# Design and delivery of protein chimeras for therapy of viral diseases in grape

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## Long-term Projects benefits to CA wine/grape Industries from the emerging vector-borne diseases

Multiple grapevine associated viruses have been discovered over the years [6]. Grapevine leafroll associated viruses (GLRaV) appear to be the most devastating of all viruses and are widespread worldwide [7]. Several mealybug species are efficient vectors of GLRaV. Among 10 different viral subtypes, GLRaV-3 is the most infectious one, which can cause up to 40% losses in yield. Grapevine red blotch associated viruses (GRBaV) emerged only recently in 2008 in California, USA **[8].** GRBaV are transmitted by leafhoppers. Survey of red blotch infected vines in the USA indicate abo.ut 15% losses in yield. Leafroll and red blotch virus infection in grapevines disrupt immune defense pathways against biotic and abiotic stress, carbohydrate metabolism, photosynthesis, respiration, electron transport, and hormonal balance. Disruptions in these pathways harm the berry quality because of irregular ripening as well as affect plant growth resulting in a delay of sprouting, reduced tolerance to stresses and even death of the chronically infected grapevines. Note that there are no cures for leafroll and red blotch viral diseases. As with many vector-borne diseases, insecticides provide, at best, only a temporary relief but do very little to eliminate the causative virus. Successful completion of this project will lead to design and testing of antiviral protein chimeras for the therapy of leafroll and red blotch viral diseases in grapevines. This therapy will be highly efficacious in clearing the virus. It will also be cost-effective and receive consumer acceptance. Finally, by the end of this project, we will be in a position to prepare data packages for the federal regulatory processes as a part of marketing so that the wine/grape industry has a quick access to our therapy.

## Objectives

1. Design anti-viral protein chimeras consisting of a grape-derived peptide targeting the viral membra.ne and a grape-derived protease targeting the viral coat protein. The peptide and protease will be joined by a flexible linker of 7-20 amino acids to facilitate the synergy of membrane targeting and protease activities and consequently, will lead to rapid clearance of the virus.
2. Overexpress and purify different chimeras in Human Kidney Embryonic (HEK) cells, test their efficacy by detached leaf assay using samples from virus-infected grapevines, and select the chimeras with the highest anti-viral activities. The chimeras with highest anti-viral activities identified by the l't round of the detached leaf assay will be further refined to improve their activity.

## Workplan and Methods

**For Objective 1** (Design of grape-derived anti-viral protein chimeras)

We will design chimeras consisting of a grape-derived peptide targeting the viral membrane and a grape-derived protease targeting the viral coat protein. Red blotch and leafroll being respectively envelop and filamentous viruses, they derive their membrane components from the host grape. Although, the viral membrane is different from the host grape membrane in terms of the presence of covalently attached and non-covalently associated protein complexity and dynamics of the membrane lipid components, we will pay special attention to select appropriate lytic peptides that may target GRBaV and GLRaV-3. For this, we will scan the anti-viral peptide database [9] to select the ones with the highest activity or the lowest ICS0 (i.e., the concentration of the peptide at which the peptide reduces the plaque forming unit, pfu, to half on a log scale of the initial untreated condition). We will then search for the closest homologs of these peptides present in the grape proteins. Finally, we will engineer helix-turn-helix (HTH) peptides using the grape homologs. Note that, we previously demonstrated that the HTH peptides possess higher antimicrobial activity than the constituent single helices because they are more efficient in membrane attachment, insertion, and rupture [1].

**For Objective 2** (Production, efficacy testing, and refine of the anti-viral chimeras)

Different chimeras will be expressed in and purified (in mg quantities) from human cells using established protocols (12]. Briefly, the DNA encoding various chimeras will be synthesized from Genscript (codon optimized for HEK293F, pUC57, Kan+). The DNA encoding the chimeras will be cloned in pcDNA3.1+/C-DYK (BamHI/EcoRI) sit, which includes a TEV (Tobacco Etch Virus) protease cleavable FLAG tag. The recombinant clone will be selected by restriction digestion and DNA sequencing. The plasmid will be prepared from overnight culture (Maxiprep Kit) and will be used for transferring HEK293F cells (30µg) following manufacturer's instructions. Following transferring, the cell suspension will be collected every day for 5 days and protein expression will be monitored by western blot analysis to determine the optimum condition for expression. The recombinant protein will be expressed under the optimum condition and will be affinity purified using FLAG tag resin. The FLAG tag from the purified protein will be removed by TEV cleavage. The viral CPs, GRBaV (ID=AMQ35562.1) will also be expressed in and purified from HEK cells.

We will determine the anti-viral efficacy of different chimeras using detached leaf assay similar for what we performed for PD treatment [2]. For this, we will collect leaves from leafroll and red blotch infected grapevines in Norther/Central California. Six top chimeras with lytic peptides with high anti-viral activity and Subtitles with high CP cleavage activity will be initially chosen for analysis. For each virus infection, 35 leaves will be tested in each biological replicate: 5 leaves each for the treatment of 6 chimeras and 5 leaves for treatment with water (control). The treatment will involve dipping of the petioles for 2 hours in 1 ml of 10-20 µM chimera [1]. Three biological replicates with 35 leaves in each replicate will be analyzed for the presence of GRBaV DNA specific loci by qPCR using virus-specific primers as reported [13-14]. A reference DNA, a grape gene encoding the endochitinase PR4-like protein (LOC100266390: XM_002274383.3) will be used to demonstrate that the reference DNA level is not affected by treatment. The level of clearance in the treated sample will be measured relative to the untreated sample. This will allow us to rank the chimeras in terms of their relative activity on GRBaV or GLRaV-3.

The anti-viral specificity and activity of the top-ranked chimera will be further improved by yeast display (15]. See Figure 3, in which different steps are shown to select Subtitlisin with improved specificity toward a red blotch virus CP. First, we will clone a library of plasmids with mSubtilisin genes (with Ser -> Ala substitution at the catalytic Asp, His, Ser triad). mSubtilisin genes will encode proteins that will be devoid of catalytic activity but will retain binding to the viral CP. Second, we will transformation with the

plasmids to express mSubtilisin non-binders (grey) and binders (green/purple) on the surface of yeast: Third, we will use CP-immobilized beads to isolate yeast variant with high affinity mSubtilisin binders (purple) on the surface. Fourth, we will revert to Ala->Ser at the catalytic site to recover and measure the protease activity using a chromogenic synthetic substrate. Fifth, we will repeat the steps to further improve the protease activity of Subtilisin on CP. Finally, after several rounds of evolution, 5-10 Subtilisin variants will be selected with high activity and specificity toward the viral CP.
The high affinity and specificity Subtilisin will replace the original grape subtilisin in the top-ranked· chimera.

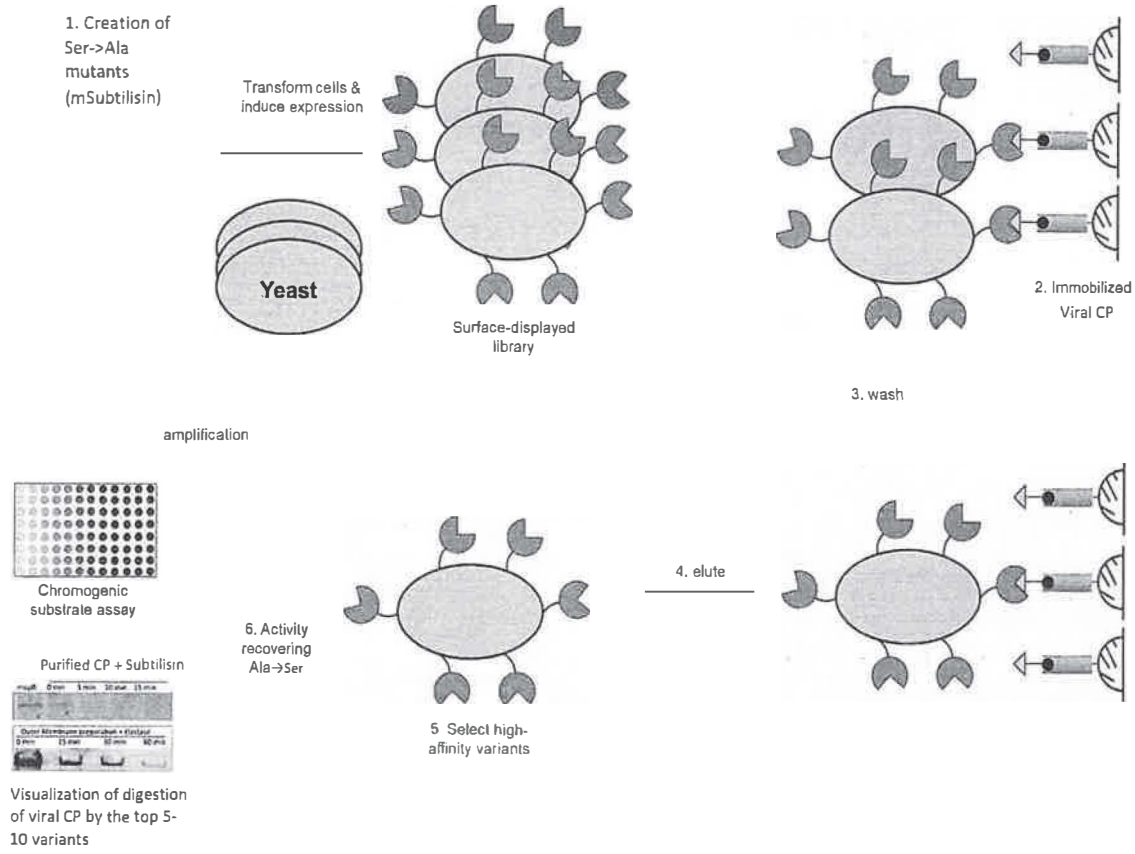


**Figure 3.** Schematic description of yeast display method for selecting Subtilisin variants with improved activity and specificity toward the viral CPs.

## Project Timetable

**At the end of this 12-month pilot project, we will identify both antiviral lytic peptides and chimeras that would be suitable for field trials.**