Independent-mobile RNA (iRNA) expression vector against HLB- Initiate operation "Lab 2 Farm"

Georgios Vidalakis, University California Riverside, vidalg@ucr.edu

Year 2 of 2 (75% Complete)

Objectives

1. Gain a deeper understanding of the iRNA biology; A. Simon
2. Continue designing anti-CLas enzybiotics; D. Nelson
3. (a) Continue developing a suite of small RNAs that target Asian citrus psyllid (ACP) and (b) define the parameters necessary for introducing iRNA into citrus phloem using Agro-infiltration and laser-mediated mechanical inoculation; J. Brown
4. Collect preliminary data on the yellow vein associated iRNA and citrus vein enation virus (CVEV) interactions; K. Debbink
5. Initiate iRNA citrus transmission experiments to study transmissibility and tree and fruit effects; G. Vidalakis

Problem and Significance

Our team discovered a new species of plant associated RNA named independent-mobile RNA (iRNA). iRNA was discovered in association with a citrus disorder named yellow vein, reported once in the 1950s in CA. The iRNA was tentatively named: citrus yellow vein associated virus (CYVaV). The National Academy of Sciences reported that a promising management of huanglongbing (HLB) in currently infected trees, which may also confer protection against future infections, is using phloem-restricted virus vectors to generate small RNAs or peptides directly in the tissue colonized by '*Candidatus* Liberibacter asiaticus' (CLas) and fed on by ACP. To date, only a single virus vector is available for delivering products into the phloem of citrus trees: citrus tristeza virus (CTV), the causal agent of catastrophic citrus diseases such as quick decline and stem pitting. CTV is phloem-limited, stable for many years, and can produce a moderate level of an inserted protein or siRNA. However, CTV accumulation is variable, which likely precludes its ability to generate sufficient small RNAs to be efficacious in the field. In addition, CTV is very large (20 kb), naturally transmitted by several aphid species, and it is not known if the currently developed attenuated strains are universally mild and will remain that way. Preliminary iRNA experiments revealed that it is small and simple to manipulate, phloem-limited, graft-transmissible to many citrus species, accumulates to extremely high levels, causes mild symptoms that fade over time, and cannot escape into the environment. Based on these properties, iRNA is an ideal expression vector for the development of commercial products for HLB and ACP management.

Benefit to Industry

This project will benefit citrus growers in California and other citrus producing states and areas around the world. This project will build the research team and generate the preliminary data to leverage the necessary non-CRB funds (federal and private) required to take this technology from the lab, through the regulatory pipeline, into the private sector for commercialization and to the hands of the growers. This process is going to be lengthy and costly. If successful, the citrus growers will have a family of commercial iRNA vector products for HLB and ACP management.

Progress Summary

1. Gain a deeper understanding of the iRNA biology

We have solved the problem of instability that plagues all virus vectors by figuring out how to design insert hairpins that CYVaV will accept as "self" and not "foreign". We can now make vectors with two inserts that are exceptionally stable. We have also identified siRNAs that target CVEV and callose synthase (60% reduction in callose deposition). We have also surprisingly found that

CYVaV alone can virtually eliminate detectable polymerized phloem protein 2 (PP2), which is the protein that clogs the veins of citrus afflicted with HLB. PP2 binds and encapsulates CYVaV, reducing the amount of free protein in the sap.

We have also successfully re-introduced CYVaV into citrus, the key experiment for using the vector. This was accomplished using a dodder "IV" connecting a tube containing sap from an infected plant directly to a Mexican lime plant. This will allow us to generate an "inoculum source tree" from which budwood can be excised for grafting (Figure 1)

Figure 1. Introduction of CYVaV back into citrus using a dodder IV.

We have also found that CYVaV generates defective non-coding RNA fragments (1 kb) that cannot generate the polymerase. We are determining if this RNA can also be used as a vector along with CYVaV for more inserts.

Finally, we have identified two siRNAs that can target critical CLas mRNAs *in vitro*. Using a surrogate system (*L. crescens*, Lcr, in papaya), we will be testing if we can successfully keep the bacteria from growing *in vivo*.

2. Continue designing anti-CLas enzybiotics

The first objective of this project was to grow Lcr in the laboratory so the antimicrobial properties of the anti-CLas protein-based antimicrobials, known collectively as "enzybiotics" can be evaluated on a close relative to CLas. We obtained Lcr strain BT-1 from two independent sources, ATCC database (https://www.atcc.org) and UC Riverside, both of whom obtained it from the University of Florida. We tested six media conditions and found one recipe worked very well.

The second objective of this project was to evaluate/design enzybiotics for expression by iRNA. To begin, we screened several enzymes for antimicrobial activity on common Gram-negative pathogens. However, as results began to flow in about potential size requirements for iRNA expression, it became clear that the genes for protein-based enzybiotics might be larger than acceptable for iRNA expression. Therefore, a decision was made to abandon the larger bacterial-derived enzybiotics and change course to focus on smaller, plant-based peptide enzybiotics.

To evaluate smaller, plant-based enzybiotics, we bioinformatically screened the entire PhytAMP database (http://phytamp.pfba-lab.org/), which contains all plant-based antimicrobial peptides (AMPs). Ultimately, we down selected to seven candidates with known antimicrobial properties that encompass a variety of sizes, surface charges, and plant sources.

3a. Continue developing a suite of small RNAs that target ACP

No progress was made on this objective last year. The work will be completed by October 2022.

3b. Define the parameters necessary for introducing iRNA into citrus phloem

Preferred delivery methods for introducing CYVaV in citrus phloem are agro-inoculation, and gene gun.

(ii) Effective doses of dsRNA, for potato psyllid (PoP) and ACP adults to achieve knockdown is 100 ng/uL (max 200 ng/ul to saturation). Knockdown persists for nine days before beginning to decline in PoP. Tests to evaluate knockdown in replicated experiments involving PoP and ACP 3rd instars and teneral adults have been completed. Till now >60 genes were tested in preliminary studies to identify differential knockdown candidates to represent expected RNAi variation depending on gene target.

(iii) Targets. The top six groups are under evaluation in ACP (interactors in biochemical pathways or homologous function in multiple isoforms). Their selection was based on efficacy in PoP in groups (3-4) and singly or in pairs for PoP and/or ACP. All experiments use 3rd instar nymphs and recently molted (teneral) adults in separate assays (3-5 replicates x 3 technical reps each). All promising ACP dsRNAs have been synthesized, with the respective qPCR probe/primers. One dsRNA target in group by laser-etching/topical experiments, and by ACP efficacy testing in feeding studies (UFL).

4. Collect preliminary data on the yellow vein associated iRNA and citrus vein enation virus (CVEV) interactions

Our previous work on this project demonstrated that CYVaV can be encapsulated by the CVEV coat protein, meaning that CVEV is a putative helper virus for CYVaV.

To identify this encapsidation sequence, we designed a series of deletion mutants, which have deletions of between 40-200 nucleotides in length in the CYVaV genome.

We have made progress on the cloning work to make the deletion mutants. After modifying our cloning strategy, we were able to successfully amplify some of the desired CYVaV deletion mutant products for those mutants with deletions near the beginning of the CYVaV genome.

5. Initiate iRNA citrus transmission experiments to study transmissibility and tree and fruit effects

(i) We tested a few wild-type and recombinant CYVaV constructs in Daisy and Tango cell lines and reported successful replication of CYVaV in both ‘Daisy’ and ‘Tango’ cell lines of citrus.

Transfected protoplasts appeared to regenerate cell walls, undergo cell division, and remain viable for up to 2 months post-transfection.

(ii) To study the CYVaV distribution in the US, five samples from Texas A&M University and 39 samples from Puerto Rico have been analyzed using qRT-PCR. These samples from old citrus orchards that have not undergone thermotherapy since 1970s were selected as they are more likely to have CYVaV. None of the 44 samples in total have been tested positive for the presence of CYVaV.

(iii) A replicated field trial with 198 trees from >15 popular rootstock scion combinations has been started at Agricultural Operations fields at UC Riverside. We studied CYVaV mobility and symptomatology at the first time point (6 months after graft-inoculation). Of the 72 graft-inoculated trees, 32% of trees (from different rootstock scion combinations) tested positive, whereas 68% trees tested negative for CYVaV. None of the 16 older trees (over 1 year old) with assorted rootstock scion combinations were positive for CYVaV (Figure 2; Table 1).

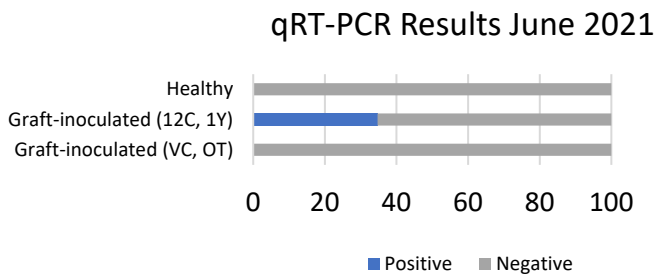

Figure 2. RT-qPCR results of 112 root and shoot samples (combined) including 24 healthy samples (2 per combination), 72 graft-inoculated samples (6 per combination), and 16 assorted combinations. 12C=12 combinations, 1Y= 1 year old trees, VC=Various (assorted) combinations, OT=old trees).

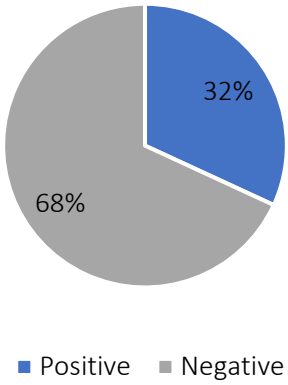

Figure 3. Overall percent infection rate of CYVaV in field

	12 commercially popular R/S combinations	Infection rate (%)
1	Limoneria 8A Lisbon Lemon/Rubidoux Trifoliate	5 of 6 (83%)
2	Limoneria 8A Lisbon Lemon/Macrophylla	2 of 6 (33%)
3	Parent Washington Navel/Carrizo Citrange	1 of 6 (17%)
4	Biogold Giulietta Mandarin/Carrizo Citrange	2 of 6 (33%)
5	Limoneria 8A Lisbon Lemon/Carrizo Citrange	5 of 6 (83%)
6	Cara Cara Navel/Carrizo Citrange	1 of 6 (17%)
7	Biogold Giulietta Mandarin/Rubidoux Trifoliate	0 of 5 (0%)
8	Cara Cara Navel/Rubidoux Trifoliate	0 of 5 (0%)
9	Tango Mandarin/Rubidoux Trifoliate	4 of 6 (67%)
10	Miho Wase Satsuma/Carrizo Citrange	0 of 6 (0%)
11	Tango Mandarin/Carrizo Citrange	2 of 6 (33%)
12	Parent Washington Navel/Rubidoux Trifoliate	0 of 5 (0%)

Table 1. CYVaV percent infection rate per rootstock scion combination in field.

Only 5 of 88 trees (including additional combinations) showed characteristic yellow vein disease symptoms (Figure 3). The survival of grafts was checked 6 months after original graft inoculation (all original grafts had survived for at least 2 weeks) and trees with both dead grafts were re-grafted in June 2021.

Figure 3. Citrus yellow vein disease symptoms in field experiment (June 2021).

Publications

Kwon, S-J, Bodaghi, S., Dang, T., Gadhave, K.R., Ho, T., Osman, F., Maher, A.R., Tzanetakis, I.E., Simon, A.E. & Vidalakis, G. 2021. Complete nucleotide sequence, genome organization and comparative genomic analyses of citrus yellow vein associated virus (CYVaV). Frontiers in Microbiology. doi: 10.3389/fmicb.2021.683130.

Saberi, E. and Brown, J.K. Differential expression of Candidatus Liberibacter solanacearum genomic and prophage genes in different instars of the potato psyllid host, Bactericera cockerelli. Phytopathology (submitted).

Paredes-Montero, J.R., Arif, U., and Brown, J.K. RNA interference-mediated knockdown of genes implicated in the synthesis of ecdysteroids, impairs molting in the potato psyllid, Bactericera cockerelli (Insecta: Hemiptera). Pest Management Science (submitted).

Presentations

K. R. Gadhave. Citrus yellow vein associated virus (CYVaV) novel RNA: 70-year-old California tale. 2021 Western Extension Research Activity (WERA) 20 Meeting, UC Riverside, May 13, 2021

K. R. Gadhave. Plant viruses and insect vectors: strong alone, but unstoppable together? Special Seminar, Seoul National University, Seoul, S. Korea, September 6, 2021

CRB Project # 5300-207