Treatments for targeting bacteria and psyllids in citrus trees

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Problem: Citrus Greening Disease

- Global threat to all types of citrus crops including oranges, lemons, limes and grapefruit.
- Infected trees show:
  - Micronutrient deficiency
  - Thin fruit set and increased fruit drop
  - Lopsided fruit, may contain aborted seeds, and unacceptable juice quality
- Eventually leading to **tree death**
- Estimated **$15 billion** loss in revenue to the citrus industry.
- Caused by phloem-restricted **pathogenic bacterium:** *Candidatus* Liberibacter asiaticus (Las).
- **Asian citrus psyllid** is the confirmed vector of Las.

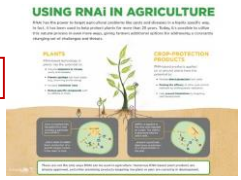
Methods for Insect Management

1. Heavy application of Chemical insecticides

Chemical resistance

Long-term cost **too high** for growers

2.
   - Targeting insects using dsRNA products. Psyllid RNAi products.
   - Producing and screening genetically improved citrus trees (USDA) .



RNAi

- Can be easily delivered topically or as soil treatments.
- **Topical Sprayable applications** can persist for **35d post treatment** of field trees. (1.5 M tall, 7 yr old Valencia) (Hunter et al, 2010;2012)
- **Soil application of dsRNA using clay** absorbents can last up to **12 months post treatment**. (Metz and Hunter in prep 2018)

However…

- Effective management of Citrus greening disease requires both management or the **vector** and **pathogen**.
- While dsRNA is good for targeting insect pests and viral pathogens…
- **Bacteria do not have homologous RNAi machinery.**
- Therefore we have investigated three emerging gene targeting technologies as potential solutions:
  - **Peptide phosphorodiamidate morpholino oligomers** (PPMOs)
  - **FANA Antisense Oligonucleotides** (F-ASOs)
  - **CRISPR-CAS9 gene-editing**

Antisense Technologies for Gene Silencing

Synthetic oligonucleotides, Non-GMO

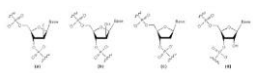
Peptide conjugated Phosphorodiamidate Morpholino Oligonucleotides (PPMO)

Cell penetrating peptides are used

(Wesolowski, et al, 2011, 2013)

FANA Antisense Oligonucleotides (F-ASOs),

- (2'-deoxy-2'-fluoro-D- arabinonucleic acid)



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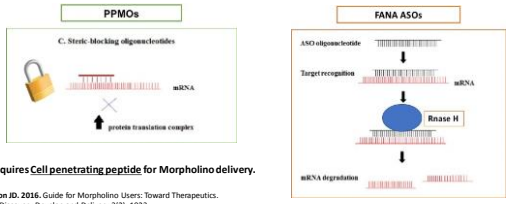
Comparing mechanisms of oligonucleotide-induced downregulation of gene expression



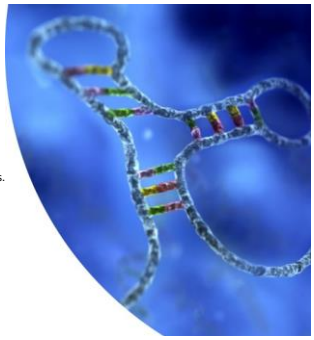
Requires Cell penetrating peptide for Morpholino delivery.

Moulton JD. 2016. Guide for Morpholino Users: Toward Therapeutics. J Drug Discovery Develop and Delivery 3(2): 1023.

The EGS/RNase P system was shown to destroy various types of RNAs, Hitrik et al, 2016. Targeted inhibition of WRN helicase by external guide sequence and RNase P RNA. Biochimica et Biophysica Acta 1859: 572–580.

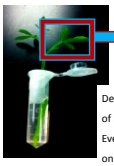
Requires no formulations, conjugates, or carriers for delivery F-ASO, antisense oligos, are singled stranded RNA, and are Capable of self delivery without coatings or carriers

Advantages of using PPMO or F-ASO for gene silencing and regulation



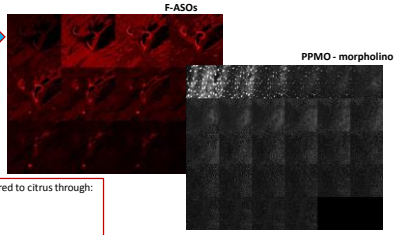
- **Efficient cellular delivery** systems for RNA silencing and regulation.
- Display **high stability** and resistance to nucleases.
- Maintain **high specificity** for RNA targets.
- **Nontoxic** to non-target species.
- Significantly **longer persistence** than uncoated dsRNA used in RNAi strategies.
- **Able to target and suppress bacteria** which *cannot be done using dsRNA*, used in RNAi

Systemic Delivery of Antisense technologies to citrus



Treated citrus

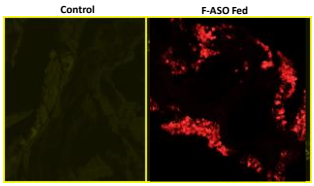
Deep scan of leaf tissue Every 2 um on average.



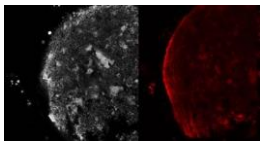
ASOs were also successfully delivered to citrus through:
- Foliar spray
- Root absorption
- Soil application
- Clay absorbents

(Hunter et al, in prep 2018)

Host delivered F-ASOs to insects



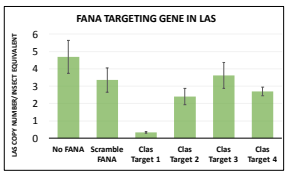
Psyllid fed on citrus absorbing water(Control-left), Psyllid fed on F-ASO citrus cutting which absorbed Scrambled control SC-ASO control (right).



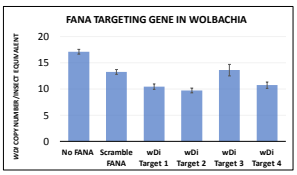
Confocal microscopy analysis: F-ASO probe with fluorescent probe ATTO633 visualized in insect brain Post feeding 8 days from treated plant.

(Hunter et al, in prep 2018)

ASO Suppression of plant pathogen and psyllid endosymbionts



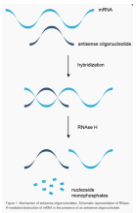
Result: F-ASO-1 resulted in the greatest suppression of bacterium, Clas, in psyllids reared on infected citrus seedlings.



Result: F-ASO-2 suppression of gene in *Wolbachia-Di*, resulted in the greatest suppression of the psyllid endosymbiont.

(Hunter and Pelz-Stelinski, in prep 2018)

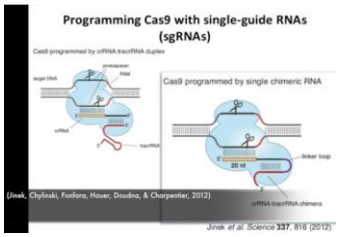
F-ASO Summary Statements:



- First evidence for successful delivery of two synthetic antisense technologies into plants for the management of insect pests and plant pathogens.
- Molecules were shown to move systemically through plant tissues as visualized with Confocal microscopy and spectrophotometry.
- Adult insects showed systemic movement and entry into cells without a carrier (F-ASO) in hemolymph and organs, and alimentary tract.
- Bacterial titers were reduced in treated plants and endosymbionts in insects without causing damage to plant tissues.
- Results suggest a role for these products in the reduction of plant pathogens like Liberibacter asiaticus, CLas, associated with Citrus greening disease; and reduction of insect vectors of pathogens, increased capabilities to target bacterial endosymbionts in insects.

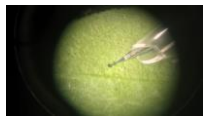
CRISPR-Cas9

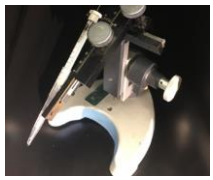
- **Goal:** Produce psyllid adults which cannot fly using CRISPR-CAS9 knockout.
- Preliminary trials: Microinjection of Adult Psyllids for proof of gene knockout.
- *Current/Future trials*:
  - Microinjections into nymphs.
  - Microinjection into eggs
  - Univ. Florida Collaborators:
  - Dr. Kirsten Pelz-Stelinski, and Dr. Andres Mojica.
  - USDA Collborator:
  - Dr. Steve Garzinsky,



Methods

- Adult psyllid were microinjected with dual single guide RNAs (sgRNAs) and Cas9 mRNA. Produced by Dharmacon™
- Psyllid which survived 8d post injection were separated into three groups.
- gDNA was extracted from psyllid adults.
- Sequencing was then performed across the desired gene target.



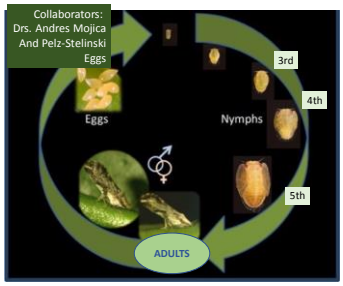


Statements are not an endorsement by the USDA to exclusion of products That may perform similarly.

Preliminary Results

Wayne Hunter, USDA,ARS
Thomson Paris, Univ. Florida
Maria Gonzalez USDA, and

- Genomic sequencing and alignments, determined that deletions occurred in the correct targeted region for TXT, Thioredoxin.
- The majority of deletions were in Gene Variant 2, (202 bp_KO).
- Only two adults had full length knockout KO at (550 bp, in Variant 1.
- ~33% survived 4 of 12 insects.
- *Future work includes:*
  - Optimization of injection methods.
  - Injection of 4th and 5th instars for phenotypic evidence of wing deformation.



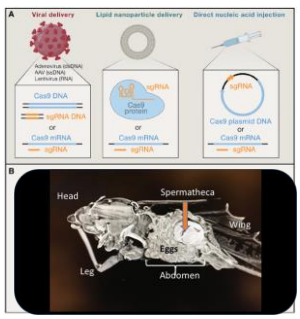


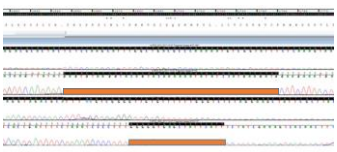
Figure--Strategies for In Vivo Delivery of CRISPR-Based Genome-Editing Agents
(A) Viral (red)-, lipid-nanoparticle (green)-, and direct-nucleic-acid-injection (blue)-mediated delivery of CRISPR-based genome-editing agents have all been successfully used to achieve in vivo genome editing.

(B) These methods have been used to deliver genome editing agents to a variety of organisms organs. The genes that were modified within each organ corresponding to the delivery method used.

Geminiviruses are a large family of plant viruses with single-stranded, circular DNA genomes (2.5–3.0 kb) and a wide host range, including monocotyledonous and dicotyledonous plants.

Upon infection, they produce numerous replicons through rolling-circle replication, which may serve as repair templates during HDR (Hanley-Bowdoin et al., 2013; Baltes et al., 2014).

Preliminary Data on CRISPR_Cas9 Asian Citrus Psyllid



Groups of 2 adults extracted, six groups each Treatment (Control or Injected)

Full length untreated—gDNA ACP Control 5 kb long.

555 nt Total region of knock-out

Knockout Full length

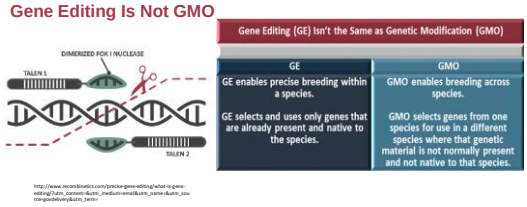
202 nt knockout--PARTIAL

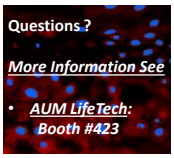
Knockout Partial , was in a second gene variant.

One group *out of three* had Complete knockout.
Two groups *out of three had* partial knockout, same region.

For the gDNA Sequencing
Purified template was prepared for sequencing using PCR primers and a BigDye™ Terminator v3.1 kit (Applied Biosystems, Waltham, Massachusetts) following manufacture's suggestions. Capillary electrophoresis was performed using a 3730XL DNA Analyzer (ABI) with a 50 cm array. USDA, ARS, U.S. Horticultural Research Laboratory, Genome Lab, Fort Pierce, FL.

Advantages





- **Poster Number:** P0467
  - Title: FANA_Antisense Oligonucleotides: Next Generation RNA Silencing Technology to Target Pathogens in Blood and Ectoparasites
- **Poster Number:** P0310
  - Title: FANA_ASO Reduces Pathogens and Pest of Fruit Crops: Citrus and Grapevine

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- Hunter et al, in prep 2017
- Metz and Hunter in prep 2017
- Pelz-Stelinski, Sandoval Mojica, UF, and Hunter, ARS, in prep2017

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- USDA, NIFA, Citrus Greening award #2015-70016-23028. National Institute of Food and Agriculture. #2014-10154. Project Lead: Dr. S. Brown, Kansas State University. Grant :**Developing an Infrastructure and Product Test Pipeline to Deliver Novel Therapies for Citrus Greening Disease".**