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

<table>
<thead>
<tr>
<th>Technique</th>
<th>Regeneration System</th>
<th>Efficiency of transgenic plant regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile epicotyl mediated</td>
<td>Organogenesis</td>
<td>High</td>
</tr>
<tr>
<td>Mature stem pieces mediated</td>
<td>Organogenesis</td>
<td>Low</td>
</tr>
<tr>
<td>Juvenile Callus mediated</td>
<td>Somatic Embryogenesis</td>
<td>High</td>
</tr>
<tr>
<td>Juvenile Protoplast mediated</td>
<td>Somatic Embryogenesis</td>
<td>Low</td>
</tr>
</tbody>
</table>
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
- Epicotyl sections after 1 month in selection medium
- PCR confirmation of presence of transgene
- Transgenic plants in the greenhouse
- PCR positive and acclimatized transgenic plants
- Transgenic plants grafted onto Carizzo rootstock

Juvenile tissue transformation using embryogenic cell suspension cultures

protoplasts 24 hours after transformation
4-6 weeks
6-8 weeks
3-4 months
5-6 months Transgenic
5-6 months non-transgenic
8-9 months
One year
Genes that have been tested in the field against HLB

1) **ANTIMICROBIAL GENE CONSTRUCTS**
   - AttacinE - Lytic peptide gene from *Hyalophora cecropia*.
   - 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) **SYSTEMIC ACQUIRED RESISTANCE RELATED GENE CONSTRUCTS**
   - 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) **Xanthomomas resistance gene from rice**
   - Xa21 – rice blight resistance gene from rice (*Oryza sativa*)

4) **INSECTICIDAL GENE CONSTRUCTS**
   - 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.
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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
</table>
| **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. |
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 \( \text{padj} < 0.05 \)

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.
Quantification of \textit{NtSABP2} in citrus transgenic lines.

(A and C) qPCR showing \textit{NtSABP2} transcript levels.

(A) Analysis of 35S-\textit{NtSABP2} and (C) \textit{AtSUC2-NtSABP2} transgenic lines.

(B and D) Western blot probed with \textit{NtSABP2} specific antibody showing \textit{NtSABP2} protein levels in (B) 35S-\textit{NtSABP2} and (D) \textit{AtSUC2-NtSABP2}.
### 35S-NtSABP2

<table>
<thead>
<tr>
<th>Line ID</th>
<th>Trees free of HLB*</th>
<th>Total number of trees</th>
<th>% of healthy trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>3</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>4</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>3</td>
<td>67</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>6</td>
<td>83</td>
</tr>
</tbody>
</table>

### AtSUC2-NtSABP2

<table>
<thead>
<tr>
<th>Line ID</th>
<th>Trees free of HLB*</th>
<th>Total number of trees</th>
<th>% of healthy trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4</td>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>2</td>
<td>50</td>
</tr>
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</table>

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.
Research Article

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

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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 disease 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.
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 $\beta$-1,3-glucanase trees in the field since 2015

August 2019
Transgenic plants start fruiting 2019
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

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2Institute of Food and Agricultural Sciences, Plant Pathology Department, University of Florida Gainesville, FL 32611, USA
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

**Citrus Canker**

*X. anoxopodis pv. citri*

**Rice Blight**

*X. oryzae pv. oryzae*

Confer resistance to more than 34 diverse strains of *Xoo*
“Hamlin” transgenic plants in the field since 2009

Xa21 MP51 and MP 49

Xa21 MP55 and MP 36
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 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** \((Vitis vinifera \text{ L.})\)
  Anthocyanin biosynthesis

- **Citrus cultivars**
  - ‘Hamlin’ sweet orange \((Citrus sinensis \text{ (L.) Osbeck})\)
  - ‘W. Murcott’ mandarin \((C. reticulata \text{ Blanco})\)
  - ‘Page’ tangelo \((C. \times \text{tangelo Ingram \\& Moore})\)

- **Construct containing an tightly regulated embryo specific promoter**
  \(Daucus carota \text{ DC3 promoter}\)
Steps in the *Agrobacterium* mediated transformation of citrus suspension cells with a construct containing the *VvmybA1* gene driven by an embryo specific DC3 promoter.

<table>
<thead>
<tr>
<th>Image</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>a) Citrus cell suspension cultures on EME-M medium at the beginning of embryonic development.</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>b) Transgenic embryos after 60 days in EME-M medium.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>c) Expression of <em>VvmybA1</em> transcriptional factor gene with non transgenic (green) embryos.</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td>d) Anthocyanin expression on the base of the transformed embryos.</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>e) Transgenic embryos germinating in the plant regeneration medium (B+ medium).</td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td>f) A normal regenerated transgenic plant micro grafted on Carrizo rootstock without expressing the <em>VvmybA1</em> gene.</td>
</tr>
</tbody>
</table>
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?

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

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Gene</th>
<th>Response / Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S</td>
<td>CFT1, CFT2 and CFT3</td>
<td>Only CFT3 was able to induce early flowering. However plants flowered in the tissue culture medium and did not survive.</td>
</tr>
<tr>
<td>35S-CsVMV bidirectional</td>
<td>CFT3</td>
<td>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.</td>
</tr>
<tr>
<td>NOS</td>
<td>CFT3</td>
<td>None of the plants have flowered even after 2 years in the greenhouse</td>
</tr>
<tr>
<td>AtHSP</td>
<td>CFT3</td>
<td>4 phenotypically normal plants flowered after 4 months in greenhouse. None of them have flowered since (2+ years)</td>
</tr>
<tr>
<td>35S reversed and fused to NOS</td>
<td>CFT3</td>
<td>4 phenotypically normal plants flowered within 6 months (2016). These did not flower again in 2017 or 2018.</td>
</tr>
<tr>
<td>AtSUC2</td>
<td>CFT3</td>
<td>6 phenotypically normal plants have flowered after a year of transfer to greenhouse (Winter 2016).</td>
</tr>
</tbody>
</table>
FT expression under the control of different promoters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter</th>
<th>Function</th>
<th>Stable transgenic lines produced</th>
<th>Lines flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT1</td>
<td>35S</td>
<td>Strong constitutive</td>
<td>201</td>
<td>0</td>
</tr>
<tr>
<td>FT2</td>
<td>35S</td>
<td>Strong constitutive</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>FT3</td>
<td>35S</td>
<td>Strong constitutive</td>
<td>11</td>
<td>11*</td>
</tr>
<tr>
<td></td>
<td>NOS</td>
<td>Weak constitutive</td>
<td>75</td>
<td>1**</td>
</tr>
<tr>
<td>FT3</td>
<td>AtHSP</td>
<td>Heat inducible</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>FT3</td>
<td>AtSUC2</td>
<td>Phloem limited</td>
<td>21</td>
<td>7</td>
</tr>
</tbody>
</table>

* 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.
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 1\textsuperscript{st} 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?

THANK YOU CRDF!