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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. Leafroll can affect *V. vinifera*, *V. labrusca*, interspecific hybrids and rootstocks (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, Ito and Nakaune 2016). These viruses belong to the genera *Ampelovirus* (GLRaV-1, -3, -4 and -13), *Closterovirus* (GLRaV-2), and *Velarivirus* (GLRaV-7) in the family *Closteroviridae*. GLRaV-1, -3 and -4 (and likely -13) are transmitted by mealybugs while no vector is known for GLRaV-2 and -7. GLRaVs are phloem-limited and GLRaV-3 is the dominant leafroll virus in vineyards, including in California (Naidu et al. 2014).

The genome of GLRaV-3 consists of 12 open reading frames (ORFs). It encodes a characteristic core of replication-associated genes, referred to as the replication gene block (RGB), at the 5' terminal portion of the genome and a more variable array of genes encoding structural and other proteins downstream of the RGB toward the 3' terminus (Naidu et al. 2015). The RGB proteins are expressed directly from the virion

RNA and other proteins are expressed from a nested set of the 3' co-terminal subgenomic RNAs. The last set of ORFs includes proteins involved in suppression of host RNA silencing, in particular p19.7 or p20B (Gouveia et al. 2012). Conserved regions in this ORF would be ideally targeted for RNAi-based control of GLRaV-3, as silencing the expression of p19.7 would enable the antiviral pathways of RNA silencing to be highly active against the virus. Distinct genetic variants of GLRaV-3 have been identified in diseased grapevines. They are referred to as genetic variant groups I to VI; they often exist in mixed infections although their biological significance is unknown (Maree et al. 2013, Naidu et al. 2015).

The transmission of GLRaV-3 by mealybugs is semi-persistent with acquisition and inoculation occurring within one-hour access period of feeding by immature stages (Almeida et al. 2013). A single mealybug is sufficient to transmit the virus and initiate infection (Naidu et al. 2014). 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 all mealybug species may disseminate all transmissible strains of GLRaV-1, -3 and -4. The attributes of the transmission process are critical to devise optimal RNAi strategies to control GM populations.

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

Current leafroll disease management options are essentially preventive and based on the use of planting material derived from clean, virus-tested certified stocks (Maliogka et al., 2014). In vineyards where infected vines are present, management strategies rely on the elimination of virus-infected vines and the reduction of mealybug populations to limit vector-mediated spread through the application of systemic insecticides, e.g. spirotetramat (Pietersen et al., 2013). The level of mealybug control needed to limit virus spread is not known, although encouraging results were recently reported with spirotetramat (Wallingford et al. 2015).

Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance (Oliver and Fuchs, 2011). Innovative technologies to breed resistant grapevine material are needed to complement current strategies and address their limitations. Resistance can be achieved by applying RNAi, a relatively new paradigm for crop protection from pathogens and arthropod pests. The approach relies on the development of RNAi constructs targeting specific pathogen or insect genes and their use to specifically down regulate the expression of the target genes in plants. There are a few examples of the successful application of RNAi against viruses, including in fruit crops such as papaya (Gonsalves et al. 2008) and plum (Hily et al. 2004), and the RNAi approach holds great promise against sap-sucking arthropods (Kumar et al. 2014, Pitino et al. 2011, Tzin et al. 2015, Zha et al. 2011).

The RNAi approach is highly specific and is anticipated to reduce hazards of chemical pesticides. The fact that mealybugs transmit leafroll viruses offers an opportunity to explore a two-pronged approach to simultaneously target virus and vector. The proposed research is to develop grapevines resistant to grapevine leafroll-associated virus 3 (GLRaV-3) and GM using RNAi. Our strategy is to combine 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.

List of Objectives

The specific objectives are to:

1. Optimize RNAi constructs against GM
2. Develop a high throughput transient expression system to test the efficacy of RNAi constructs
3. Characterize stably transformed RNAi grapevines
4. 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 by 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 grape mealybugs. Using colony specimens, the grape mealybug osmoregulation genes aquaporin (*AQP1*) and sucrase (*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 grape mealybugs. The cloned *AQP1* fragment is 490-bp in size and the cloned *SUC1* fragment is 394-bp in size. The sequences of *AQP1* and *SUC1* expressed in the gut will be used to design RNAi constructs.

To further the efficacy of RNAi, a non-specific nuclease was identified by RT-PCR with overlapping degenerate primers designed from highly conserved region of *NUC* sequences of other hemipterans, particularly the citrus mealybug, and total RNA from the GM colony population. The cloned *NUC* fragment from GM is 877-bp in size. Sequence analysis of the cloned PCR amplicons validated the nature of the DNA products obtained. It has been shown that non-specific nucleases act as RNAi suppressors in many insects, including phloem-feeders, for which *NUC* is expressed in the gut and functions to degrade ingested dsRNA. Therefore, the *NUC* sequence will be applied to design dsRNA constructs against *NUC*, which will be stacked with RNAi against *AQP1* and *SUC1*.

The cloned *AQP1, SUC1* and *NUC* fragments from GM were used to engineer dsRNA constructs. Stacked genes were favored for dsRNA constructs design to interfere with osmoregulatory genes. Since GM 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). This will enhance the efficacy of RNAi against GM by co-targeting two molecular functions with linked physiological function (protection against dehydration) and a RNAi suppressor.

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 explored.

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

First, we monitored the behavior of GM 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 transfer from Pixie grapes to tissue culture-grown plantles, as shown by repeated counts within 2-3 weeks.

Since tissue culture grape material is suboptimal for transient assays with dsRNA constructs, we investigated the potential of detached leaves of Pixie grapes. 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**).

Fig. 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 throughout the leaf, particularly to its very small veins. This is encouraging for the future delivery of dsRNA constructs against the grape mealybug in transient assays. Additionally, grape mealybugs deposited on detached Pixie leaves showed a high survival rate (more than 80%) even after two weeks of exposure (**Figure 2**). Such conditions are anticipated to be well adapted to evaluate the effect of stacked *AQP1, SUC1* and *NUC* dsRNA constructs against grape mealybugs in a transient assay.

Experiments were initiated to monitor the uptake of dsRNA through detached leaves of Pixie, as illustrated (**Figure 3**). This work was relying on the use of dsRNA designed against the green fluorescent protein (GFP) gene. A solution of dsRNA-GFP was exposed to the petiole of a detached leaves of Pixie. Uptake and transfer of GFP-dsRNA was verified by Northern blot hybridization using a ^{32}P-labeled GFP probe and total RNA isolated from detached leaves of Pixie. Results documented uptake and accumulation of dsRNA in detached Pixie leaves, as shown by Northern blot hybridization. This work

validated our experimental setting and demonstrated that a detached Pixie leaf immersed into a solution of dsRNA can uptake and accumulate dsRNA. This work is critical to validate stacked *AQP1, SUC1* and *NUC* dsRNA constructs.

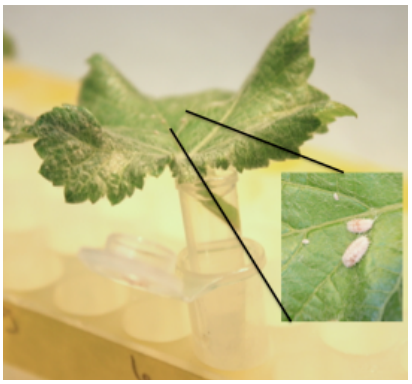

Fig. 3. Detached Pixie leaf assay with feeding mealybug adults.

Subsequently, the processing of GFP-dsRNA by the RNAi machinery in detached Pixie leaves was assessed by characterizing the accumulation of siRNA. Results revealed the processing of GFP-dsRNA into GFP-siRNA, as shown by Northern blot hybridization using total plant RNA enriched in small RNAs and a ^{32}P-labeled GFP probe. Based on these preliminary findings, similar work will soon be carried out with stacked *AQP1, SUC1* and *NUC* dsRNA constructs. Anticipating these bioassays, GM crawlers and eggs were collected in local vineyards to expand the existing GM colony on Pixie grapes. This work is critical for future bioassays to determine the anti-GM activities of dsRNA constructs against *AQP1, SUC1* and *NUC* using detached leaves of Pixie, as it is anticipated that this research will require numerous GM at varied development stage.

<u>*To address objective #3*</u> - Characterize stably transformed RNAi grapevines -, plantlets following *Agrobacterium tumefaciens*-mediated transformation with pGA482G-LR3p19.7-4, a plasmid containing an inverted-repeat p19.7 construct from GLRaV-3, were transferred from a growth chamber to soil in the greenhouse. DNA was extracted from actively growing putative transgenic plants plantlets to characterize transgene insertion by PCR using appropriate primers. Results showed an DNA amplicon of the expected size in some of the putative transgenic plants. Additional confirmatory work will be done by Northern blot hybridization.

As one of the major objectives of the project is to engineer dsRNA constructs against GM and GLRaV-3 and use them to achieve resistance via RNAi, the use of p19.7, the RNA silencing suppressor of GLRaV-3, is not suitable. This is because RNAi is desired for resistance to both GM and GLRaV-3, and silencing the silencing suppressor would be counterproductive. For this reason, the genomic sequence of GLRaV-3 isolates that is available in public databases was mined to identified conserved nucleotide regions outside of the p19.7 open reading frame. Regions of interest were identified in the open reading frames encoding the coat protein (*CP*) and RNA-dependent RNA polymerase (*RdRp*). These conserved regions will be used as template to engineer dsRNA constructs by PCR for future *in planta* transformation experiments. Other conserved GLRaV-3 genomic regions will be investigated. Following validation in bioassays, stacked *AQP1, SUC1* and *NUC* dsRNA constructs will be fused with stacked GLRaV-3 *CP* and *RdRp* dsRNA constructs with the goal of achieving resistance to GM and GLRaV-3.

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. A total of 740 participants were exposed to the project and the concept of RNAi for resistance to GM and GLRaV-3. Targeted venues and groups in 2017 were:

- Sustainable Ag Expo on November 13, 2017 in San Luis Obispo, CA (550 participants)
- Cornell Center for Technology Licensing, Innovations in Food Systems on May 7, 2017 in Ithaca, NY (100 participants)
- Master Gardeners on August 15, 2017 in Geneva, NY (20 participants)
- USDA-FAS and Bosnian Cochran Fellows on June 20-21, 2016 in Geneva, NY (10 participants)

- Finger Lakes Forum on January 18, 2016 in Geneva, NY (60 participants)

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

Publications

Douglas, A. 2017. Strategies for enhanced crop resistance to insect pests. Annual Review of Plant Biology. 69:637-660.

Fuchs, M. 2017. Grapevine Viruses: Molecular Biology, Diagnostics and Management. Meng, B., Martelli, G.P., Golino, D.A. and Fuchs, M.F (eds). Springer Verlag, Cham, Switzerland, pp. 1-691.

Presentations

- Sustainable Ag Expo in San Luis Obispo, CA
- Cornell Center for Technology Licensing, Innovations in Food Systems in Ithaca, NY
- Master Gardeners in Geneva, NY
- USDA-FAS and Bosnian Cochran Fellows in Geneva, NY
- Finger Lakes Forum in Geneva, NY

Research relevance statement, indication how this research will contribute towards finding solutions to the pest and disease being studied

A colony of grape mealybug was established on Pixie grapes. Using colony specimens, osmoregulatory genes *AQP1* and *SUC1* that are expressed in the gut and are required for water balance and survival were identified and characterized by RT-PCR with total RNA extracted from grape mealybugs. A nonspecific nuclease gene (*NUC*) was similarly obtained by RT-PCR. Sequence analysis of the cloned PCR amplicons validated the nature of the *AQP1, SUC1* and *NUC* products obtained. In parallel, a transient assay using detached Pixie grape leaves was developed to evaluate the performed of dsRNA constructs against the grape mealybug. Preliminary results suggested that uptake, accumulation and processing of dsRNA constructs is occurring in detached grape leaves. This foundational work is setting the conditions for the assessment of the anti-GM effect of stacked *AQP1, SUC1* and *NUC* dsRNA constructs. 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) and RNA-dependent RNA polymerase of GLRaV-3 were identified and characterized by RT-PCR and sequencing. Some transgenic plants of rootstock 110R containing an inverted-repeat p19.7 construct were obtained, paving the way for future transformation experiments with stacked dsRNA constructs.

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. We are exploring RNAi technologies for achieving resistance. A a colony of grape mealybug was established 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 transient RNAi assay based on detached Pixie grape leaves was used to determine the uptake, accumulation and processing of dsRNA. This system will be further used to assessed the effect of stacked *AQP1, SUC1* and *NUC* dsRNA constructs against GM. 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) and RNA-dependent RNA polymerase (RdRp) of GLRaV-3 were identified. A set of overlapping primer pairs covering conserved regions of the p19.7, CP and RdRP 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 plants that were established in the greenhouse.

Status of funds

Funds were spent for salaries of key personnel (postdoctoral associates and technicians) involved in the research, supplies and greenhouse rent, travel from labs to and from vineyards for sample collection, and travel to grower's meetings and other venues to present research progress.

Summary and status of intellectual property associated with the project

No intellectual property is associated with the project.

Literature cited

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