RESISTANCE TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 AND THE GRAPE MEALYBUG

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ABSTRACT
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. This virus is 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. Therefore, we are exploring RNA interference (RNAi), a technology that has been successfully applied against viruses of fruit crops and phloem feeding insects, to achieve resistance against GLRaV-3 and the grape mealybug. To apply RNAi against the grape mealybug, the osmoregulation genes *AQP1* and *SUC1*, as well as a nonspecific nuclease (*NUC*), were characterized by reverse transcription (RT)-polymerase chain reaction (PCR) using total RNA from specimens from a colony maintained in the greenhouse with overlapping degenerate primer pairs designed in conserved regions of the genes of interest based on alignments of similar sequences of other hemipterans. The cloned *AQP1, SUC1,* and *NUC* fragments are 490-, 394-, and 877-bp in size, respectively. Sequence analysis of the cloned PCR amplicons validated the nature of the *AQP1, SUC1* and *NUC* products obtained. To evaluate the performance of dsRNA constructs against the grape mealybug, a transient assay based on artificial diet was developed. This bioassay was selected because a detached grape leaf assay proved suboptimal to test the effect of dsRNA constructs on the survival of grape mealybugs. Preliminary bioassay results revealed a 20% reduction in the survival of *P. maritimus* nymphs on an artificial diet containing dsRNA constructs to *AQP1, SUC1* and *NUC* relative to a control diet. This effect was significant (p=0.0436). Additional work is underway to verify these very encouraging results. In parallel, RNAi against GLRaV-3 focused on conserved nucleotide regions within the open reading frame coding for protein p19.7 (*p19.7*), a viral RNA silencing suppressor, the coat protein (*CP*), the RNA-dependent RNA polymerase (*RdRp*) and the heat shock 70 homolog (*HSP70h*) of GLRaV-3. Sets of overlapping primer pairs covering conserved regions of *p19.7*, *CP*, *RdRp* and *HSP70* 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. Next, pyramided GLRaV-3 dsRNA constructs will be stacked with grape mealybug dsRNA constructs. It is anticipated that a pyramided approach for the simultaneous engineering of resistance against GLRaV-3 and the grape mealybug will protect grapevines against the major virus of leafroll disease and its widely distributed insect vector.

LAYPERSON SUMMARY
Leafroll disease affects yield, fruit ripening and aromatic profiles of wines. Grapevine leafroll-associated virus 3 (GLRaV-3) is the predominant virus associated with leafroll disease in vineyards. This virus is transmitted by several species of mealybugs, including the grape mealybug, which is its most abundant and widely distributed

vector, as well as, a pest of grapes. Management of leafroll viruses and their mealybug vectors is challenging due to a lack of recognized host resistance. We explore RNA interference (RNAi) technologies to achieve resistance against GLRaV-3 and the grape mealybug by simultaneously interfering with the expression of key genes of the virus and its major vector. For RNAi against the grape mealybug, our targets are osmoregulatory genes that are expressed in the gut and required for water balance and survival. Two osmoregulation genes from the grape mealybug, as well as another gene that is essential for RNAi efficacy, were isolated and characterized. In parallel, a transient assay based on artificial diet was developed, as a detached grape leaf assay proved sub-optimal. Preliminary results revealed a 20% reduction in the survival of *P. maritimus* nymphs on an artificial diet containing dsRNA constructs to *AQP1, SUC1* and *NUC* relative to a control diet. This effect was significant ($p=0.0436$). For RNAi against the virus, conserved nucleotide sequence regions within four coding viral regions were identified and characterized. Among these four regions, an inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *Agrobacterium tumefaciens*-mediated transformation. Next, GLRaV-3 and grape mealybug dsRNA constructs will be stacked since we anticipate that combining resistance against GLRaV-3 and the grape mealybug will protect grapevines against the major virus of leafroll disease and its widely distributed insect vector.

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 (Ito and Nakaune, 2016; 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 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 (Pietersen et al., 2013). 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 double stranded (ds) RNA 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 (Fuchs, 2017).

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). Perturbing the expression of osmoregulatory genes required for water balance, specifically *AQP1* and *SUC1*, in the gut of phloem-feeding insects causes the insects to lose water from the body fluids and dehydrate, dying within 2-3 days (Karley et al. 2005, Shakesby et al. 2009, Tzin et al. 2015). Second, the functions of *AQP1* and *SUC1* 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 grape mealybug using RNAi by pyramiding dsRNA constructs against several 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

Our specific objectives are to:

1. Optimize RNAi constructs against the grape mealybug
2. Develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug
3. Characterize stably transformed RNAi grapevines
4. Disseminate information to stakeholders through presentations at conventions and workshops

RESULTS AND DISCUSSION

To address objective #1 and optimize RNAi constructs against the grape mealybug, *AQP1* and *SUC1* have been characterized by RT-PCR using total RNAs isolated from crawlers of a grape mealybug colony maintained on Pixie grapes in the greenhouse and overlapping primers. 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* were used to design dsRNA constructs which were cloned in a binary plasmid for expression *in planta*.

To enhance the efficacy of RNAi against the grape mealybug, dsRNA constructs against the osmoregulation genes *AQP1* and *SUC1* were stacked. Additionally, we identified *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) by reverse transcription (RT)-polymerase chain reaction (PCR) using overlapping primers and total RNA from crawlers. A dsRNA *NUC1* construct should protect dsRNA against degradation and dramatically increase insect mortality by stacking dsRNA against the osmoregulation genes with dsRNA against the nuclease, as recently documented (Luo et al. 2017). The *NUC1* dsRNA construct was stacked with dsRNA constructs to *AQP1* and *SUC1*. The feasibility of a gene stacking approach is assured by our previous research, in which up to five dsRNA constructs for *in planta* delivery were used with no effect on plant growth or development but with high mortality of psyllid and whitefly pests (Luo et al. 2017, Tzin et al. 2015).

For GLRaV-3, dsRNA constructs to the suppressor of RNA silencing *p19.7* and the coat protein (*CP*) open reading frame were engineered. Additional dsRNA constructs from conserved regions of the viral genome were developed by analysis of aligned virus nucleotide sequences available in GenBank and identification of short stretches of conserved regions. Emphasis was placed 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*. We retrieved full-length GRLaV-3 genome sequences available in GenBank and analyzed them to identify highly conserved nucleotide sequence regions. Search outputs revealed conserved nucleotide stretches of 100-300 nucleotides in size for *CP*, *RdRp* and *HSP70h*. Individual conserved regions were amplified by RT-PCR using specific primers and total RNA from GLRaV 3-infected grapevines as template. The integrity of these constructs was verified by restriction digestions and sequencing. Each of these fragments was cloned into the plasmid pEPT8 - a plasmid derived from pUC19 that contains the cauliflower mosaic virus 35S promoter sequence and nopaline synthase terminator sequence - and subsequently into binary plasmid pGA482G for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. DsRNA constructs to GLRaV-3 *RdRp* and *HSP70h* complete the *CP* and *p19.7* dsRNA constructs previously engineered.

Anticipating the engineering of stacked dsRNA constructs to the grape mealybug and GLRaV-3 for combined resistance to the virus and its most abundant vector, targeting the viral silencing suppressor *p19.7* (Gouveia et al. 2012) is not optimal. This is because RNAi should be fully effective and no silencing suppressor should be used for maximal efficacy. Therefore, dsRNA constructs of GLRaV-3 *CP*, *RdRp* and *HSP70h* will be stacked first; and these constructs will then 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 *p19.7*) will continue to be used but only for resistance against GLRaV-3. Expression of pyramided dsRNA constructs to *AQP1, SUC1*, NUC, *CP*, *RdRp*, and *HSP70* will be driven by the phloem-specific promoter s*ucrose-H+ symporter* (*SUC2)* to target RNAi expression to the grape phloem, the preferred feeding sites of the grape mealybug, and preferred localization tissue of GLRaV-3 in grape.

To address objective #2 and develop a high throughput transient expression system to test the efficacy of RNAi constructs against the grape mealybug, optimizing the delivery of dsRNA constructs to grape tissue was an initial priority. This work is critical for the future development of RNAi transient bioassays to identify the most promising dsRNA constructs against the grape mealybug. Efforts included the monitoring of the behavior of the grape mealybug on tissue culture-grown grape plantlets, anticipating that transient assays will be carried out on this type of plant material, perhaps via vacuum-assisted infiltration (Yepes et al. 2018). Crawlers were deposited on leaves and stems of tissue culture-grown *Vitis vinifera* or rootstock plantlets and observed over time (Figure 1). Unfortunately, this new habitat was not optimal for crawlers, as the majority of specimens did not survive the transfer from Pixie grapes onto stems or leaves of tissue culture-grown grapevines, regardless of the nature of the plant material, i.e., *Vitis vinifera* cultivars or rootstock genotype, as shown by repeated counts within 2-3 weeks.

Fig. 1. Grape mealybug crawlers on a stem of a tissue culture-grown *V. vinifera* cv. Syrah grape plantlet.

Since tissue culture grape material was shown to be suboptimal for transient assays with dsRNA constructs based on the behavior of the grape mealybug, the use of detached leaves of Pixie grapes was investigated. Pixie is a natural dwarf grapevine derived from the periclinal chimera of *Vitis vinifera* cv. Pinot Meunier. It has short internodes and is a preferred host of the grape mealybug. To test the feasibility of a detached leaf assay, we excised young Pixie leaves and placed them in microfuge tubes containing distilled water or a red food dye (10%). Red pigmentation was visible in the veins of Pixie leaves within 1 hour and more pigment continued to disperse is subsequent hours (Figure 2). This initial work revealed that a food dye spreads from the stem of a detached Pixie grape petiole throughout the leaf, particularly to its very small veins. This result was very encouraging for the delivery of dsRNA constructs against the grape mealybug in transient assays.

Fig. 2. Absorption of red food coloring by detached leaves of Pixie grape. Left panel: A subset of leaves are 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) versus red food coloring (right). Pictures were taken 18h after exposure.

Next grape mealybugs from a colony maintained on potted Pixie vines in the greenhouse were deposited on detached Pixie leaves to evaluate their behavior on this new habitat. A high survival rate (more than 80%) of grape mealybugs was consistently obtained in replicated assays even after two weeks of exposure (Figure 3).

Fig. 3. Close-up the excised a Pixie leaf with its petiole immersed in water and mealybug adults feeding on secondary veins.

Such conditions are anticipated to be well adapted to evaluate the effect of dsRNA constructs against the grape mealybug in a transient assay based on excised Pixie leaves. The next step was to determine if a dsRNA construct can be administered to an excised petiole of a Pixie leaf. We used a dsRNA construct to the green fluorescent protein (GFP) as a proxy for dsRNA constructs to the grape mealybug. First, we tested the stability of the GFP dsRNA construct in water over time. No degradation was observed for the GFP dsRNA construct over the course of the experiment (0 to 24 hrs), as shown by electrophoresis on an agarose gel (Figure 4).

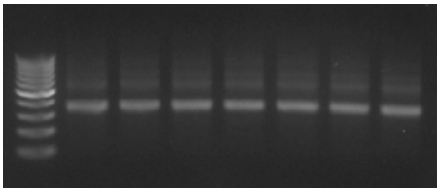

Fig. 4. Analysis of the stability of a GFP dsRNA construct kept in water after 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 6 (lane 6), 12 (lane 7) and 24 (lane 8) hours by electrophoresis on an agarose gel.

Then the GFP dsRNA construct (0.05 µg/µl in 200 µl solution) was added to the microfuge tubes contained excised Pixie leaves and its presence was tested by Northern blot hybridization in tissue collected from Pixie leaves at 24 hours post-soaking using a specific ^{32}P-labled probe (Figure 5). Analysis of the Northern blot image showed an uptake of the GFP dsRNA construct by excised Pixie leaves. Results were also consistent with the integrity of the GFP dsRNA detected in leaf tissue and some degradation 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.

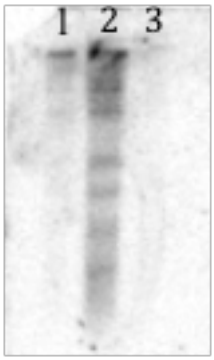

Fig. 5. Northern blot hybridization of total RNA extracted from excised Pixie leaves for which the petiole was immersed into a GFP dsRNA solution for 24 hours (lane 2) or water (lane 3). Lane 1 is the GFP dsRNA construct in water as positive control.

Based on these encouraging preliminary results, we initiated 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. This analysis is critical to determine whether the 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 dsRNA constructs and testing their effect on the survival of grape mealybugs. Unfortunately, the detached grape leaf assay provided inconsistent data in terms of the efficiency of the dsRNA construct uptake in Pixie leaves, and subsequently in mealybugs. Therefore, this assay was abandoned and the use of a diet to be supplemented with dsRNA constructs of interest was investigated. First, we determined if adults and nymphs that were reared on Pixie grapes could survive on an artificial diet. The artificial diet used in this study was similar to the one that is routinely used for work with other hemipterans. The objective was to see if this type of food source could sustain grape mealybugs for a few days. Results should that at least 70% of the specimens survived for 3-5 days when exposed to the artificial diet. These conditions were deemed appropriate for the testing of the effect of dsRNA constructs on the survival of grape mealybugs.

Experiments with dsRNA constructs focused on nymphs as they are the most efficient stage for GLRaV-3 transmission (Almeida et al., 2013). Approximately twenty 1-10-days-old *Pseudococcus maritimus* mealybug nymphs were exposed to the artificial diet and their survival was measured at three days post-exposure. Mealybugs were fed first an artificial diet (without any dsRNA construct) or the same diet supplemented with the dsRNA construct against *NUC* at 0.2 µg/µl. Then, mealybugs were allowed to feed for 72 hours on the control diet or the same diet supplemented with various dsRNA constructs. Experiments were triplicated. Results showed a 20% reduction of the survival of grape mealybugs exposed to a diet supplemented with dsRNA

constructs against *AQP1* (0.1 µg/µl), *SUC1* (0.1 µg/µl), and *NUC* (0.3 µg/µl) relative to control diets, i.e. diet without any supplements and diet supplemented with a dsRNA construct against *GFP* (0.4 µg/µl) (Figure 6). This reduction was significant (p=0.0436). The survival of nymphs on a diet supplemented with dsRNA constructs against *AQP1* (0.2 µg/µl), and *SUC1* (0.2 µg/µl) was also significantly reduced relative to the two control diets (p=0.0824) (Figure 6). As expected, the effect of dsRNA constructs against *AQP1*, *SUC1* and *NUC* on mealybug mortality was more pronounced than the dsRNA constructs against *AQP1* and *SUC1*. Similarly, as expected, the survival of nymphs exposed to a diet supplemented with *NUC* (0.2 µg/µl) and GFP (0.2 µg/µl) was identical to that of nymphs on the control diets (p=0.3184) (Figure 6). These results are very encouraging. New experiments to verify these trends are underway.

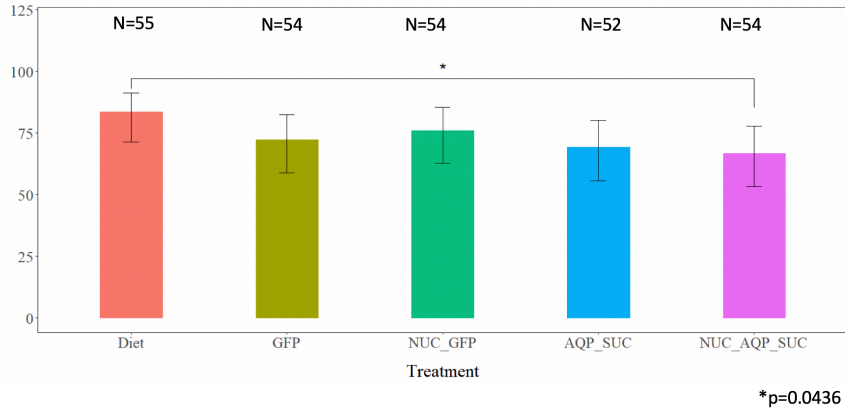

Fig. 6. Survival of *Pseudococcus maritimus* nymphs at three days-post exposure to an artificial diet supplemented with various dsRNA constructs.

To address objective #3 and characterize stably transformed RNAi grapevines, an inverted-repeat p19.7 construct was engineered and used for the production of transgenic grapevines via *Agrobacterium tumefaciens*-mediated transformation. Embryogenic cultures of rootstock genotypes 110R and 101-14 were used for stable transformation experiments. Following transformation with *A. tumefaciens*, elongation of embryogenic cultures was observed with the highest efficacy obtained with 110R followed by 101-14. A few plants of the rootstock genotypes 110R that were subjected to transformation experiments were regenerated and micropropagated in tissue culture. Some putative transgenic plantlets were transferred to soil in the greenhouse by removing them from test tubes or polylethylene tissue culture bags using forceps, rinsing roots in water and trimming roots to about one third in length to stimulate growth prior to transfer to Cornell mix in individual plastic pots. Plants were covered with plastic bags to avoid dehydration. Plastic bags were gradually opened following active growth in the greenhouse. Transgene insertion will be characterized by PCR and Southern blot hybridization using total plant DNA isolated from leaves of actively-growing putative transgenic plants. In the near future, RT-PCR and Northern blot hybridization will be carried out to confirm transgene expression and the accumulation of siRNA, respectively. 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 engineer stable grapevine transformants with other GLRaV-3 dsRNA constructs will focus on stacked dsRNA constructs of *CP*, *RdRp* and *HSP70h*. These dsRNA constructs are vital for combining resistance to the virus and the grape mealybug, as the dsRNA *p19.7* construct, which is coding for a silencing suppressor, would not be optimal for inclusion as one of the stacked constructs. Next, pyramided GLRaV-3 dsRNA constructs will be stacked with dsRNA *AQP1, SUC1* and *NUC* constructs since bioassays validated the RNAi approach against the grape mealybug in transient assays. In parallel, we initiated efforts to transform *Vitis vinifera* cvs. Cabernet franc and eventually Pinot noir.

To address objective #4 and disseminate information to the industry, research results were communicated to 380 growers, vineyard managers, vintners, farm advisors, extension educators, crop consultants, researchers, and regulators in California, North Carolina and Ontario, Canada at the following meetings:

- Fuchs, M. 2019. Biology of grapevine viruses. Mealybug and virus outreach meeting, April 4, Stockton, CA (participants = 250).
- Fuchs, M. 2019. Impact of leafroll and red blotch diseases. Vinedresser meeting, March 28, Dobson, NC (participants = 20).
- Cieniewicz, E. and Fuchs, M. 2018. Virus diseases: Why should I care and what can I do? California State University - Fresno, October 3, Jordan College of Agriculture Sciences and technology, Department of Viticulture and Enology, Fresno, CA (participants = 30).
- Fuchs, M. 2018. Grape virus research updates. Biennial Grape Research Tailgate Tour, August 30, Niagara-on-the-Lake, Ontario, Canada (participants = 80).

These presentations provided opportunities to communicate on research progress and discuss the future of RNAi technology for leafroll and mealybug management.

CONCLUSIONS

Leafroll is one of the most devastating and widespread viral diseases of grapevines. Grapevine leafroll-associated virus 3 (GLRaV-3) is the dominant virus in leafroll diseased vineyards. This virus is transmitted by several species of mealybugs, including the grape mealybug (*Pseudococcus maritimus*), which is its most abundant and widely distributed vector in vineyards, and a pest of grapes. We are exploring RNAi to protect grapevines against GLRaV-3 and the grape mealybug. For RNAi to GLRaV-3, conserved nucleotide sequence regions of *p19.7*, *CP*, *RdRp* and *HSP70* were used to engineer dsRNA constructs. Putative transgenic plants of the rootstock genotype 110R were obtained following *Agrobacterium tumefaciens*-mediated transformation with a dsRNA *p19.7* construct and established in the greenhouse. Transgene insertion was confirmed in a few transgenic 110R plants. For the grape mealybug, key osmoregulatory genes *AQP1* and *SUC1* and the nonspecific nuclease *NUC* were obtained from crawlers of a grape mealybug colony established on Pixie grapes in the greenhouse. A bioassay based on artificial diet was developed. This assay revealed a 20% reduction in the survival of *P. maritimus* nymphs that fed on an artificial diet containing dsRNA constructs to *AQP1, SUC1* and *NUC* relative to a control diet. This effect was significant ($p=0.0436$). These results are very encouraging. Therefore, we will pyramid dsRNA constructs against several targets of the virus and the insect vector, anticipating a greater efficacy in disease management and greater opportunities in impeding the development of virus and insect vector populations capable of overcoming the resistance.

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