Towards the Ultimate Solution: Genetic Resistance to HLB in Commercial Citrus

Greening Summit
Florida Citrus Growers Institute
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IFAS

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THE PROBLEM:

NO RESISTANCE TO GREENING EXISTS IN COMMERCIAL CITRUS GERMPLASM

-Even if tolerant citrus species are found (papedas?), tolerance cannot be moved into commercial scions by conventional breeding

Resistance to HLB

Citrus greening or Huanglongbing (HLB) is an important disease of citrus affecting all commercial citrus cultivars. In Florida, the disease is caused by *Candidatus* Liberibacter asiaticus, a phloem limited bacterium transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama.

Genetic engineering of citrus permits the incorporation of gene(s) encoding anti-microbial peptides into the plant's genome. Anti-microbial peptides are usually small peptides and are present in plants, insects and humans. In general, these peptides inhibit the synthesis of the invading pathogen's cell membrane and prevent its further replication.

Leaf with mottled leaf symptom of HLB

Primary Approach

Genetic engineering via *Agrobacterium* mediated or protoplast transformation of important citrus cultivars with antimicrobial genes and evaluation for resistance to HLB disease. We are also attempting to target the expression of the foreign antimicrobial genes to the phloem tissue where the bacteria reside, thereby minimizing foreign gene products in the juice or fruit.

ANTIMICROBIAL GENE CONSTRUCTS

Constructs containing natural or synthetic antimicrobial peptide genes were constructed. The genes were driven either by a **constitutive d35s promoter** or a **phloem specific *Arabidopsis* sucrose synthase promoter.** A green fluorescent protein/neomycin phosphotransferase II (EGFP/NPTII) fusion gene under control of a cassava mosaic virus promoter was used to monitor and select transformed cells.

Currently, the following genes are being used for transformation into citrus.

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

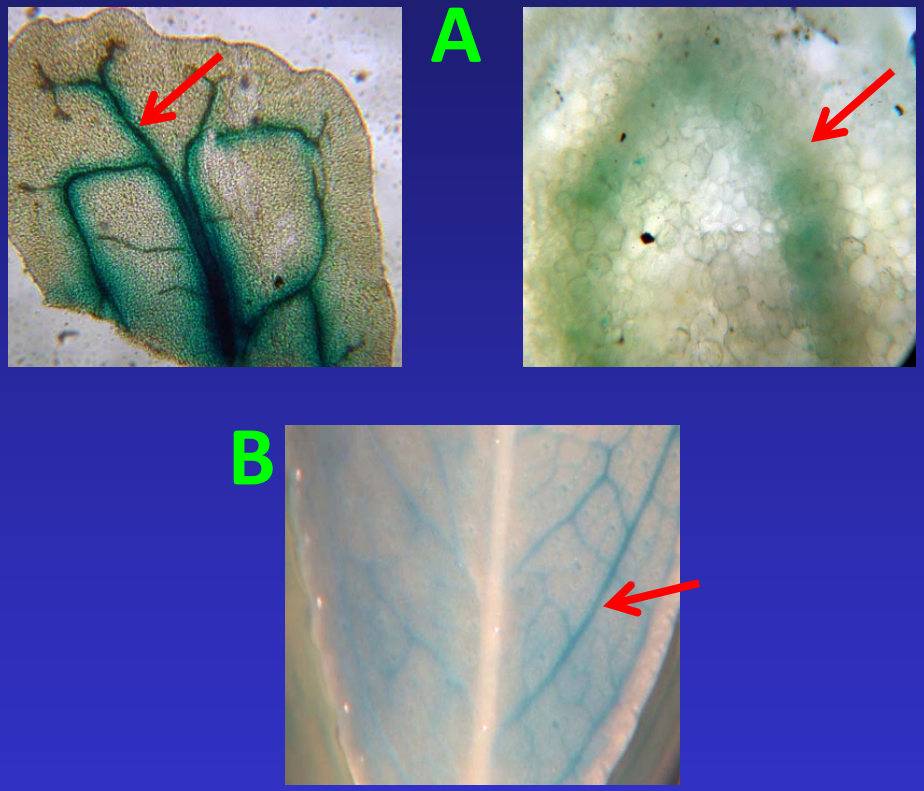
CEME-Codon optimized cecropin A-melittin lytic peptide gene

LIMA-Lytic peptide gene obtained from Dr. Dennis Gray, MREC, UF/IFAS – works against Xyella in grape!

PTA-Codon optimized N terminally modified Temporin A gene

GUS EXPRESSION IN THE PHLOEM

The HLB bacterium is a phloem limited bacterium. To evaluate the effectiveness of promoters in targeting gene expression into the phloem, the phloem specific *Arabidopsis* sucrose synthase and Rice sucrose synthase promoters were evaluated for their activity in Citrus. Citrus plants were transformed with a GUS gene driven by either of the promoters and transgenic plants were histochemically stained to locate GUS activity.

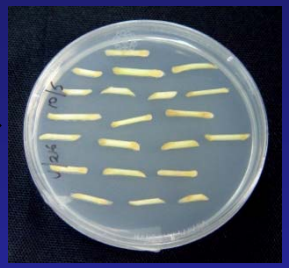


Phloem specific GUS expression by the *Arabidopsis* (A) and Rice (B) sucrose synthase promoters

AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

Etiolated seedlings

Epicotyl sections in *Agrobacterium*



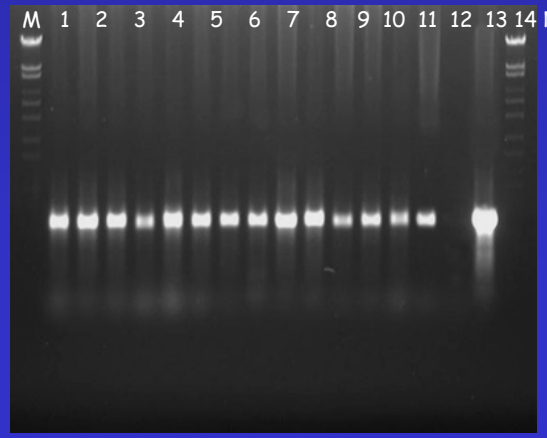
Epicotyl sections in Co-cultivation medium

Epicotyl sections with regenerated shoots after 1 month in selection medium

Transgenic plants grafted onto Carizzo rootstock

Transgenic plants in the greenhouse

PCR positive and acclimated transgenic plants



PCR confirmation of presence of transgene

TRANSGENIC LINES REGENERATED USING THE DIFFERENT CONSTRUCTS (Drs. Manjul Dutt and Ahmad Omar)

Transgenic Summary			
Cultivar	**Plasmid(S)**	**Gene(s)**	**No. of lines with plants in soil**
Duncan	**d35s-AttacinE**	AttacinE	27
Misc Grapefruit	**d35s-LIMA**	LIMA	43
Misc	**d35s -PTA**	N-Terminally modified TemporinA gene	18
Valencia	**d35s-CEMA**	Cecropin-Melittin Fusion gene	4
Misc	**d35s-CEME**	Cecropin-Melittin Fusion gene. Differs from CEMA by containing different amino acids in the C terminus	14
Grapefruit	**d35s –CEAD**	CecropinA-CecropinD fusion gene	2
Key lime	**OSS-GUS**	Rice sucrose synthase promoter	8
Key lime	**Rolc-GUS**	Agrobacterium RolC Promoter	6

TRANSGENIC LINES REGENERATED USING THE DIFFERENT CONSTRUCTS (Drs. Manjul Dutt and Ahmad Omar)

Transgenic Summary				
Cultivar	**Plasmid(S)**	**Gene(s)**	**No. of lines with plants in soil**	**No. of lines in Vitro**
Carrizo	pC1391-AO1	GUS under AtSuc2 promoter	6	9
Carrizo	pC1391-AO1 + pCit101	Co-transformation GUS and GFP	2	5
Carrizo	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	15	20
Valencia	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	4	10
Hamlin	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	2	9
Key Lime	pC1391-AO1	GUS under AtSuc2 promoter	2	8
Key Lime	pC1391-AO1 + pCit101	Co-transformation GUS and GFP	1	4
Key Lime	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	2	15
March	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	0	6
Duncan	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	1	5
OLL#8	pSuc2-LIMA-vec2	LIMA under AtSuc2 promoter and GFP	0	2

ALTERNATIVE CITRUS TRANSFORMATION TECHNOLOGY

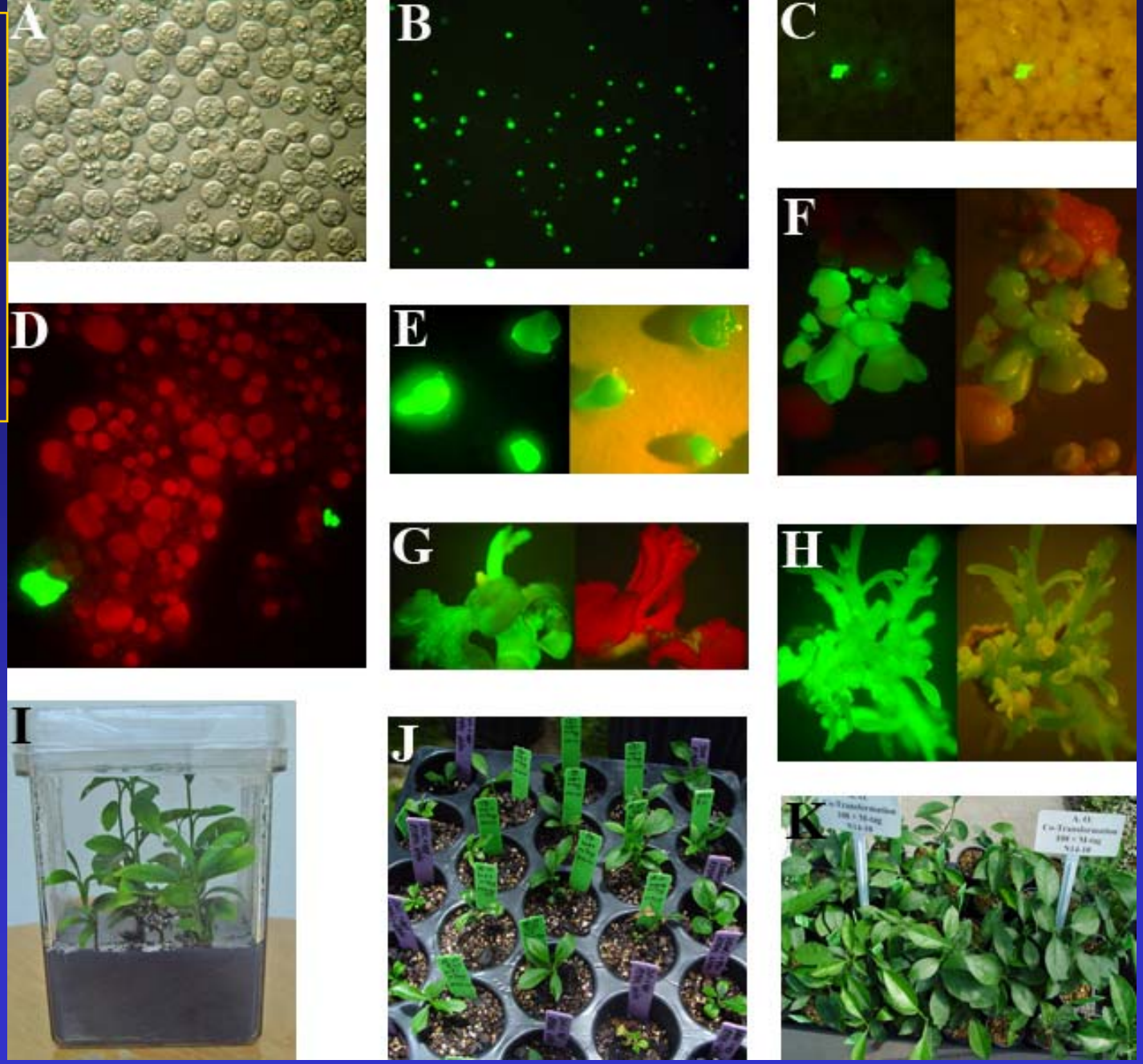
-protoplast/GFP method, regeneration via somatic embryogenesis

-does not require an antibiotic resistance gene like the Agro-system (the European Union is highly opposed to transgenics containing antibiotic resistance genes)

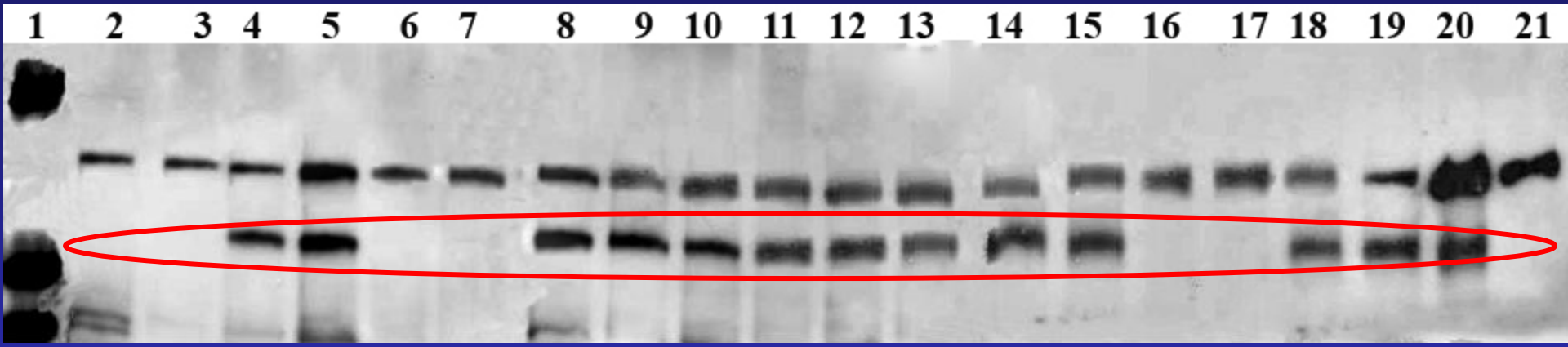
-now working for tangerine cultivar W. Murcott (tangerines and tangelos are recalcitrant in the Agro-system) – potential for transforming other fresh fruit cultivars!

Citrus protoplast transformation using GFP with regeneration via somatic embryogenesis

(Dr. Ahmad Omar)



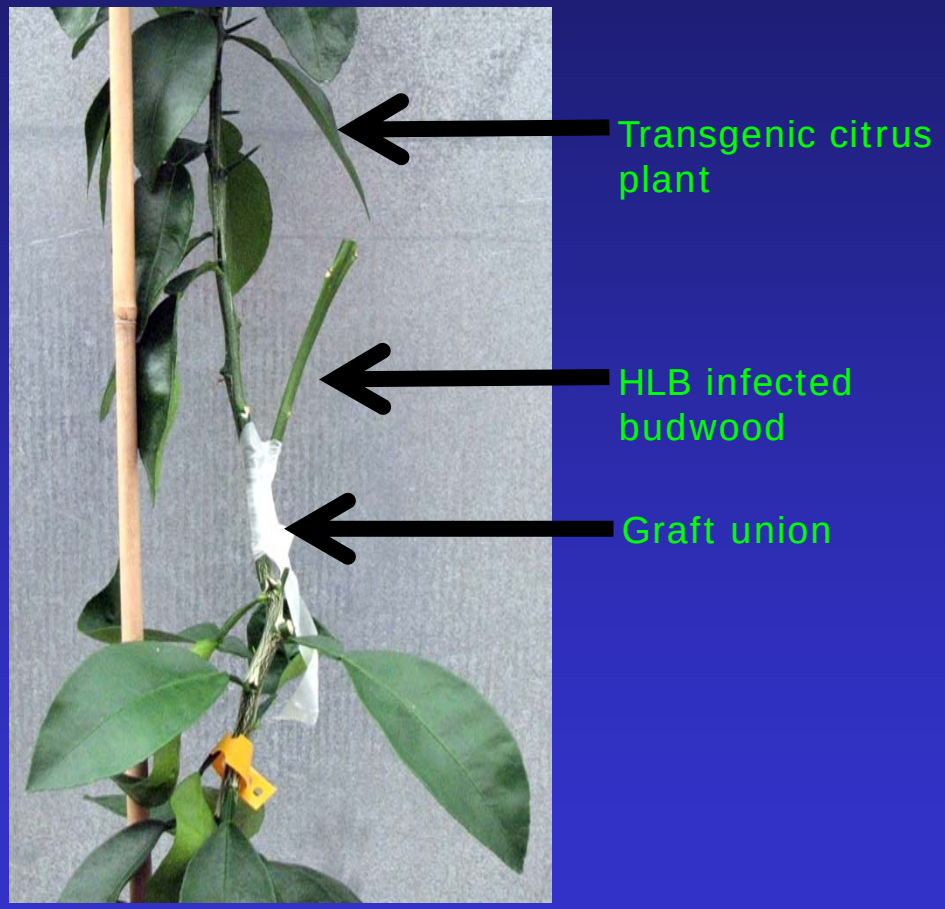
Western analysis



Soluble extracts of the transgenic rice expressing the wild-type *Xa21* gene (lane 20), non-transgenic rice (lane 21), transgenic citrus expressing the *GFP* gene only (lane 2), non-transgenic citrus (lane 3), transgenic citrus positive for *GFP* and *Xa21* genes (lanes 4-19) were subjected to Western blotting with reference to molecular mass markers (indicated in lane 1)

TRANSGENIC PLANT CHALLENGE

Transgenic plants were graft challenged with HLB infected sweet orange budwood. Plants are currently under evaluation. Transgenic plants will be evaluated for HLB symptoms and infection will be verified by qRT-PCR.



Continued R&D to Improve Transformation Efficiency

- Selection of quality sweet orange clones with reduced juvenility to expedite subsequent plant evaluation
- Identification of OLL#8 sweet orange (Valencia-like) that transforms an order of magnitude better than standard Hamlin or Valencia
- Extending methodology to recalcitrant commercial cultivars

Transgenic W. Murcott

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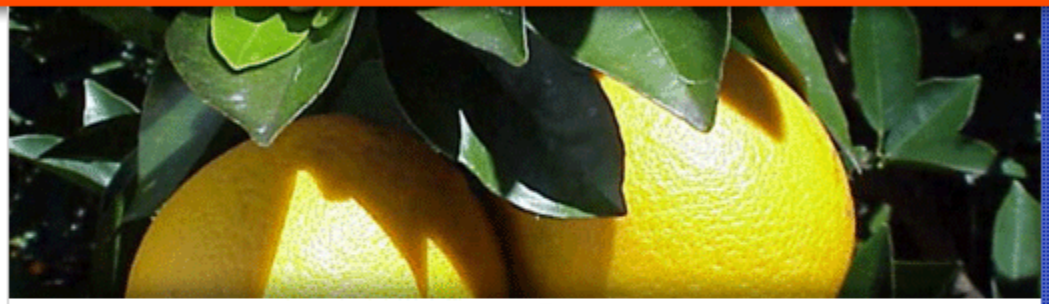


Transformation Lab

Mission

The Core Citrus Transformation Facility was established to expedite integration of recombinant DNA technologies and genetic engineering into basic research from other related disciplines, including biochemistry, food science/processing, post harvest physiology, horticulture, plant pathology, and entomology by providing a service that will allow researchers from all disciplines to have their genes of interest transferred and tested in the appropriate citrus cultivars. A functional core citrus transformation laboratory should benefit nearly all phases of the Florida citrus industry and will no doubt play a major role in the long-term improvement of citrus.

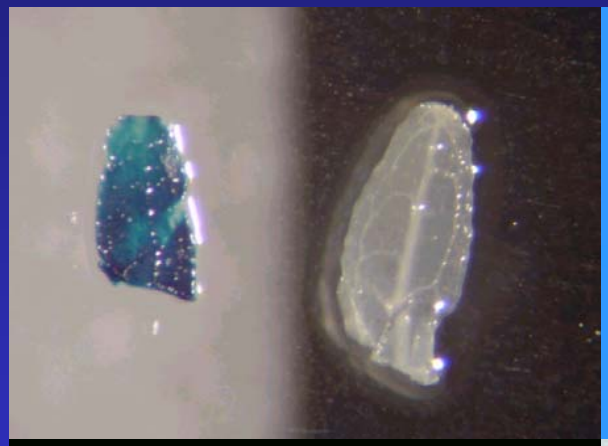
General information on CCTF

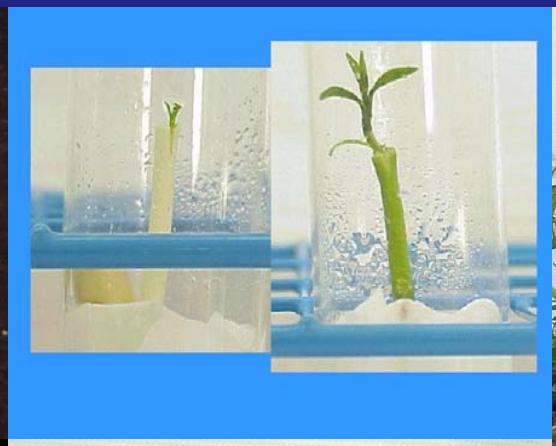
UF UNIVERSITY of FLORIDA
IFAS Research
Citrus Research and Education Center

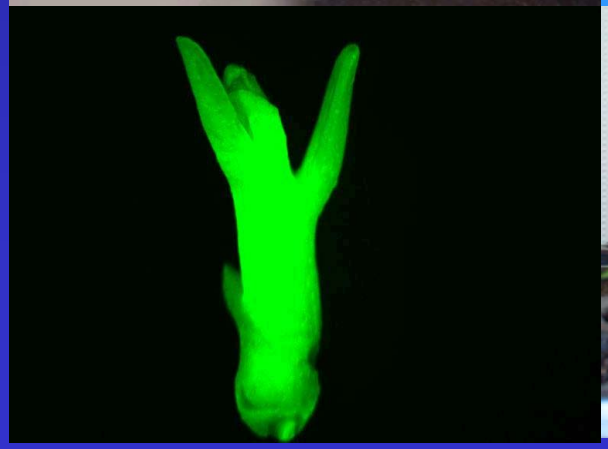
CCTF in operation for more than 5 years; visit our website at: www.crec.ifas.ufl.edu/facilities/transformation/index.htm

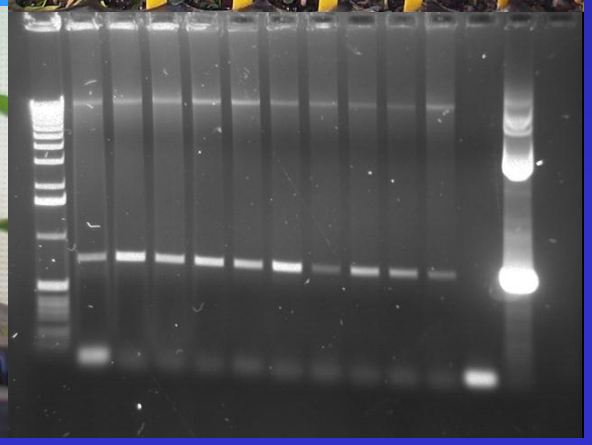
Sole purpose of the facility is to produce transgenic Citrus seedlings according to consumers orders - including those associated with greening

Managed by Dr. Vladimir Orbovic









THANKS FOR YOUR SUPPORT!

FCPRAC & USDA-CSREES

Industry Partners

Collaborators

IFAS Administration

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"When the going gets tough, The tough get going" – Sister Esther, 6th grade