INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 17-0416-000-SA

Title of Project: Resistance to grapevine leafroll-associated virus 3 and the grape mealybug

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July, 1 2018 to March 15, 2019

List of objectives

- Complete the development of stacked RNAi constructs against the grape mealybug and grapevine leafroll-associated virus 3
- Develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug
- Produce and characterize stably transformed grapevines with RNAi constructs against grapevine leafroll-associated virus 3
- Disseminate information to stakeholders through presentations at conventions and workshops

Description of activities conducted to accomplish each objective, and summary of accomplishments and results of each objective

To address objective #1 and complete the development of stacked RNAi constructs against the grape mealybug and grapevine leafroll-associated virus 3, we concatenated dsRNA constructs of *AQP1, SUC1*, and *NUC1*. These three dsRNA constructs are derived from the grape mealybug. The first two gene are required for water balance in the gut of phloem-feeding insects. We hypothesize that perturbing their expression through RNAi causes the grape mealybugs to lose water from the body fluids and to dehydrate, dying within 2-3 days, as previously shown for other insects (Karley et al. 2005, Shakesby et al. 2009, Tzin et al. 2015). To enhance the efficacy of the *AQP1* and *SUC1* dsRNA constructs, *NUC1,* a non-specific nuclease that is expressed in the gut and functions to degrade ingested dsRNA (Christiaens et al. 2014, Luo et al. 2013), was characterized, engineered as dsRNA construct, and added to the other two grape mealybug dsRNA construct. It is anticipated that the addition of *NUC1* will protect dsRNA against degradation and dramatically increase insect mortality, as recently documented (Luo et al. 2017). Expression of the concatenate *AQP1*, *SUC1* and *NUC1* dsRNA construct is presently driven by the T7 promoter. The next step will be to clone the concatenate dsRNA construct in a plasmid for expression driven by the phloem-specific promoter s*ucrose-H+ symporter* (*SUC2)* to target RNAi expression to the preferred feeding sites of the grape mealybug.

For GLRaV-3, dsRNA constructs to the suppressor of RNA silencing *p19.7* (Gouveia et al. 2012) and the coat protein (*CP*) open reading frame were engineered. To identify new dsRNA constructs, the GLRaV-3 genome was mined for relatively short stretches of conserved regions with a major emphasis on the RNA-dependent RNA polymerase (*RdRp*) and the heat shock protein 70 homolog (*HSP70h*) open reading frames of GLRaV-3. Conserved regions were identified for *RdRp* and *HSP70h*. Additionally, a highly conserved short stretch in the 3'end untranslated region (UTR) of the GLRaV-3 genome was identified. These conserved GLRaV-3 sequence regions were characterized by RT-PCR using overlapping primer pairs. For resistance against GLRaV-3, efforts will focus on the 3' UTR region, *RdRp*, and *HSP70h* of GLRaV-3. These dsRNA constructs will complete the *CP* and *p19.7* dsRNA constructs previously engineered.

Targeting of the GLRaV-3 silencing suppressor p19.7 is not optimal for combined resistance to the grape mealybug and GLRaV-3. This is because the inclusion of a virus-encoded silencing suppressor in a concatenated dsRNA construct will interference with the efficacy of stacked dsRNA constructs against GLRaV-3 and the grape mealybug. Therefore, dsRNA constructs of GLRaV-3 *CP*, *RdRp* and *HSP70h*, and 3'UTR will be stacked first; then, these constructs will be stacked with dsRNA constructs of *AQP1, SUC1* and *NUC1* from the grape mealybug. The GLRaV-3 dsRNA construct pGA482G-LR3p19.7-4 (against the viral silencing suppressor) will continue to be used but only for resistance against GLRaV-3.

To address objective #2 and develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug, we established a colony of grape mealybug on Pixie grape in insect-proof screen cages in a greenhouse with egg sacks and first instar crawlers collected in a vineyard in the Finger Lakes region of New York. Pixie is a dwarf grape accession that cycles rapidly through flowering and fruit maturation. It is derived from a chimera of *Vitis vinifera* cv. Pinot Meunier. Pixie has short internodes, which results in a small size vine (Figure 1). This dwarf growth habit creates a good microhabitat for grape mealybugs.

Figure 1. Potted Pixie grape in a greenhouse.

We used Pixie grapes to develop a high throughput transient expression system for the testing of the efficacy of dsRNA constructs against the grape mealybug. First, we used a dsRNA construct to the green fluorescent protein (*GFP*) gene as a proxy for the concatenate *AQP1, SUC1* and *NUC1* dsRNA construct against the grape mealybug. The *GFP* dsRNA (0.05 µg/µl in 200 µl solution) was administered to excised petioles of Pixie leaves that were placed in microfuge tubes (Figure 2). We predicted that the dsRNA construct taken up by the leaf transpiration stream will be translocated to other tissues, including the phloem sap, for ingestion by the mealybugs when feeding on the leaves.

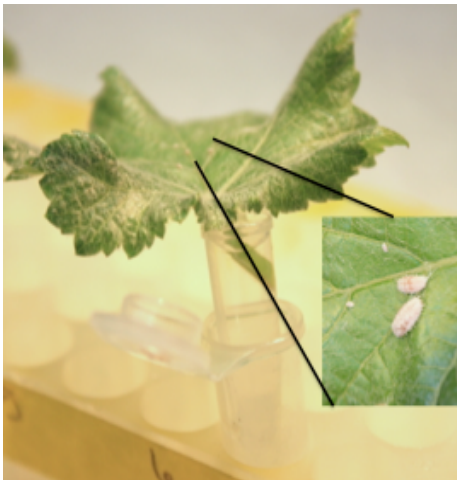

Figure 2. Close-up the detached Pixie leaf assay with mealybug adults feeding on secondary veins.

Monitoring the translocation of *GFP* dsRNA by Northern blot hybridization image showed an uptake of the contructs by excised Pixie leaves (Figure 3). This result validated the exclised leaf bioassay for uptake of dsRNA. Results were also consistent with the integrity of the *GFP* dsRNA detected in leaf tissue and some degradation, as expected, possibly due to the plant RNAi machinery since several DNA products of lower molecular mass than the 0.4kb full-length *GFP* dsRNA construct were detected (Figure 3). Northern blot hybridization experiments to determine whether the *GFP* dsRNA construct can be detected in grape mealybugs exposed to excised Pixie leaves soaked in a *GFP* dsRNA construct for 24-48 hours are underway. This analysis is critical to determine whether intact dsRNA construct is delivered to the insect and diced by the RNAi machinery of the insect to 21 nt siRNA, with minimal nonspecific degradation. Optimizing such conditions is vital prior to running separate experiments with the concatenate *AQP1, SUC1,* and *NUC1* dsRNA constructs to test its effect on the survival of grape mealybugs.

Next, we will quantify the impact of dsRNA treatments on survival of grape mealybugs. Separate experiments with different dsRNA constructs will be run sequentially. The efficacy of RNAi against the grape mealybug will be evaluated by (i) Assessing survival of insects, (ii) Testing insects for predicted reduction in the expression of osmoregulatory genes by RT-qPCR, normalized to grape mealybug housekeeping genes (ribosomal protein RPL7 and β-tubulin), (iii) Quantifying the osmotic pressure of insect hemolymph. Data on grape mealybug survival will be analyzed statistically to identify the most efficient dsRNA construct or dsRNA construct combination at impeding grape mealybug feeding or survival.

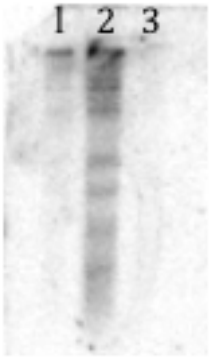

Figure 3. Northern blot hybridization of total RNA extracted from excised Pixie leaves for which the petiole was immersed into a *GFP* dsRNA solution for 24 hours (lane 2) or water (lane 3). *GFP* dsRNA in water was used as positive control (lane 1).

Grape mealybugs feeding on the excised Pixie leaves (with dsRNA constructs against osmoregulatory genes) are predicted to display reduced expression of these genes, elevated hemolymph osmotic pressure and depressed performance, relative to grape mealybugs on control plants (with *GFP* dsRNA). The experiments will be conducted over a time course (up to 6-10 days). By analogy with other insects, the reduced gene expression and elevated hemolymph osmotic pressure are predicted at least 1-2 days prior to onset of mortality, and likely within 24 h of initiation of feeding.

Analyses of bioassays using excised Pixie leaves to evaluate grape mealybug dsRNAs will inform the selection of the most promising constructs for stable transformation of grapevine, ensuring that we have the full set of controls: empty vector; ds*GFP*; and ds*NUC1*, to discriminate any negative effects of the transformation *per se*, production of a non-specific dsRNA, and suppression of the gut nuclease, respectively.

To address objective #3 and produce and characterize stably transformed grapevines with RNAi constructs against grapevine leafroll-associated virus 3, embryogenic cultures of rootstock genotypes 110R and 101-14 were used for stable transformation experiments with the dsRNA *p19.7* construct from GLRaV-3. Following transformation with *A. tumefaciens*, elongation of embryogenic cultures was observed with the highest efficacy obtained with 110R followed by 101-14. A few putative transformants of the rootstock genotype 110R were regenerated and transferred to soil in the greenhouse. Transgene insertion was characterized by PCR and Southern blot hybridization using total plant DNA isolated from leaves. Additional putative transgenic 110R and 101-14 rootstock plants were transferred from tissue culture to the greenhouse for characterization of transgene insertion and expression. Efforts to transform *Vitis vinifera* cvs. Cabernet franc and eventually Pinot noir will be pursued.

Additional efforts to produce stable grapevine transformants with GLRaV-3 dsRNA constructs will focus on stacked dsRNA constructs of 3'UTR, *CP*, *RdRp* and *HSP70h*. These dsRNA constructs are vital for combining resistance to the virus and the grape mealybug, as a dsRNA *p19.7* construct, which is coding for a silencing suppressor, would not be optimal for inclusion as

one of the stacked constructs. Efforts to pyramid 3'UTR, *CP*, *RdRp* and *HSP70h* are under way. Pyramided GLRaV-3 dsRNA constructs will be stacked with dsRNA *AQP1, SUC1* and *NUC* constructs as soon as the dsRNA constructs to the grape mealybug are validated in transient assays. Expression of these concatenated dsRNA constructs will be driven by the *SUC2* promoter to target phloem cells.

To address objective #4 and disseminate information to stakeholders through presentations at conventions and workshops, we presented research results at the 2018 CDFA Pierce's Disease Research Symposium in San Diego, CA. Research results will be further communicated to varied audiences, including farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at meetings in California and New York.

Publication produced and pending, and presentations made that relate to the funded project

Fuchs, M. 2018. Ecology of grapevine red blotch virus, and resistance to grapevine leafroll-associated virus 3 and the grape mealybug. California Department of Food and Agriculture Pierce's Disease Research Symposium, December 19-20, San Diego, CA.

Relevance statement, indicating how this project will contribute towards finding solutions to, or dealing with, the pest or disease being addressed

Leafroll is one of the most devastating and widespread viral diseases of grapevines. It reduces yield, delays fruit ripening, increases titratable acidity, lowers sugar content in fruit juices, modifies aromatic profiles of wines, and shortens the productive lifespan of vineyards (Almeida et al. 2013, Naidu et al. 2014). The economic cost of leafroll is estimated to range from $12,000 to $92,000 per acre in California (Ricketts et al. 2015) and from $10,000 to $16,000 in New York (Atallah et al. 2012).

Six major viruses named grapevine leafroll-associated viruses (GLRaVs), e.g. GLRaV-1, -2, -3, -4, -7, and -13, have been identified in diseased vines (Naidu et al. 2014, Naidu et al. 2015). Among these viruses GLRaV-3 is the dominant leafroll virus in vineyards, including in California (Maree et al. 2013, Naidu et al. 2014, Naidu et al. 2015). This virus is phloem-limited and semi-persistently transmitted by several species of mealybugs with acquisition and inoculation occurring within one-hour access period of feeding by immature mealybug stages (Almeida et al. 2013). There is no significant effect of host plant tissue on transmission efficiency; nor is there specificity of transmission (Almeida et al. 2013, Naidu et al. 2014), indicating that many mealybug species may disseminate all strains of GLRaV-3.

Mealybugs are sap-sucking insects in the family Pseudococcidae. They are pests of grapes and many other important crops. At high densities, mealybugs can cause complete crop loss, rejection of fruit loads at wineries, and death of spurs, although small infestations may not inflict significant direct damage. In the feeding process on plant sap, mealybugs excrete honeydew that often becomes covered with a black sooty mold, which additionally damages fruit clusters. Several mealybug species feed on vines but the grape mealybug is the most abundant and

widespread in US vineyards (Almeida et al. 2013). Unassisted, mealybugs have limited mobility but first instar immatures (crawlers) can be dispersed over long distances by wind and other means (Almeida et al. 2013).

In diseased vineyards, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations through the application of systemic insecticides, primarily spirotetramat. However, managing leafroll viruses and their mealybug vectors remains challenging due to several factors, including a lack of recognized host resistance (Oliver and Fuchs, 2011). Resistance can be achieved by applying RNA interference (RNAi) technologies. The approach relies on the development of RNAi constructs targeting specific pathogen or insect genes and their use to specifically down regulate their expression upon infection or feeding. The RNAi approach is highly specific and anticipated to reduce hazards of chemical pesticide applications. The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target virus and vector.

The goal of our research is to develop a robust RNAi-based strategy against GLRaV-3 and the grape mealybug. The basis for our approach is three-fold. First, mealybug survival depends on two gene functions localized to the gut that prevent osmotic collapse and dehydration of the insect, as it feeds on its sugar-rich diet of plant phloem sap. These genes are the water channel aquaporin *AQP1* and the sucrase-transglucosidase *SUC1* (Jing et al. 2016), with evidence that insect mortality is enhanced by co-targeting these two genes with different molecular function but related physiological role (Tzin et al. 2015). Second, these gene functions can be targeted by *in planta* RNAi, with evidence from related phloem feeding insects that RNAi efficacy is enhanced by stacking these RNAi constructs with RNAi against the gut nuclease (*NUC1*) (Luo et al. 2017). Third, RNAi is being successfully applied against viruses of fruit crops such as papaya and plum.

No source of resistance to GLRaV-3 or the grape mealybug is identified in cultivated or wild *Vitis* spp. Our research is exploring innovative methodologies to achieve resistance to GLRaV-3 and the grape mealybug via RNAi, a technology that has been successfully applied against viruses of fruit crops and phloem feeding insects. It is anticipated that (i) incorporating GLRaV-3 RNAi constructs into some of the major rootstocks and scion cultivars of interest to the California grape industry will confer resistance to leafroll disease and protect grafted vines from virus infection in the vineyard, and (ii) expressing RNAi constructs against key osmoregulatory genes expressed in the gut of the grape mealybug will suppress grape mealybug populations feeding on transgenic grapevines, and (iii) pyramiding RNAi constructs against GLRaV-3 and the grape mealybug will confer durable protection of grafted vines in vineyards.

Layperson summary of project accomplishments

Leafroll is one of the most devastating and widespread viral diseases of grapevines. It causes economic losses by reducing yield, delaying fruit ripening, increasing titratable acidity, lowering sugar content in fruit juices, modifying aromatic profiles of wines, and shortening the productive lifespan of vineyards. Among the viruses associated with leafroll disease, grapevine leafroll-

associated virus 3 (GLRaV-3) is dominant in vineyards. GLRaV-3 is phloem restricted and transmitted by several species of mealybugs, including the grape mealybug (*Pseudococcus maritimus*), which is its most abundant and widely distributed vector, and a pest of grapes. Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance. Our goal is to develop grapes with resistance against GLRaV-3 and the grape mealybug. To achieve this goal, we first established a colony of grape mealybug on grape in screen cages in a greenhouse. Using colony specimens, osmoregulatory genes that are expressed in the gut of the grape mealybug and are required for water balance and survival were identified. These grape mealybug osmoregulation genes were isolated and characterized. We hypothesize that interfereing with the expression of these genes will a genetic approach will have a detrimental effect on the performance of grape mealybug, thus, reduding their potential as vector of GLRaV-3. Similarly, another grape mealybug gene was isolated and characterized for use to enhance the efficacy of our methodology to achieve resistance. Genetic analyses of the three grape mealybug genes of interest validated the nature of the products obtained. In parallel, a bioassay based on detached grape leaves was developed to evaluate the efficacy of our resistance approach against the grape mealybug. Bioassays are under way. This assay is critical to identify the best performing gene or gene combination to achieve resistance. In addition, conserved genetic regions of GLRaV-3 were identified and characterized. We predict that interfering with the expression of these viral genomic regions will affect the viability of GLRaV-3, thus protecting grapevines from virus infection. One of these GLRaV-3 genes was transferred into cells of rootstock 110R for the production of grapevines to be characterized and tested for resistance to GLRaV-3.

Status of funds

Approximately 64% of the allocated budget has been expended at the time of the interim report. Funds were spent for salaries of key personnel (postdoctoral associate, and technicians) involved in the research, materials and supplies, greenhouse rent, and travel from labs to and from vineyards for grape mealybug collection.

Summary and status of intellectual property associated with the project

No intellectual property is associated with the project.

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