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

Genetic Strategies for Citrus Disease Management: HLB

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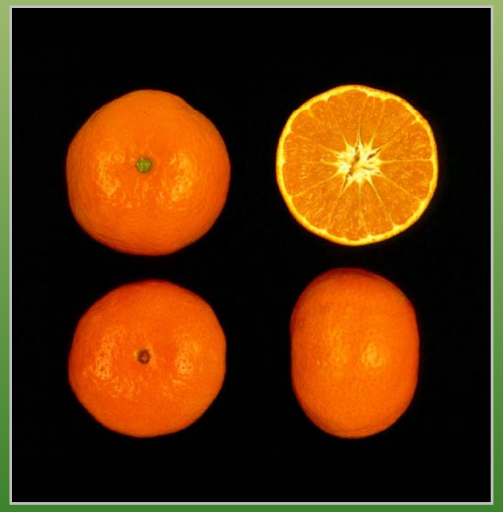


ARS Citrus Improvement

The oldest citrus breeding program in the world

W. T. Swingle

H.J. Webber

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

USDA Citrus Scion Releases

	Year	Release	Pedigree
Swingle and Webber era	1930	Minneola Orlando	Duncan x Dancy
	1959	Robinson Osceola Lee	Clementine x Orlando
Reece and Gardner era	1963	Page	Clementine x Minneola
	1964	Nova	Clementine x Orlando
Hearn era	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

Scion Improvement Objectives

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

Earlier USDA Released Rootstock Varieties

Variety	**Year**	**Industry %**
Carrizo/Troyer citrange	1934	30
Swingle citrumelo	1974	46
Sun Chu Sha mandarin	1988	< 1%

Total = >75%

New Rootstocks from USDA

- **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 2009**

Hamlin
sweet orange

Osceola County
Trees 16 years old

US-802 rootstock

US-897

New Florida Rootstocks Need

Favorable effect on:

- Fruit yield
- Tree size
- Fruit sweetness
- Fruit size/shape
- Propagation
- Tree anchorage
- Tree cold tolerance
- Tree longevity
- More . . .

Tolerance to:

- Phytophthora
- Tristeza virus
- Diaprepes weevil
- Citrus Blight
- Nematodes
- Flooding
- Salinity
- High pH
- Greening/HLB

Focus on Developing HLB-and Canker Resistant Citrus

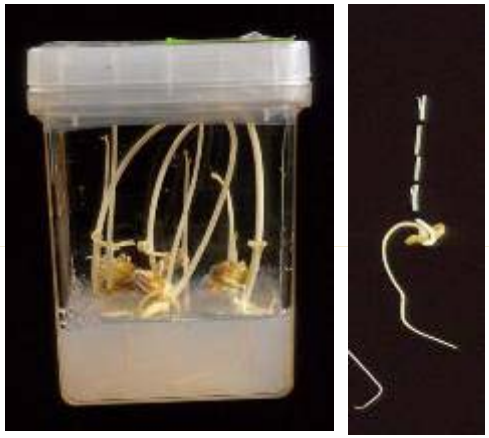
- No HLB resistance identified in cultivated Citrus scion varieties
- Transgenics appear to be the only medium term solution for HLB resistance
- Incorporate genes to reduce survival, growth, and/or virulence of causal pathogens, as well as genes to deter psyllid vector
- With little known about host /pathogen interaction, antimicrobial peptides have been the focus
- Additional or alternative transgenes will be used based on virulence mechanisms and host responses

Antimicrobial Peptides

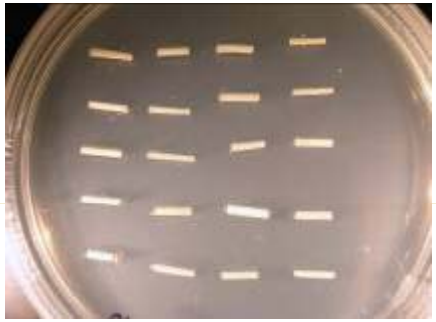
- Broadly active against groups of micro-organisms
- Widespread in multicellular organisms
- First line of active defense to combat infection
- Most are very small molecules
- Numerous distinct AMPs produced in each organism, with somewhat different activities
- Most function by inserting into microbial membranes causing leakage etc.
- Results in microbial death or prevents growth

Citrus Transformation

Transformation



Selection

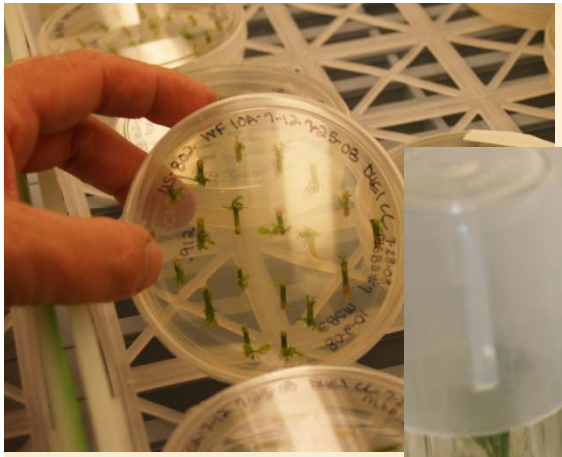


Regeneration

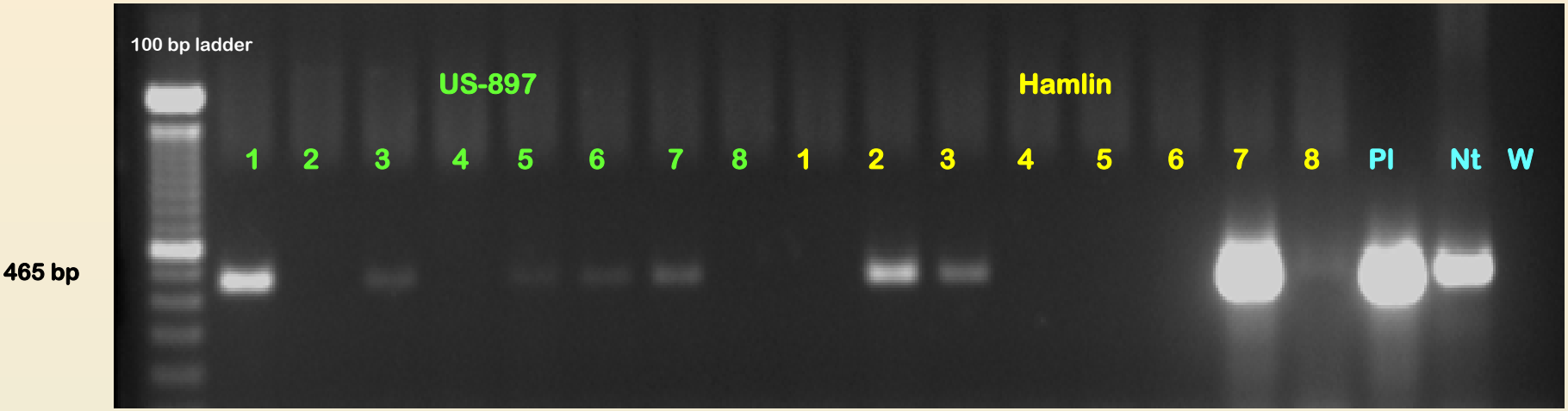
Micro-grafting

Grow out

Evaluation



- more than 10,000 putative transformants micrografted or rooted
- individuals will vary based on insertion point & #
- first transgenics HLB challenged in December



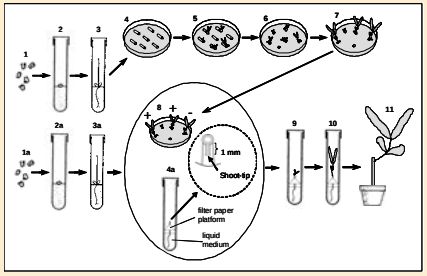
Pl = plasmid control

Nt = tobacco control

W = water control

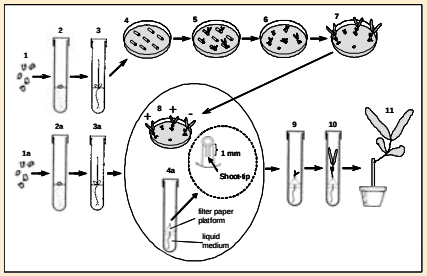
- Test for presence of transgene
- Ultimately mRNA and peptides where possible

Transgenic Project: Parallel Tracks



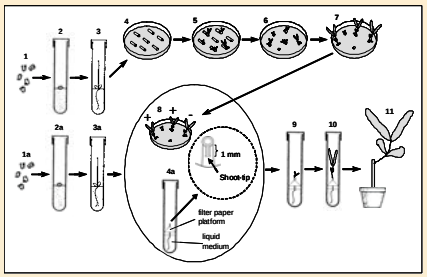
- Fastest track- possible “home run” using double 35S promoter/D4E1/kanamycin and other AMPs on rootstocks, sweet orange and grapefruit
  - Emphasizing components which are deregulated in crop plants
- Experiments to overcome transformation bottlenecks
- Identifying new targets for transgenes
- Using other promoters etc.

Selection of AMPs



- Plant-derived or synthetic for greater consumer acceptance
- Low potential for adverse health effects
- Reports of effectiveness against related bacteria
- Screening *in-vitro*, model systems (tomato), and citrus rootstocks

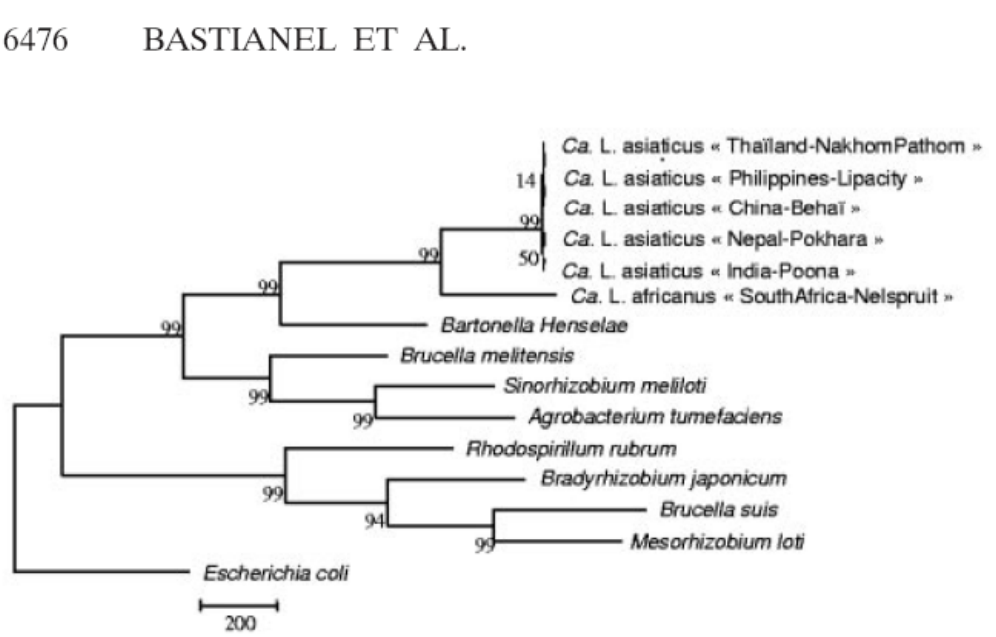
Selection of AMP Transgenes

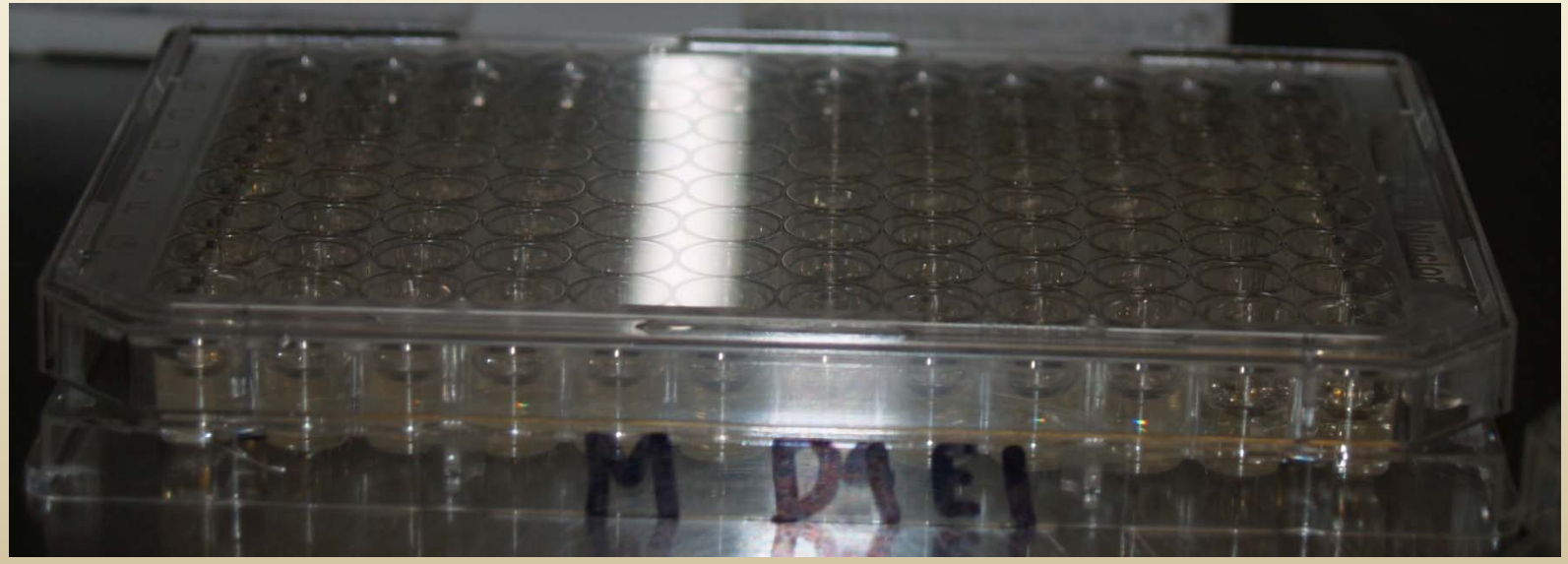


- D4E1: a synthetic AMP of 17 amino acids
  - active against Agrobacterium in poplar
  - undergoing extensive tests for use in human medicine- should help fast-track deregulation
- May be desirable to pyramid several AMPs with very different modes of action
- New cooperative agreement to identify new synthetic AMPs

In –Vitro AMP Screening

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





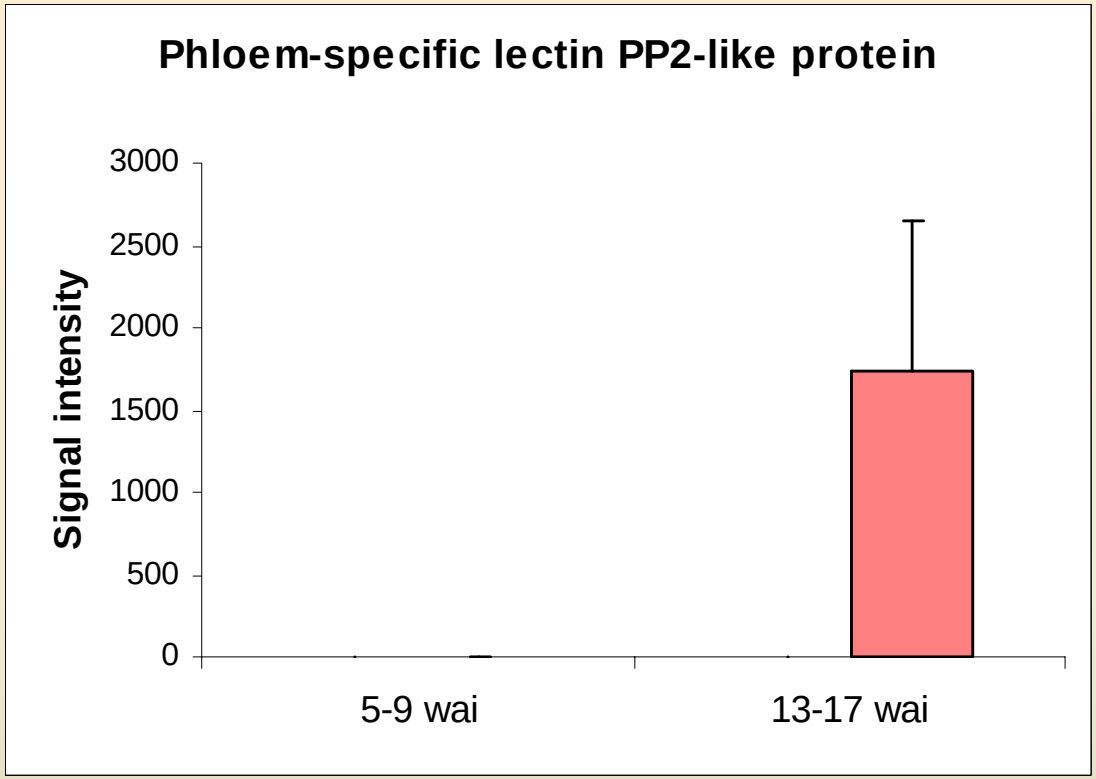
Test Compound	MIC of Bacteria Exposed to Test Compound (µM)		
	A. tumefaciens	*S. meliloti*	*X. s. citri*
	>30	>30	>30
Cecropin A	3	3	10
Cecropin B	10	3	10
D4E1*	1	0.3	1
D2A21*	1	0.3	1
Drosocin	>30	>30	>30
Histatin-5	>30	>30	>30
Indolicidin	10	3	3
LL-37	1	1	1
Magainin I	>30	>30	>30
Magainin II	>30	>30	>30
Melittin	1	1	1
α-Purothionin	30	10	1
Pyrrhocoricin	>30	10	>30
SMAP-29	1	0.3	1
Tachyplesin I	0.3	0.3	0.3

***Agromed LLC**

Other Transgenes for HLB Resistance

- Working with Duan group at USHRL to identify targets based on newly sequenced Liberibacter asiaticus genome
- Hailing Jin at UCR and Kim Bowman generated data on microRNA profile changes resulting from HLB infection
- Numerous other opportunities have been initiated

Leaves from ‘Valencia’ orange plants non-infected (green) and infected (red) with Ca. L. asiaticus 5-9 weeks and 13-17 weeks after inoculation.



A phloem-specific protein is induced later during infection and appears be an attempt of the host to seal sieve tubes as a barrier against an increasing bacterial population.

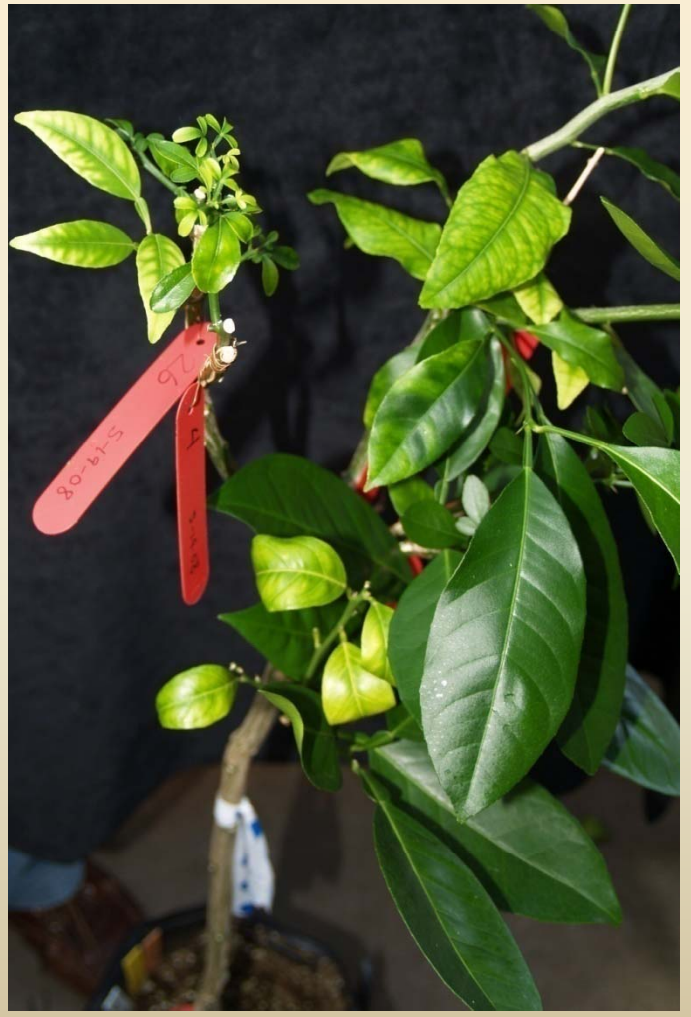
From: Plant Science 2008; 175(3): 291-306, Albrecht and Bowman

Testing for HLB Resistance: Have series of AP24 (Osmotin) transformed Carrizo from earlier project

Transformed lines grafted onto series of HLB+ plants

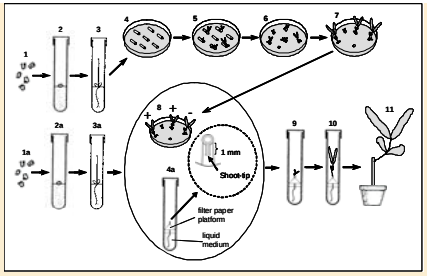
In both cases here, clear HLB symptoms on leaves below graft

However, these are grafted with the same source transformant



Testing for HLB Resistance

- Still slow going from transformant to multiple plants suitable for testing
- Routinely get >90% infection with graft inoculation- 2 buds and 2 leaf midribs per plants
- PCR detection and symptoms within 6+ weeks
- Likely will need 50 independent transformants/ genotype/ construct to have high probability of strong expression
- Comparing graft vs. caged psyllid vs. “natural” psyllid - confidence vs. efficiency vs. overwhelming titer
- Will select 10 mostly high AMP lines for further testing in greenhouse



Transgenic Project Plan- *Major Bottlenecks*

- Juvenility- standard protocols use seeds.
- Transformation is VERY poor with mature tissues, and will absolutely need for monoembryonic and seedless types, earlier fruiting for all
- Trifoliate types have 10X transformation rate unifoliates
- Transformation of mandarins is much more difficult than sweet orange or grapefruit
- Rapid throughput- need higher transformation % and quicker passage from Agro to grafted plant
- Will each citrus genotype be regulated separately?

Evaluating distant citrus and citrus relatives for HLB-resistance

- published reports of HLB resistance in different species in the Aurantiodeae
- some clearly reflect different strain host range
- however, broad search may yield true resistance
- experiments in progress include more than 50 genotypes.- collaboration R. Lee

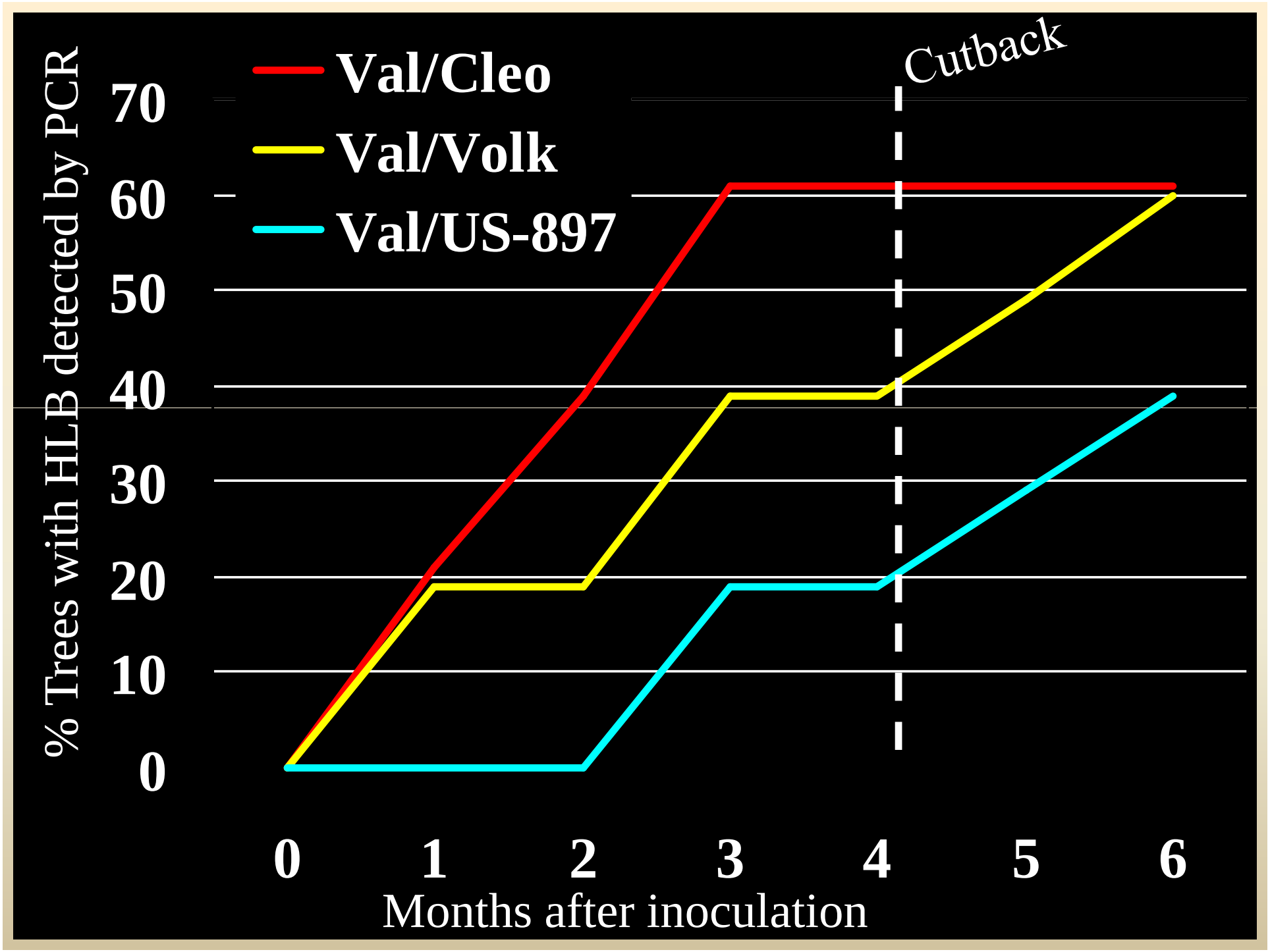
Evaluating distant citrus and citrus relatives for HLB-resistance

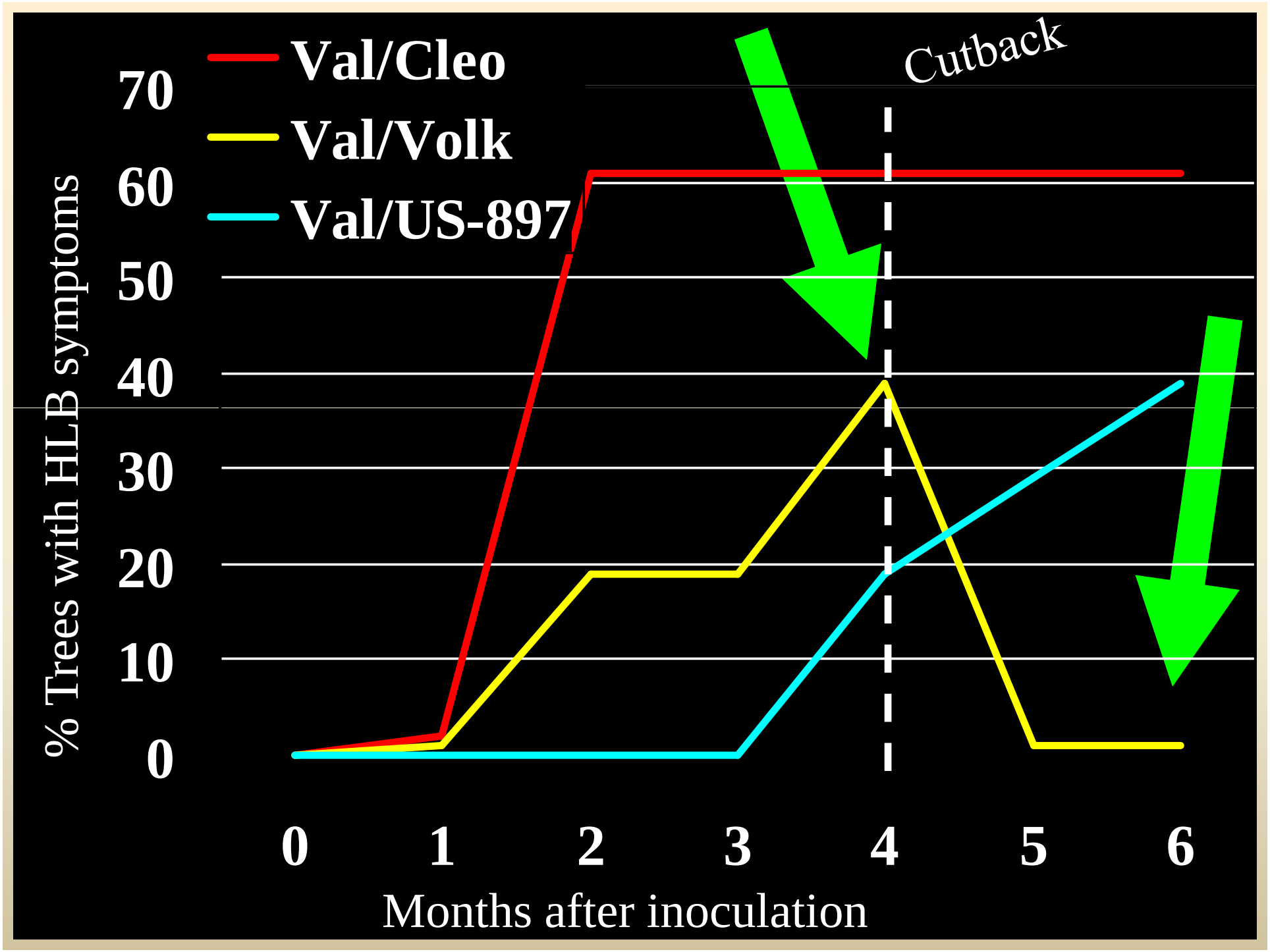
- May identify genes which can then be used to transform commercial citrus
- Some possibility of using hybrids directly as rootstocks….
  - >Rootstock alone MAY confer resistance?
- Transforming citrus with FT gene to permit flowering within year of seed germination
  - >Will permit rapid introgression of genes from distant relatives into commercial citrus rootstocks and scions

In our work and the work presented at the Dec 2008 HLB Conference:

So far, exploration of existing resistance in citrus and relatives appears to reveal tolerance but not resistance

Could be basis for future industry, but would provide “typhoid Marys” alongside existing trees


Val/Cleo
Val/Volk
Val/US-897
Cutback
% Trees with HLB detected by PCR
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Months after inoculation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Thanks!

- Florida Citrus Production Research Advisory Council
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