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

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Year 1 of 2 (30% Complete)

Objectives

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

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 (citrus yellow vein associated virus-CYVaV), reported once in the 1950s in California. A promising management approach for huanglongbing (HLB), 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 Asian citrus psyllid (ACP). Our iRNA experiments revealed that this novel RNA 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, has no known natural vector (i.e. cannot move freely into the environment), and generates a sub genomic RNA (sgRNA). Based on these properties, iRNA is an ideal expression vector, that is very different and could complement the developed citrus tristeza virus (CTV) vector, for the development of commercial products for HLB and ACP management.

Benefit to Industry

This project will generate the preliminary data to leverage the necessary non-CRB funds (federal and private) required to take this technology from the lab, into the private sector and through the regulatory pipeline, for commercialization and into the hands of the growers. This process is going to be lengthy and costly and will extend beyond the 2 years of this project but it is this support of the CRB that will kick start the process. If successful, the citrus growers will have a family of commercial iRNA vector products for HLB and ACP management and potentially for management of other emerging diseases.

Plans and Procedures

1. Gain a deeper understanding of the iRNA biology:

The structure of the right end of the iRNA sequence will be defined and the promising insertion sites, the size limit of inserted sequences for accumulation in single cells and in citrus trees will be determined. The possibility of the iRNA replicating inside of sieve elements will be explored.

2. Continue designing anti-CLas enzybiotics:

Using bioinformatic tools, the CLas genome will be explored to identify the embedded enzybiotics (anti-CLas protein-based antimicrobials), as well as develop a collection of enzybiotic catalytic domains from phages (bacterial viruses) known to infect Gram-negative bacteria. The best enzybiotic candidates, the modifying peptides, and chimeras of enzybiotics with peptides will be tested for expression in the iRNA vector.

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

The candidate targets for RNA interference (RNAi) having a high likelihood of knockdown with measurable ACP phenotypes will be determined.

(b) define the parameters necessary for introducing iRNA into citrus phloem:

Different approaches for delivering the iRNA vector expressing double stranded (ds)RNAs or peptides to the phloem of tomato and citrus seedlings will be pursued, including bacteria mediated infiltration, mechanical delivery via laser-wounding, and particle bombardment inoculation.

4. Collect preliminary data on the yellow vein associated iRNA and CVEV interactions:

The possibility of CVEV serving as a helper virus for the iRNA will be explored and if so, the genetic sequence that allows for virus particle formation by the CVEV coat protein will be determined.

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

The transmissibility of iRNA will be studied using various iRNA delivery platforms (e.g. protoplast, agro-infiltration etc.). Geographical distribution of iRNA in the US will be studied. The effects of iRNA on citrus tree growth, yield and fruit quality will be studied in commercially used citrus scions and rootstocks in field conditions.

Progress Summary

1. Gain a deeper understanding of the iRNA biology

We have discovered five locations that can accept different types of inserts. To increase stability of the inserts, we have designed specific scaffolds that we call “Lock and Dock”. Nucleotide sequences inserted into a lock and dock become completely stable in several locations. We have also found that the CYVaV-iRNA can accept multiple inserts, which is important as our goal is to generate a virus vector for virus induced gene silencing (VIGS) targeting CTV, CLas, CVEV, callose synthase and the psyllid.

We were able to demonstrate the proof of principal with targeting a reporter gene (i.e., green fluorescent protein [GFP]) and CTV using iRNA in tobacco plants. We performed three successful tests. One of them (#6) reduced CTV-GFP to near undetectable levels. Being able to “vaccinate” citrus against CTV using iRNA may allow growers to start using sour orange rootstock again.

We also initiated experiments on targeting Callose Synthase 7 gene expression. Trees infected with HLB have reduced movement of sugars from leaves to roots, which is likely the cause of tree death. Our initial results show that we can decrease the levels of callose in the phloem using CYVaV targeting the enzyme.

Our team also secured a USDA-Specialty Crop Research Initiative (SCRI) grant and continued working with the startup Silvec Biologics Inc. that has licensed the iRNA technology. Silvec also continued fundraising efforts and moved to an incubator laboratory at the University of Maryland.

2. Continue designing anti-CLas enzybiotics:

The first focus of this objective is to grow *Liberibacter crescens* (Lcr), a CLas surrogate, in the laboratory so potential antimicrobial properties of enzybiotics can be evaluated. We obtained Lcr strain BT-1 from two independent sources, and we tested six media conditions and found one recipe the worked very well and we have identified a set of conditions for improved bacterial growth.

The second focus area is to design anti-CLas protein-based antimicrobials, known collectively as “enzybiotics”, for expression by iRNA. We screened several enzymes for antimicrobial activity on Gram-negative bacteria (the type of bacteria that CLas belongs to) and we found four (4) enzymes to possess antimicrobial activity. It is anticipated that they will likewise have antimicrobial activity toward Lcr and ultimately CLas, as these organisms are also Gram-negative. Now that growth conditions for Lcr have been worked out, testing these enzymes against Lcr is the next step.

The last focus area is to evaluate/design anti-CLas peptide-based antimicrobials. This is important because as results began flowing in about the size requirements for iRNA expression, we realize that our protein-based enzybiotics are ~15 kilo Daltons in size, which may be too big for iRNA expression. Additionally, given that they are derived from bacteria (i.e. non-plant sources), there is concern that we may run into regulatory issues down the road. Therefore, we shifted focus to develop smaller, plant-based enzybiotics. Peptides are also more likely to have better stability profile compared to larger protein enzymes. Ultimately, we will down-select and provide the top 2-3 candidates from both the protein enzybiotics and the smaller plant defensin-like peptides to Dr. Simon's group for cloning into the iRNA vector.

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

(b) define the parameters necessary for introducing iRNA into citrus phloem

We identified agro-inoculation and gene gun as the preferred delivery methods and we identified 100 ng/uL as the effective knockdown dose of dsRNA, based on results from the potato psyllid (PoP) model system. Knockdown persists for nine days before beginning to decline in potato psyllids. Initially six genes were tested in preliminary studies to identify differential knockdown candidates to represent expected RNAi variation depending on gene target. The top four groups (n=14 targets) are under evaluation with PoP as single genes. ACP dsRNAs have been synthesized, with the respective qPCR probe/primers. dsRNAs are ready to begin laser-etching/topical experiments, and by ACP efficacy testing in feeding studies.

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

We tested whether the iRNA was able to be encapsidated by CVEV, another virus that infects citrus. This is important to know because aphids would likely be able to transmit the encapsidated iRNA from plant-to-plant. Our results in this objective confirmed our hypothesis that the iRNA can be encapsidated by the CVEV coat protein using the model system of tobacco plants with either the iRNA only, CVEV only, or both the iRNA and CVEV.

Because the encapsidated iRNA could potentially be transmitted from plant-to-plant by aphids, it is important to know how this encapsidation occurs so that it can be controlled. The next part of our objective is to identify the genetic sequence within the iRNA that facilitates encapsidation by the CVEV coat protein. We have designed 10 iRNA mutants that will be used in experiments to identify this sequence during the remainder of the grant. Once identified, this sequence can be manipulated to prevent or facilitate CVEV encapsidation of the iRNA.

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

To test the transmissibility of CYVaV iRNA expression vector to commercial citrus, the first efforts will involve regeneration of citrus plants from protoplasts of embryonic suspension cultures of citrus transfected with yellow vein RNA at the plant transformation facility at UCR. To achieve this goal, 5 cell lines of commercial citrus have been established and will be used in the subsequent transfection experiments. The protoplast protocol for citrus is currently being standardized. We have applied for a BRS ePermit to be able to receive the recombinant CYVaV-iRNA constructs from the University of Maryland.

To study the potential natural distribution of CYVaV across the USA, an APHIS permit has been obtained and the citrus samples from old orchards that were propagated from sources that have not undergone therapy for removal of citrus pathogens, have been requested from multiple universities across the USA. So far, five samples from Texas A&M University and 33 samples from Puerto Rico have been received (currently being processed).

To study the effect of CYVaV RNA on commercial citrus in field conditions, a replicated field trial with 198 trees from 15 popular rootstock scion

combinations was planted at the Agricultural Operations fields at UC Riverside. Six (6) trees per combination will be graft-inoculated with CYVaV and 6 will remain uninoculated as negative controls.

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Publications and Presentations

Publications:

1. Molki, B., Ha, P.-T., Cohen, A.L., Crowder, D.W., Gang D.R., Omsland, A., Brown, J.K., and Beyenal, H. 2019. The infection of bacterial plant pathogen Candidatus Liberibacter solanacearum is associated with altered physiology of its insect host. Enzyme Microb Technol. 129:109358.