Final Report for CDFA Agreement Number 16-0612-SA

RESISTANCE TO GRAPEVINE FANLEAF VIRUS IN ROOTSTOCKS

Principal Investigator:	**Collaborator:**
Marc Fuchs	Deborah Golino
Plant Pathology and Plant-Microbe Biology	Foundation Plant Services
Cornell University	University of California
Geneva, NY 14456	Davis, CA 95616
mf13@cornell.edu	dagolino@ucdavis.edu

Reporting Period: The results reported here are from work conducted from July 1, 2016 to June 30, 2018

ABSTRACT

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration, a detrimental disease of grapevines. Management of fanleaf currently relies on the use of rootstocks with resistance to *Xiphinema index*, the dagger nematode vector of GFLV, and eventually soil disinfection. No source of resistance to GFLV has been identified in wild or cultivated *Vitis* species. Therefore, we are exploring RNA interference (RNAi) to confer resistance to GFLV in rootstocks. Several RNAi constructs were designed in conserved regions of the two GFLV genomic RNAs, including from the recently recognized viral suppressor of silencing, and tested their anti-GFLV potential in transient assays with *Nicotiana benthamiana*, a systemic herbaceous host. A few promising RNAi constructs were identified and transferred into embryogenic calli of grapevine rootstock genotypes 101-14 MGT, 110R, 3309C and 5C via *Agrobacterium tumefaciens*-mediated transformation. Putative transgenic plants of 101-14 MGT and 3309C were obtained and transferred to soil in the greenhouse. The integration and expression of RNAi constructs in transgenic rootstocks was confirmed by PCR, Northern blot hybridization and reverse transcription-PCR, respectively. Screening these rootstocks clones for resistant to GFLV will be the next step. Information on research progress was disseminated to stakeholders through presentations at conventions and workshops.

LAYPERSON SUMMARY

Grapevine fanleaf virus (GFLV) is one of the most devastating viruses of grapevines worldwide. The virus is transmitted by the dagger nematode *Xiphinema index* and is primarily managed in diseased vineyards through the use of rootstocks that are resistant to *X. index*. Such rootstocks delay the debilitating effect of GFLV on vine health and production but do not prevent GFLV infection. Since no source of resistance to GFLV is known in wild or cultivated *Vitis* species, we are exploiting the anti-viral pathways of RNA interference (RNAi), an innate plant defense system, to confer resistance to GFLV infection in grapevine rootstocks. Several RNAi constructs derived from different conserved regions of the GFLV genome, including a recently recognized viral suppressor of RNAi, were identified and engineered for expression *in planta*. These constructs were concatenated to substantially reduce the probability that genetically diverse GFLV variants from vineyard populations will defeat the resistance. These constructs were used in transformation experiments of rootstocks and putative transgenic 101-14 and 3309C plants were established in the greenhouse. The insertion and expression of RNAi constructs were verified in transgenic plants. Research progress on the development of fanleaf-resistant rootstocks was communicated to grape growers, farm advisors and service providers at varied venues.

INTRODUCTION

Fanleaf is one of the most devastating viral diseases of grapevines (Andret-Link et al. 2004). It causes serious economic losses by reducing vigor and yield, altering fruit juice chemistries, shortening the productive life of vineyards or causing vine death. The causal agent, grapevine fanleaf virus (GFLV), is specifically transmitted from vine to vine by the soil-borne, ectoparasitic dagger nematode, *Xiphinema index* (Andret-Link et al. 2004, Fuchs et al. 2017).

GFLV belongs to the genus *Nepovirus* in the family *Secoviridae*. It has a bipartite, positive-sense single-stranded RNA genome. The two genomic RNAs are expressed as a polyprotein that is cleaved into individual proteins at specific proteolytic cleavage sites. RNA1 (7,342 nts) codes for five proteins: 1A (unknown function), $1B^{Hel}$ (putative helicase), $1C^{VPg}$ (viral protein genome-linked), $1D^{Pro}$ (proteinase) and $1E^{Pol}$ (putative RNA-dependent RNA polymerase). These proteins are involved in proteolytic processing and replication (Andret-Link et al. 2004, Fuchs et al. 2017). RNA2 (3,774 nts) codes for three proteins: $2A^{HP}$ (homing protein), $2B^{MP}$ (movement protein) and $2C^{CP}$ (coat protein) that are involved in RNA2 replication, movement and virion formation, respectively. Both GFLV RNA1 and RNA2 are required for systemic plant infection (Andret-Link et al. 2004, Fuchs et al. 2017).

Fanleaf management primarily relies on prophylactic measures through sanitation and certification that facilitate the production of planting material derived from clean, virus-tested stocks (Andret-Link et al. 2004, Maliogka et al. 2014, Fuchs and Lemaire 2017). Control of the nematode vector *X. index* is another component of the GFLV management portfolio, however, this approach can be challenging due to the relative lack of effective nematicides and harsh environmental consequences related to their use. Prolonged fallow periods (up to 10 years) can reduce nematode populations in infested soils, but lengthy fallow periods are not practical in high-value grape-growing areas (Andret-Link et al. 2004). Grapevines with resistance to *X. index* have been identified and rootstocks resistant to this dagger nematode have been developed (Andret-Link et al. 2004, Oliver and Fuchs 2011).

Fanleaf is primarily managed in diseased vineyards by the use of rootstocks that are resistant to *X. index*, including 039-16. These rootstocks are extensively used in grape-growing regions where GFLV is a major threat to productivity, including the Central Coast, North Coast, Sacramento Valley and San Joaquin Valley in California. They substantially delay the debilitating effect of GFLV on vine health and production but do not prevent GFLV infection (Andret-Link et al. 2004, Oliver and Fuchs 2011). As a result, vines become infected through translocation of the virus from rootstocks to scions and the productive lifespan of vineyards is substantially reduced. In addition to conferring a limited long-term protection of grapevines from GFLV, some of the *X. index*-resistant rootstocks have undesired viticultural characteristics such as high vigor and poor rooting ability or susceptibility to lime-induced chlorosis (Oliver and Fuchs 2011). Resistance to GFLV in rootstocks would be desirable for fanleaf control; however, no source of resistance to this virus has been identified in wild or cultivated *Vitis* species (Oliver and Fuchs 2011).

Exploiting the anti-viral pathways of RNA interference (RNAi), an innate plant defense system, and use RNAi constructs derived from conserved regions of the GFLV genome to transform some of the most popular grapevine rootstocks is an elegant approach to engineer resistance. RNAi is an innate immune defense mechanism against plant viruses. It is a post-transcriptional process that is triggered by dsRNA for the silencing of gene expression in a nucleotide sequence-specific manner through the production of small dsRNAs called small interfering (si) RNAs, for which the guide strand is incorporated into the RNA-induced silencing complex to find mRNAs that have a complementary nucleotide sequence, resulting in their endonucleolytic cleavage. Silencing is associated with the production of 21 to 24 nt dsRNA duplexes called small interfering RNAs (siRNAs) and are generated from dsRNA precursors by ribonuclease III-type Dicer-like enzymes. The siRNAs are then incorporated and converted to single stranded RNAs (ssRNAs) in an Argonaute-containing RNA induced silencing complex. This complex targets RNAs for cleavage in particular mRNAs that are complementary to siRNAs, i.e. viral RNAs of an invading virus, by inducing their post-transcriptional gene silencing processing through endonucleolytic cleavage. As a result, viral RNAs are chopped and nonfunctional, hence, resistance to virus infection. The formation of dsRNAs by hairpin (hp) RNAs facilitates the silencing of target viral mRNAs via RNAi, resulting in the accumulation of virus-specific siRNAs that guide the destruction of complementary viral RNA.

Viruses encode proteins that act as suppressors of RNA silencing (Fuchs et al. 2017). Their role is to counter-act the innate defense system of the plant by interfering with critical steps of the antiviral pathways of RNA silencing. Thus, an RNAi strategy designed against viral RNA silencing suppressors (VRS) should be optimal to confer resistance to virus infection in plants. In the case of GFLV, a VRS remains elusive. Thus, research is needed to identify and characterize a VRS for GFLV and translate the corresponding information to engineer resistance against GFLV in rootstock.

Single or multiple virus gene sequences can be used to develop resistant plants (Fuchs, 2017; Fuchs and Lemaire, 2017). However, pyramiding sources of resistance is essential for achieving broad-spectrum and durable resistance. Stacking resistance-conferring gene sequences into single crop genotypes; i.e. grape rootstock genotypes, is paramount for protection against commonly occurring infections by genetically diverse virus strains across diverse ecosystems. In addition, pyramiding sequences from different viral coding regions, particularly highly conversed segments that are involved in various steps of the virus infectious cycle, i.e. replication, cell-to-cell movement, virion assembly, and/or acquisition by a vector will favor broad-spectrum and durable resistance (Fuchs, 2017). Indeed, by stacking polygenic resistance sources into a single crop genotype, for example into grape rootstock 101-14, the probability of genetically diverse virus variants overcoming multiple resistance-conferring gene sequences is substantially reduced compared to monogenic resistance sources (Fuchs, 2017). This is because many mutations with a low probability of occurrence and a high fitness penalty would be required for virus adaptation to pyramided resistance genes. As a result, populations of viruses are less likely to defeat the resistance (Fuchs, 2017).

OBJECTIVES

The major objective of our research was to explore RNAi to confer resistance to GFLV in rootstocks. Our hypothesis is that silencing several GFLV-encoded genes, including a VRS, in rootstocks will confer practical resistance to GFLV. The specific objectives of our research were to:

1. Develop RNAi constructs from conserved genomic regions of GFLV
2. Test RNAi constructs for reduction of GFLV accumulation in transient assays
3. Transfer promising RNAi constructs into grapevine rootstock embryogenic calli and develop transgenic clones
4. Initiate phenotyping of transgenic RNAi grapevine rootstock clones by agroinfiltration with infectious GFLV constructs
5. Disseminate information to stakeholders through presentations at conventions and workshops

RESULTS AND DISCUSSION

Objective 1: *Develop RNAi constructs from conserved genomic regions of GFLV*. The goal was to mine the GFLV genome sequence and identify highly conserved genomic nucleotide (nt) sequence regions for the engineering of RNAi constructs. The complete GFLV nucleotide sequences available in GenBank were downloaded and mined for short conserved nucleotide regions. Search parameters were 25 nts stretches in length for which 85% of the positions were conserved amongst at least 95% of the sequences. Search outputs revealed 10 conserved regions throughout the GFLV genome (**Fig. 1**). These conserved nucleotide stretches of 100-300 nucleotides in size are located on RNA1 (five conserved regions) and RNA2 (five conversed regions) (**Fig. 1**). The conserved RNA1 regions are located in the 1A, $1B^{Hel}$ and $1E^{Pol}$ coding regions. The conserved RNA2 regions are located in the $2A^{HP}$, $2B^{MP}$, and $2C^{CP}$ coding regions, as well as in the 3'untranslated region (**Fig. 1**).

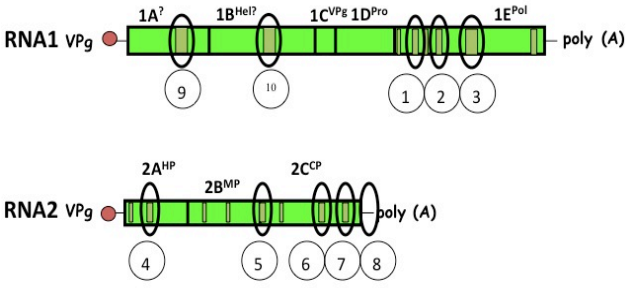

Figure 1. Mapping of conserved nucleotide sequences on the GFLV genome. Conserved sequences are represented with light brown stripes. Fragments used for the production of concatenate RNAi constructs are circled and labeled 1-10. RNA1 coding regions are: $1A^{?}$, $1B^{Hel?}$, $1C^{VPg}$, $1D^{Pro}$, $1E^{Pol}$. RNA2 coding regions are: $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$.

Individual conserved regions were amplified by PCR using specific primers and full-length cDNAs of GFLV RNA1 and RNA2 as template. Then, concatenate constructs resulting from the ligation of PCR products from different coding regions were produced (**Table 1**). Most concatenates were generated with fragments from different GFLV coding regions rather than from within a single coding region. This was done in expectation of broad-spectrum and durable resistance (Fuchs, 2017).

Table 1. Concatenate constructs (100-300 nts in size) designed in conserved regions of the GFLV genome.

Concatenate	Gene	Letter
5+8+2	$2B^{MP}+2C^{CP}+1E^{Pol}$	A
7+1+4	$2C^{CP}+1E^{Pol}+2A^{HP}$	B
4+6+3	$2A^{HP}+2C^{CP}+1E^{Pol}$	C
3+7+5+1+6+8	$1E^{Pol}+2C^{CP}+2B^{MP}+1E^{Pol}+2C^{CP}+2C^{CP}$	D
2+4+5	$1E^{Pol}+2A^{HP}+2C^{CP}$	E
1+6+8	$1E^{Pol}+2C^{CP}+2C^{CP}$	F
6+7+8	$2C^{CP}+2C^{CP}+2C^{CP}$	G
3+7+5	$1E^{Pol}+2C^{CP}+2B^{MP}$	H
1+2+3	$1E^{Pol}+1E^{Pol}+1E^{Pol}$	I

For example, fragment 245 encompasses conserved fragments of $1E^{Pol}$ (conserved region #2 on figure 1) $2A^{HP}$ (conserved region #4 on figure 1) and $2B^{MP}/2C^{CP}$ (conserved region #5 on figure 1). Similarly, fragment 375 encompasses conserved fragments of $1E^{Pol}$ (conserved region #3 on figure 1), $2C^{CP}$ (conserved region #7 on figure 1) and $2B^{MP}/2C^{CP}$ (conserved region #5 on figure 1). To facility their designation, each concatenate construct was assigned a capital letter (**Table 1**). For example, concatenate construct 245 was named E and concatenate construct 375 was named H. Each of these fragments was cloned into the plasmid pEPT8 - a plasmid derived from pUC19 that contains the cauliflower mosaic virus 35 promoter sequence and nopaline synthase terminator sequence - and subsequently into binary plasmid pGA482G for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. The integrity of all cloned concatenate constructs in pEPT8 and PGA482G was verified by restriction digestions and by sequencing at the Cornell Biotechnology Resource Center.

Advancing our understanding of GFLV-host interactions will undoubtly provide new insights into how the virus highjacks the plant machinery and which viral protein domains are key to the plant-virus interactome. Along this vein, three additional RNAi constructs were engineered.

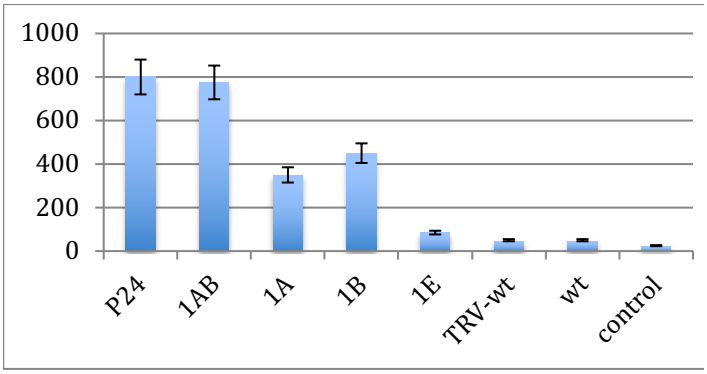

Figure 2. Expression of GFP in transgenic *Nicotiana benthamiana* plants expressing GFP that were agroinoculated first with a chimeric tobacco rattle virus (TRV) containing GFP and then with different GFLV constructs. Measurements of GFP expression were taken in apical leaves at six days post-agroinoculation with GFLV constructs. P24: silencing suppressor of grapevine leafroll-associated virus 2; 1AB: a fusion construction of GFLV 1A-$1B^{Hel}$; 1A; GFLV 1A; 1B; GFLV $1B^{Hel}$; 1E: GFLV RNA1-encoded RNA-dependent RNA polymerase; TRV-wt: wild-type TRV; wt: TRV-GFP; and control. Average values represent measurements from 5-10 plants per treatment. Error bars are shown.

These RNAi constructs were designed in the RNA1-encoded 1A and 1B^{Hel} coding regions. The impetus for the RNAi constructs is that parallel research revealed that each of these two coding regions has a weak VRS function while the fusion product 1A-1B^{Hel} acts as a strong VRS (**Fig. 2**). The GFLV fusion product 1A-1B^{Hel} acts as a VRS at the systemic level, as shown by suppression of green fluorescence protein (GFP) expression in apical leaves of transgenic *Nicotiana benthamiana* expression GFP (**Fig. 2**).

The VRS activity of GFLV 1A-1B^{Hel} was confirmed by fluorescence measurement of detached leaves of transgenic *N. benthamiana* expressing GFP under UV illumination following agroinfiltration (**Fig. 3**).

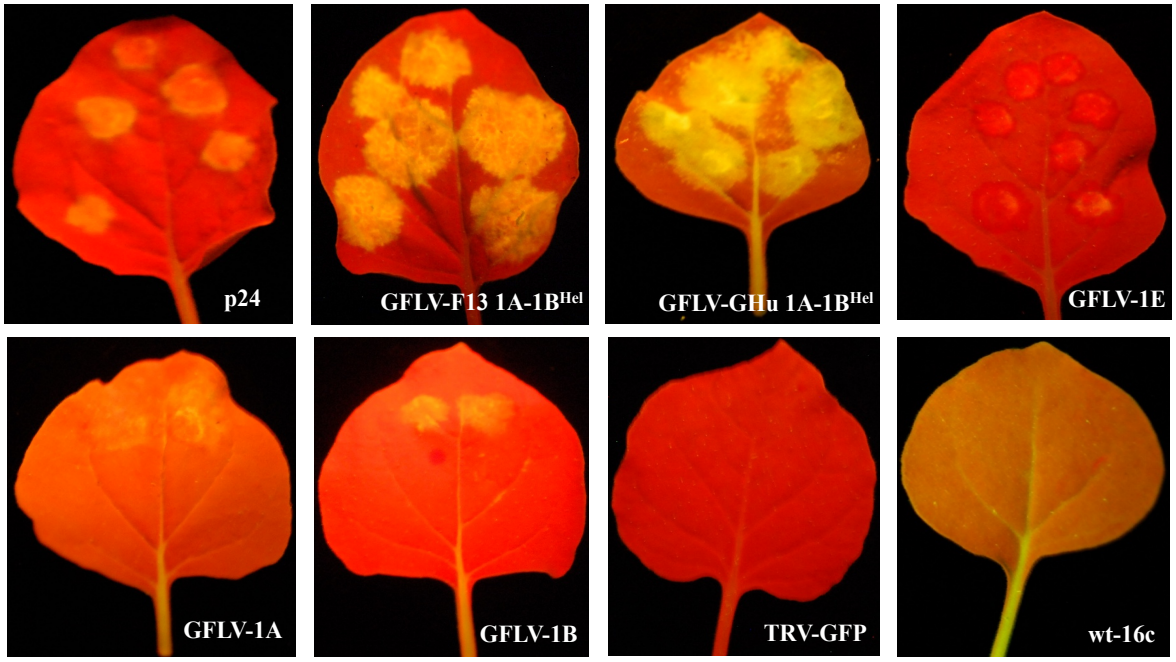

Figure 3. Expression of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in transgenic *Nicotiana benthamiana* expressing GFP that were agroinoculated first with a chimeric tobacco rattle virus (TRV) containing GFP and then with different GFLV constructs, including the GFLV strain F13 1A-1B^{Hel} fusion product, the GFLV strain GHu 1A-1B^{Hel} fusion product, the GFLV strain F13 1E^{Pol}, the GFLV strain F13 1A, and the GFLV strain F13 1B^{Hel}. Controls were p24 of GLRaV-2, 16c agroinoculated with TRV-GFP, and wild-type 16c. Detached leaves of transgenic *N. benthamiana* expressing GFP were photographed under UV illumination at 4-6 days post-agroinoculation.

The VRS activity of GFLV 1A-1B^{Hel} was as strong as that of p24, the VRS of grapevine leafroll-associated virus 2 (**Fig. 2 and 3**). It is anticipated that RNAi 1A-1B^{Hel} will have a strong anti-GFLV effect by interfering with RNAi. Similar VRS features were assigned to the 1A, 1B^{Hel}, and 1A-1B^{Hel} fusion product of GFLV strains F13 and GHu (**Fig. 3**).

Objective 2: *Test RNAi constructs for reduction of GFLV accumulation in transient assays*. The goal of this objective is to use a transient assay to screen the potential of RNAi constructs at interfering with GFLV multiplication. The development of grapevine rootstocks and the screening for resistance to GFLV is time consuming. Therefore, resistance to GFLV was evaluated first in the systemic herbaceous hosts *N. benthamiana* prior to its application to grapevines. Herbaceous hosts such as *N. benthamiana* offer the benefits of mechanical inoculation for resistance evaluation, short time to achieve systemic infection, and more expedient and high-throughput options to streamline the screening for resistance.

Agroinfiltration was explored as a high-throughput and fast system for testing the capacity of RNAi constructs to interfere with GFLV multiplication following their transient expression. Infiltration was carried out using a needle-less syringe in two lower true leaves per *N. benthamiana* plant, one of which received a control treatment (enhanced green fluorescent protein - eGFP -) and the other of which received a GFLV RNAi construct. Other plants receiving eGFP treatments to both lower leaves were used for control comparisons. Experiments were repeated at least three times. Five days after lower leaves were agroinfiltrated, upper leaves of *N. benthamiana*

plants were mechanically inoculated with GFLV using 1:50 dilutions of crude extracts of infected *N. benthamiana* leaves. Six days after mechanical inoculations with GFLV, leaf samples were collected and tested for GFLV accumulation by DAS-ELISA using specific antibodies. Thirteen days post-GFLV infection an additional leaf sample consisting of a single apical leaf was tested by DAS-ELISA using specific antibodies to verify systemic infection.

Results suggested reduced levels of GFLV accumulation in agroinfiltrated leaves receiving the RNAi construct, particularly RNAi constructs A, F, G and H, versus those agroinfiltrated with *A. tumefaciens* containing an eGFP construct at six days post-inoculation (**Fig. 4**). Plants that were not infiltrated with *A. tumefaciens*, but infected with GFLV, indicated the highest virus titers in all experiments. The next highest relative virus titers were observed in leaves receiving the eGFP control treatment, as expected. In contrast, several GFLV RNAi constructs, including A, F, G and H, showed relatively lower virus titers versus control treatments. Particularly, construct H had potent anti-GFLV activity in repeated experiments (**Fig. 4**).

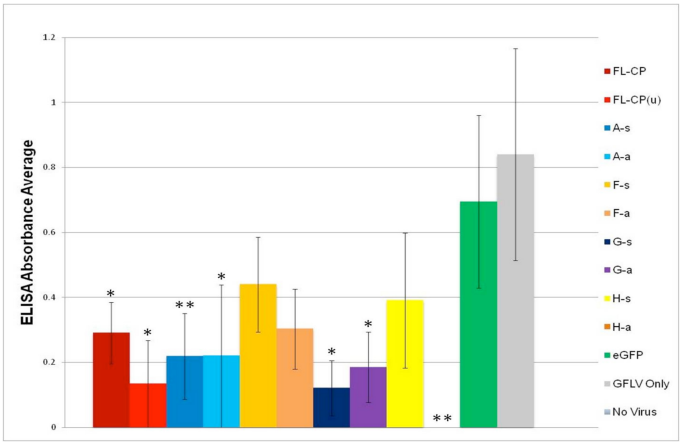

Figure 4. Relative GFLV titer measured by ELISA at six days post-inoculation in leaves agroinfiltrated with varied GFLV RNAi constructs. Absorbance value averages obtained across four experiments with 5 plants each are shown. Significant differences compared to control treatments are indicated * ($P<0.05$) and ** ($P<0.01$).

Among the RNAi constructs tested so far, those with a consistent high anti-GFLV effect were H and G, followed by A. Interestingly, RNAi construct H showed no detectable virus in any of the plants in all four experiments (**Fig. 4**). The effect of RNAi construct F on GFLV accumulation was not significant. These results were consistent with the fact that some GFLV RNAi constructs suppressed virus accumulation in agroinfiltrated leaf patches. Nonetheless, GFLV was detected in apical leaves at 13 days post-inoculation, regardless of the level of interference with GFLV accumulation in agroinfiltrated leaves. This suggested that expression of GFLV RNAi constructs should be stable in order to confer resistance.

Additionally, semi-quantitative RT-PCR was carried out on total RNA extracted from leaf disks of agroinfiltrated *N. benthamiana* leaves to further analyze the effect of RNAi constructs on GFLV accumulation. The ribulose 1,5-biphosphate carboxylase gene (*Rcb1*) was used as a housekeeping gene. A reduced GFLV RNA2 abundance was revealed in leaves that received RNAi constructs as compared to eGFP-infiltrated leaves from the same plant (**Fig. 5**). These results confirmed the trend observed with the DAS-ELISA testing. It should be noted that primers used to detect GFLV were designed to bind within GFLV RNA2 in such a way that they did not yield a product in RT-PCR from the transgene constructs, allowing for specific detection of viral transcripts only. Agroinfiltration of *N. benthamiana* was validated as a high-throughput and fast system for testing the capacity of RNAi constructs to interfere with GFLV multiplication following their transient expression. This assay highlighted the potential of RNAi constructs H, G and A at interfering with GFLV multiplication. The screening of additional GFLV RNAi and hp RNAi constructs, particularly the RNAi 1A-1B^{Hel} construct, is underway using the transient assay in *N. benthamiana*.

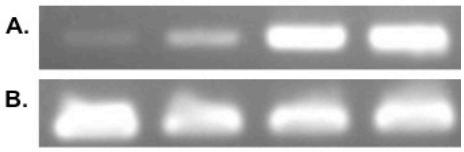

Figure 5. Semi-quantitative RT-PCR showing (A) lower relative GFLV RNA2 abundance in a *N. benthamiana* leaf agroinfiltrated with constructs A (two left lanes) versus a control infiltrated leaf at six days post-inoculation (two right lanes), (B) *Rcb1* internal RT-PCR control.

Objective 3: *Transfer promising RNAi constructs into grapevine rootstock embryogenic calli and develop transgenic clones*. The goal of this objective is to transform embryogenic cultures with GFLV RNAi constructs and regenerate putative transgenic plants. Embryogenic cultures of rootstock genotypes 101-14 MGT, 3309C, 110R and 5C were used for stable transformation experiments. GFLV RNAi constructs H, G, A and 1A-1B^{Hel} were transferred into rootstock embryogenic cultures (**Fig. 6**).

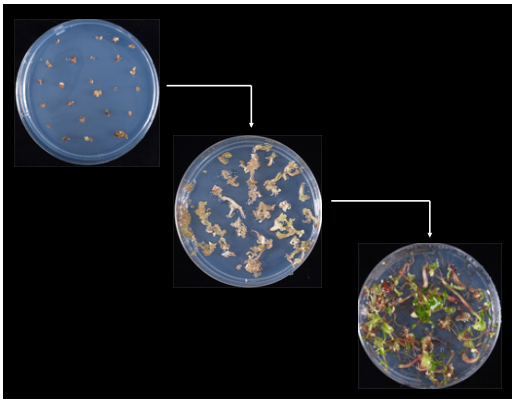

Figure 6. Embryogenic calli of rootstock genotype 101-14 MGT following exposure to *Agrobacterium tumefaciens* strain C58 containing GFLV RNAi construct H (left), elongating in the dark on a specific medium (middle) and regenerating into small plantlets (right).

Following transformation with *A. tumefaciens*, different degrees of elongation of embryogenic cultures were observed with the highest efficacy obtained with 101-14 MGT followed by 110R and 3309C and 5C. Additional transformation experiments of the four rootstocks are under way. Several plants of the rootstock genotypes 101-14 MGT and 3309C that were subjected to transformation experiments were transferred to soil in the greenhouse (**Fig. 7**). Plants were removed from test tubes or polylethylene tissue culture bags using forceps and roots were rinsed in water and trimmed 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 (**Fig. 7**). Plastic bags were gradually opened following active growth, usually within 2-3 weeks at 20±5° and 75-150 $\mu Em^{-2}s^{-1}$.

Figure 7. Plant of a putative transgenic rootstock genotype 101-14 MGT established in soil in the greenhouse.

Transgene insertion was characterized by PCR in actively growing rootstock plants that were established in the greenhouse (**Fig. 8**). Results revealed DNA amplicons of the expected size for concatenate GFLV constructs H, G, A and 1A-1B^{Hel} using appropriate primers and total plant DNA isolated from leaves of putative transgenic rootstock genotypes 101-14 MGT and 3309C with the E.Z.N.A SP Plant DNA Kit (Omega Bio-tek). Sequencing DNA amplicons confirmed the nature of the products and the integrity of the inserted transgenic constructs in the different rootstock plants analyzed with 100% nucleotide identity between the sequence obtained and the expected sequence.

Figure 8. Population of putative transgenic grape rootstock clones of genotypes 101-14 and 3309C in a greenhouse.

Additionally, Northern blot hybridization confirmed the insertion of concatenate GFLV constructs H and G in *XbaI*-digested total DNA of some putative transgenic plants of rootstock genotypes 101-14 MGT and 3309C using a DIG-labeled probe specific to the GFLV-F13 1D^{Pro} coding region (**Fig. 9**). Analysis of hybridization patterns revealed that transgene insertion loci were independent in the different transgenic clones analyzed, as illustrated by hybridization bands of different sizes obtained by Northern blot assays (**Fig. 9**). Additional putative transgenic rootstock plants, including some that were transformed with the RNAi construct 1A-1B^{Hel}, are characterized for RNAi transgene insertion by PCR and Northern blot hybridization using appropriate primers and an appropriate DIG-labeled probe, respectively.

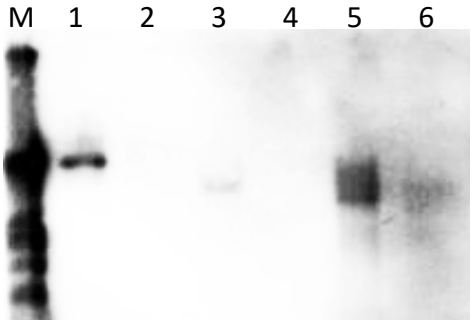

Figure 9. Northern blot characterization of RNAi constructs in transgenic rootstock genotype 101-14 MGT clones 6 (lane 2) and 17 (lane 4) and 3309C clones 3 (lane 6) and 5 lane 7). Lanes 2 and 4: DNA from nontransgenic rootstock 101-14 MGT and 3309C, respectively. M: 1-10kb DNA ladder.

Transgene expression was characterized in in actively growing rootstock plants that were established in the greenhouse by reverse transcription (RT)-PCR. Results indicated the accumulation of corresponding mRNA in total RNA isolated from leaves of some of the putative transgenic plants containing concatenate GFLV constructs H, G and 1A-1B^{Hel} with the E.Z.N.A SP Plant RNA Kit (Omega Bio-tek) using appropriate primers. Sequencing DNA amplicons obtained by RT-PCR validated the nature of the mRNA and the integrity of the RNAi constructs in the different transgenic rootstocks clones analyzed as a 100% nucleotide identity with the expected sequence was obtained.

Objective 4: *Initiate phenotyping of transgenic RNAi grapevine rootstock clones by agroinfiltration with infectious GFLV constructs.* Transgenic grapevine rootstock clones, for which the insertion and expression of RNAi construct were validated by PCR, Northern blot hybridization and RT-PCR, respectively, and control plants were maintained in the greenhouse for phenotyping work. The goal of this objective is to agroinfiltrate transgenic rootstock clones with infectious clones of GFLV strains F13 and/or GHu to identify resistant lines. Infectious GFLV clones were prepared as described (Osterbaan et al. 2018) and agroinilftrated into young leaves of greenhouse-grown plants. In spite of several attempts, we repeatedly failed to establish GFLV infection in transgenic and nontransgenic, control plants, as shown by consistently negative RT-PCR tests using total RNA from agroinfiltrated leaf areas and appropriate primers or DAS-ELISA with specific GFLV antibodies. Failure to establish infection was experienced regardless if infectious clones of GFLV strain F13 or strain GHu were used. The reason for this failure is unclear; perhaps the infectious GFLV clones, which are capable of establishing systemic infection in *N. benthamiana* (Osterbaan et al. 2018), are not infectious in grape tissue. Alternative explanations might be that experimental conditions for grapevine agroinfiltration are suboptimal for GFLV infection or greenhouse-grown grapevines are not amendable to agroinfiltration using needless-syringes. More efforts are needed to address this issue and develop a reliable system for phenotyping grape rootstocks by agroinfiltration with infectious GFLV clones.

Objective 5: *Disseminate information to stakeholders through presentations at conventions and workshops.* Research progress on the development of fanleaf resistant rootstocks was disseminated to grape growers at varied venues. Communication efforts reached over 400 growers, extension educators and service providers in California and New York:

Fuchs, M. 2017. Viruses: Biology, ecology and management. Sustainable Ag Expo, November 13, San Luis Obispo (participants = 250).

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).

Fuchs, M. 2016. Genetically modified organisms. Finger Lakes Forum, January 18, Geneva, NY (participants = 60).

Conclusions

Several RNAi constructs derived from conserved regions of the two GFLV genomic RNAs were engineered and stacked to facilitate durable and broad-spectrum resistance against GFLV populations in vineyards. Several putative transgenic rootstocks of genotypes 101-14 MGT and 3309C were obtained and established in the greenhouse. Transgene insertion and expression was verified by PCR, Northern blot hybridization and RT-PCR in transgenic rootstock plants. Evaluating resistance to GFLV of selected transgenic plants will be the next important steps of our study. This research is anticipated to provide innovative solutions to manage GFLV in diseased vineyards.

References Cited

Andret-Link, P., Laporte, C., Valat, L., Laval, V., Ritzenthaler, C., Demangeat, G., Vigne, E., Pfeiffer, P., Stussi-Garaud, C. and Fuchs, M. 2004. Grapevine *fanleaf virus*: Still a major threat to the grapevine industry. Journal of Plant Pathology, 86:183-195.

Fuchs, M. 2017. Pyramiding resistance-conferring gene sequences in crops. Current Opinion in Virology, 26: 36-42.

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.

Fuchs, M. Schmitt-Keichinger, C. and Sanfaçon, H. 2017. A renaissance in nepovirus research provides new insights into their molecular interface with hosts and vectors. In: Advances in Virus Research, M. Kielian, K. Maramorosch, T.C Mettenleiter and M.J Roosinck (eds.), pp. 61-105.

Maliogka, V., Martelli, G.P., Fuchs, M. and Katis, N. 2014. Control of viruses infecting grapevine. In: Control of Plant Viruses, Advances in Virus Research, G. Loebenstein and N. Katis (eds.), Elsevier, 91:175-227.

Oliver, J.E. and Fuchs, M. 2011. Tolerance and resistance to viruses and their vectors in *Vitis* sp.: A virologist's perspective of the literature. American Journal of Enology and Viticulture, 62:438-451.

Osterbaan, L.J., Vigne, E., Schmitt-Keichinger, C. and Fuchs, M. 2018. Optimization of an agroinoculation system for establishment of systemic GFLV infection in *Nicotiana benthamiana*. Journal of Virological Methods 257:16-21.

Funding Agencies

Funding for this project was provided by the California Department of Food and Agriculture's Pierce's Disease/Glassy-winged Sharpshooter and Other Designated Pests and Diseases of Wine Grapes Program.