IDENTIFICATION AND UTILIZATION OF COLD TEMPERATURE INDUCED GRAPEVINE METABOLITES TO MANAGE PIERCE'S DISEASE.

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Reporting Period: The results reported here are from work conducted January 2011 to October 2011.

ABSTRACT
This work builds on discoveries made in the past seven years of research on better understanding the mechanism(s) responsible for the Pierce's disease (PD)-cold curing phenomenon. A thaumatin-like (TLP) grape protein was found in elevated levels in the xylem sap from cold-exposed vines and we cloned and expressed TLP in *E.coli*. We found that TLP protein possesses higher levels of *Xylella fastidiosa (Xf)* toxicity *in vitro* compared to *E. coli* protein extracts from cells containing the cloning vector but no TLP gene. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. In collaboration with the Waterhouse lab we are characterizing these phenolic compounds and assessing their potential *anti-Xf* activity *in vitro*. One phenolic compound, trans-resveratrol, which only occurred in Pinot noir grapevines exposed to cold temperatures, and was shown by two different labs to be toxic to *Xf* cells grown *in vitro* Previously, greenhouse grown Pinot Noir and Cabernet Sauvignon vines treated with commercial abscisic acid (ABA) were shown to have higher levels of recovery from PD than non-treated vines, as well as producing higher levels of polyphenolic compounds. In fall 2010 we treated Riesling vines growing in a vineyard in Napa that had light to moderate PD symptoms vines with a foliar spray or a soil drench treatment of ABA. The severity of PD symptoms in the treated vines will be compared with the severity of symptoms in non-treated PD-affected vines in fall, 2010.

LAYPERSON SUMMARY
We have succeeded in producing recombinant thaumatiin-like grape protein (TLP) in *E.* coli. W*e* have also observed that grapevine TLP produced by *E. coli* has a deleterious effect on *Xylella fastidiosa (Xf)* when it is grown *in vitro* in the laboratory. This supports our rationale for over- expressing TLP in grapevines as a potentially promising approach to decreasing the size of *Xf* populations in Pierce's disease (PD)-affected grapevines. We have been characterizing the phenolic compounds in cold xylem sap and comparing these to warm xylem sap. We have noticed a number of differences, specifically the presence of the phenolic compound trans-resveratrol in cold sap and its absence in warm sap in some grape varieties. This suggests that trans-resveratrol may play a role in the cold curing process. In previously described research we added trans-resveratrol to solid media used to grow *Xf* and observed that the Temecula strain is inhibited at trans-resveratrol concentrations lower than those concentrations that inhibit the Fetzer strain. This further suggests that phenolic compounds play a role in the cold curing process. Ongoing field trials are examining if root or foliar applications of the plant hormone ABA could stimulate the synthesis of phenolic compounds in field grown vines infected with *Xf* and possibly decrease the severity of PD symptoms in the field vines.

INTRODUCTION
Previous research conducted in the Purcell laboratory at UC Berkeley definitively demonstrated that *Vitis vinifera* grapevines that were infected with *Xylella fastidiosa (Xf)* the bacterial pathogen that causes Pierce's disease (PD) could often be cured of the infection if exposed to freezing temperatures for some period of time. This "cold curing" phenomenon likely explains why PD is restricted to areas that have mild winter temperatures. Research conducted in our laboratory by Dr. Melody Meyer confirmed and expanded the work performed by Purcell, et. al.. She found that grapevines exposed to cold temperatures had elevated levels of a thaumatin-like protein (TLP) that has been shown to have antimicrobial properties in other plant host/pathogen interactions. We cloned and expressed the grapevine TLP in *E. coli* and showed that incubation of the cloned TLP with cultured *Xf* cells considerably decreased the viability of the *Xf* cells compared to incubating the *Xf* cells with other appropriate controls. We are now cloning the TLP gene in an Agrobacterium binary vector with the intention of over-expressing the TLP in transgenic grapevines. Once TLP-transgenic grapevines are obtained and characterized they will be inoculated with *Xf* using mechanical and insect inoculation. The vines will then be rated for symptom development and compared to non-transgenic *Xf*-inoculated vines.

Dr. Meyer's research also showed elevated levels of polyphenolic compounds in xylem sap extracted from cold exposed grapevines. In collaboration with the Waterhouse lab we have been characterizing the phenolic compounds in the xylem fluid. One phenolic compound, trans-resveratrol, only occurred in Pinot noir exposed to cold temperatures, and our lab, as well as another, showed that resveratrol was toxic to *Xf* cells *in vitro*. Dr. Meyer's research also showed elevated levels of the plant hormone abscisic acid (ABA) in xylem sap of cold exposed grapevines. She showed that exogenous application of ABA greatly increased the PD curing rates of potted grapevines exposed to the comparatively mild winter temperatures in Davis. In 2010 we applied ABA in the fall to PD-infected vines growing in a vineyard in Napa. The severity of PD symptoms in the ABA-treated vines will be compared to non-treated controls in October, 2011.

OBJECTIVES

1. Over express the grapevine TLP in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA.
2. Inoculate TLP-expressing grapevines with *Xf* and determine the incidence and severity of PD in transgenic versus non-transgenic *V. vinifera*.
3. a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
   b. Compare the phenolic content of xylem sap of grapevines treated with ABA under non-freezing conditions to phenolics in cold-exposed xylem sap.
   c. Determine if these compounds affect *Xf* growth/survival *in vitro.*
4. Determine if foliar or drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

RESULTS AND DISCUSSION

We successfully cloned and expressed grapevine TLP in *E. coli* (**Figures 1 and 2**). We sequenced the grape TLP gene and are currently inserting it into an *Agrobacterium* binary vector system for over-expressing TLP in transgenic grapevines. Our initial plan was to use the same vector we used to generate our hemagglutinin transgenic lines, however our results using these plasmids, which use two 35S promoters to express the HA gene and uses hygromycin resistance, produced a number of lines in which crossing over by the two promoters ended up deleting all or part of the HA gene construct while maintaining the antibiotic selection. To avoid these deletion events we have decided to change the binary system to a neomycin (kanamycin) resistant plasmid driven by the nptII promoter to eliminate the chance of promoter crossing over/deletion events. We used the pUNCB5omega plasmid for initial cloning of the grape TLP gene in *E. coli*. We are now moving the TLP construct into the low copy pCB4NN plasmid. This plasmid provides the neomycin resistance and is in low copy in case there are any toxicity issues resulting from expression of TLP in Agrobacterium. We plan on submitting the appropriate TLP transformation constructs to the UC Davis plant transformation facility at the end of October, 2011. Once we get transformed plants back from the plant transformation facility we will be able to proceed with the *Xf* pathogenicity portion of this project.

We are also in the process of making a larger quantity of purified, recombinant TLP in *E. coli* which we will give to the Comparative Pathology Laboratory at UC Davis for polyclonal antibody production. The TLP-specific antibodies will be used for quantifying TLP in the transgenic grapevines.

We performed a time course experiment in which *Xf* was combined with dialyzed TLP, and then plated onto PD3 media at intervals over two days. As controls we also performed the same incubation procedure with *Xf* and water, *Xf* with empty vector supernatant (*E. coli* lysate that was not transformed with TLP), and *Xf* with a potassium buffer. We plated these suspensions directly after combining (0 hours), 16 hours, 24 hours, 40 hours and 48 hours of incubation. The plates were then incubated at 28C for 10-14 days. Our results showed that the early time course platings had no differences in *Xf* growth. However, after 48 hours post combination, the *Xf* and water control as well as the *Xf* and potassium buffer control still grew *Xf* colonies. The *Xf* combined with the empty vector supernatant showed growth, but less than the water and potassium buffer. The *Xf* that had been combined with dialyzed TLP did not show any growth.

We have analyzed sap samples collected from Placerville, CA (during the months of January and February) where cold curing occurs, as well as sap from Winters, CA where cold curing does not take place. In collaboration with the Waterhouse lab at UC Davis, we are determining accurate polyphenolic profiles for Cabernet Sauvignon clone 8 on 110R rootstock and Pinot Noir clone 2A on 101-14 rootstock. In the Placerville (cold) Pinot Noir samples, a number of phenolic compounds were identified: B procyanidins, catechin, epicatechin, trans-resveratrol, caftaric acid, and a resveratrol tetramer. Cabernet Sauvignon samples produced an identical polyphenolic profile except that the resveratrol tetramer was not present. Interestingly, the warm Pinot Noir sap lacked characteristic peaks for trans-resveratrol as well as the resveratrol tetramer. The fact that trans-resveratrol is present in vines that experience "cold curing" while it is absent in vines that do not undergo "cold curing" suggests that resveratrol may play a role in the curing process. We will analyze the MS/HPLC data after two winters of collecting sap, and should be able to provide an accurate picture of what happens with regards to polyphenolic concentrations during the winter.

We also added purified trans-resveratrol to solid media used to grow *Xf* and observed that the Temecula strain was inhibited at concentrations significantly lower than concentrations of trans-resveratrol which inhibited growth of the *Xf* Fetzer strain. While the reason for this differential sensitivity is not known, it is interesting to note that the Temecula strain was isolated in a location with comparatively mild winters while Fetzer was isolated from PD-affected vines growing in N. California. It could be possible that the Fetzer strains evolved mechanisms to detoxify low levels of phenolic compounds that were synthesized in vine exposed to the colder winter temperatures of N. California, while the Temecula strain was not subjected to elevated levels of phenolics growing in S. California.

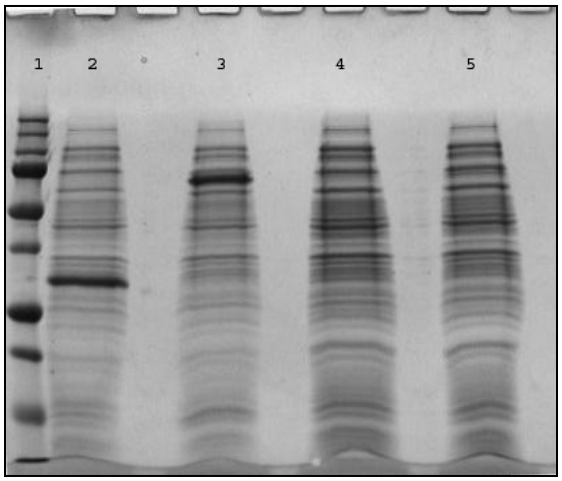

Figure 1. Recombinant *Vitis vinifera* 'Cabernet Sauvignon' TLP protein, expressed in *E. coli* and analyzed by SDS-PAGE. Lane 1: Dual Color SDS-ladder (lower band-25 kD; upper band-75 kD); Lane 2: Arrow denotes induced recombinant Cabernet Sauvignon TLP; Lane 3: Induced recombinant polygalacturonase (PG) (positive control); Lane 4: non-induced recombinant Cabernet Sauvignon TLP; Lane 5: non-induced recombinant polygalacturonase (PG).

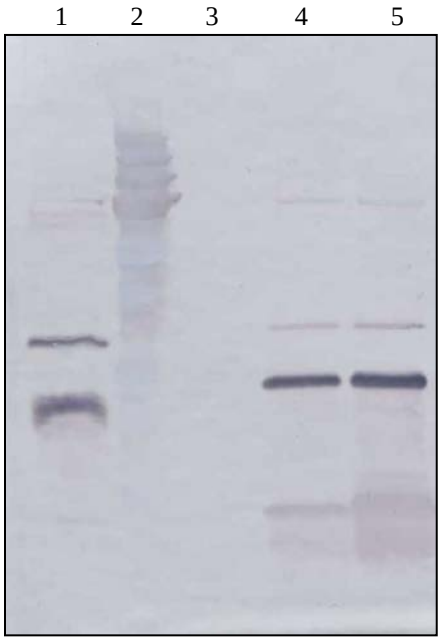

Figure 2. Western blot of SDS-PAGE of recombinant *Vitis vinifera* 'Cabernet Sauvignon' TLP protein expression using anti-His tagged antibody. Lane 1: CS3 raw lysate pellet (positive control); Lane 2: SDS Dual color ladder; Lane 3: Non-induced CS3 (negative control); Lane 4: CS3 dialysis purified pellet; Lane 5: CS3 dialysis purified supernatant.

Our previous research showed that ABA applications to greenhouse grown Cabernet Sauvignon and Pinot Noir grapevines infected with *Xf* increased overwinter curing rates in Davis, CA., a location which has relatively warm winter temperatures that induces only low rates of PD cold curing. Our previous work also showed that ABA-treated vines had higher levels of polyphenolics than vines growing in Davis which did not receive ABA applications. In October of 2010 we applied foliar sprays and root drenches of ABA to *Xf*-infected Riesling vines growing in a Napa vineyard. We will rate the severity of PD symptoms in October 2011 and compare the severity of treated versus non-treated vines.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.