California Department of Food and Agriculture - Interim Progress Report

CDFA Agreement Number 16-0612-SA

Title of Project: Resistance to grapevine fanleaf virus in rootstocks

Principal Investigators
Marc Fuchs
Department of Plant Pathology and Plant-Microbe Biology
Cornell University,
Geneva, NY 14456
Phone: 315-787-2487
E-mail: mf13@cornell.edu)

Principal Cooperator
Deborah Golino
University of California
Foundation Plant Services
One Shields Avenue
Davis, CA 95616
Phone: 530 752-3590
E-mail: dagolino@ucdavis.edu

Reporting period: The results reported here are from work conducted from March to July 2017

Introduction

Fanleaf is one of the most devastating viral diseases of grapevines (Andret-Link et al. 2004). Fanleaf disease causes serious economic losses by reducing vigor and yield, altering fruit juice chemistries, shortening the productive life of vineyards or causing vine death. Its 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* (Fuchs et al. 2017). 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. 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 schemes that facilitate the production of planting material derived from clean, virus-tested stocks. 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. 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 (Oliver and Fuchs 2011).

Fanleaf is primarily managed in diseased vineyards by the use of rootstocks that are resistant to *X. index*. 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 (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 RNA 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. 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.

Objectives

The research is designed to engineer resistance to GFLV in grape rootstocks through RNAi. The specific objectives are 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

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

Objective 1: Develop RNAi constructs from conserved genomic regions of GFLV
The goal of this objective is 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**).

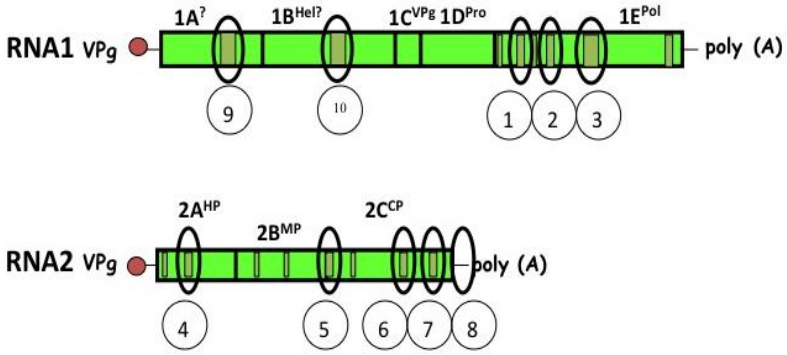

Figure 1. Mapping of conserved nucleotide sequences on the GFLV genome. Conserved sequences are represented with light brown stripes. Fragments used for the production of concatenate RNAi constructs are circled and labeled 1-10. RNA1 coding regions are: $1A^{?}$ (unknown function), $1B^{Hel?}$ (putative helicase), $1C^{VPg}$ (viral genomic-linked protein), $1D^{Pro}$ (protease), $1E^{Pol}$ (RNA dependent-RNA polymerase). RNA2 coding regions are: $2A^{HP}$ (homing protein), $2M^{MP}$ (movement protein) and $2C^{CP}$ (coat protein).

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**).

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). 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). These fragments were cloned into the plasmid pEPT8 and

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	$2C^{CP}+2C^{CP}+C^{CP}$	G
3+7+5	$1E^{Pol}+2C^{CP}+2B^{MP}$	H
1+2+3	$1E^{Pol}+1E^{Pol}+1E^{Pol}$	I

subsequently in binary plasmid pGA482G (Ling et al. 1997) for mobilization into *Agrobacterium tumefaciens* strain C58 for plant transformation. The integrity of all cloned concatenate constructs was verified by restriction digestions and by sequencing at the Cornell Biotechnology Resource Center.

Two additional hp RNAi constructs were engineered. These hp RNAi constructs were designed in the RNA1-encoded 1A and $1B^{Hel}$ coding regions. The impetus for the hp RNAi constructs is that each of these coding regions has an RNAi silencing suppressor function while the fusion product $1A\text{-}1B^{Hel}$ acts as a strong silencing suppressor (**Fig. 2**).

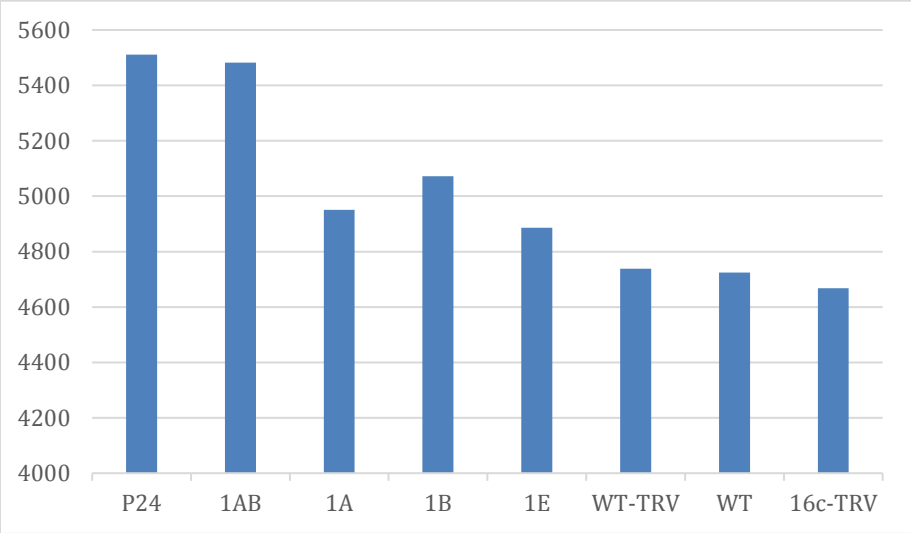

Figure 2. Expression of green fluorescent protein (GFP) in transgenic *N. benthamiana* expressing GFP that were agroinoculated first with a chimeric tobacco rattle virus (TRV) containing GFP and then with different GFLV constructs. Measurements of GFP expression were taken at six days post-agroinoculation with GFLV constructs. P24: silencing suppressor of grapevine leafroll-associated virus 2; 1AB: a fusion construction of GFLV $1A\text{-}1B^{Hel}$; 1A; GFLV RNA-1encoded 1A; 1B; GFLV RNA1-encoded 1BHel; 1E: GFLV RNA1-encoded RNA-dependent RNA polymerase; WT-TRV: wild-type TRV; WT: wild type *N. benthamiana* expressing GFP; and 16c-TRV: nonagroinoculated transgenic *N. benthamiana* expressing GFP.

The VRS activity of the fusion $1A\text{-}1B^{Hel}$ was as strong as p24, the VRS of grapevine leafroll-associated virus 2. It is anticipated that GFLV hp RNAi 1A and $1B^{Hel}$ will have a strong anti-GFLV effect by interfering with RNAi silencing. GFLV hp RNAi constructs of 1A and $1B^{Hel}$ will be used alongside other GFLV RNAi constructs for rootstock transformation.

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 *Nicotiana 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 to verify systemic infection.

Results suggested relatively reduced levels of GFLV accumulation in agroinfiltrated leaves receiving the GFLV RNAi construct versus those agroinfiltrated with *A. tumefaciens* containing an eGFP construct at six days post-inoculation (**Fig. 3**). 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 showed relatively lower virus titers versus control treatments.

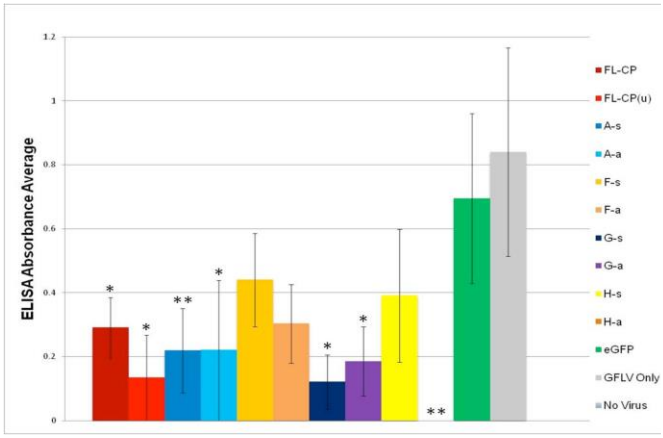

Figure 3. Relative GFLV titer measured by ELISA at six days post-inoculation in leaves agroinfiltrated with varied GFLV RNAi constructs. Absorbance value averages obtained across four experiments with 5 plants each are shown. Significant differences compared to control treatments are indicated * ($P<0.05$) 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. 3**). 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.

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. 4**). These results confirmed the trend observed from 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. The transient assays will be further used to screen additional GFLV RNAi and hp RNAi constructs.

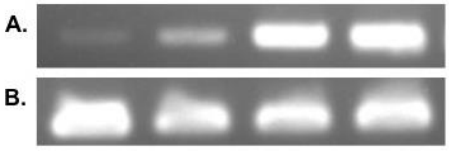

Figure 4. Semi-quantitative RT-PCR showing (A) lower relative GFLV RNA2 abundance in a *N. benthamiana* leaf agroinfiltrated with constructs A (two left lanes) versus a control infiltrated leaf at six days post-inoculation (two right lanes), (B) *Rcb1* internal RT-PCR control.

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 RNAi constructs and regenerate putative transgenic plants.

RNAi constructs H and G and hp RNAi constructs 1A and $1B^{Hel}$ will be transferred shortly into rootstock embryogenic cultures.

Objective 4: Initiate phenotyping of transgenic RNAi grapevine rootstock clones by agroinfiltration with infectious GFLV constructs
The goal of this objective is to characterize the insertion and expression of RNAi constructs in putative transgenic rootstocks, and agroinfiltrate transgenic plants with GFLV to identify resistant lines.

This objective will be met once transgenic rootstocks are developed and available for resistance screening.

Objective 5: Disseminate information to stakeholders through presentations at conventions and workshops
The goal of this objective is to report research progress to grape growers.

Information on the development of fanleaf-resistant rootstocks was disseminated at the following venues:
1. 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).
2. Fuchs, M. 2016. Genetically modified organisms. Finger Lakes Forum, January 18, Geneva, NY (participants = 60).
3. Fuchs, M. 2015. Genetic engineering as a tool to develop fanleaf-resistant grapevine rootstocks, Rootstock, The Future of Genomics & Genetics Tools in Plant Breeding and Pest & Disease Resistance – with Comparative Tasting, Napa, CA, November 12 (participants = 150).

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

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

Osterbaan, L.J., Schmitt-Keichinger, C. and Fuchs, M. 2017. Optimization of an agroinoculation system for establishment of systemic GFLV infection in *Nicotiana benthamiana*. Journal of Virological methods, submitted.

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

The research is anticipated to provide an innovative solution to manage grapevine fanleaf virus in diseased vineyards.

Layperson summary of project accomplishments

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 (Oliver and Fuchs 2011), we explored the anti-viral pathways of RNA interference (RNAi), an innate plant defense system, to confer resistance to GFLV infection in grapevine rootstocks. Different RNAi constructs derived from different conserved regions of the GFLV genome were identified and engineered for expression *in planta*. These constructs will be used in grape rootstock transformation experiments.

Status of funds

Funds were spent for salaries of key personnel (technicians) involved in the research and for supplies in molecular biology and tissue culture.

Summary and status of intellectual property associated with the project

Intellectual property associated with the use of some the GFLV RNAi constructs used in this project is owned by Cornell University.

Literature 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.

Ling, K-S., Zhu, H-Y., Alvizo, H., Hu, J.S., Drong, R.F., Slightom, J.L. and D. Gonsalves, D. 1997. The coat protein gene of grapevine leafroll associated closterovirus-3: cloning, nucleotide sequencing and expression in transgenic plants. *Arch Virol.* 142:1101-1116.

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.