

California Department of Food and Agriculture - Interim Progress Report

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Title of Project: Resistance to Grapevine Leafroll-Associated Virus 3 and the Grape Mealybug

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Introduction

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, Fuchs et al. 2017).

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 transmissible 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 (GM) (*Pseudococcus maritimus*) 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 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 GM. 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 has been being successfully applied against viruses of fruit crops such as papaya (Gonsalves et al. 2008) and plum (Hily et al. 2004). The proposed research is to develop grapevines resistant to GLRaV-3 and the GM using RNAi by combining RNAi against targets of the virus and the insect vector, providing for greater efficacy in disease management and greater opportunities in impeding the development of virus and insect vector populations capable of overcoming the resistance.

Objectives

Specific objectives are to:

- Optimize RNAi constructs against GM
- Develop a high throughput transient expression system to test the efficacy of RNAi constructs
- Characterize stably transformed RNAi grapevines
- Disseminate information to stakeholders through presentations at conventions and workshops

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

To address objective #1 - Optimize RNAi constructs against GM -, a colony of grape mealybug was established on Pixie grape in screen cages in a greenhouse and in a growth chamber using 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 has short internodes, which results in a small size vine. This dwarf growth habit creates a good microhabitat for GMs. Pixie is derived from a chimera of *Vitis vinifera* cv. Pinot Meunier. Using colony specimens, the GM osmoregulation genes *AQP1* and *SUC1* were identified by RT-PCR with overlapping degenerate primer pairs designed in conserved regions of the genes of interest based on alignments of similar sequences of other hemipterans, and subsequently cloned in *Escherichia coli* plasmids. Sequence analysis of the cloned PCR amplicons validated the nature of the DNA products obtained from GMs. The cloned *AQP1* fragment is 490-bp in size and the cloned *SUC1* fragment is 394-bp in size.

For RNAi efficacy, it is important to use highly conserved dsRNA constructs. Therefore, the sequence identity of *AQP1* and *SUC1* of GM from populations in the Finger Lakes region of New York and populations from other geographic areas were determined. Specimens of grape mealybugs were collected in Long Island vineyards in New York and in Rogue Valley vineyards in Southern Oregon. These specimens were characterized by RT-PCR and sequencing to assess the relatedness of *AQP1* and *SUC1* in grape mealybugs from varied locals. Results showed a high nucleotide sequence identity. Similarly, for RNAi efficacy, it is critical to identify nonspecific nuclease (*NUC*) in GMs. Such nucleases have been shown to contribute to the degradation of dsRNA constructs in other hemipterans. Based on sequence alignments of *NUC* of other hemipterans, particularly the citrus mealybug, overlapping degenerate primer pairs were designed in highly conserved regions and used in RT-PCR with total RNA from the colony population. The cloned *NUC1* fragment from the grape mealybug is 877-bp in size. Sequence analysis of the cloned PCR amplicons validated the nature of the DNA products obtained.

The cloned *AQP1*, *SUC1* and *NUC* fragments from the GM were used to engineer dsRNA constructs. Stacked genes were favored for dsRNA constructs design to interfere with osmoregulatory genes. Since GMs are phloem feeders, the expression of stacked genes will be driven by a phloem-specific plant promoter such as *AtSUC2* (*A. thaliana* sucrose transporter 2).

To address objective #2 - Develop a high throughput transient expression system to test the efficacy of RNAi constructs -, efforts to optimize the delivery of dsRNA constructs to grape tissue were initiated. This is critical for the future development of RNAi transient bioassays to identify the most promising dsRNA constructs against the GM. Efforts included the monitoring of the behavior of GMs on tissue culture-grown grape plantlets, anticipating that transient assays will be carried out on this type of plant material. Crawlers were deposited on leaves and stems of tissue culture-grown plantlets, and observed over time (Figure 1). Unfortunately, this new habitat was not optimal for crawlers, as the majority did not survive the

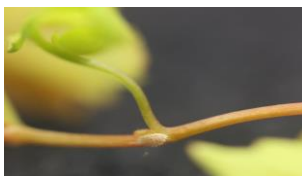


Figure 1. Grape mealybug crawlers on a stem of a tissue culture-grown grape plantlet.

transfer from Pixie grapes onto stems or leaves of tissue culture-grown grapevines, as shown by repeated counts within 2-3 weeks.

Since tissue culture grape material is suboptimal for transient assays with dsRNA constructs, the use of detached leaves of Pixie grapes was investigated. Young Pixie grape leaves were placed in microfuge tubes with half receiving 10% red food coloring and half received distilled water. Red pigment was visible in the veins within 1hr and more pigment continued to disperse in subsequent hours (Figure 2).



Figure 2. Absorption of red food coloring by detached leaves of Pixie grape. Left panel: A subset of leaves exposed to red food coloring (top) vs. distilled water (bottom). Middle and right panels: Close-up of primary, secondary and tertiary veins of leaves exposed to water (left) vs red food coloring (right). Pictures were taken 18h after exposure.

This initial work revealed that a food dye spreads from the stem of a detached grape leaf through out the leaf, particularly to its very small veins. This is encouraging for the future delivery of dsRNA constructs against the GM in transient assays. Additionally, GMs deposited on detached Pixie leaves showed a high survival rate (more than 80%) even after two weeks of exposure (Figure 3). Such conditions are anticipated to be well adapted to evaluate the effect of stacked *AQP1*, *SUC1* and *NUC1* dsRNA constructs against GMs in a transient assay.

To address objective #3 - Characterize stably transformed RNAi grapevines -, the genomic sequence of GLRaV-3 isolates available in public databases was analyzed. Conserved nucleotide regions within the open reading frame coding for protein p19.7, a well-characterized RNA silencing suppressor, and for the coat protein (CP) were identified. Mining efforts in other regions of the GLRaV-3 genome are under way with the goal of identifying other highly conserved segments. A set of overlapping primer pairs covering conserved regions of the p19.7 and CP open reading frames were designed and used in RT-PCR. Amplicons of the expected size were obtained, cloned in *Escherichia coli* plasmids and validated by sequencing. One inverted-repeat p19.7 construct was engineered and transferred into a plant expression vector for RNAi expression *in planta*. The correct insertion of the construct of interest was verified by restriction digestion and sequencing, and subsequently mobilized into *Agrobacterium tumefaciens* strain C58 for grape transformation. This construct was named pGA482G-LR3p19.7-4. Embryogenic calli of rootstock 110R (*V. rupestris* x *V.*

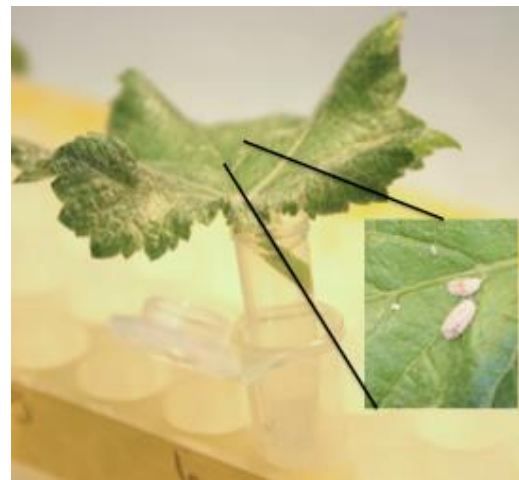


Figure 3. Close-up the detached Pixie leaf assay with mealybug adults feeding on secondary veins.

berlandieri) were exposed to recombinant agrobacteria for *A. tumefaciens*-mediated transformation and the production of transgenic grapevines expressing *p19.7*. Transformed calli are developing on kanamycin-selective medium. The development of other inverted-repeat constructs of *p19.7* and *CP* is on going. After thorough validation, this new set of dsRNA constructs will be used in new transformation experiments.

To address objective #4 - Disseminate information to stakeholders through presentations at conventions and workshops -, research results were communicated to varied audiences, including farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at winter school meetings in California and New York. Targeted venues and groups in 2017 were (i) Sustainable Ag Expo on November 13 in San Luis Obispo, CA (550 participants), (ii) Cornell Center for Technology Licensing, Innovations in Food systems on May 7 in Ithaca, NY (100 participants), (iii) Master Gardeners on August 15 in Geneva, NY (20 participants), (iv) USDA-FAS and Bosnian Cochran Fellows on June 20-21 in Geneva, NY (10 participants), and (v) Finger Lakes Forum on January 18 in Geneva, NY (60 participants).

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

Publications:

1. Fuchs, M. and Lemaire, O. 2017. Novel approaches for virus disease management. In: Grapevine Viruses: Molecular Biology, Diagnostics and Management. Meng, B., Martelli, G.P., Golino, D.A. and Fuchs, M.F (eds). Springer Verlag, pp. 599-621.

Presentations:

1. Fuchs, M. 2017. Viruses: Biology, ecology and management. Sustainable Ag Expo on Nov. 13, San Luis Obispo, CA (participants = 550).
2. Fuchs, M. 2017. Innovations and insights in plant breeding. Cornell Center for Technology Licensing, Innovations in Food systems: Feeding a growing world, May 7, Ithaca, NY (participants = 100).
3. Fuchs, M. 2016. Genetically modified organisms in agriculture. Master Gardeners, August 15, Geneva, NY (participants = 20, length in hours = 0.5, total contact hours = 10).
4. Fuchs, M. 2017. HoneySweet in New York. Bosnian Cochran Fellows – USDA-FAS, June 20-21, Geneva NY (participants = 10).
5. Fuchs, M. 2017. Genetically modified organisms. Finger Lakes Forum, January 18, Geneva, NY (participants = 60).

Research relevance statement, indicating how this research will contribute towards finding solutions to red blotch disease in California

No source of resistance to GLRaV-3 or the GM is identified in cultivated or wild *Vitis* spp. Our research is exploring innovative methodologies to achieve resistance to GLRaV-3 and GM 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 GM will suppress GM populations feeding on transgenic grapevines, and (iii) pyramiding RNAi constructs against GLRaV-3 and GM 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. To explore RNAi technologies for achieving resistance, we first established a colony of grape mealybug on Pixie grape in screen cages in a greenhouse and a growth chamber using egg sacks and first instar crawlers collected in a vineyard in New York. Using colony specimens, osmoregulatory genes that are expressed in the gut and are required for water balance and survival were identified. The grape mealybug osmoregulation genes *AQP1* and *SUC1* were characterized by RT-PCR with overlapping degenerate primer pairs designed in conserved regions of the genes of interest based on alignments of similar sequences of other hemipterans, and subsequently cloned. The cloned *AQP1* fragment is 490-bp in size and the cloned *SUC1* fragment is 394-bp in size. For RNAi efficacy, it is critical to use a RNAi construct against nonspecific nuclease (*NUC*) to prevent the degradation of dsRNA constructs. Based on sequence alignments of *NUC* of other hemipterans, overlapping degenerate primer pairs were designed in highly conserved regions and used in RT-PCR with total RNA from the colony population. The cloned *NUC* fragment is 877-bp in size. Sequence analysis of the cloned PCR amplicons validated the nature of the *AQP1*, *SUC1* and *NUC* products obtained. In parallel, a RNAi transient assay based on detached Pixie grape leaves was examined to evaluate the performance of dsRNA constructs against the grape mealybug. In addition, conserved nucleotide regions within the open reading frame coding for protein p19.7, a viral RNA silencing suppressor, and for the coat protein (CP) of GLRaV-3 were identified. A set of overlapping primer pairs covering conserved regions of the p19.7 and CP open reading frames were designed and used in RT-PCR. Amplicons of the expected size were obtained, cloned and validated by sequencing. One inverted-repeat p19.7 construct was engineered and transferred into embryogenic calli of rootstock 110R via *Agrobacterium tumefaciens*-mediated transformation for the production of transgenic grapevines.

Status of funds

Funds were spent for salaries of key personnel (postdoctoral associate, graduate student and technicians) involved in the research, materials and supplies, greenhouse rent, and travel to grower's meetings to present research progress.

Summary and status of intellectual property associated with the project

No intellectual property is associated with the project.

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