Genetic Engineering of Citrus to defeat HLB –

Highlights from the UF/CREC Breeding Program

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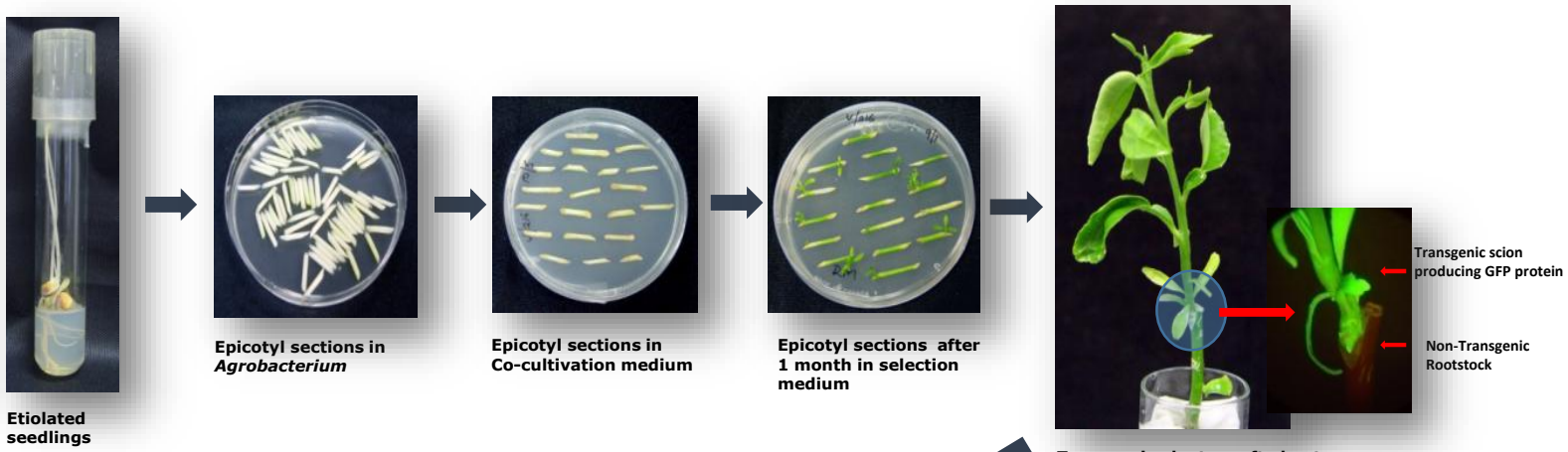
Key Points from our transgenic research:

- UF-CREC Breeding Program instrumental in developing citrus genetic engineering technology
- Production of HLB tolerant transgenic citrus
- Development of all citrus transgenic plants with no GMO signature – may be required for successful CRISPR application in citrus!
- Development of early Flowering citrus – overcoming juvenility
- Gene stacking
- Future trends

Major citrus genetic transformation systems

Technique	Regeneration System	Efficiency of transgenic plant regeneration
Juvenile epicotyl mediated	Organogenesis	High
Mature stem pieces mediated	Organogenesis	Low
Juvenile Callus mediated	Somatic Embryogenesis	High
Juvenile Protoplast mediated	Somatic Embryogenesis	Low

Juvenile tissue transformation using epicotyl explants



Etiolated seedlings

Epicotyl sections in *Agrobacterium*

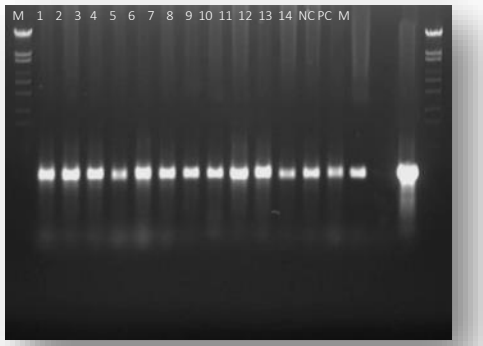
Epicotyl sections in Co-cultivation medium

Epicotyl sections after 1 month in selection medium

Transgenic scion producing GFP protein

Non-Transgenic Rootstock

Transgenic plants grafted onto Carizzo rootstock



PCR confirmation of presence of transgene

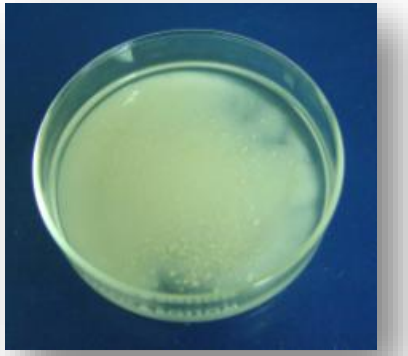
PCR positive and acclimatized transgenic plants

Transgenic plants in the greenhouse

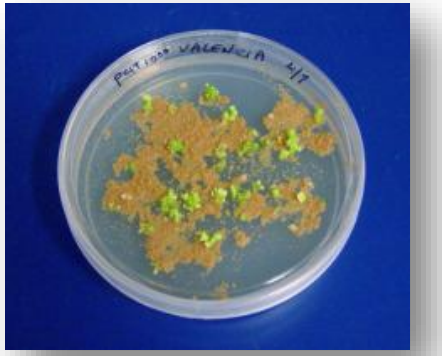
Dutt, M. and J.W. Grosser.2009. Evaluation of parameters affecting *Agrobacterium*-mediated transformation of Citrus. Plant Cell, Tissue and Organ Culture 98: 331-340

Juvenile tissue transformation using embryogenic cell suspension cultures

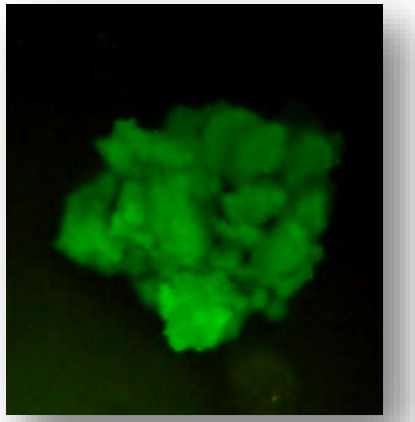
Citrus cell suspension cultures



Cell suspension with *Agro*



Transgenic callus / embryos regenerating on selection medium

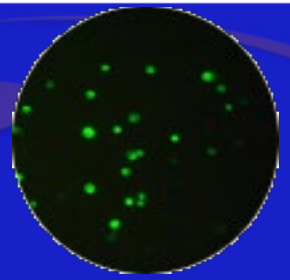


GFP expressing transgenic citrus callus

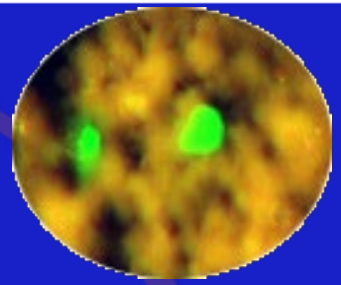
Close-up of GFP expressing transgenic citrus somatic embryos

Transgenic citrus plants

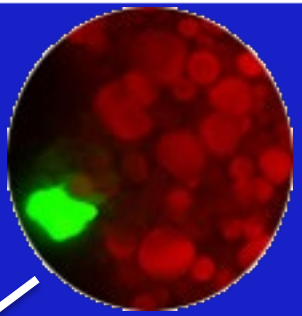
protoplasts



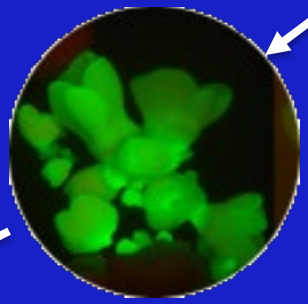
24 hours after transformation



4-6 weeks



6-8 weeks



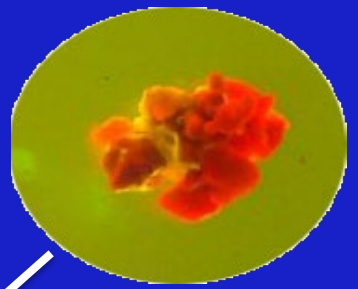
3-4 months

5-6 months Transgenic

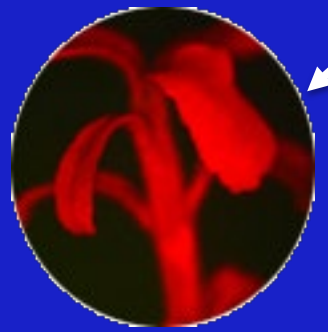
8-9 months

8-9 months

One year



3-4 months



5-6 months non-transgenic

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Genes that have been tested in the field against HLB

1) <u>ANTIMICROBIAL GENE CONSTRUCTS</u>

- AttacinE - Lytic peptide gene from *Hyalophora cecropia*.
- CEAD - Codon optimized cecropin A-cecropin D lytic peptide gene.
- CEAD3 - Codon optimized cecropin A-cecropin D lytic peptide gene variant 1 with Pr1B signal peptide.
- CEAD17- Codon optimized cecropin A-cecropin D lytic peptide gene variant 2 with Pr1B signal peptide.
- CEMA - Codon optimized cecropin A-melittin lytic peptide gene.
- CEME - Codon optimized cecropin A-melittin lytic peptide gene.
- LIMA-A and LIMA-B - Lytic peptide genes kindly provided by Dr. Dennis Gray, MREC, UF/IFAS .
- PTA - Codon optimized N terminally modified Temporin A gene.

2) <u>SYSTEMIC ACQUIRED RESISTANCE RELATED GENE CONSTRUCTS</u>

- SABP2 - Salicylic Acid Binding Protein 2 gene from Tobacco (*Nicotiana tabacum).*
- NPR1 - Non-expressor of Pathogenesis Related 1 gene from *Arabidopsis thaliana.*
- BG - beta-1,3-glucanase from *Citrus sinensis.*

3) <u>Xanthomomas resistance gene from rice</u>

- Xa21 – rice blight resistance gene from rice *(Oryza sativa)*

4) <u>INSECTICIDAL GENE CONSTRUCTS</u>

- GNA - Snowdrop lectin gene from the snowdrop plant (*Galanthus nivalis*).

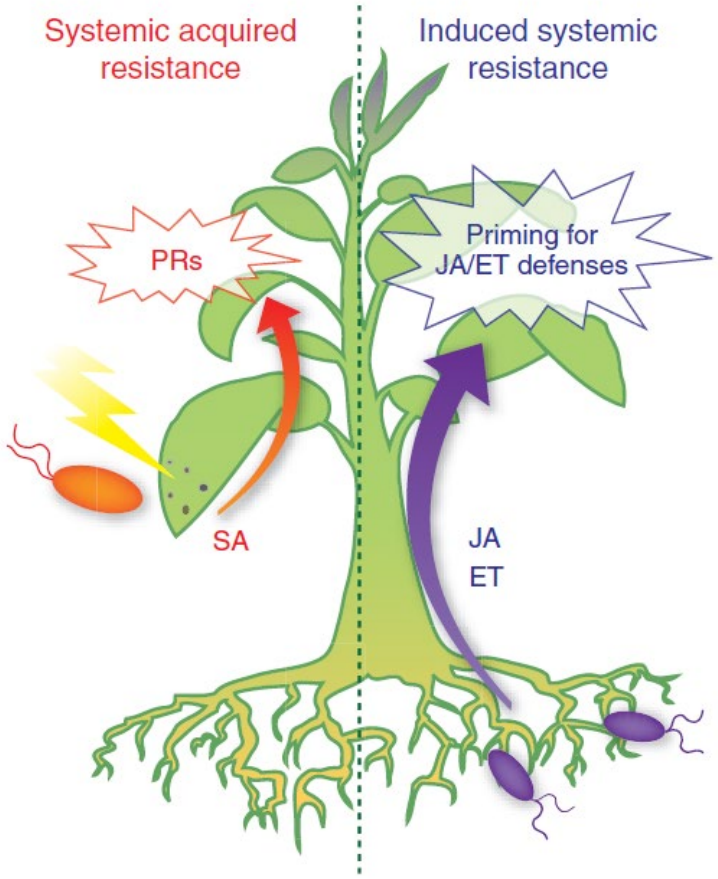
Anti-microbrial peptide (AMP) genes looked promising for 2-3 years, but then mostly faded, thus our focus on:

Systemic Acquired resistance (SAR) for HLB tolerance

Utilizing Systemic Acquired resistance (SAR) for HLB tolerance

- **SAR** can be defined as:
  - a defense response resulting in the systemic expression of a subset of defense genes
  - Causes the plant to subsequently be systemically “immunized” so that further infection will either exhibit increased resistance or reduced disease symptoms.

Systemic acquired resistance (SAR)



- Engineered plants that overexpress a SAR inducing gene could potentially be permanently kept in the primed state.
- Because of constitutive priming, the plant should be able to rapidly and effectively induce their various cellular defense responses, thus leading to enhanced defense responses following *Clas* attack.

Upon pathogen infection, there is activation of cellular defense responses.

Some genes involved in the SAR process

NPR1

- **receptor for the plant defense hormone salicylic acid**
- **activates SA-dependent defense genes**

SABP2

- **catalyzes the conversion of methyl salicylic acid (MeSA) into salicylic acid (SA)**
- **binds SA with high affinity**

SARDI

- **major node in SA signaling**
- **Responsible for ICS1 (Isochorismate Synthase 1) induction and salicylic acid (SA) synthesis**

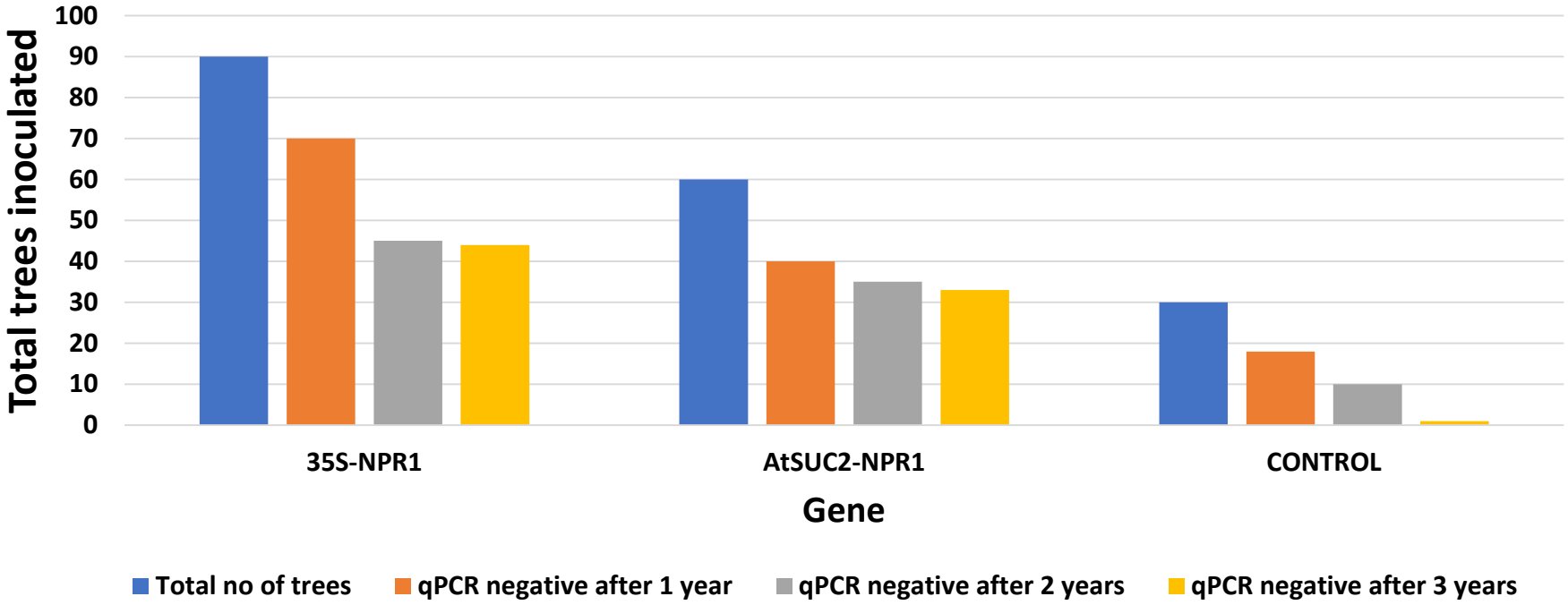
OBF5

- **interacts with NPR1 to promote expression of salicylic acid induced genes**

DIR1

- **Encodes a putative apoplastic lipid transfer protein that is involved in SAR.**

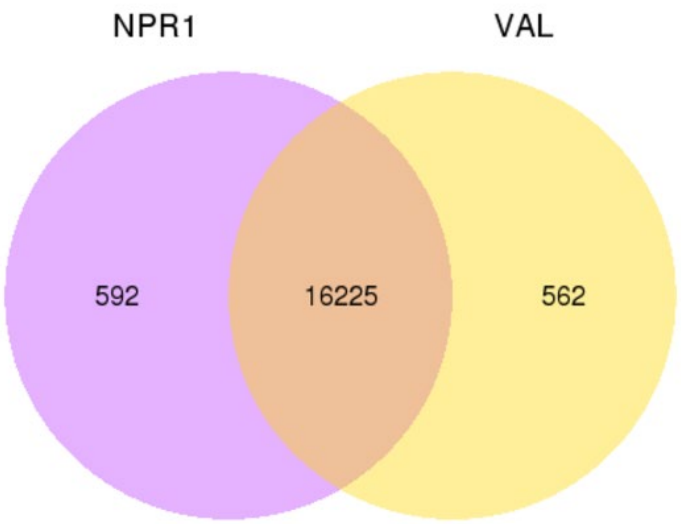
Results from Field trials



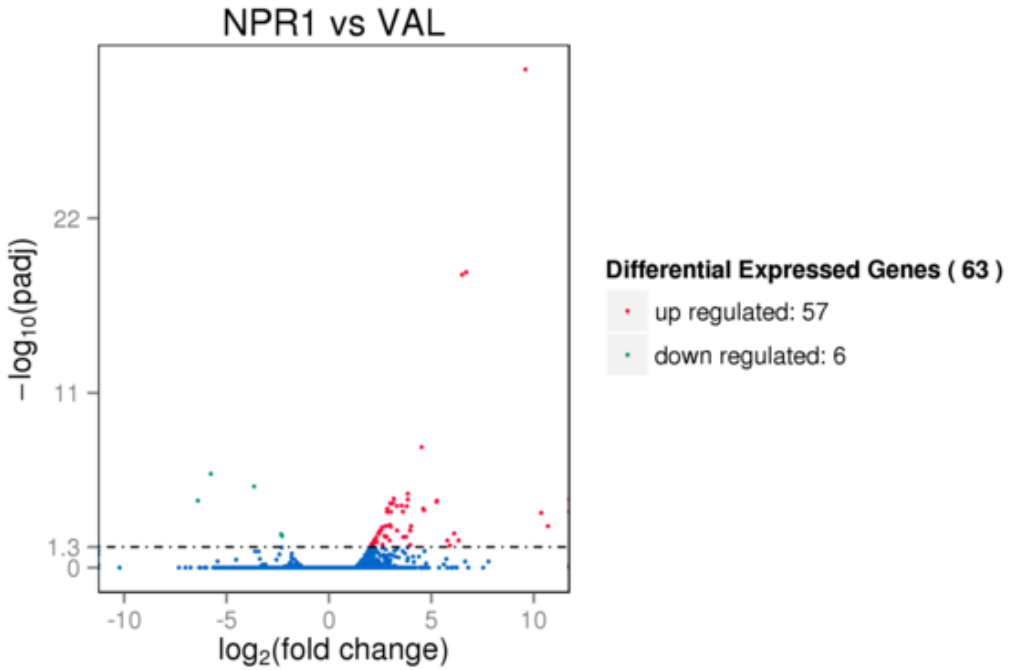
Transgenic trees and controls were planted in two sites, both in South Florida counties with a 80 - 90+ HLB infection rate. Samples were collected at yearly intervals and analyzed using qPCR.

Differential gene expression between AtNPR1 overexpressing lines and Valencia non transgenic control

Differentially expressed genes



Significant differentially expressed genes padj < 0.05

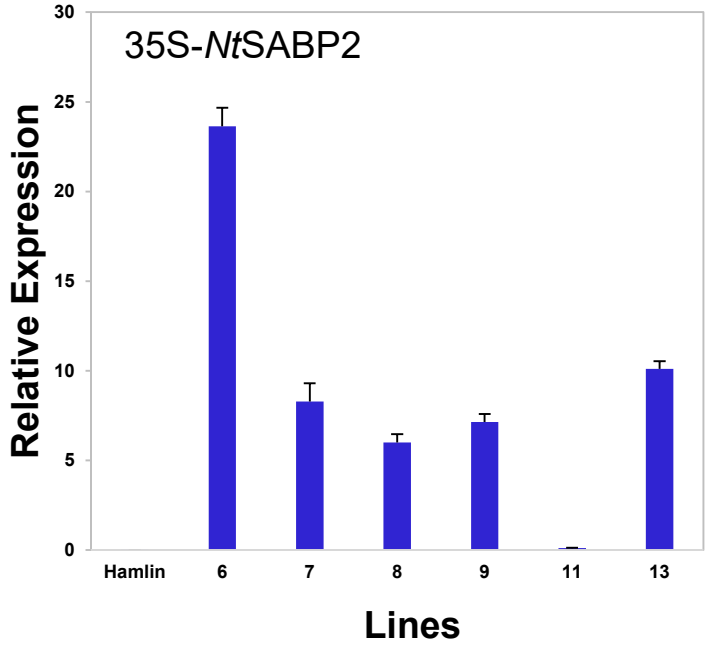


DEGseq, HTSq used for differential genes quantification

Salicylic acid binding protein 2 (SABP2)

- The inactive form of salicylic acid (SA), or methyl salicylic acid (MeSA), function as SAR signal.
- The *Nicotiana tabacum derived* SABP2 is a methyl salicylate esterase required for SAR.

A



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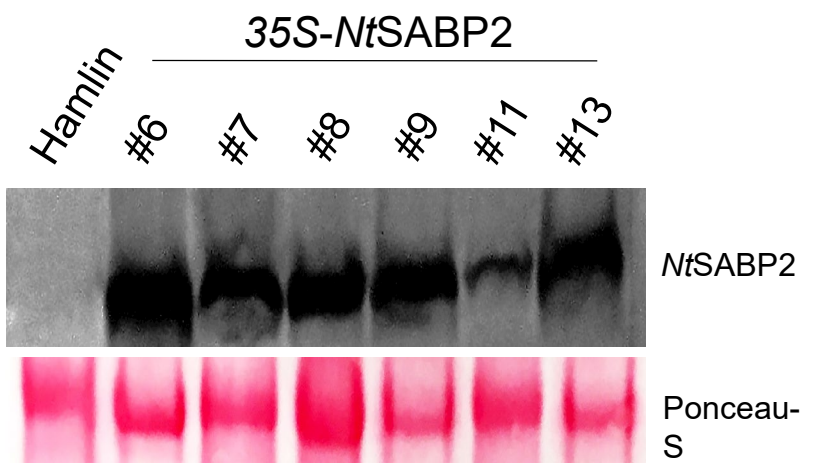

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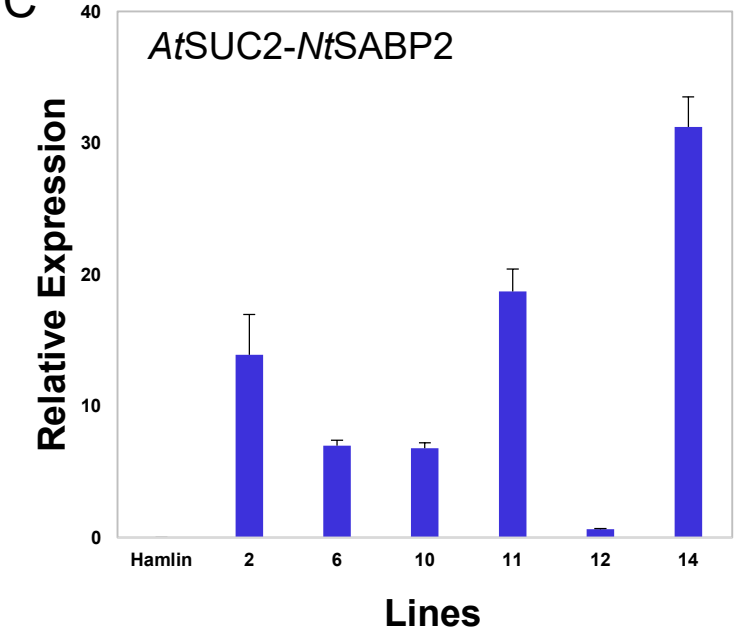

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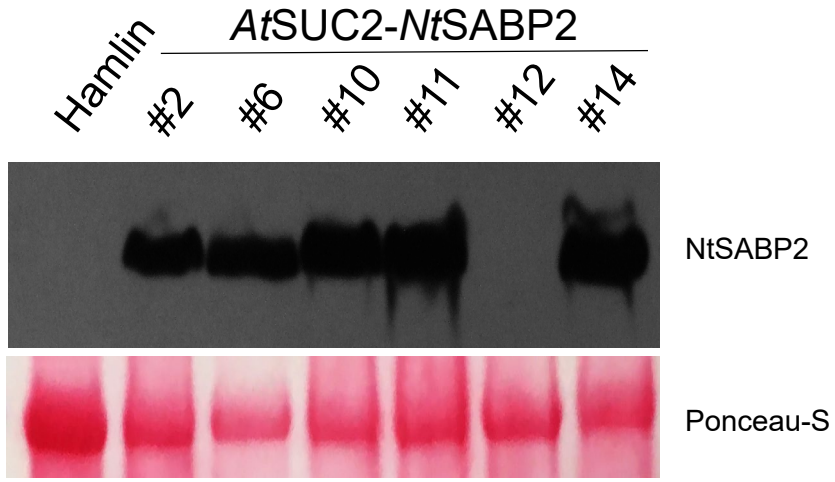

Quantification of *Nt*SABP2 in citrus transgenic lines.

(A and C) qPCR showing *NtSABP2* transcript levels. (A) Analysis of 35S-*Nt*SABP2 and (C) *At*SUC2-*Nt*SABP2 transgenic lines

(B and D) Western blot probed with *Nt*SABP2 specific antibody showing *Nt*SABP2 protein levels in (B) 35S-*Nt*SABP2 and (D) *At*SUC2-*Nt*SABP2.

35S-NtSABP2			
Line ID	Trees free of HLB*	Total number of trees	% of healthy trees
6	3	5	60
7	3	4	75
8	2	4	50
11	2	3	67
13	5	6	83

AtSUC2-NtSABP2			
Line ID	Trees free of HLB*	Total number of trees	% of healthy trees
2	4	6	67
6	4	6	67
10	2	7	29
11	1	4	25
12	2	2	100
14	1	2	50

HLB resistance assessment of transgenic lines planted in the field.

Number of trees with Ct value of 32 or higher after 4+ years in the field are indicated.

HLB (–) tree expressing the NPR1 SAR inducing gene.

Hamlin with SABP2

Hamlin with NPR1

Dutt M., G. Barthe, M. Irey, J. Grosser. 2015. Transgenic Citrus Expressing an *Arabidopsis* NPR1 Gene Exhibit Enhanced Resistance against Huanglongbing (HLB; Citrus Greening). PLoS ONE 10(9): e0137134. doi: 10.1371/journal.pone.0137134.

PLOS | ONE

RESEARCH ARTICLE

Transgenic Citrus Expressing an *Arabidopsis* NPR1 Gene Exhibit Enhanced Resistance against Huanglongbing (HLB; Citrus Greening)

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OPEN ACCESS

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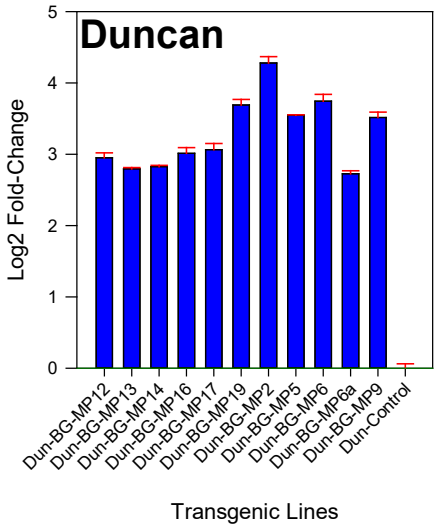
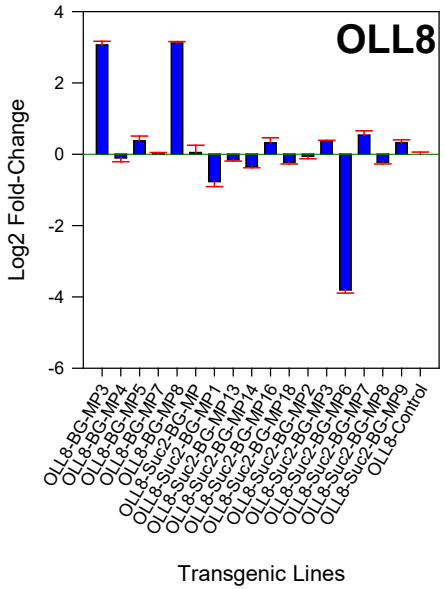
Abstract

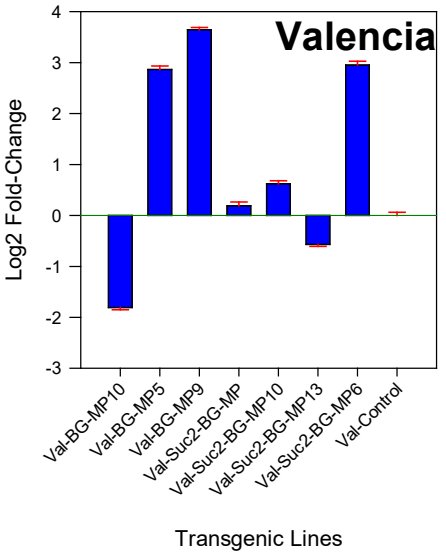
Commercial sweet orange cultivars lack resistance to Huanglongbing (HLB), a serious phloem limited bacterial disease that is usually fatal. In order to develop sustained disease resistance to HLB, transgenic sweet orange cultivars 'Hamlin' and 'Valencia' expressing an *Arabidopsis thaliana NPR1* gene under the control of a constitutive CaMV 35S promoter or a phloem specific *Arabidopsis* SUC2 (*AtSUC2*) promoter were produced. Overexpression of *AtNPR1* resulted in trees with normal phenotypes that exhibited enhanced resistance to HLB. Phloem specific expression of NPR1 was equally effective for enhancing disease resistance. Transgenic trees exhibited reduced diseased severity and a few lines remained disease-free even after 36 months of planting in a high-disease pressure field site. Expression of the *NPR1* gene induced expression of several native genes involved in the plant defense signaling pathways. The *AtNPR1* gene being plant derived can serve as a component for the development of an all plant T-DNA derived consumer friendly GM tree.

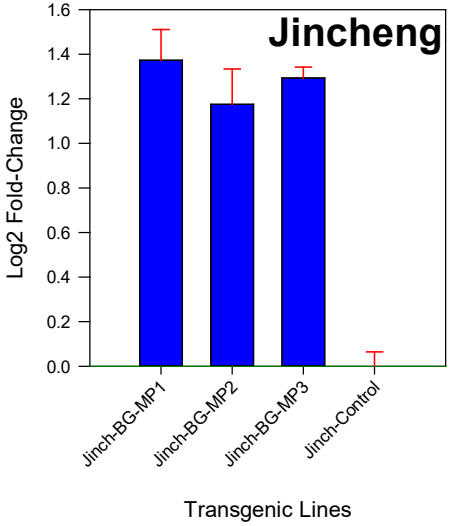
β-1,3-glucanase gene

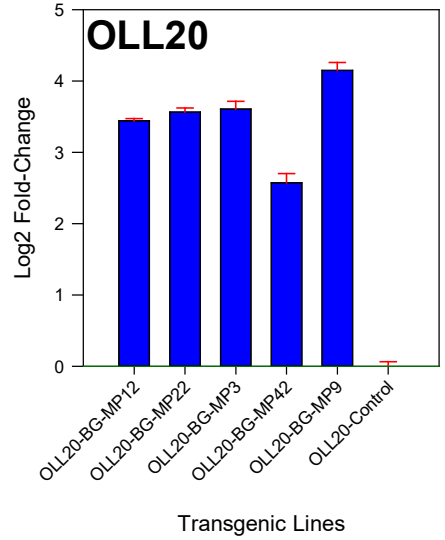
Transgenic lines containing the *β-1,3-glucanase* gene under either 35S promoter or AtSuc2 promoter (Phloem specific) were analyzed for mRNA production through qRT-PCR. qPCR are showing a different level of *β-1,3-glucanase* gene expression among the generated transgenic lines. Transgenic lines with either construct that had a 1-fold higher level of expression were considered to exhibit a relatively high level of expression of *β-1,3-glucanase* gene. Some lines are showing low expression compare to the non-transgenic plant.

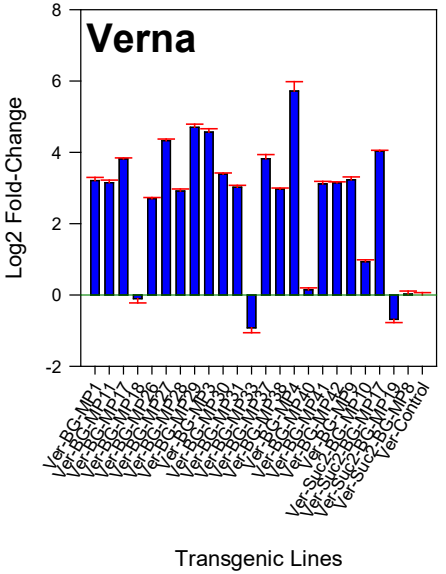












Transgenic ***β-1,3-glucanase*** trees in the field since 2015

August 2019

UF|IFAS UNIVERSITY of FLORIDA

Transgenic plants start fruiting 2019

Journal of Horticultural Science & Biotechnology (2007) **82** (6) 914–923

Introduction of *Xa21*, a *Xanthomonas*-resistance gene from rice, into 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] using protoplast-GFP co-transformation or single plasmid transformation

By A. A. OMAR,[1] W.-Y. SONG[2] and J. W. GROSSER[1*]

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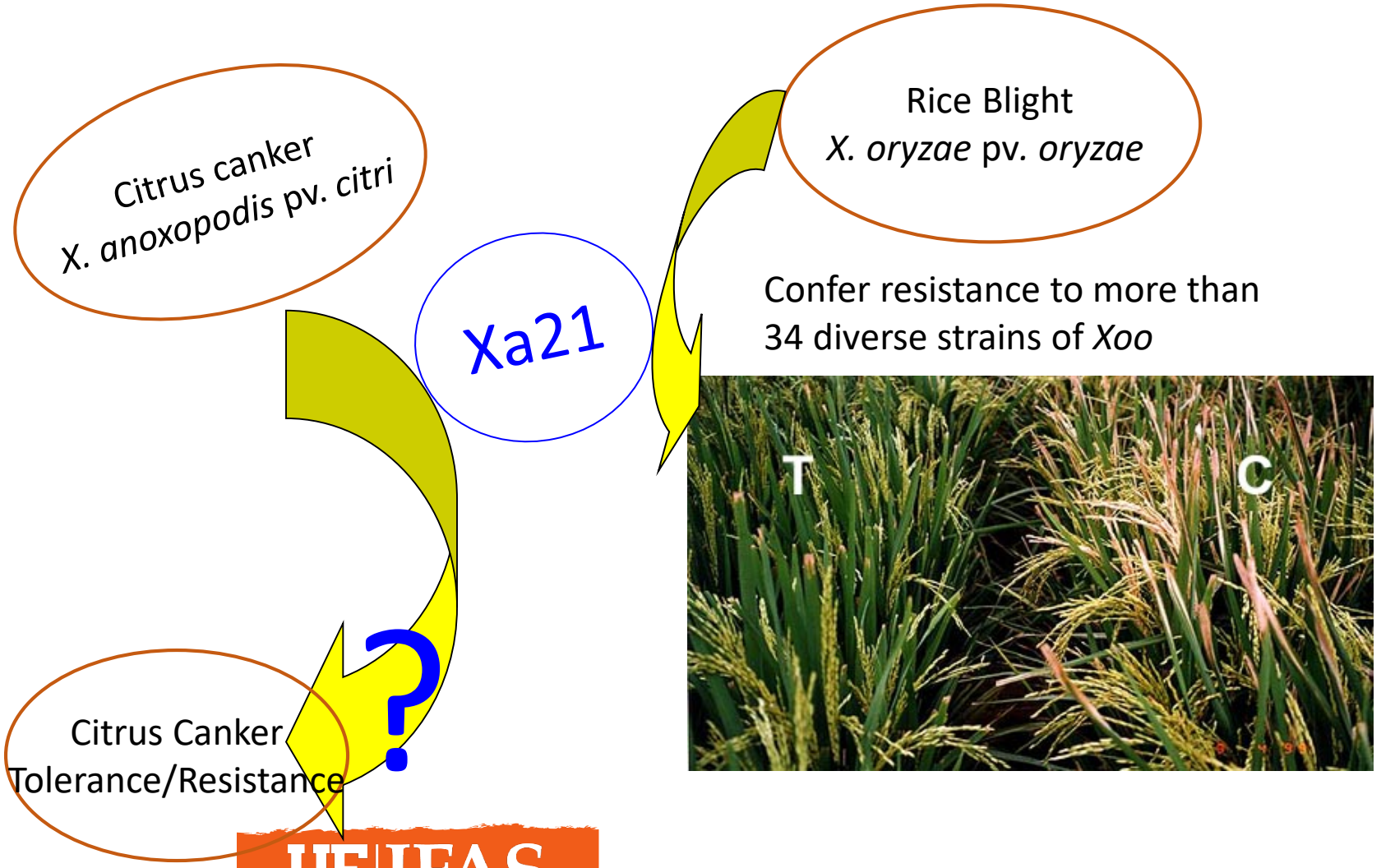
Transfer of the *Xa21 xanthomonas* resistance gene from rice into 'Hamlin' sweet orange using a protoplast/*GFP* transformation system

Why Hamlin?

- ✓ 'Hamlin' is one of the leading commercial cultivars in Florida
  - high yield potential and early maturity
- ✓ 'Hamlin' has a high regeneration capacity from protoplasts and stability in culture
- ✓ 'Hamlin' is one of the highly susceptible cultivar to canker



“Hamlin” transgenic plants in the field since 2009

Xa21 MP51 and MP 49

Xa21 MP55 and MP 36

‘W. Murcott’ mandarin transgenic Xa21 trees in the field since 2015

Transgene Stacking- Why do we need multiple genes if NPR1 works??

- Citrus is a long-lived perennial tree and there is always the possibility of resistance breakdown.
- Gene stacking with sequences offering differing modes of resistance mechanism can provide a viable solution for long term resistance.
- Gene stacking can potentially target both ACP and the bacterium in the same plant.

What is currently available for the benefit of the stakeholders?

Transgenic EV1, Valencia, OLL4 and Hamlin scions have been produced using the mature tissue transformation facility to express either the NPR1, the SABP2 or NPR1 stacked with SABP2.

Development of consumer friendly citrus

Development of consumer friendly citrus

- Develop transgenic citrus without any viral or bacterial component.
- Develop strategies to produce an all plant and eventually an all citrus DNA containing intragenic plant.
- Deliver DNA into the citrus cell by bypassing the conventional Agrobacterium mediated transformation system.

Regulatory friendly?

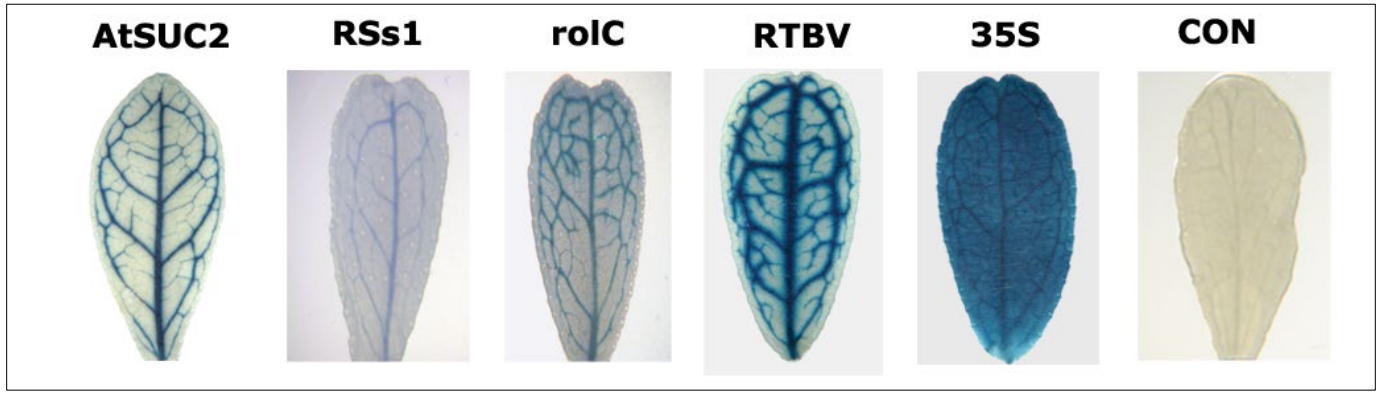
Based on the information cited in your letter, APHIS has determined that tobacco itself is not a plant pest, no organisms used as sources of the genetic material to create the GE tobacco are plant pests, and the method used to genetically engineer the tobacco did not involve plant pests. Therefore, consistent with previous responses to similar letters of inquiry, APHIS does not consider your GE tobacco as described in your December 14, 2015 letter to be regulated under 7 CFR part 340. Additionally, cultivated tobacco is not listed as a Federal noxious weed under 7 CFR part 360, and APHIS has no reason to believe that the increased biomass phenotype of your GE tobacco would increase the weediness of tobacco.

Excerpt of a letter from APHIS to Bayer confirming non-regulated status of Tobacco plants created without sequences originally derived from a plant pest / pathogen.

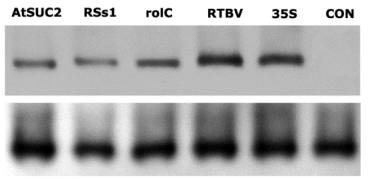
Identification of strong citrus derived promoters

- The promoter region of three constitutively expressed citrus genes were identified, cloned and inserted into a transformation vector fused to the reporter gene uidA (GUS).
  - GAPDH (glyceraldehyde-3-phosphate dehydrogenase)
  - EF1-α (elongation factor 1-α) and
  - CYP (cyclophilin)
- Deletion analyses were performed to identify the appropriate fragment for optimum gene expression.

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



- HLB resides in the phloem.
- Targeting the trans-protein in the phloem potentially resolves issues of the presence of the protein in the fruit and juice.
- Four phloem specific promoters have been evaluated
  - ✔ **1) *Arabidopsis* Sucrose synthase promoter.**
  - **2) Rice Sucrose synthase promoter.**
  - **3) *Agrobacterium* rolC promoter.**
  - **4) Rice Tungro Bacilliform Virus promoter.**



Citrus HLB responsive PP2 promoter???

Dutt M., G. Ananthakrishnan, M.K. Jaromin, R.H. Brlansky and J.W. Grosser. 2012. Evaluation of four phloem-specific promoters in vegetative tissues of transgenic citrus plants. Tree Physiology 32(1):83-93.

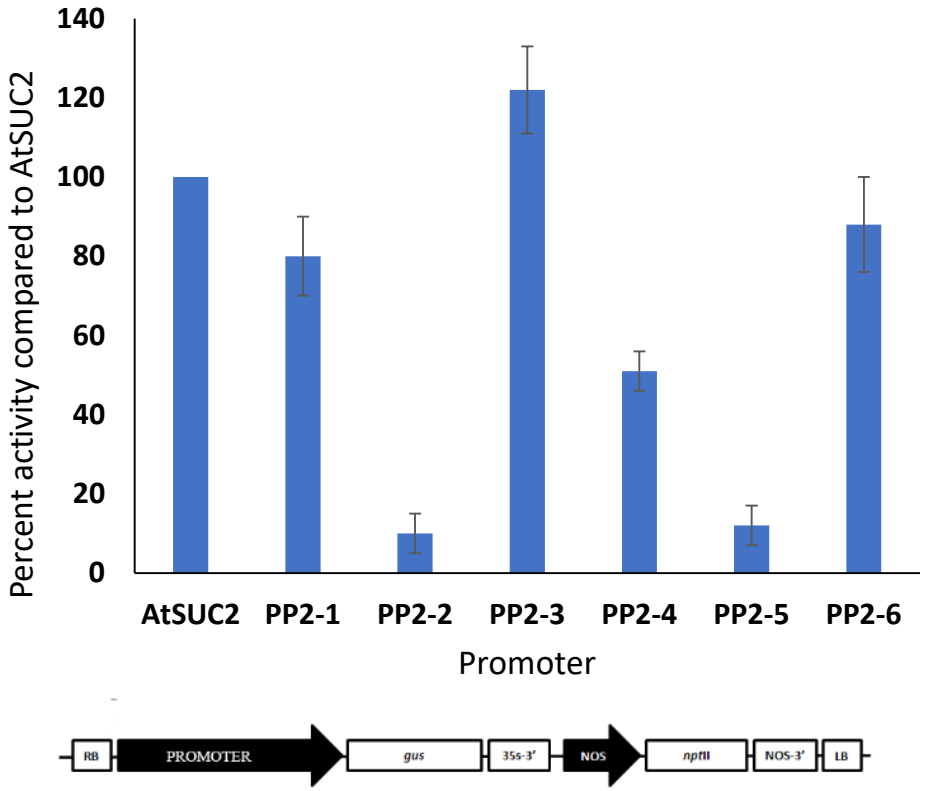
Citrus derived phloem specific promoters

Comparison between 6 endogenous Phloem Protein 2 derived phloem specific promoters

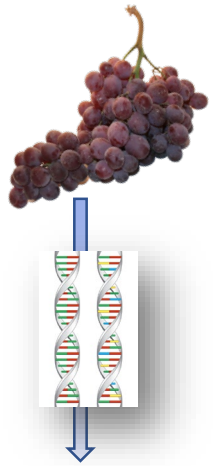
All promoters were identified and isolated from *Citrus sinensis* 'Valencia' and cloned upstream of a *gus* gene in the pCAMBIA derived binary vector pC2300-GUS.

Agrobacterium mediated transformation of Mexican Lime was carried out.

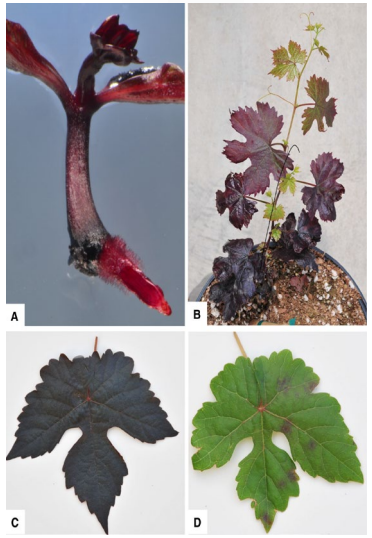
GUS activity was tested on stable transformed lines.



Develop a transformation system utilizing a plant derived reporter gene

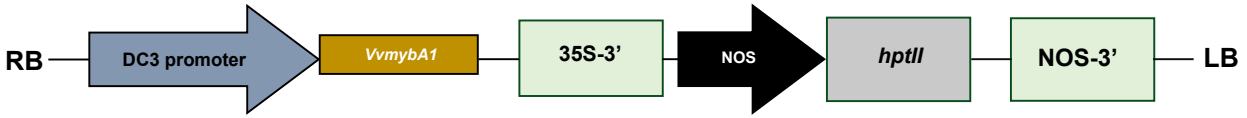


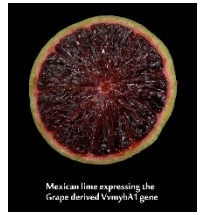
- ***VvmybA1* transcriptional factor gene of grapevine (*Vitis vinifera* L.)**
  Anthocyanin biosynthesis



- **Citrus cultivars**
  'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck)
  'W. Murcott' mandarin (*C. reticulata* Blanco)
  'Page' tangelo (*C*. x tangelo Ingram & Moore)

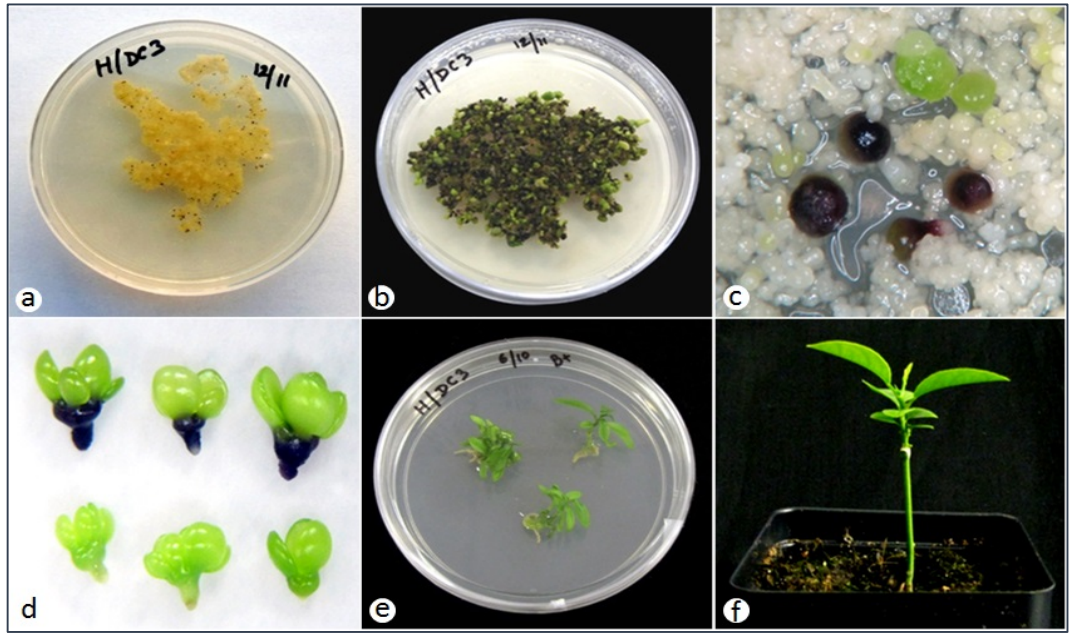
- **Construct containing an tightly regulated embryo specific promoter**
  Daucus carota DC3 promoter





Kandel, R., et al. (2016) Evaluation of a grapevine-derived reporter gene system for precision breeding of Vitis. Plant Cell, Tissue and Organ Culture **124**(3): 599-609.
Dutt, M. et al. (2016) Ornacitrus: Development of Genetically Modified Anthocyanin Expressing Citrus with both Ornamental and Fresh Fruit Potential. JASHS 141:54-61.

Steps in the *Agrobacterium* mediated transformation of citrus suspension cells with a construct containing the *VvmybA1* gene driven by an embryo specific DC3 promoter.



a) Citrus cell suspension cultures on EME-M medium at the beginning of embryonic development.
b) Transgenic embryos after 60 days in EME-M medium.
c) Expression of *VvmybA1* transcriptional factor gene with non transgenic (green) embryos.
d) Anthocyanin expression on the base of the transformed embryos.
e) Transgenic embryos germinating in the plant regeneration medium (B+ medium).
f) A normal regenerated transgenic plant micro grafted on Carrizo rootstock without expressing the *VvmybA1* gene

PLOS ONE

RESEARCH ARTICLE

Embryo-specific expression of a visual reporter gene as a selection system for citrus transformation

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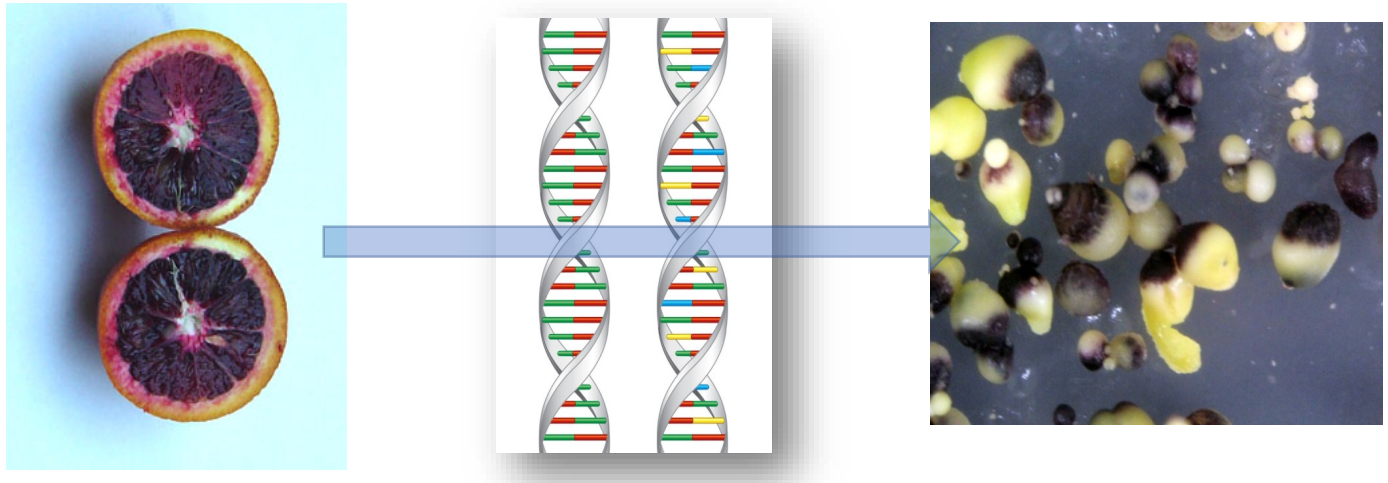
Abstract

The embryo-specific *Dc3* gene promoter driving the *VvMybA1* anthocyanin regulatory gene was used to develop a visual selection system for the genetic transformation of citrus. *Agrobacterium*-mediated transformation of cell suspension cultures resulted in the production of purple transgenic somatic embryos that could be easily separated from the green non-transgenic embryos. The somatic embryos produced phenotypically normal plants devoid of any visual purple coloration. These results were also confirmed using protoplast transformation. There was minimal gene expression in unstressed one-year-old transgenic lines. Cold and drought stress did not have any effect on gene expression, while exogenous ABA and NaCl application resulted in a minor change in gene expression in several transgenic lines. When gas exchange was measured in intact leaves, the transgenic lines were similar to controls under the same environment. Our results provide conclusive evidence for the utilization of a plant-derived, embryo-specific visual reporter system for the genetic transformation of citrus. Such a system could aid in the development of an all-plant, consumer-friendly GM citrus tree.

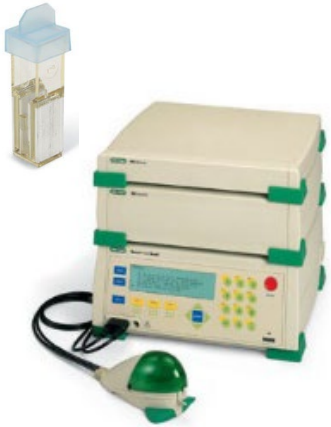
Development of citrus based visual reporter systems



The RUBY anthocyanin gene was isolated from the blood orange ‘Moro’ cultivar

Produce an all citrus DNA containing intragenic citrus without the use of Agrobacterium.

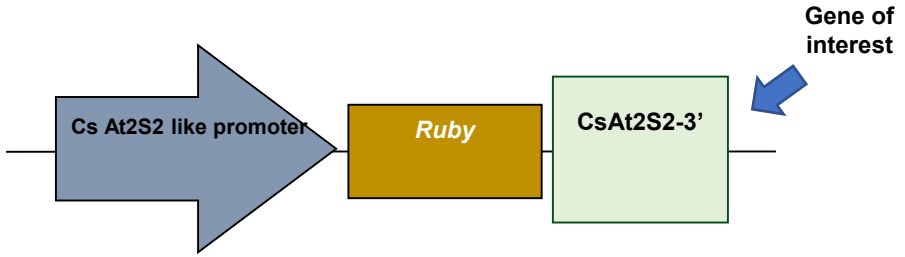
The *RUBY* transcription factor gene was cloned from the ‘Moro’ Blood Orange.



Several embryo specific promoters were identified from the Sweet Orange genome and evaluated.

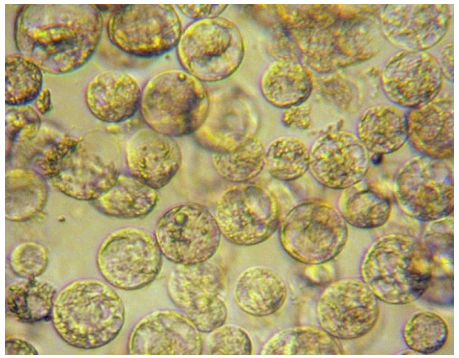
A promoter fragment with >90% homology to the *Arabidopsis* embryo specific At2S2 gene promoter was selected.

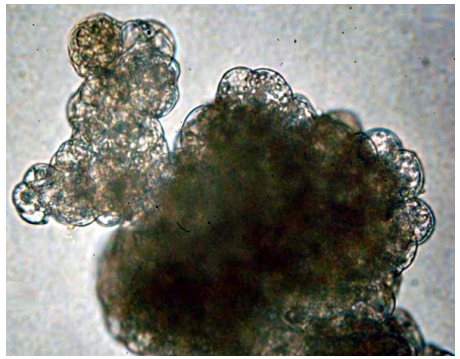
A construct containing the Ruby gene driven by the Cs At2s2 like embryo specific promoter was produced.

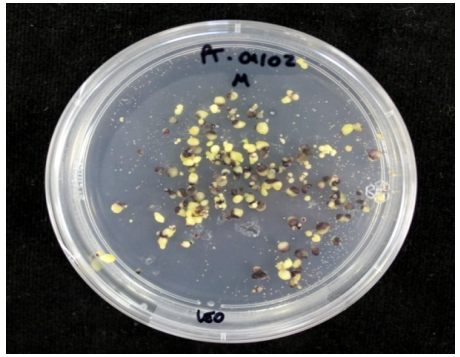


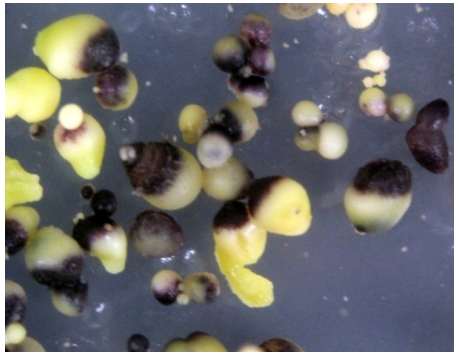
Resulting plants contain all citrus DNA and no GMO-signature; may be required for successful applications and consumer acceptance of CRISPR technology in citrus!

Protoplast mediated Transformation







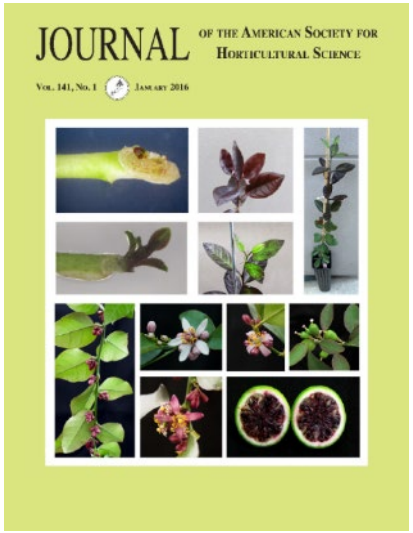


- Protoplast transformation offers the ability to bypass the *Agrobacterium* mediated transformation process.
- It enables the use of linear DNA pieces.
- No GFP or any viral / bacterial components.

Citrus Biofortification

- Grape derived transcription factor VvMybA1.
- Citrus derived transcription factor Ruby.
- Used as novel ornamentals or released for fresh fruit consumption.

- **Develop modified Persian Lime cultivars for South Florida or sweet orange / grapefruit cultivars adapted to the tropical / subtropical environment.**



Anti-oxidant, anti-cancer Purple Margaritas could certainly help sway public attitudes towards GMO citrus!

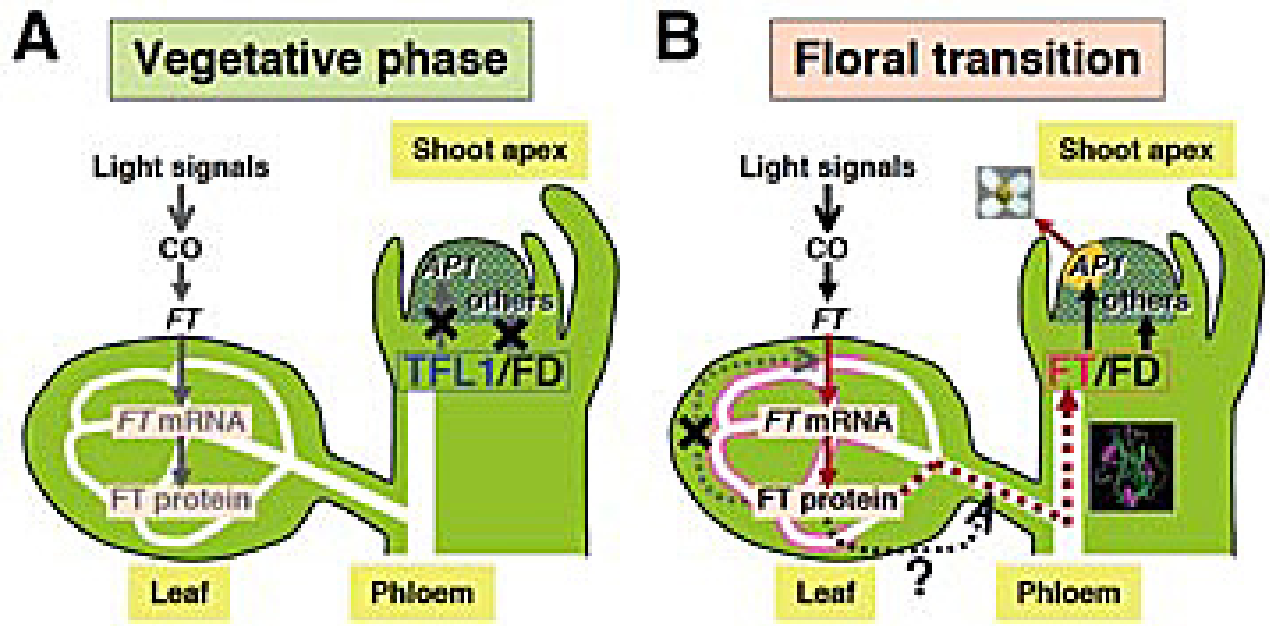
Reducing the juvenile phase in immature citrus

What is FT?

- **FLOWERING LOCUS T gene.**
- Component of the mobile flower-promoting signal.
- Promotes the transition from vegetative growth to flowering.

How does the FT protein work?

- Translated in leaves and then transported to the shoot apical meristem.
- Acts in the shoot apical meristem to induce target meristem identity genes such as APETALA1 (AP1) and initiates floral morphogenesis.



Utilizing transgenic techniques to aid conventional and GM breeding

- Juvenile citrus takes 3 to 12 years to flower.
- FT protein is graft transmissible (Corbesier et al., 2007).
- Will grafting the juvenile seedlings obtained in our breeding program to FT overexpressing rootstocks significantly decrease the evaluation time for the new hybrids and juvenile explant transgenics?

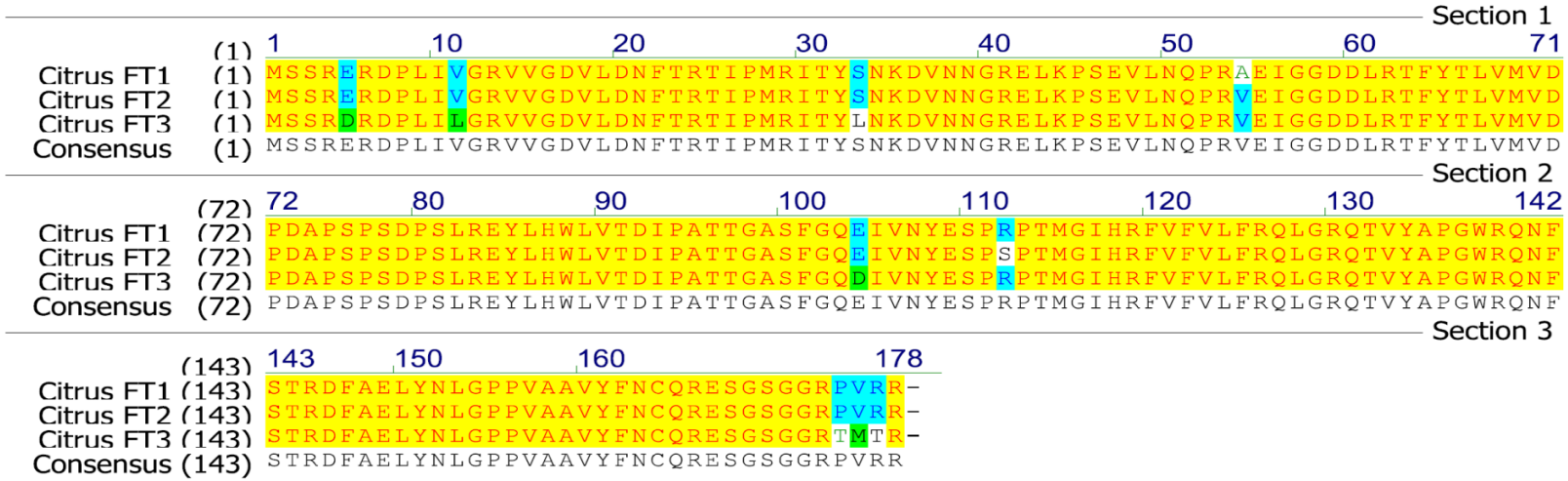
Corbesier L., Vincent C., Jang S., Fornara F., Fan Q., Searle I., et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis. Science* 316 1030–1033

Why develop a transgenic rootstock overexpressing FT??

- Citrus has a long juvenile phase, and the breeding cycle in citrus ranges from 5 to 10 years depending on the hybrid.
- The FT protein is transmissible through a graft junction and can be transported from the rootstock to the scion.
- Production of rapid cycling plants can reduce the time needed to obtain pollen to advance to the next generation.

All FT proteins are not equal!

- There are 3 FT homologs in citrus (CiFT1, CiFT2 and CiFT3)
- CiFT1 and CiFT2 are considered to be alleles of the same locus.
- CiFT1 and CiFT2 overexpression does not result in precocious flowering!



Tested constructs in citrus

Promoter	Gene	Response / Status
35S	CFT1, CFT2 and CFT3	Only CFT3 was able to induce early flowering. However plants flowered in the tissue culture medium and did not survive.
35S-CsVMV bidirectional	CFT3	Several plants flowered within months of hardening to the greenhouse. However, vigor of these plants were low and there was a problem of terminal dieback; potentially from the side effects of enhanced FT protein production.
NOS	CFT3	None of the plants have flowered even after 2 years in the greenhouse
AtHSP	CFT3	4 phenotypically normal plants flowered after 4 months in greenhouse. None of them have flowered since (2+ years)
35S reversed and fused to NOS	CFT3	4 phenotypically normal plants flowered within 6 months (2016). These did not flower again in 2017 or 2018.
AtSUC2	CFT3	6 phenotypically normal plants have flowered after a year of transfer to greenhouse (Winter 2016).

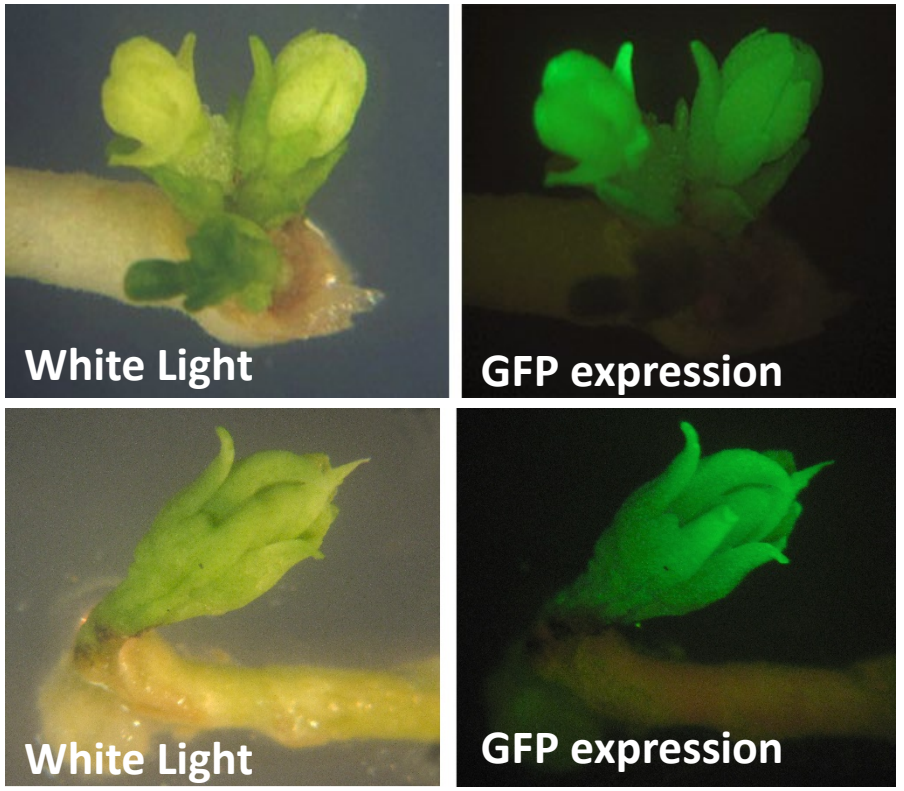
FT expression under the control of different promoters

Gene	Promoter	Function	Stable transgenic lines produced	Lines flowering
FT1	35S	Strong constitutive	201	0
FT2	35S	Strong constitutive	34	0
FT3	35S	Strong constitutive	11	11*
FT3	NOS	Weak constitutive	75	1**
FT3	AtHSP	Heat inducible	40	0
FT3	AtSUC2	Phloem limited	21	7

* These lines could be successfully acclimated to soil.
** Plant flowered just once.

Effect of the 35S promoter on the FT3 expression

- In vitro flowering.
- Cessation of terminal meristem growth.
- Plants lost apical dominance and were bushy in appearance.



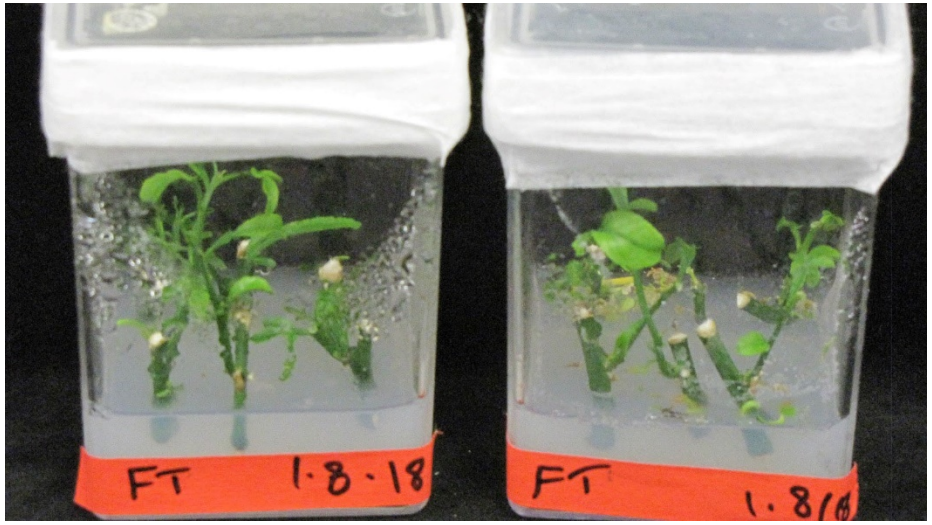
FT3 overexpressing 16 month old transgenic line

Next steps???

Tissue culture and mist bed propagation of selected FT3 overexpressing lines.

- Mother plants
- Seedlings

Budding rootstock liners with juvenile budwood.




FT H 3% 10.2.17
WESTCOTT

Transgenic FT3 seedling flowering after 8 months of germination in the greenhouse.

Can transgenic Carrizo FT3 rootstock be used to facilitate overcoming juvenility in the breeding program?

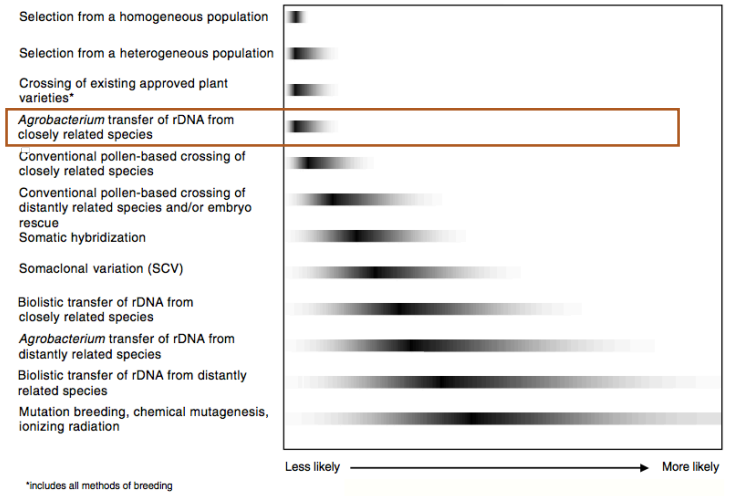
1. Sugar belle x Succari B4/17/39 grafted onto transgenic Carrizo FT3 clone # 3 August 1st 2018

Conclusions

- The *Citrus clementina* derived FT3 gene when overexpressed in Carrizo citrange resulted in early flowering citrus.
- Enhanced gene expression is stable across generations.
- Transgenic lines are being clonally propagated for FT protein transmission studies.
- Ability to induce early flowering in grafted juvenile scions is being investigated with some positive results

Unanswered questions on our GM plants..

- Resistance or Avoidance?
- Consumer acceptance?
- Long term transgene stability
- Nutritional assessments
- Deregulation? Who pays for it?



Source: National Research Council. *Safety of Genetically Engineered Foods: Approaches to Assessing Unintended Health Effects*. Washington, DC: The National Academies Press, 2004.

THANK YOU CRDF!

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