may be a new “season opener” variety.

Ftp-6-47-100

Cold-hardiness breeding has long been a component of the USDA citrus program. Many hybrids near commercial quality with Poncirus grandparent, or great-grandparent. Now looks like Poncirus is greatest source of HLB resistance! Maybe we'll get lucky with these hybrids….

1-4-59 This selection is very orange-like with little trifoliate taste or smell when fully ripe. Parentage is Ambersweet X US-119 ((Duncan X P. trifoliata) X Succory). This selection is highly resistant to tristeza and may have some tolerance to HLB. It may also have some level of cold tolerance acquired from the Poncirus grandparent.

Thanks!

- Florida Citrus Research and Development Foundation
- New Varieties Development and Management Corporation
- Florida Citrus Research Foundation (Whitmore)
- USDA / ARS base funds

Jodi Avila	Robyn Baber	Scott Ciliento
Regina Conley	Daniel Davis	Jacqueline Depaz
Emily Domagtoy	Lynn Faulkner	Lorri Hutchinson
Diane Helseth	Steve Mayo	Jerry Mozuruk
Mike Rutherford	James Salvatore	Lindsay Turnbull
Kerry Worton	Jon Worton	Eldridge Wynn

Overview of Breeding Genetics Presentations at 2011 Orlando, FL, HLB Conference

- 3 full days, 400 participants from 20 countries, 75 oral presentations, 96 posters,
- Urgency of HLB as a threat to citrus production and the engine of substantial grower investment has fully engaged numerous researchers to find solutions

Loading the tool box!

- Developing tools that will open up cutting edge technologies which make this "a golden age for biology". Steve Lindow (UC Berkeley) noted that "HLB is a tough nut to crack, and you are lucky you didn't get it even a few years ago"
- Tremendous progress in genomics of host, pathogen, and vector- FINALLY we will have the full wiring diagram to fix "what's broken"
- Identifying genes that may confer resistance, better understanding of HLB biology and gene expression in resistant and susceptible Citrus. Targets for action!

Other important points…………..

- Items I didn't discuss earlier
  - Evidence that some rootstocks may enhance HLB-tolerance of scions (Grosser; Stuchi; Albrecht/Bowman)
  - Strong sense in Florida industry that aggressive foliar nutrients permit continued production in HLB-infected trees: contraversial and not proven

Take home?

- If knowledge is power........
  - Our knowledge of HLB, Liberibacter, ACP and their interaction with Citrus have grown greatly over the last two years
  - The total understanding is reaching a critical mass that will soon reveal great tools for living with HLB
  - And a series of ever-better solutions will emerge over the coming years