California Citrus Nursery Board (CCNB) -Proposal of Research

Project Title: Optimizing the timing and plant introduction of Transmissible small nuclear RNAs (TsnRNAs) for dwarfed citrus

Fiscal Year: 2019

Project Duration: Year 3 of 5-7 years

Project Leaders:

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Contractor's Name: The Regents of the University of California, on behalf of its Riverside campus.

Current and Pending sources for this project: none
Due: November 15, 2018

Executive Summary

Development of commercial dwarfed trees, excellent candidates for high-density plantings and citrus production under protective structures (CUPS), will be critical to meet challenges posed by Huanglongbing (HLB), water shortages, and labor costs.

'Transmissible small nuclear RNAs' (TsnRNAs) are well-characterized viroid RNA species that modify tree performance without inducing disease. TsnRNA-IIIb reduces the canopy volume of navel orange trees on trifoliate rootstock (approx. 50% in high-density planting), increases the yield per land surface unit, and concentrates fruit in the optimum canopy zone for harvest without affecting fruit quality. These findings indicate a potential value of such technology in Huanglongbing (HLB) and general orchard management. The commercial use of TsnRNA for citrus dwarfing has been approved by the CDFA since 2001. However, citrus nurseries have indicated to us that to deploy this technology in a commercial setting, the optimal time and method of plant introduction of the dwarfing TsnRNA is an aspect that needs to be investigated. This is a critical aspect so that citrus nurseries will not jeopardize production (i.e. contamination with transmissible viroid RNAs) and can optimize their TsnRNA/Dwarfing workflow. This project aims to introduce the dwarfing agent TsnRNA-IIIb into Parent Washington navel orange trees of different ages with different techniques, and evaluate tree growth in the field over time. This year's request for funding is to allow the treatments to proceed for the replicated trials at two locations and two time points for proper statistical analysis of the trees measurements (AgOps, Riverside and Lindcove Research and Extension Center in 2017 and 2018).

Project's Benefit to Citrus Nursery Industry

The introduction and potential spread of HLB in CA makes it imperative to develop effective HLB management plans to ensure that the CA citrus industry continues to thrive. HLB is a serious threat to the CA citrus industry as there is no known cure and all commonly grown citrus varieties are susceptible to the disease. HLB causes losses of over $950 million per year in Florida (http://www.fred.ifas.ufl.edu/pdf/economic-impact-analysis/Economic_Impacts_Florida_Citrus_Industry_2012-13.pdf). To continue thriving, the CA citrus industry must act now.

High-density groves have been reported to yield better despite being infected with HLB (Belasque et al., 2010). Dwarf trees are a prerequisite for high-density plantings (avoid overlapping trees resulting in competition, reduced yields, and high production costs) and CUPS, which probably will be one of the most efficient ways to manage HLB. To survive HLB, the CA citrus industry must invest in management of the disease. Eradication of diseased plants is a huge economic setback. In addition, the difficulty in detecting HLB in the field makes it more likely that numerous plants will be infected and that the disease will spread as it has happened in China, Brazil, and Florida. Employing the TsnRNA dwarfing technology at the commercial level to allow efficient citrus production in high-density planting in CUPS will be key to meet challenges posed by HLB, water shortages, labor costs, and to reduce the environmental impact of insecticides.

The efficient, safe and cost-effective production of such trees by nurseries is an absolute prerogative to allow TsnRNA dwarfed citrus trees deployment to a commercial level. Ideally, nurseries would want to be able to introduce (e.g. graft, slash, or inject) the dwarfing agent(s) 1 week before shipment so that if a client cancels an order, trees can be sold to someone else. Typically, 12-18 months old trees are sold to growers but whether this is the optimum tree age for TsnRNA introduction for optimum dwarfing results remains to be established.

This project addresses the following CCNB's Program Priorities:

1. Growing Citrus in screenhouses/greenhouses in containers.
   - New ways to control citrus pests & pre shipment treatments.

TsnRNA dwarfed citrus trees were originally developed at the University of California, Riverside (Semancik et al., 1997). At the time, pathogen-tested Valencia orange scions were budded to trifoliate rootstock seedlings. At the time of budding, the rootstock seedlings were also graft-inoculated with citron sources of TsnRNA-IIIb isolates (syn. citrus dwarfing viroid, CVd-IIIb). Following one year in a glasshouse, trees were planted at the Lindcove Research and Extension Center (Exeter, CA). Measurements of tree height and canopy circumference showed a dramatic reduction in tree size in response to the presence of TsnRNA-IIIb. In another experiment, pathogen-tested Parent Washington navel orange scions were budded on 12-month-old trifoliate Rich 16-6 rootstock seedlings grown from seed produced from disease tested mother plants. At the time of budding, the seedling rootstocks were also grafted with two blind buds from the source tree of the CDFA approved growth-modifying TsnRNA-IIIb (GenBank deposit AF18147) (Semancik et al., 1997;), an approach that resulted in dwarfed citrus trees in high-density plantings (Vidalakis et al., 2011). However, in a commercial nursery setting, introduction of the dwarfing agent should ideally take place just prior to shipping or in the field right after planting rather than at the time of budding in the nursery. Whether this is a feasible option needs to be investigated.

The citrus industry (i.e. CRB) has already funded: 1) A long-term project to investigate the mechanisms and use of TsnRNAs for citrus trees dwarfing and 2) Research on CUPS production systems. CRB has also invested in the hormonal and gene expression profiling of existing TsnRNA-dwarfed trees. A citrus dwarfing ad hoc committee has been established and members are currently finalizing the design of large field trials that aim to explore if different combinations of rootstocks and scions can result in dwarfing in response to TsnRNAs treatments, and researching CUPS structures with dwarfed citrus. In May 2016, the committee recommended that an experiment investigating the optimum tree age and method of TsnRNA-IIIb introduction should be conducted so that the citrus nurseries can safely and effectively use this technology at a commercial scale. The citrus dwarfing ad hoc committee identified the CCNB as a potential sponsor for this study. A proof of concept is needed before the technology can be applied at a large scale so that the citrus nurseries can offer the industry dwarfed trees for high density plantings or CUPS that will allow the growers to deal with pressing issues such as HLB, water shortages, reduced farmland availability, and increasing labor costs.

Objectives

Research:

The project aims to assess the dwarfing efficiency 1. Of different TsnRNA introduction methods 2. In trees of different ages. By introducing the dwarfing agent TsnRNA-IIIb with a range of techniques into trees of different ages and planting them in the field, we will monitor the difference in tree size in 4-7 years, and identify the best protocol.

Educational:

Results will be shared with citrus nurseries, growers and other stakeholders (regulators, extension specialists and the general public) during CCPP, CCNB, CRB meetings, and other public citrus events such as the CCPP Walkthrough, UC Riverside Citrus Day, and the LREC Fruit Display. Information will also be disseminated via ccpp.ucr.edu and facebook.com/UCRCCPP/. Results will be published in scientific journals and the Citrograph magazine, and presented at professional conferences and growers seminars.

Workplans and Methods

Years 1-2 (2017-2018). –

1. Navel oranges

   Disease-tested Parent Washington navel orange trees on *P. trifoliata* rootstock provided by Tree Source nursery were treated with TsnRNA-IIIb as indicated in Table 1. Two replicated trials at AgOps-Riverside and Lindcove Research and Extension Center-LREC, Exeter were established and, in this reporting period, the third time point treatments were performed (i.e. 12 months, highlighted in gray, Table 1).

Table 1: Experimental design for TsnRNA-IIIb's optimal inoculation studies for navel orange trees.

Time of inoculation expressed in # months post grafting	TsnRNA-IIIb inoculation method	Location of tree at the time of inoculation	# trees
0 May 2017	Control - NA	Glasshouse	10
0 May 2017	Grafting	Glasshouse	10
0 May 2017	Slashing	Glasshouse	10
0 May 2017	Vaccination gun	Glasshouse	10
6 November 2017	Grafting	Glasshouse	10

6	November 2017	Slashing	Glasshouse	10
6	November 2017	Vaccination gun	Glasshouse	10
12	May 2018	Bud grafting	Glasshouse	10
12	May 2018	Slashing	Glasshouse	10
12	May 2018	Vaccination gun	Glasshouse	10
18	November 2018	Grafting	Glasshouse	10
18	November 2018	Slashing	Glasshouse	10
18	November 2018	Vaccination gun	Glasshouse	10

We previously planned to plant these trees in the field in the Fall 2018 but they are still too small (See pictures below). We delayed planting until appropriate size is reached, tentatively Spring 2019.

Navel/Trifoliate

We observed some uneven growth of the trees. As the time points for inoculation proceeded, it became increasingly difficult to choose trees that displayed comparable growth rates. Thus, we decided we will stop these inoculations at the 18 month-time point (i.e. November 2018) was the last). We will not proceed with further time points for navel oranges to avoid that the original size of the tree affects the final observation/tree measurements at the end of the experiment. In addition, after August 2017 we were not able to move citrus materials, in this case budwood containing TsnRNA-IIIb to LREC from Riverside therefore, the 2017 (year 1) experiment did not have uniform inoculum sources.

2. <u>Tango</u>

Tango/Trifoliate

1500 Tango/Trifoliate trees were ordered from TreeSource and received in August 2018. 750 trees are at LREC and the other 750 in Riverside (See pictures below). They were transplanted into larger pots with coconut core. LREC and Riverside are coordinating to perform the same operations at the same time (tentatively February 2019).

3. <u>Inoculum quantification by qPCR</u>

A qPCR assay was established to quantify TsnRNA-IIIb inoculum. Other primers available in the lab for this specific TsnRNA were tested but efficiency was too low as these primers had not been designed for quantification purposes. New primers and a plasmid were

ordered and the new developed assay (in collaboration with Dr Fatima Osman, UC Davis) can now be used to quantify inoculum every time a treatment is performed. This will be important for publications and to prove that at least a certain minimum amount was used for each treatment.

Year 3-7 - Proposed for 2019

Due to the observed some uneven growth of the navel trees in year 1, and higher commercial interest in Tango voiced at the CCNB Meeting in December 2017, we decided to repeat the experiment in year 2 with Tango/trifoliate with a much larger pool of trees to choose from at each inoculation time point to ensure comparable growth.

We are proposing for year 3-2019 to start with the staggered inoculation of 280 pathogen-tested uniform Tango scions budded on *P. trifoliata* rootstock to establish two fully replicated trials at the two locations (AgOps, Riverside and LREC, Exeter). 280 uniform trees for each of the two sites will be treated with TsnRNA-IIIb as presented in Table 2. More specifically, trees will be inoculated with TsnRNA-IIIb at different ages (10 trees per age) and with different techniques: grafting, slashing and with a vaccination gun (10 trees per technique). Non-inoculated (control) trees will be included (10 trees) (Table 1). The TsnRNA-IIIb treated trees along with untreated controls will be kept in a glasshouse for one year, and then planted at high-density (10ft x 22ft; i.e. approx. 3 x 6m) on raised beds in AgOps, Riverside and LREC, Exeter beginning Spring 2020 with the trees of year 1-2017.

We would welcome any input from the CCNB committee members on the proposed work for year 3-2019.

Table 2: Experimental design for TsnRNA-IIIb's optimal inoculation studies for Tango/Trifoliate

Time of inoculation expressed in # months post grafting	TsnRNA-IIIb inoculation method	Location of tree at the time of inoculation	# trees
0	Control - NA	Glasshouse	10
0	Grafting	Glasshouse	10
0	Slashing	Glasshouse	10
0	Vaccination gun	Glasshouse	10
6	Grafting	Glasshouse	10
6	Slashing	Glasshouse	10
6	Vaccination gun	Glasshouse	10
12	Bud grafting	Glasshouse	10
12	Slashing	Glasshouse	10
12	Vaccination gun	Glasshouse	10
18	Grafting	Field	10
18	Slashing	Field	10
18	Vaccination gun	Field	10
24	Grafting	Field	10
24	Slashing	Field	10
24	Vaccination gun	Field	10
30	Grafting	Field	10
30	Slashing	Field	10

30	Vaccination gun	Field	10
36	Grafting	Field	10
36	Slashing	Field	10
36	Vaccination gun	Field	10
42	Grafting	Field	10
42	Slashing	Field	10
42	Vaccination gun	Field	10
48	Grafting	Field	10
48	Slashing	Field	10
48	Vaccination gun	Field	10

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Year 4-10

Field evaluations to assess the efficacy of the inoculation timing and technique will be performed at the field. Trees will never be pruned. Tree height and canopy circumference measurements will be taken each year during the winter season and used to estimate tree volume. Data will be subjected to statistical analysis. Given the very noticeable phenotype observed in previous experiments (Semancik et al. 1997, Vidalakis at al., 2010; Vidalakis et al. 2011) we predict differences will be obvious after 4-5 years in the field.

Project Management and Evaluation

Irene Lavagi and Georgios Vidalakis will direct the experiments. Greg Greer, Rock Christiano, Irene Lavagi will produce the experimental trees. Irene Lavagi and Rock Christiano will monitor growth of the trees and analyze data. An annual report to the CCNB on the status of the project will be provided and presentations will be given as requested by the CCNB. Field visits for all interested parties will be encouraged at any time and at events such as the UC Riverside Citrus day and the CCPP Walkthrough at LREC.

Literature Review

Belasque J. Jr, Bassanezi R., Yamamoto P., Ayres A., Tachibana A., Violante A., Tank A. Jr, Di Giorgi F., Tersi F., Menezes G. 2010 Lesson from huanglongbing management in São Paulo State, Brazil. Journal of Plant Pathology, 92, 285–302.

Semancik J., Rakowski A., Bash J., Gumpf D. 1997. Application of selected viroids for dwarfing and enhancement of production of 'Valencia'orange. Journal of Horticultural Science, 72: 563-570.

Vidalakis, G., Pagliaccia, D., Bash, J.A., Semancik, J.S. 2010. Effects of mixtures of citrus viroids as transmissible small nuclear RNA (TsnRNA) on tree dwarfing and commercial scion performance on carrizo citrange rootstock. Annals of Applied Biology, 157:415-423.

Vidalakis, G., Pagliaccia, D., Bash, J.A., Afunian, M., Semancik, J.S. 2011. Effects of Citrus Dwarfing viroid, a transmissible small nuclear RNA on tree size and scion performance specific to poncirus trifoliata rootstock with applications for high density planting. Annals of Applied Biology, 158: 204-217.

ATTACHMENT #1

BUDGET PROPOSAL

Project Title/Description: Optimizing the timing and plant introduction of Transmissible small nuclear RNAs (TsnRNAs) for dwarfed citrus

Project Leader: Irene Lavagi and Georgios Vidalakis

Proposed Fiscal Year: 01/01/2019 – 12/31/2019

A. PERSONNEL SERVICES:

Student salaries		$ 5,000.00
Staff Benefits =	%	$

TOTAL PERSONNEL SERVICES $ 0

B. OPERATING EXPENSES:

Laboratory Supplies (qPCR assays, inoculum prep)	$ 3,000
Travel	$ 500
Postage	$ 500
Other: (land prep, planting, irrigation)	$ 4,000.00
Cultural Care ($308 for 2 ½ acres, monthly)	$

C. TOTAL OPERATING EXPENSES: $ 13,000

D. TOTAL BUDGET REQUESTED: $ 13,000

Note:
A CRB project to further study TsnRNAs was awarded this fall. The CRB project aims to screen for different scion varieties that can be dwarfed and analyze existing TsnRNA-dwarfed trees to assess the potential to reduce cost production and environmental impact offered by this technology. The optimization of TsnRNA's inoculation proposed here to the CCNB is a separate and well-delimited project that complements the CRB.

Signature of Requestor: I Lavagi Date: 9th Nov 2018

Signature of Requestor: Date: 9th Nov 2018

Department Chair: _____ Date: _____