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

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ABSTRACT
This work builds on discoveries made in the past six 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. We have cloned and expressed TLP in *E .coli* and our preliminary findings show that crude TLP protein extract possesses anti-*Xylella fastidiosa* (*Xf*) activity *in vitro*. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. We are beginning to characterize these phenolic compounds, and assess their potential anti-*Xf* activity *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. This fall applied ABA, both soil drench and foliar sprays, to PD-affected field grown Riesling vines in the Napa Valley.

LAYPERSON SUMMARY
Previous work on "cold curing" of Pierce's disease (PD) affected grapevines led to the identification of thaumatin-like protein (TLP) in grapevine xylem sap. TLP is expressed in greater amounts in vines that have been exposed to cold temperatures and may be associated with the cold curing phenomenon. Currently we have cloned and expressed TLP in *E. coli*. Producing TLP in *E. coli* should allow us to produce enough protein to better evaluate the role of TLP in curing of PD. Crude extracts of *E. coli* expressing TLP were applied to PD3 medium plates and initial results showed adding TLP to PD3 growth media greatly inhibited the growth of one strain of *Xylella fastidiosa*. We are currently preparing to make transgenic grapevines that express this protein at higher levels. Previous work also identified polyphenolic compounds as a possible mediator of the "cold curing" phenomenon. Here, we show that a specific polyphenolic compound, resveratrol, is produced in vines that experience cold curing, while it is absent from grapevines grown in warmer environments. In a previous project, we were able to induce "cold curing" of PD in Davis, CA (an area that traditionally does not show extensive PD "cold curing") by applying the plant hormone ABA to the vines. This fall we applied ABA to PD-affected, field-grown vines in Napa.

INTRODUCTION
In our previous work we characterized many biological parameters of xylem sap from cold-exposed (freezing temperatures) and "warm," (non-freezing) temperatures in both field grown and cold chamber exposed grapevines. We found that *Xylella fastidiosa (Xf)*-infected potted grapevines that were exposed to freezing temperatures at several sites in Northern California and vines exposed to -5C in cold chamber emerged pathogen free the following summer (Meyer and Kirkpatrick, 2004-2008). We measured many different biological parameters, such as pH, organic acid, sugar and ion concentrations, and osomolarity in Pierce's disease (PD)-susceptible *Vitis vinifera* 'Pinot Noir' and PD-less susceptible *V. vinifera* 'Cabernet Sauvignon' grapevines over three winters.

One of the parameters determined in these previous studies was the protein profiles of cold- and warm-treated xylem sap. One of these proteins, a thaumatin-like protein (TLP), was significantly up regulated in cold exposed vines. We have cloned

and expressed the *V. vinifera* TLP protein and showed some inhibition of *Xf* growth when crude protein extracts from TLP-expressing *E. coli* were applied to PD3 medium plates.
We have also been assessing the potential role that xylem sap phenolic compounds may play in the "cold curing" process. In collaboration with the Waterhouse lab at UC Davis, we are characterizing the phenolic compounds in cold and warm xylem sap by HPLC/MS, and have identified that a major polyphenol in cold-exposed xylem sap is trans-resveratrol.

OBJECTIVES

1. Over-express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA and western blot analyses.
2. Inoculate TLP-expressing grapevines with Xf and determine the incidence and severity of PD in TLP-transgenic versus non-transgenic *V. vinifera*.
   a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
   b. Compare the phenolic compound composition and concentration in xylem sap extracted from cold- and warm-exposed *V.vinifera* grapevines as well as grapevines treated with ABA under non-freezing conditions.
   c. Determine if these compounds affect Xf growth/survival *in vitro.*
3. Determine if foliar and drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezingconditions.

RESULTS AND DISCUSSION

We have cloned and expressed *Vitis vinifera* thaumatin-like protein using the pet-30b (Invitrogen) protein expression vector in Novagen BL21(DE3) *E. coli* (**Figure 1**). To obtain recombinant TLP for anti *Xf* experiments, BL21 cells were grown overnight and harvested by centrifugation. The supernatant was discarded and the pellet was resuspended in 5 ml of 20 mM phosphate buffer pH 7.0. Lysozyme (2 mg/mL) was added and incubated for 30 minutes at room temperature on a shaker. The solution was then sonicated eight times at 10 second bursts. This suspension was centrifuged at 5000rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was purified in a modified protocol originally described by Daniell et al. (2000). The pellet was resuspended in 5ml of solubilisation buffer (100 mM Tris/Acetate, pH 8.6, 6 M guanidine-HCl, and 0.5mM EDTA). The solution was then passed through a 0.22 um filter.

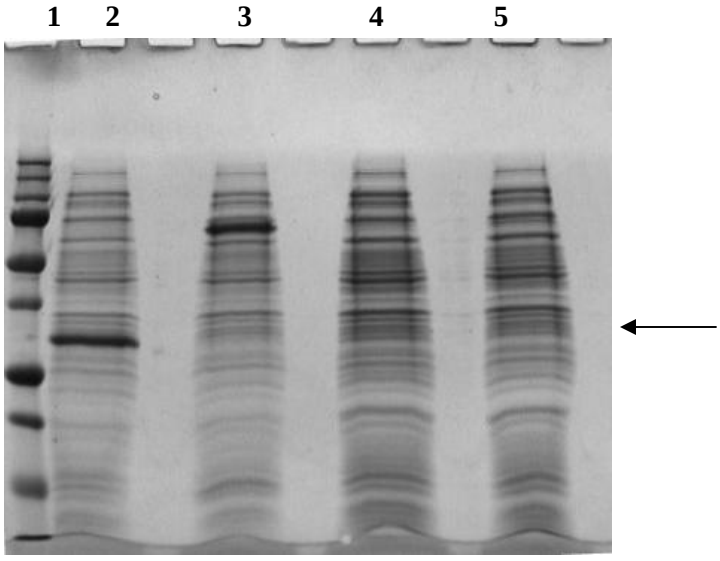

Figure 1. Thaumatin-like Protein (TLP) that was cloned and expressed using a *E.coli* expression vector. Note that arrow points to the correct size of grapevine TLP protein (~35 kD).

Lane 1: Dual color SDS ladder.
Lane 2: Cell lysate from IPTG induced *E.coli* with TLP construct.
Lane 3: Cell lysate from IPTG induced *E.coli* with a polygalacturonase (PG) construct (positive control).
Lane 4: Cell lysate from *E.coli* with TLP construct, not induced.
Lane 5: Cell lysate from *E.coli* with PG construct, not induced.

The filtered solution was then dialyzed for 16 hours at 4°C in 1 L of stirred phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.2), with 0.306 g/l oxidized glutathiones and 0.307g/l reduced glutathiones. The solution in the dialysis tubing was collected and centrifuged at 5000rpm for 30 minutes. The pellet and supernatant were separated. The pellet was resuspended in 5ml of phosphate buffer and passed through a sterile 0.22 um filter. The supernatant was also filter sterilized.

Sterile dialysis prepared protein suspensions and potassium phosphate buffer solutions were combined at a 1:1 ratio with 10-14 day old liquid grown *Xf*. *Xf* and deionized water combined at a 1:1 ratio, applied at 200ul/plate, provided baseline control for treatment comparisons. *Xf* was added at a ratio of 1:1 with the following suspensions: supernatant from the dialysis product for both the empty vector (Pet30b with no TLP insert) and the Cabernet TLP protein (CS3), pellet from the dialysis product resuspended in 20mM phosphate buffer (pH 7.0) for both the empty vector and the CS3 protein, and 20mM phosphate buffer (pH 7.0) only.

Survival of *Xf* was measured at by plating the bacterial suspensions at the following five time points: immediately after combining the buffer or protein suspension with the bacterial cells, 16 hours after the first sampling, 24 hours after the first sampling, 40 hours after the first sampling, and 48 hours after the first sampling. The plates were incubated at 28°C for 11-14 days, photographed, and the effectiveness of the treatments was determined visually (**Figure 3**).

Xylem sap total phenolics from ABA-treated and non-treated controls have been measured. Trends showing that total phenolics were found in higher concentrations in cold-exposed vines were also seen in the ABA-treated vines (Meyer and Kirkpatrick, 2008). In addition, the phenolic content in ABA-treated vines was higher than non-treated vines (**Figure 2**).

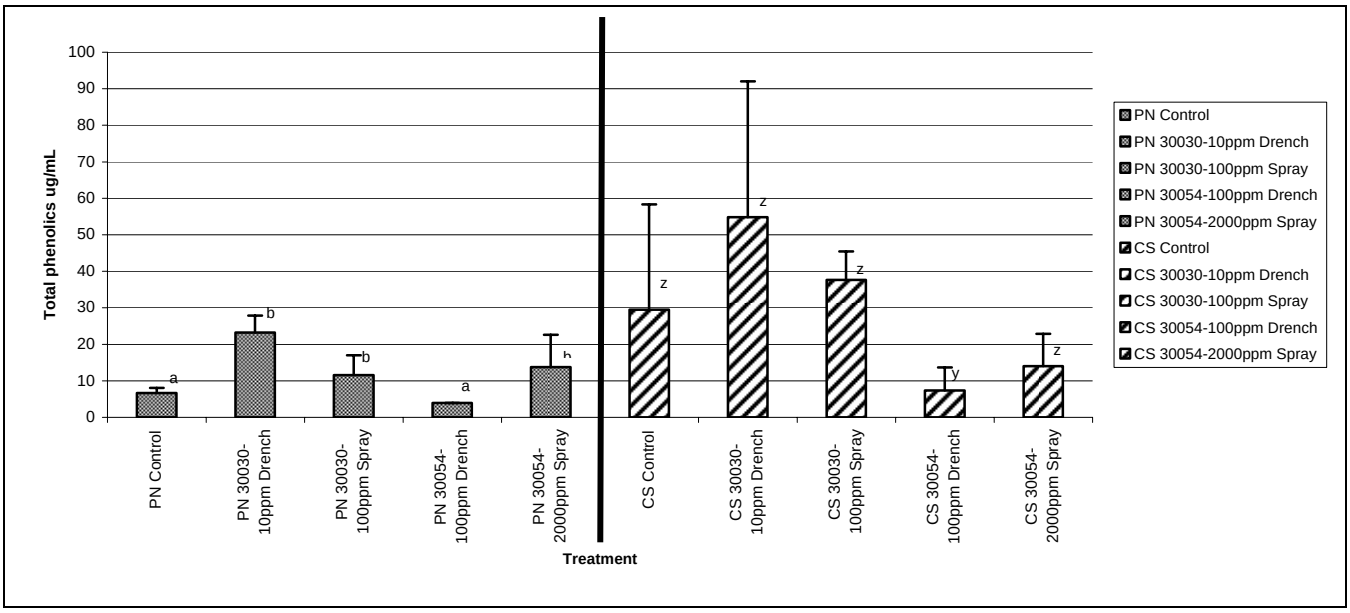

Figure 2. Total phenolic content of xylem sap from ABA treated vines as measured by a gallic acid colorometric assay. PN = Pinot noir xylem; CS = Cabernet sauvignon xylem sap. 30054 = a natural ABA, 30030 a chemically modified ABA. Different letters are significantly different by unpaired t-test with a 2-tailed p-value<=0.05

Xylem sap was expressed from dormant 'Cabernet Sauvignon' and 'Pinot Noir' grape vine canes obtained from the Chateau Leidigh Estate Winery located in Placer County in February, 2009. Previous work has shown that grape vines infected with *Xf* show significant recovery when exposed to cold winter temperatures, such as those experienced in this Placer county vineyard. As a control, we also collected sap from Davis grown vines where curing, due to warmer temperatures, is significantly less than that observed in Placerville. Sap was expressed by placing canes in a "pressure bomb", allowing one end of the cane to protrude from the cylinder, and then pressurizing the chamber with air to pressures between 300 and 400 psi, to collect the xylem sap exudate. These samples were kept frozen at -80C until they were analyzed by High Performance Liquid Chromatography/Mass Spectrometry by Mauri Anderson of the Waterhouse Lab.

The xylem sap samples were chromatographed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with electrospray ionization (ESI) mass spectrometry (MS), which produced good resolution. Phenolic compounds were identified based on retention time, UV spectra from diode array detection, and MS using commercially available reference standards. 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 polyphenol 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 resveratrol is present in vines that experience "cold curing" while it is absent in vines that do no undergo "cold curing" suggests that resveratrol may play a role in the curing process. Over the next two winters we will continue to make sap collections and hope to make a clear profile of what polyphenolic conmpounds are present in cold sap, as well as when in the year these polyphenolics appear. Last year we reported on the anti-*Xf* activity of trans-resveratrol *in vitro*. These results have been subsequently supported by Maddox et al. (2009).

In 2010 we began an effort to identify and quantify different polyphenolic compounds in xylem sap over the winter months. We collected sap from Pinot Noir and Cabernet Sauvingon vines (not infected with PD) in Winters, Ca (warm environment) and Placervlle, Ca (cold environment) during the months of January, February, March, and April. As of this writing, we have only been able to analyze the January Pinot Noir samples. In the cold samples we found B procyanidins (flavanoids), Catechin, Epicatechin, Caftaric acid, Coutaric acid, and Quercetin 3-glucuronide. The warm sap had the same polyphenolic profile. The the amount of each phenolic compound in these sap xylem samples have yet to be quantified, but based on relative signal strength, quercetin 3-glucuronide is present at concentrations seven fold higher in cold sap than in warm sap. We are currently working on analyzing the rest of the samples in order to gain a clearer understanding of what phenolic compounds are present in xylem sap, and when exactly they appear. We are also in the process of studying the effect on viability of *Xf* grown in these sap samples *in vitro*.

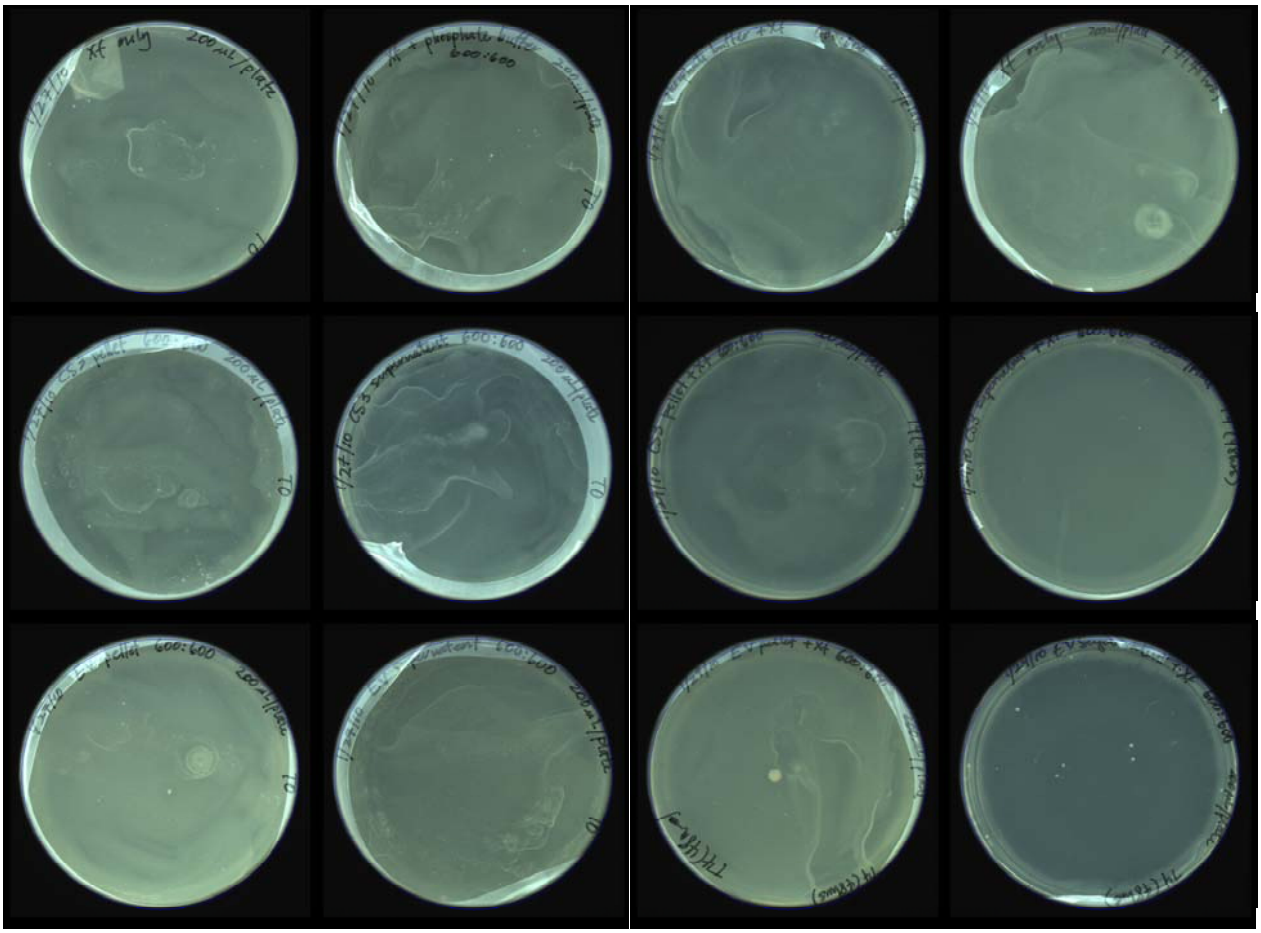

Figure 3. Dialyzed protein product plate assay results. Time 0=buffer/protein combined initially; 48 hours=48 hours after time 0. Treatments from the top left corner: column 1: *Xf* and water 10^8 CFU- time 0; CS3 pellet and *Xf*- time 0; Empty vector pellet-time 0; column 2: *Xf* and 0.2M phosphate buffer- time 0; CS3 supernatent and *Xf*- time 0; Empty vector supernatent and *Xf*- time 0; column 3: *Xf* and water- 48 hours; CS3 pellet and *Xf*- 48 hours; Empty vector pellet- 48 hours; column 4: *Xf* and 0.2M phosphate buffer-48 hours; CS3 supernatent and *Xf*- 48 hours; Empty vector supernatent and *Xf*- 48 hours.

CONCLUSIONS

The expression of recombinant *Vitis vinifera* TLP, and the subsequent plating experiments suggest that this protein may play a role in the cold curing process. We are currently preparing the appropriate constructs in order to make transgenic grapevines that over express TLP under non-freezing temperatures.

Phenolic compounds, specifically trans-resveratrol, show promise as agents that are harmful to the growth of *Xf*. The results of our plate assays are supported both in the literature and by the fact that we detected no resveratrol in warm winter sap collected in Davis, where we observe significantly less overwinter curing than in Placerville. It has been previously reported that resveratrol production in *Vitis vinifera* can be up-regulated by several diverse factors such as plant injury, UV light exposure, and pathogen invasion (Pryce et al. 1976, and Gautheron et al. 1991). It is possible that cold temperatures may serve as an external stress that increases the production of trans-resveratrol. Based on our preliminary 2010 data, in which higher concentrations of xylem sap phenolics were found in areas that experience cold curing suggest that elevated levels of xylem sap phenolic compounds may play a role in the cold curing process.

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