BIOTECHNOLOGY TO GENERATE DISEASE RESISTANT, MATURE CITRUS AS A SERVICE

UF/IFAS, CREC, CRDF
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IFAS

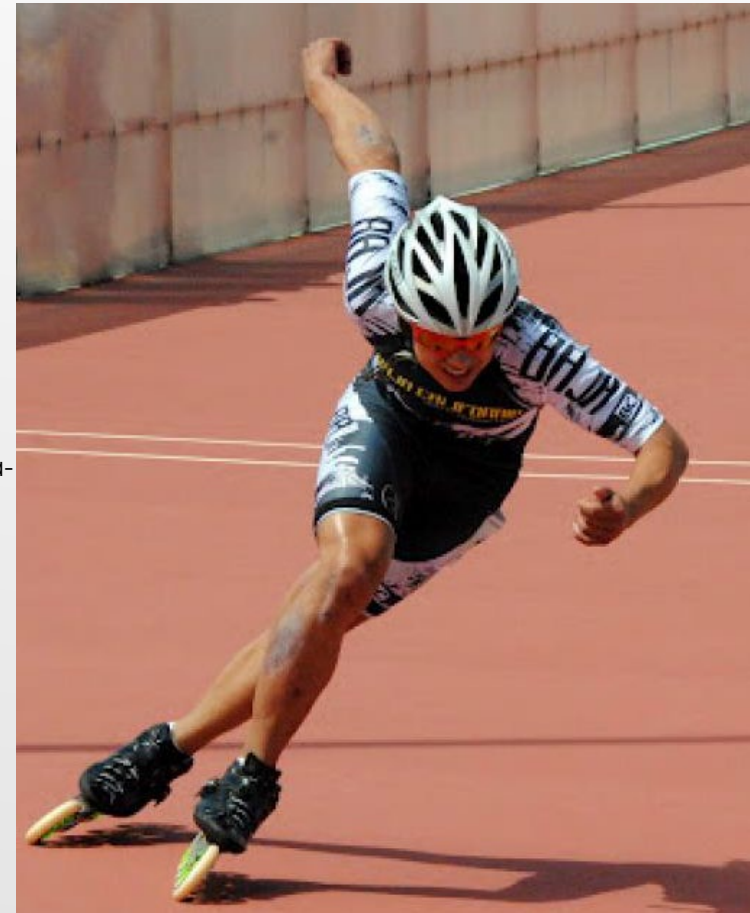
CRDF Citrus Research and Development Foundation, Inc.

THE MATURE CITRUS FACILITY (MCF) TEAM

BRIEF BIO

- Dual American, Canadian citizen
- Originally from Alberta & British Columbia
- From a mixed family farm
- > 25 years experience in Plant Biotech, Genetics & Improvement
- Former figure skater/current inline skater
- Sense of humor

https://zonalider.com/deportes/baja-california-fuera-del-medallero-en-patinaje-velocidad



TERMINOLOGY

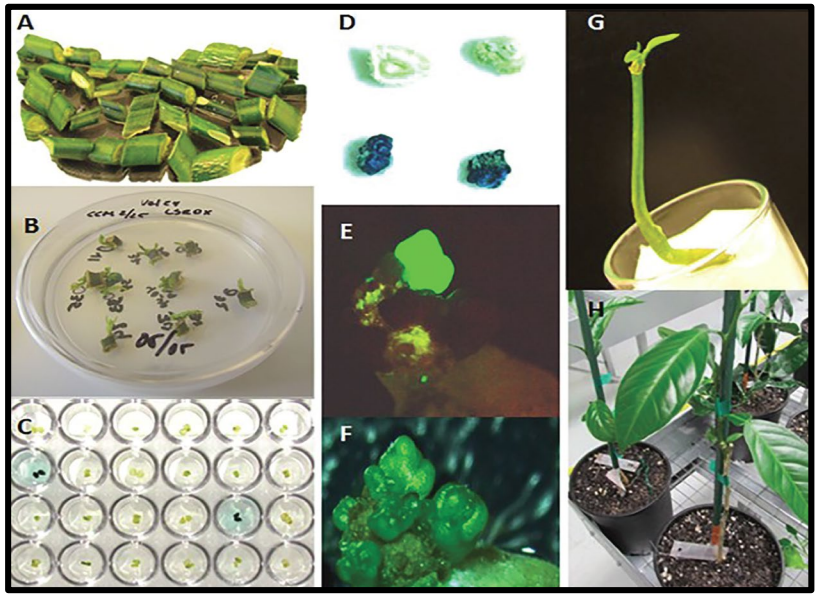
- Transgenic = gene can come from any organism.
- Transformation = process of producing transgenics.
- Cisgenic = gene comes from citrus, considered similar to traditional breeding in US (but not Europe).
- Subgenic = gene editing technology that knocks-out (deletes) a gene & its function. Considered similar to traditional breeding in US (but not Europe).
- Gene gun = biolistic transformation.
- Reporter gene = a gene that permits identification of transgenic event, usually a color change.
- Selectable marker = a gene that enables selection of transgenic event (non-transgenics killed).
- Overexpression=gene product (a protein) produced in abundance.

To transform a plant, a gene of interest, a reporter & a selectable marker typically required

WHAT IS THE MCF?

- Citrus nursery in which clean, mature citrus is introduced, budded, & used in plant improvement
- CRDF, UF/IFAS, & Dr. C. Zapata set up the MCF (~2010 - 2013) after NAS recommendations (2010) for the purpose of optimizing *Agrobacterium* transformation for Florida cultivars to produce transgenic, disease resistant cultivars for field testing & potential commercialization
- Free service to scientists who provide disease or psyllid resistance genes from ~2011-2016
- The process is dependent on budding mature citrus to immature rootstock
- The science has changed, therefore new services added: biolistic-mediated cisgenic (all citrus sequences) & biolistic-mediated subgenic (deletions) plant production following NAS (2018) report.
- One of a kind facility in North America
- 100% aligned with NAS recommendations

THE MCF NURSERY AND LABORATORY PROTOCOL



Disease testing occurs annually

HEADER HOUSE

LAB

Duties in growth facility: planting seed, transplanting, budding mature scion, watering, fertilizing, pesticide applications, etc.

Duties in lab: transformation, screening for transformants, gene editing, PCR, sub-cloning, microscopy, etc.

DIFFERENCES BETWEEN IMMATURE LAB & MCF

Immature Lab

- Mostly research (some commercialization)
- Laboratory
- Immature citrus (uses seed)
- Won't flower or fruit anytime soon
- Rootstock cultivars & Duncan grapefruit seed w/ higher transformation efficiency
- Higher throughput
- Uses different reporter genes enabling rapid identification of transgenics
- Easier, faster, & inexpensive

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MCF

- Mostly commercialization (some research)
- Nursery to grow citrus for production
- Mature citrus (uses mature budwood = stems)
- Naturally flowers & fruits 12-19 months after culture*
- Economically important budwood of scion cultivars w/ lower transformation efficiency
- Lower throughput
- Uses reporter genes for commercialization that entail more work
- Complicated, labor intensive, & expensive
- But, we're better looking

* Without the addition of extra genes

CULTIVARS IN THE MCF

- Hamlin, Valencia, Early Valencia 1 (EV1), Vernia, OLL8, OLL4, Valquarius, Ruby Red Grapefruit, Swingle, Carrizo, Kuharski
- Have not yet tried OLL4, Valquarius, & Ruby Red Grapefruit in transformations
- Mature transformation protocol developed for Pineapple sweet orange in Spain, but Pineapple not economically important in Florida
- Clementine mandarin (protocol exists), pummelo (no protocol)
- New UF & USDA rootstocks for which seed is scarce

WHAT IS PLANT TRANSFORMATION?

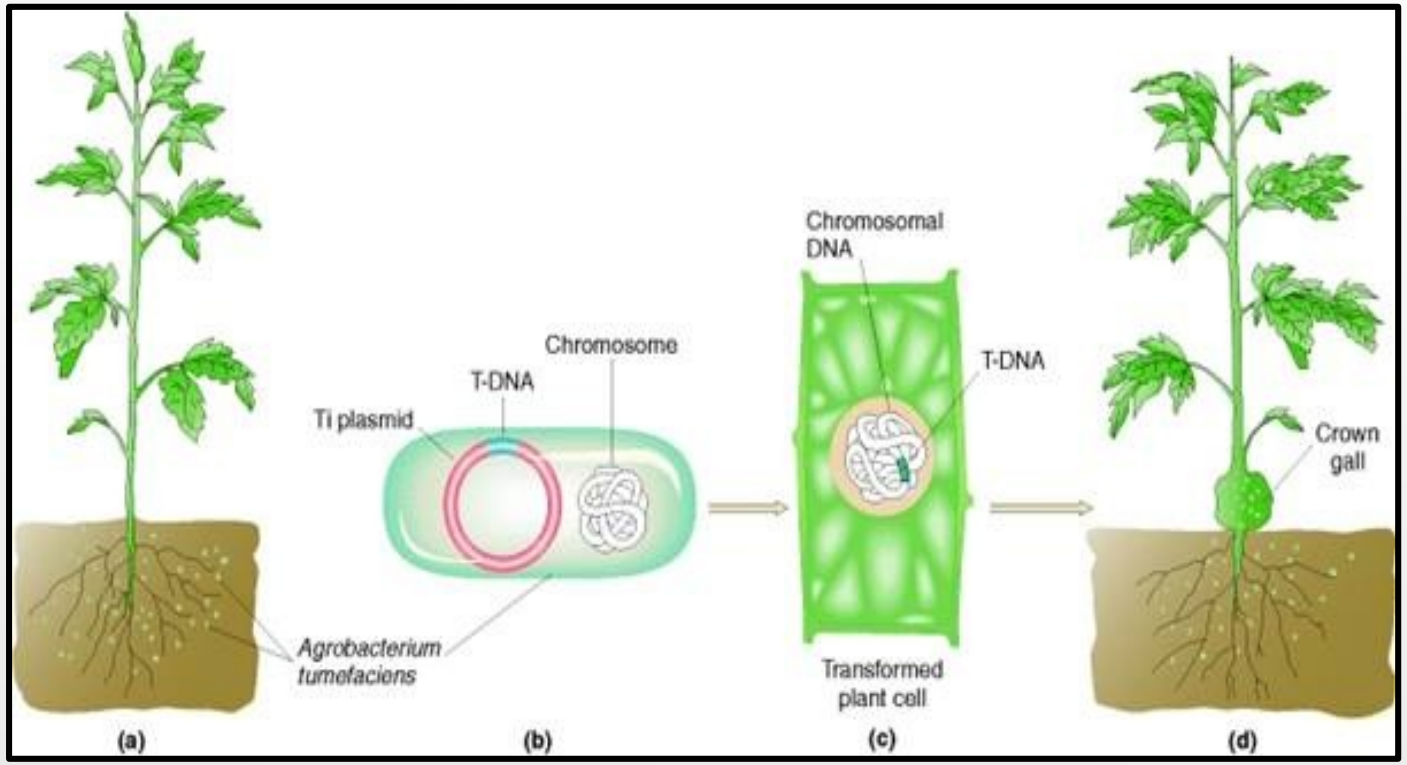
- Gene transfer
- *Agrobacterium* is a plant pest, but it can be used for gene transfer
- Products made from *Agrobacterium* are regulated like plant pests

Crown Gall caused by *Agrobacterium*

https://www.sciencedirect.com/science/article/pii/S0885576511000580

GENE TRANSFER FROM *AGROBACTERIUM* TO THE PLANT CELL

- Crops produced with *Agrobacterium* must be de-regulated by USDA APHIS, FDA, & EPA because of plant pest DNA sequences
- Genes that contain plant viral genetic elements must also be de-regulated as plant pests
- This takes ~ 8 -12 years & costs $ millions
- Most citrus transformed w/ *Agrobacterium*



https://www.biologyexams4u.com/2012/12/steps-involved-in-agrobacterium.html#.W-X6QpNKiUl

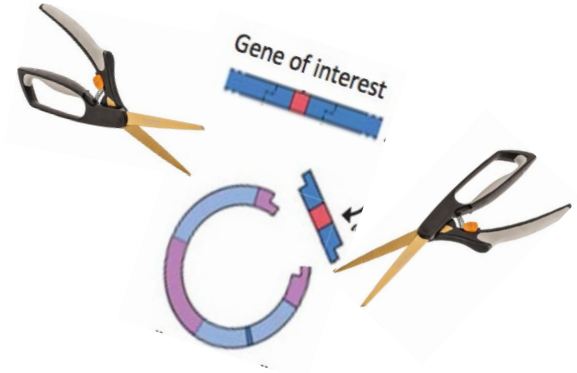
NATURALLY TRANSGENIC SWEET POTATO

https://en.wikipedia.org/wiki/Tuber#/media/File:Ipomoea_batatasL_ja01.jpg

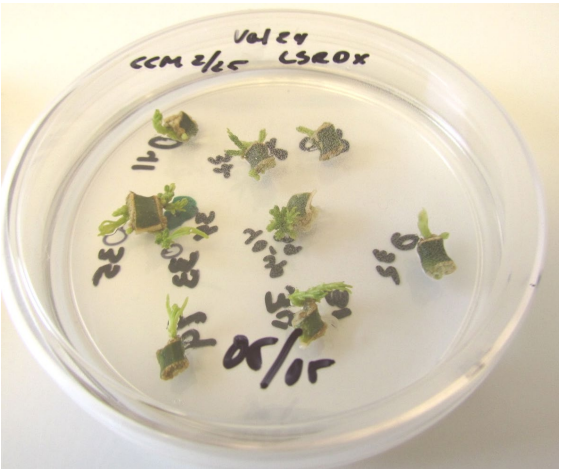
Among 291 cultivars & ancestors of sweet potato tested, all contain one or more *Agrobacterium* transfer sequences that occurred naturally (Kyndt et al. 2015)

https://www.sciencedirect.com/science/article/pii/S0885576511000580

AN ALTERNATIVE TO *AGROBACTERIUM* IS THE GENE GUN=BIOLISTICS



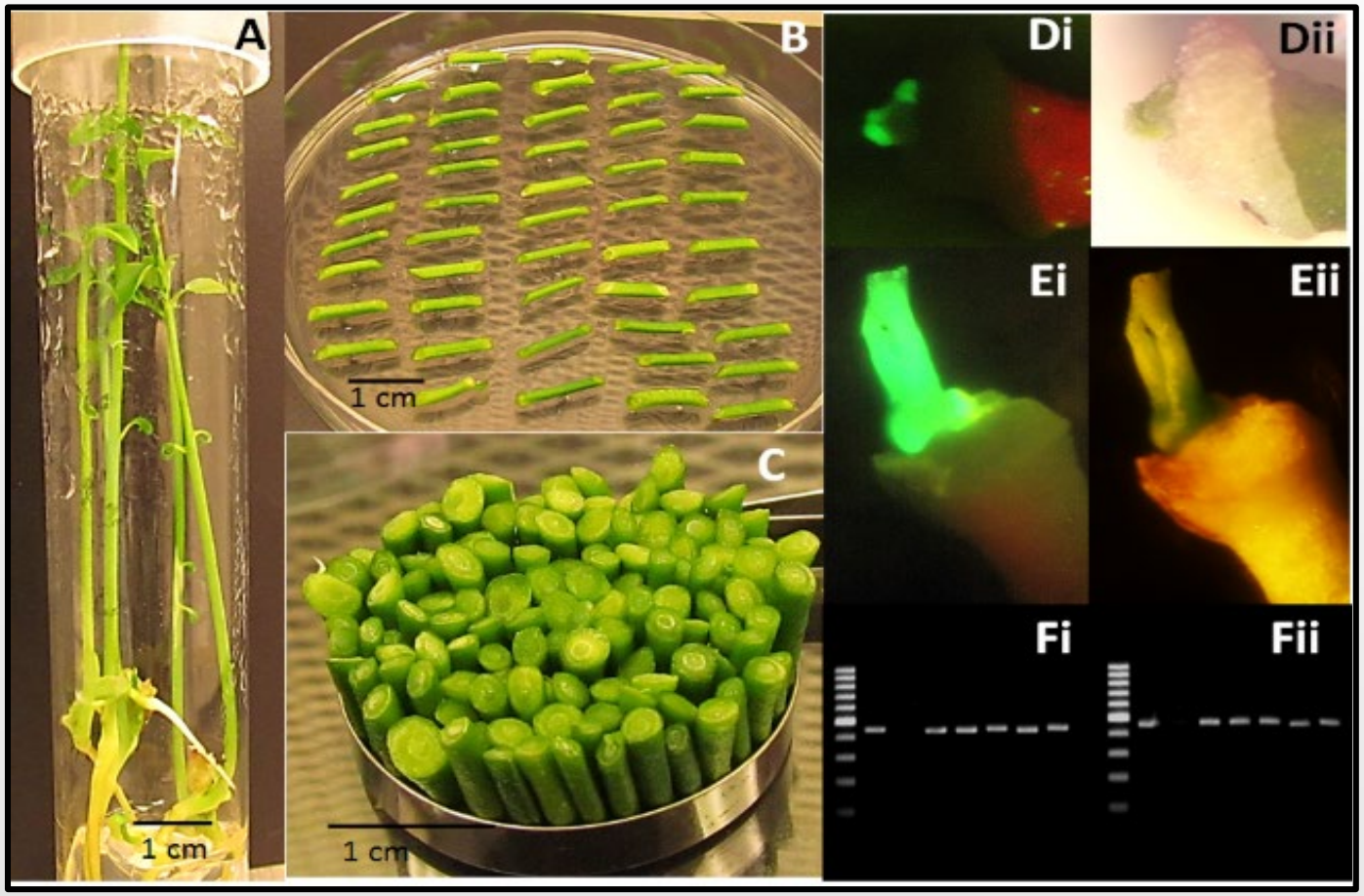
- Can produce stable (permanent) cisgenic & subgenic plants using a gene gun & DNA fragment
- No USDA APHIS oversight because no plant pest sequences. Less de-regulation, expense, & time (~ <4 years)
- Must still meet FDA & EPA approval
- Can get these trees to industry sooner
- If cisgenic or subgenic, more consumer friendly



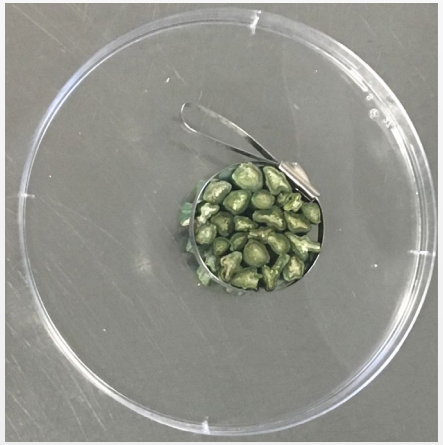
TECHNOLOGY DEVELOPMENT

FIRST REPORT OF STABLE TRANSFORMATION W/ GENE GUN & PLANT REGENERATION FROM THE MCF

- Increased efficiency to 1.9 transgenics per shot
- Immature & mature



Wu et al. (2016)



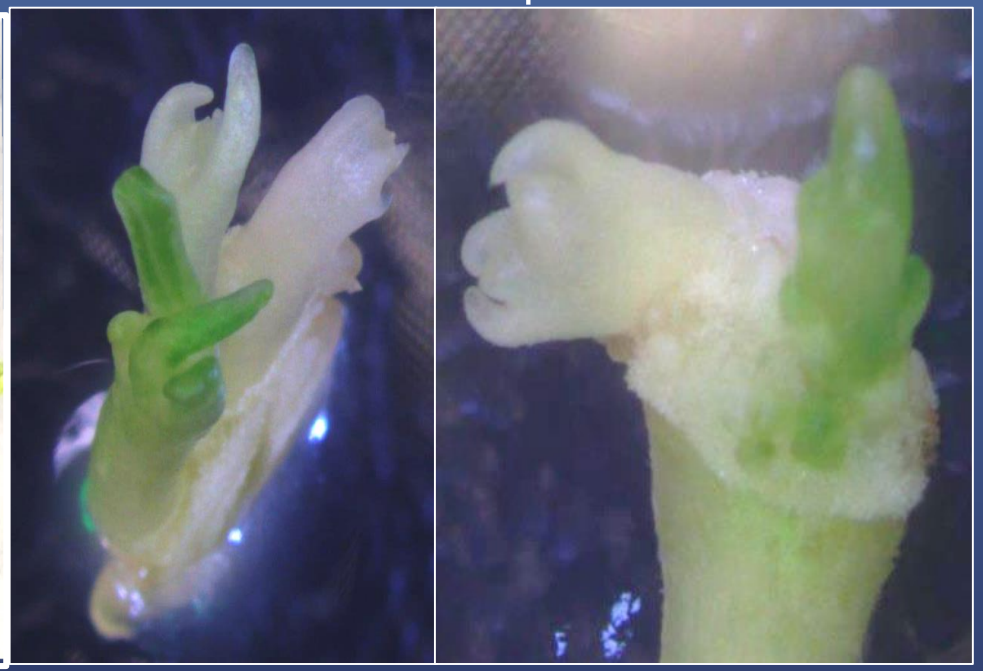
CISGENICS

Citrus anthocyanin reporter in Hamlin to visualize event

Health benefits to anthocyanins

SUBGENICS

Biolistics mediated PDS gene editing in immature Carrizo (L) & Valencia (R) explants



Both technologies considered similar to traditional breeding by USDA APHIS

TRANSFORMATION EFFICIENCIES

- Prior to my arrival, *Agrobacterium* transformation efficiency was ~ 3.5% (mean of 4 scion cultivars)(positive shoots/total number of shoots).
- Increased efficiency to ~ 7.6% (Valencia & Hamlin), but this is NOT consistent, particularly Valencia. (NB: Immature Valencia transformation also problematic).
- Highest efficiency occurs in spring, & budding influences efficiency.
- Highest *Agrobacterium* transformation efficiencies with Hamlin=EV1> Glenn Navel>Valencia >Vernia=OLL8.
- Currently determining transformation efficiency of each mature cultivar w/ the gene gun.
- OLL8 relatively recalcitrant w/ *Agrobacterium,* but can produce transgenics using gene gun!!

PLANT PRODUCTION & CUSTOMERS

- >540 transgenics produced (disease resistance genes, technology development, & increasing efficiency) (CPCD #15-045C)
- Propagated ~500 additional plants for scientists
- Current customers: Dr. Mou, Dr. Nian Wang, Drs. Dutt, Grosser, Rogers, Louzada (Texas A&M), Dr. McNellis (Penn State)
- Our mature transgenics will be included in 3 new field trials
- Potential new customers: Dr. Bonning (UF), Dr. Jeff Jones (UF), Dr. Wenbo Ma (UCR)
- Previously, Two Blades, but their genetic constructs were problematic & their study was strictly research, so Dr. Orbovic's lab recommended
- Collaborator for increasing efficiency Dr. Yi Li (UConn)
- No control over when CRDF funded scientists screen their plants & report to CRDF.

ALTERNATIVES TO MATURE TRANSFORMATION

Transformation with *Flowering Locus T (FLT)* or *Apetala* genes to induce flowering

Additional flowering genes might compromise long-term fitness of perennial trees in the grove (personal commun. L. Pena). Useful for breeding if pollen & ovaries are not sterile.

PRICES FOR TRANSGENIC AND CISGENIC PLANTS

Reporter Gene	Purpose	Price for Public Institution	Price for Private Company
Agrobacterium transformation of mature citrus (requires more deregulation)			
GUS reporter	Research or Commercialization	$500	$1,000
Anthocyanin reporter	Research or Commercialization	$300	$ 600
GFP	Research	$300	$ 600
Biolistic transformation of mature citrus (requires less deregulation)			
GUS reporter	Research or Commercialization	$1,700	$3,400
Anthocyanin reporter	Research or Commercialization	$1,500	$3,000
GFP	Research	$1,500	$3,000

Prices in this table reflect the amount of work involved in providing the service (e.g. budding mature plants, restriction digests of DNA, gel purification of minimal expression cassettes, biolistic transformation).

New service for biolistics-mediated gene editing announced in 2019!

FUTURE RESEARCH, IF FUNDED

Objectives

1. *Plant production as a service using Agrobacterium and biolistic transformation.*
2. *Increase Agrobacterium and biolistic-mediated transformation efficiencies.*
3. *Improve selection for cisgenic plant production.*
4. *Gene editing with biolistics.*

ISSUES

- Some scientists did not pay for transgenics because they had no funding & the research was a CREC priority (two disease resistance genes).
- In the past, there were no charges for transformation attempts (in which no transgenics were produced), because cultivars were untested (e.g. Grosser & Nian Wang's plant introductions). Similarly, sometimes one must work with scientists to solve issues of gene expression.
- *Agrobacterium* transformation is cultivar dependent. There is less cultivar dependency with the gene gun (if you can regenerate plants).
- For cisgenics: CRDF & UF must encourage scientists to make genetic constructs for the gene gun for faster & cheaper deregulation. A biolistics vector must be provided into which they can sub-clone genes.

THANK YOU FOR YOUR SUPPORT!

QUESTIONS?