Transgenic Solutions at CREC

Jude Grosser
Manjul Dutt, Ahmad Omar, Vladimir Orbovic and Gary Barthe
University of Florida,
Citrus Research and Education Center,
Lake Alfred, FL USA

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Antimicrobial peptides and HLB/Canker/CVC

- No natural resistance to HLB in any commercially cultivated sweet orange, grapefruit or tangerine.

- Antimicrobial peptides have been shown to provide resistance to bacterial diseases.

  — LIMA gene to control *Xylella fastidiosa*, the causal organism for Pierce's disease in grapes. (Dr. Dennis Gray, MREC, UF/IFAS)

- Same gene can theoretically be used to combat all three diseases.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

What are antimicrobial peptides?

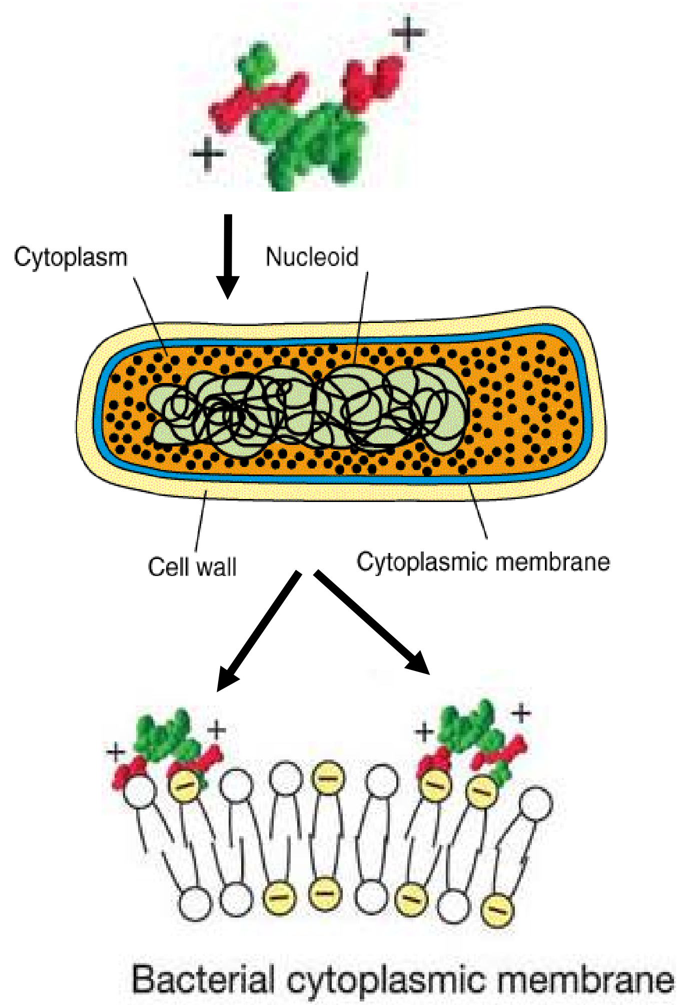
- Antimicrobial peptides are usually small proteins, usually 12 and 50 amino acids long.
- They form the first line of host defense against pathogenic infections and are a key component of the innate immune system
- Antimicrobial peptides are involved in the antimicrobial defense system among all classes of life.
  - Plants
  - Insects
  - Amphibians and
  - Mammals including humans

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Mode of action of Antimicrobial peptides

- The net charge of antimicrobial peptides is positive. Also, they are hydrophobic and they are membrane active.
- The outer surface of bacteria is negatively charged.
- These peptides are mobilized shortly after microbial infection, and act rapidly to neutralize a broad range of microbes.
- The positively charged antimicrobial peptides bind to the negatively charged bacterial membrane
- The membrane is disrupted and antimicrobial peptides inflict damage that is difficult to repair.



Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Incorporation of antimicrobial peptides for disease resistance in citrus

Objectives

- Design codon optimized antimicrobial peptides genes for citrus to combat HLB and Canker.
- Target trans-protein in phloem tissue where HLB resides.
- Produce a large number of transgenic lines using *Agrobacterium* and protoplast mediated transformation.
- Challenge plants with disease causing bacteria to evaluate resistance.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

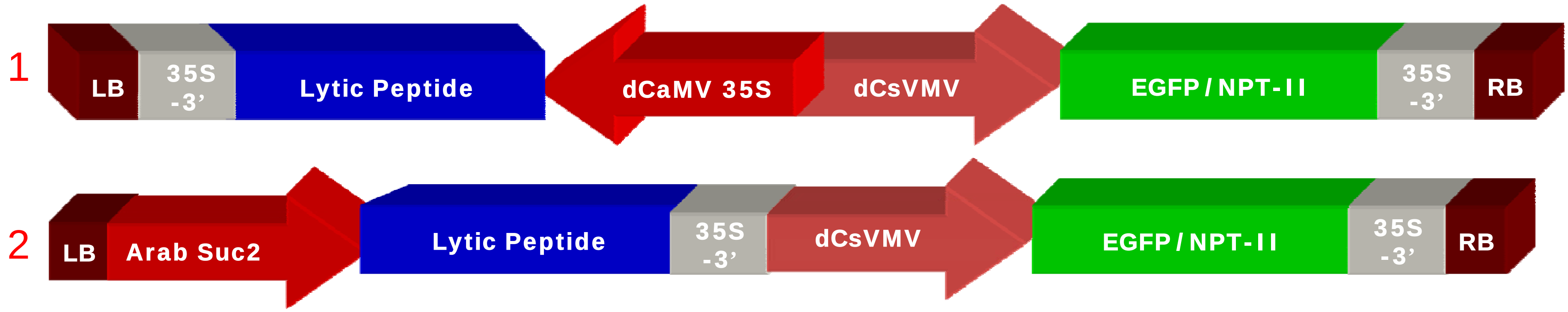
Antimicrobial gene(s) currently under evaluation

- AttacinE - Lytic peptide gene from *Hyalophora cecropia*.
- CEAD - Codon optimized cecropin A-cecropin D lytic peptide gene.
- CEMA - Codon optimized cecropin A-melittin lytic peptide gene.
- CEME - Codon optimized cecropin A-melittin lytic peptide gene (differs at the C terminus from CEMA).
- LIMA - Lytic peptide gene obtained from Dr. Dennis Gray, MREC, UF/IFAS.
- PTA - Codon optimized N terminally modified Temporin A gene.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Gene Construct(s) used for Transformation



The antimicrobial gene(s) were driven by

1. Constitutive doubly enhanced 35s promoter or
2. Phloem specific sucrose synthase promoter.

- A **green** fluorescent protein/neomycin phosphotransferase II (EGFP/NPT II) bi-functional fusion gene under control of a cassava mosaic virus promoter was used to monitor and select transformed cells.

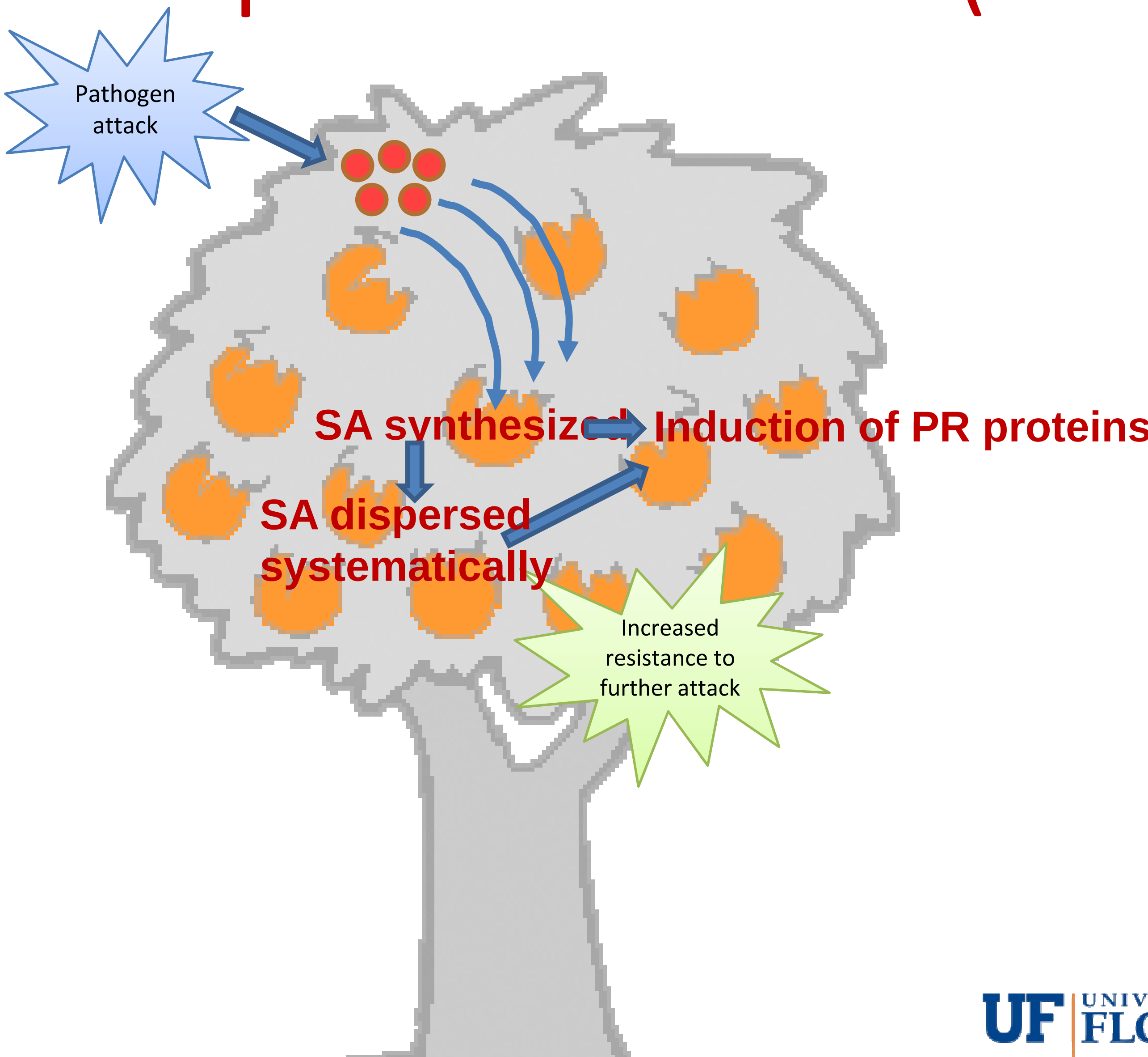
- A pBIN19 backbone was used to clone all gene constructs.

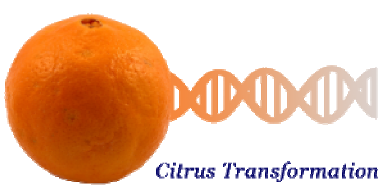
- Plasmid DNA was incorporated into *Agrobacterium* EHA105.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Systemic acquired resistance (SAR)





UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Genes for SAR

- **SABP2** (Salicylic Acid-Binding Protein 2 gene from tobacco)
  - – Isolated from the Tobacco plant.
  - – High Affinity for SA.
  - – It may be required to convert Methyl Salicylate to SA as part of the signal transduction pathways that activate systemic acquired resistance.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

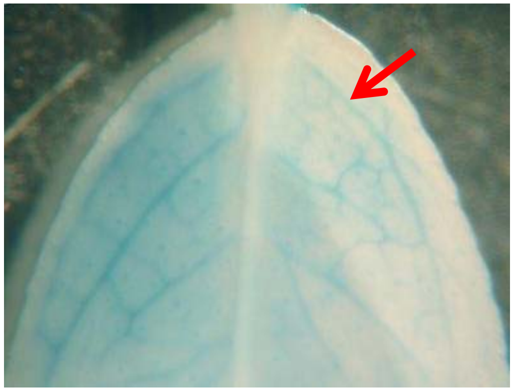
Genes for SAR

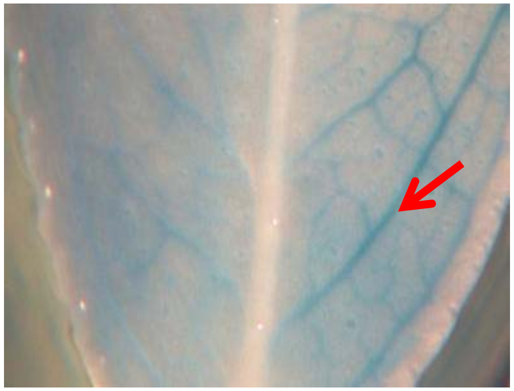
- **NPR1** (Nonexpresser of PR Genes1 gene from *Arabidopsis*)
  - Isolated from the Model plant *Arabidopsis thaliana.*
  - NPR1 is a key regulator in the signal transduction pathway that leads to SAR.
  - Mediates the salicylic acid induced expression of pathogenesis-related (PR) genes and systemic acquired resistance.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Use of phloem specific promoters to restrict trans-protein in phloem tissues





GUS expression in citrus leaf phloem tissue using the Rice Sucrose Synthase promoter

- HLB resides in the phloem.
- Targeting the trans-protein in the phloem resolves issues of the presence of the protein in the fruit and juice.
- Two phloem specific promoters are currently under evaluation
  1) *Arabidopsis* Sucrose synthase promoter and
  2) Rice Sucrose synthase promoter.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Genetic transformation of citrus

- Two methods now available
  - Agrobacterium mediated transformation
  - PEG mediated Protoplast transformation

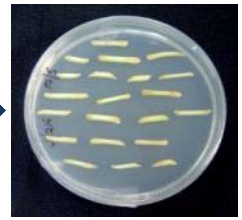
Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

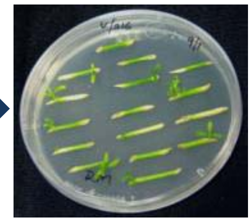
Agrobacterium Mediated Transformation

Etiolated seedlings

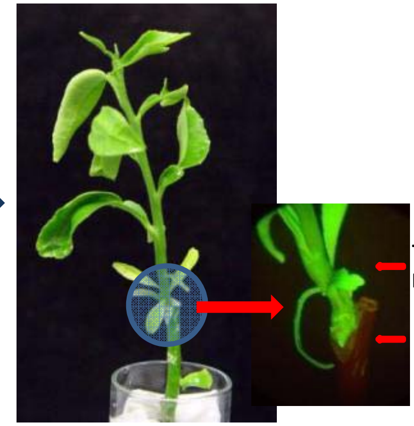
Epicotyl sections in *Agrobacterium*



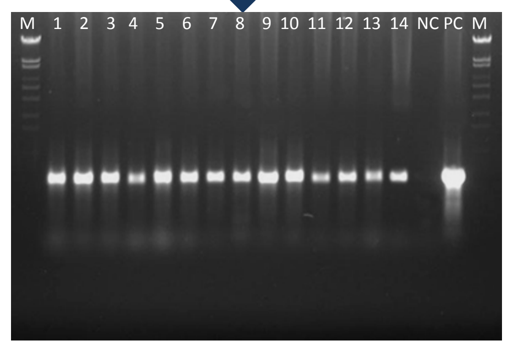
Epicotyl sections in Co-cultivation medium



Epicotyl sections after 1 month in selection medium



Transgenic plants grafted onto Carizzo rootstock



PCR confirmation of presence of transgene

PCR positive and acclimatized transgenic plants

Transgenic plants in the greenhouse

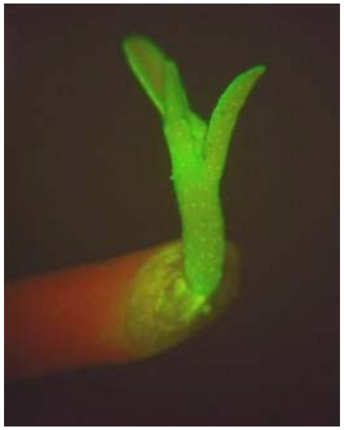
Citrus Transformation

UF UNIVERSITY of FLORIDA

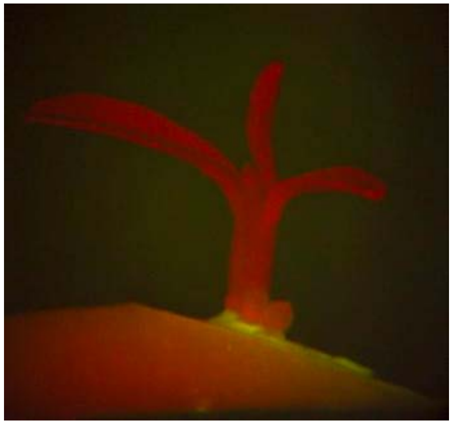
IFAS

Citrus Research and Education Center

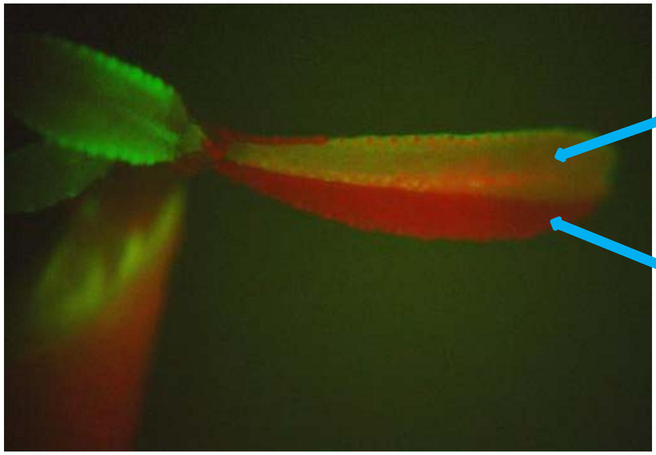
Selection of transgenic tissue



Transgenic tissue glows green!



Non-Transgenic tissue stays red



Transgenic tissue

Non-Transgenic tissue

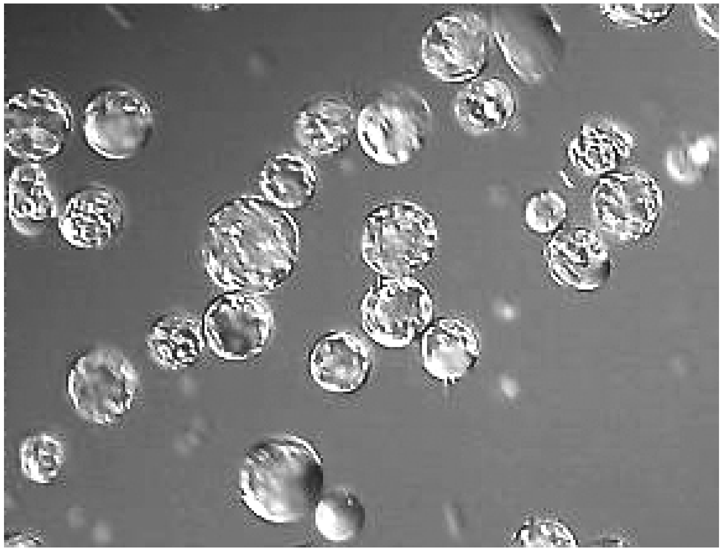
Chimeric tissue (a mix of transgenic and non transgenic)

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Citrus Protoplast Transformation

- A plant cell that had its cell wall completely removed using enzymatic means.
- Naked Plasmid DNA is incorporated into Protoplast DNA by Electroporation or PEG mediated transformation.
- Complete plants are regenerated from transformed protoplasts using Tissue culture techniques.



Citrus Transformation

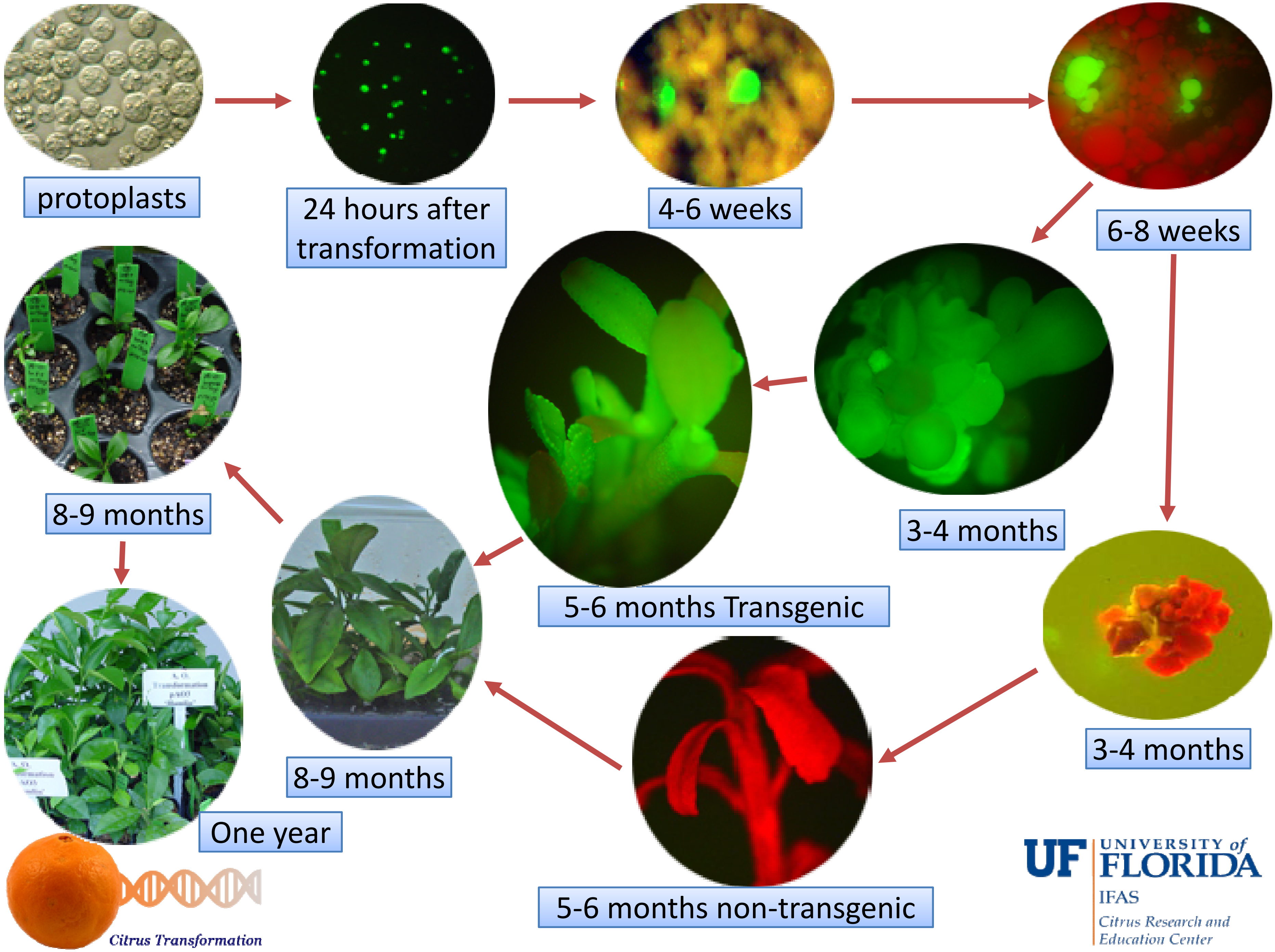
UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Protoplast transformation

- Can be used to transform polyembryonic seedless cultivars that are difficult to transform using Agrobacterium.
- Can be used to transform some polyembryonic mandarin cultivars that are often more recalcitrant to Agro-infection (i.e. W. murcott)
- Can be used to engineer plants not containing an antibiotic resistance gene.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center



protoplasts
24 hours after transformation
4-6 weeks
6-8 weeks
3-4 months
5-6 months Transgenic
8-9 months
3-4 months
5-6 months non-transgenic
8-9 months
One year
Citrus Transformation
UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Transgenic plant regeneration

Cultivar	Gene	No. of plants in soil
Duncan	AttacinE	27
Hamlin	AttacinE	15
Misc Grapefruit	LIMA	45
Valencia, Hamlin,OLL-8	LIMA	56
Carrizo	LIMA	8
Flame	LIMA-SN	10
Misc Grapefruit	PTA	12
Valencia, Hamlin, OLL8	CEMA	21
Carrizo	CEMA	20
Key Lime	CEMA	6
Misc Grapefruit	CEME	18
Hamlin	CEME	6
Valencia	CEAD	14
Carrizo	CEAD	12
Carrizo	LIMA under AtSuc2 promoter	25
Valencia, Hamlin, OLL-8	LIMA under AtSuc2 promoter	23
Key Lime	LIMA under AtSuc2 promoter	17
Misc Grapefruit	LIMA under AtSuc2 promoter	12

Citrus Transformation

Citrus Research and Education Center

Results with SAR genes

Cultivar	Gene construct	No of Transgenic plants in soil
Valencia, Hamlin, Flame	35s - SABP2	30
Valencia, Hamlin	35s - NPR1	20
Hamlin	AtSUC2 – SABP2	26
Hamlin	AtSUC2 – NPR1	10
Hamlin	35s – NPR1 + 35s - LIMA	2

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Rapid propagation of transgenics

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Young budded transgenic plants for field testing

Citrus Transformation

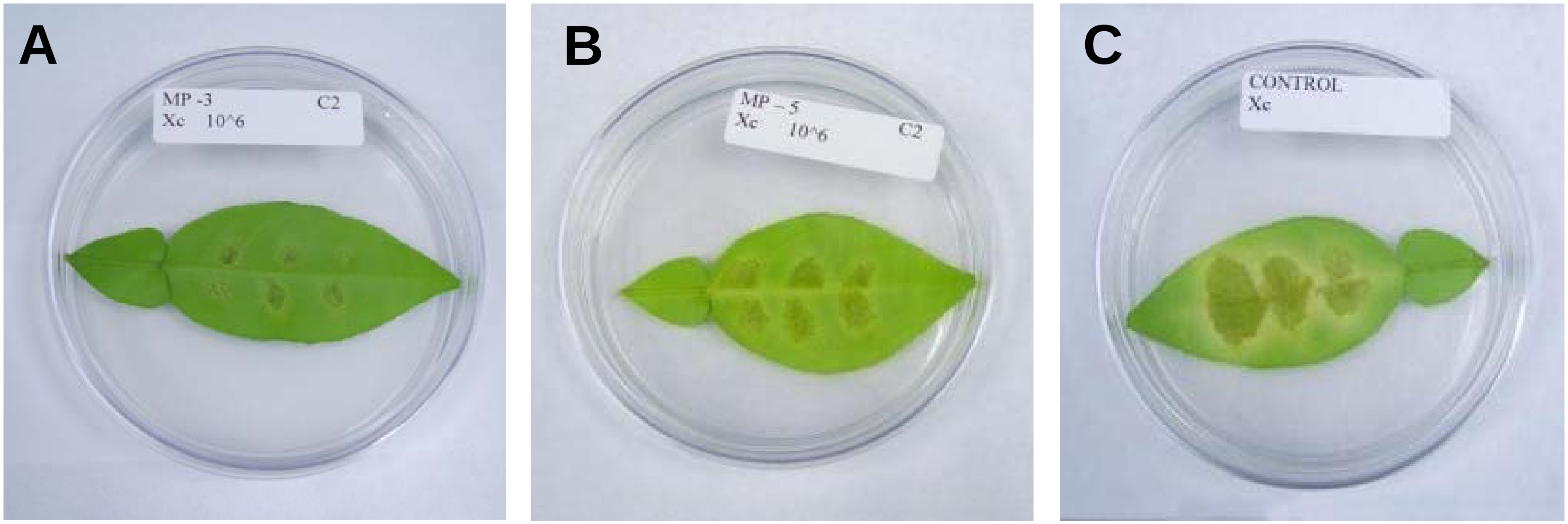
UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Transgenic trees ready for testing

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Canker (Xcc) assay on transgenic Duncan leaves containing the lytic peptide gene(s)



A – Mild tissue hypertrophy in transgenic LIMA leaf
B – Moderate tissue hypertrophy in transgenic Attacin leaf
C – Severe water-soaking and tissue hypertrophy in non-transgenic control leaf

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

qRT-PCR analysis of selected transgenic lines infiltrated with Xcc by the detached leaf assay method

Transgenic line	AttacinE		LIMA	
	Mean cT	Bacterial cells / mg tissue*	Mean cT	Bacterial cells / mg tissue
MP1	14.775	194975	18.285	18623
MP2	16.565	61453	18.362	17619
MP3	14.180	318000	26.625	70
MP4	15.935	90065	23.365	629
MP5	18.670	14369	14.040	324000
Control	15.560	115031	15.560	115031

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Transgenic plant challenge with HLB

- Carried out in an approved secure facility.
- Transgenic plants graft challenged with HLB infected sweet orange budwood.
- Several Transgenic lines are currently under evaluation.
- Transgenic plants evaluated for HLB symptoms and infection verified by qRT-PCR.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Graft and bud inoculation with HLB

HLB positive Bud wood

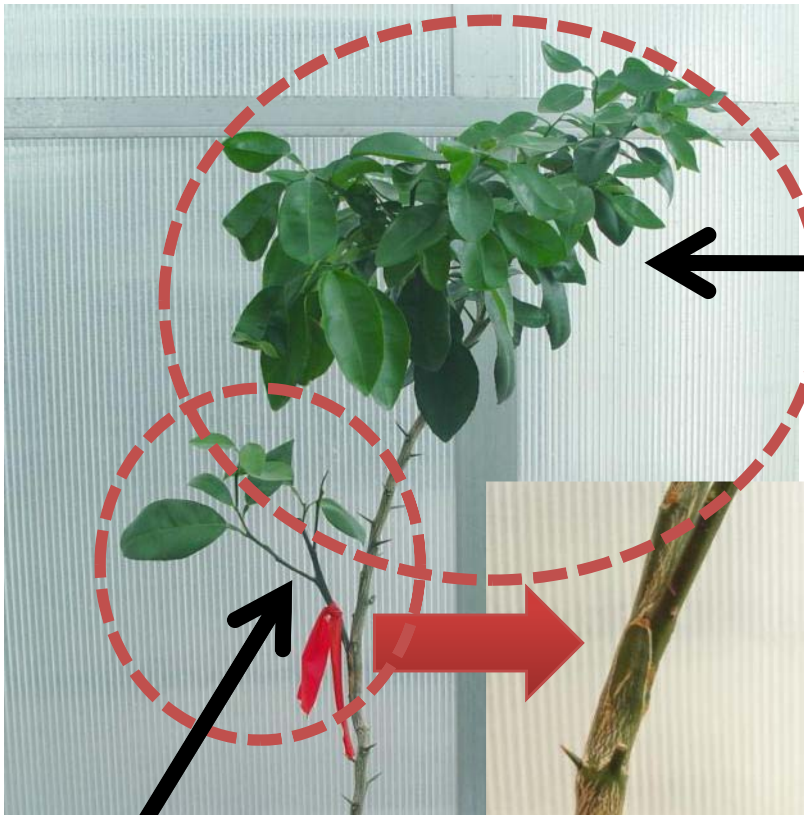
HLB positive Bud

Transgenic Citrus

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and
Education Center

Transgenic plant challenge via grafting with HLB infected budwood



Transgenic citrus plant after 13 months of inoculation with HLB + sweet orange bud stick

HLB infected budstick

Explant	qRT-PCR using HLB specific primers
Transgenic plant	Negative
Budstick	Positive

Citrus Transformation

UF UNIVERSITY of FLORIDA IFAS Citrus Research and Education Center

HLB inoculation results

- Wide range of symptoms observed after inoculation with HLB + budwood
- General mottle on top of leaf
- Mottle and subsequent yellowing

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

THANKS!

- FCPRAC block grant entitled "**SURVIVING HLB AND CANKER: GENETIC STRATEGIES FOR IMPROVED SCION AND ROOTSTOCK VARIETIES" -** Fred Gmitter, Jude Grosser, Bill Castle and Gloria Moore
- USDA-CSREES
- UF/CREC Core Citrus Transformation Facility
- CREC Faculty and Staff

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Psyllid control

- Production of Transgenic citrus plants to combat psyllids
- Duncan Grapefruit used as a model.
- Constitutive expression of a Snowdrop Lectin gene from the Snowdrop Plant (*Galanthus nivalis*)
- Several plants are currently being propagated for resistance studies.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Future approaches for genetic resistance studies

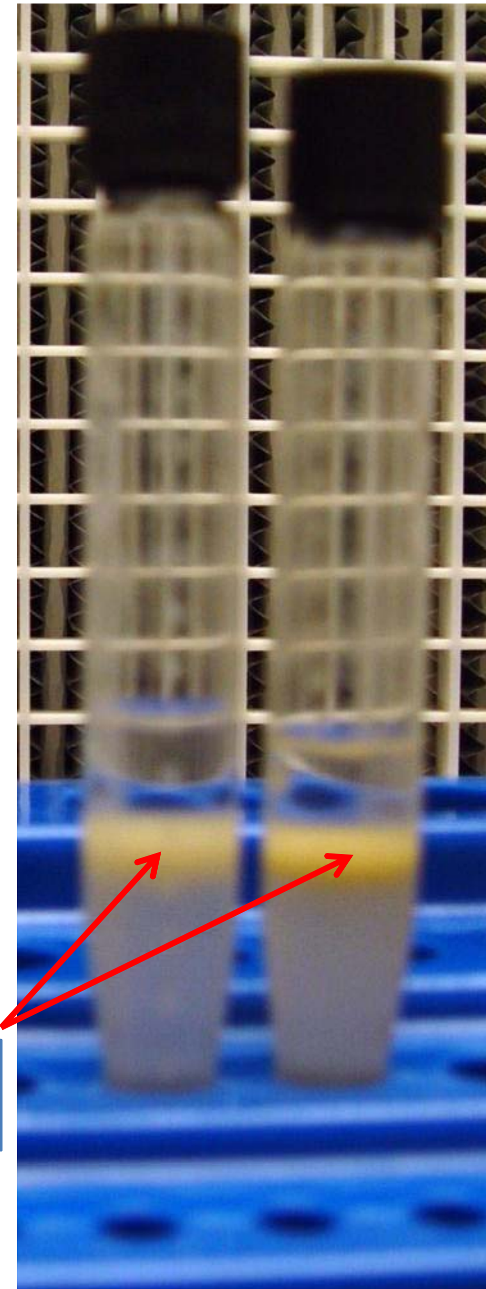
- Screen a large number of transgenic trees to select individuals with the highest level of resistance.
- Challenge putative resistant plants with psyllids.
- Field testing in a high disease pressure environment.

Citrus Transformation

UF UNIVERSITY of FLORIDA
IFAS
Citrus Research and Education Center

Cellulase

Macerozyme



Protoplast ring


Shoot tip grafting
In vitro micrografting
BELLCO
Citrus Transformation
Education Center