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

Greening Summit
Florida Citrus Growers Institute
2008

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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
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.
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
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.
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
- PCR confirmation of presence of transgene
- PCR positive and acclimated transgenic plants
- Transgenic plants in the greenhouse
- Transgenic plants grafted onto Carizzo rootstock
## Transgenic Summary

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Plasmid(S)</th>
<th>Gene(s)</th>
<th>No. of lines with plants in soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duncan</td>
<td>d35s-AttacinE</td>
<td>AttacinE</td>
<td>27</td>
</tr>
<tr>
<td>Misc</td>
<td>d35s-LIMA</td>
<td>LIMA</td>
<td>43</td>
</tr>
<tr>
<td>Misc</td>
<td>d35s-PTA</td>
<td>N-Terminally modified TemporinA gene</td>
<td>18</td>
</tr>
<tr>
<td>Valencia</td>
<td>d35s-CEMA</td>
<td>Cecropin-Melittin Fusion gene</td>
<td>4</td>
</tr>
<tr>
<td>Misc</td>
<td>d35s-CEME</td>
<td>Cecropin-Melittin Fusion gene. Differences from CEMA by containing different amino acids in the C terminus</td>
<td>14</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>d35s –CEAD</td>
<td>CecropinA-CecropinD fusion gene</td>
<td>2</td>
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<tr>
<td>Key lime</td>
<td>OSS-GUS</td>
<td>Rice sucrose synthase promoter</td>
<td>8</td>
</tr>
<tr>
<td>Key lime</td>
<td>Rolc-GUS</td>
<td>Agrobacterium RolC Promoter</td>
<td>6</td>
</tr>
</tbody>
</table>
### Transgenic Lines Regenerated Using the Different Constructs

(Drs. Manjul Dutt and Ahmad Omar)

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<thead>
<tr>
<th>Cultivar</th>
<th>Plasmid(S)</th>
<th>Gene(s)</th>
<th>No. of lines with plants in soil</th>
<th>No. of lines in Vitro</th>
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<tbody>
<tr>
<td>Carrizo</td>
<td>pC1391-AO1</td>
<td>GUS under AtSuc2 promoter</td>
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<td>9</td>
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<tr>
<td>Carrizo</td>
<td>pC1391-AO1 + pCit101</td>
<td>Co-transformation GUS and GFP</td>
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<td>5</td>
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<td>Carrizo</td>
<td>pSuc2-LIMA-vec2</td>
<td>LIMA under AtSuc2 promoter and GFP</td>
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<td>20</td>
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<tr>
<td>Valencia</td>
<td>pSuc2-LIMA-vec2</td>
<td>LIMA under AtSuc2 promoter and GFP</td>
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<td>10</td>
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<tr>
<td>Hamlin</td>
<td>pSuc2-LIMA-vec2</td>
<td>LIMA under AtSuc2 promoter and GFP</td>
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<tr>
<td>Key Lime</td>
<td>pC1391-AO1</td>
<td>GUS under AtSuc2 promoter</td>
<td>2</td>
<td>8</td>
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<tr>
<td>Key Lime</td>
<td>pC1391-AO1 + pCit101</td>
<td>Co-transformation GUS and GFP</td>
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<td>Key Lime</td>
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<td>March</td>
<td>pSuc2-LIMA-vec2</td>
<td>LIMA under AtSuc2 promoter and GFP</td>
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<tr>
<td>Duncan</td>
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<td>LIMA under AtSuc2 promoter and GFP</td>
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<tr>
<td>OLL#8</td>
<td>pSuc2-LIMA-vec2</td>
<td>LIMA under AtSuc2 promoter and GFP</td>
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</tbody>
</table>
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)
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 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
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

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
CREC Staff

“When the going gets tough, The tough get going” - Sister Esther, 6th grade