Title of Report: Interim Progress report for CDFA Agreement Number 04-0486

Project Title:
IDENTIFICATION AND UTILIZATION OF COLD TEMPERATURE INDUCED GRAPEVINE METABOLITES TO MANAGE PIERCE'S DISEASE

Principal Investor and Cooperators:

Principal Investigator
Bruce Kirkpatrick
Dept. of Plant pathology
University of California
Davis, CA 95616
bckirkpatrick@ucdavis.edu

Cooperator
Abhaya Dandekar
Dept of Plant Science
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Cooperator
Jim Lincoln
Dept. of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

Cooperator
George Kasun
Dept. of Plant Pathology
University of California
Davis, CA 95616
gekasun@ucdavis.edu

Cooperator
Andrew Waterhouse
Dept. Enology & Viticulture
University of California
Davis, CA 95616
Alwaterhouse@ucdavis.edu

Reporting Period: The results reported here are from work conducted December 2013 to April 2014

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 then cloned the TLP gene in an Agrobacterium binary vector and with the assistance of the UC Davis Plant Transformation facility we produced 13 independent Thompson seedless lines that were vegetatively propagated in the greenhouse. qRT PCR analysis of the transgenic lines showed good levels of TLP mRNA expression while non-transgenic Thompson seedless had very low levels of TLP expression. These vines were further propagated

and 12 reps of each transgenic line have been mechanically inoculated with wild type Xf. The severity of PD symptoms in the TLP transgenic vines will be compared with the severity of symptoms in non-transgenic controls and Xf cells will be quantified by culture and qPCR. .

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 characterized the phenolic compounds in the xylem fluid from cold and warm-exposed grapevines. Results of the phenolic compound analyses were reported in previous PD/GWSS Proceeding Reports.

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 and 2011 we applied ABA in the fall to mildly PD-infected Riesling vines growing in a vineyard in Napa. The severity of PD symptoms in the ABA-treated vines was compared to non-treated controls in October, 2011 and 2012. No statistically significant differences in the severity of PD symptoms was found between ABA-treated and non-treated vines.

OBJECTIVES

1) Over express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Characterize the levels of TLP expression in the TLP-transgenic lines using qRT-PCR.

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.*

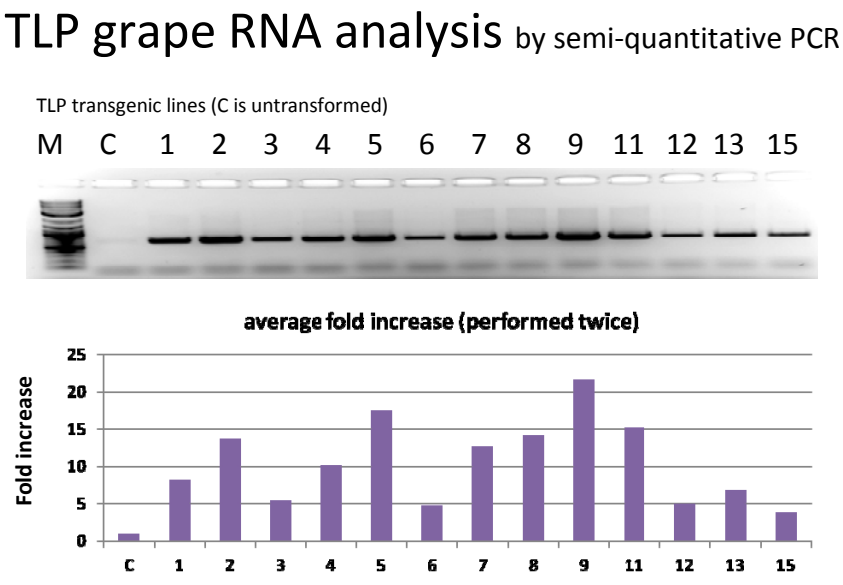
4) Determine if foliar or drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

Summary of Accomplishments and Results

Objective 1) Over-express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Characterize the levels of TLP expression in the TLP-transgenic lines using qRT-PCR.

The wt TLP gene from Thompson seedless grapevines was cloned into a Agrobacterium transformation vector that was developed in the Dandekar lab. This vector has signal peptide sequences that facilitate the expression and translocation of the TLP into grapevine xylem. We received 15 independently transformed TLP transgenic lines from the UC Davis Plant Transformation facility, 2 of those lines died before they could be vegetatively propagated. RNA was extracted from the remaining 13 lines as well as non-

transformed Thompson seedless grapevine controls using standard procedures. Equal amounts of total RNA were analyzed by a semi-quantitative RT PCR (qRT PCR) using primers specific for the grapevine TLP. Figure 1 below show the results of this analysis:



All of our lines show over-expression of TLP mRNA as compared to non-transgenic, wild type plants. We were please to find that TLP expression in greenhouse grown non-transgenic grapevines was quite low compared to the transgenic lines so if there is any benefit to over expressing TLP in "warm" temperature grown vines we should be able to see some phenotypic differences especially in the 7 lines whose TLP expression was 10 fold or greater than the non-transgenic control vines.

Objective 2) Inoculate TLP-expressing grapevines with Xf and determine the incidence and severity of PD in transgenic versus non-transgenic *V. vinifera*. Quantify Xf populations in transgenic lines and non-transgenic controls.

Ten reps of the TLP transgenic vines (13 distinct lines) and wild-type Thompson Seedless grapevines were mechanically inoculated using two 20µl drops of 10^7 (as measured by optical density) *Xf* Fetzer cells suspended in PBS. Control Thompson Seedless and TLP vines were inoculated with PBS. These vines were kept in the greenhouse and were observed for PD symptom development over 17 weeks. When symptoms first became apparent two of the TLP lines appeared to develop symptoms more rapidly than the Thompson Seedless plants. These differences proved to be not statistically significant. The Thompson Seedless and TLP progressed through symptom development with no significant differences (Figures 4 and 5). At 15 weeks bacterial cells

were isolated from the point of inoculation (POI) as well as 25cm above POI. There was no significant difference in Xf populations in TLP transgenics versus non-transgenic controls.

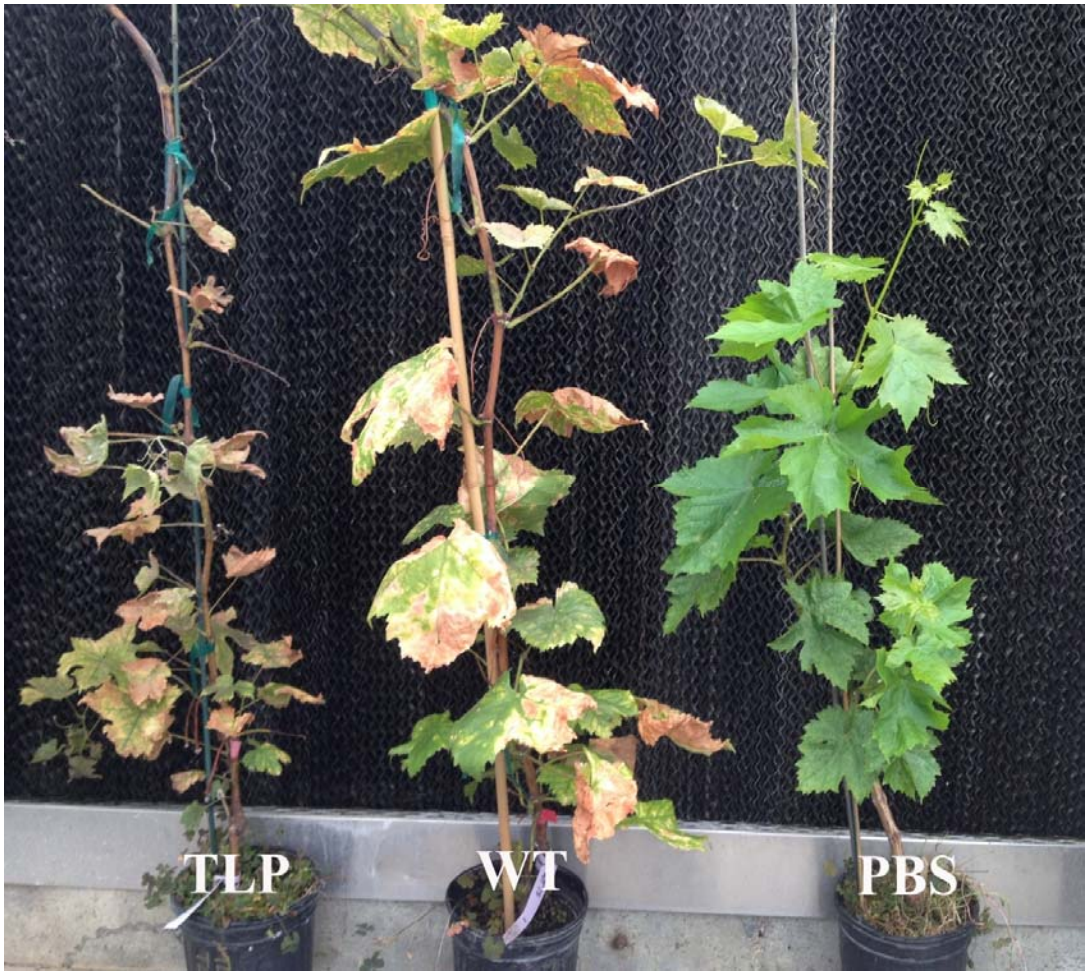


Figure 4: Representative PD symptoms in TLP-transgenic and non-transgenic Thompson seedless vines mechanically inoculated with wt Fetzer Xf or PBS controls 15 weeks post inoculation.

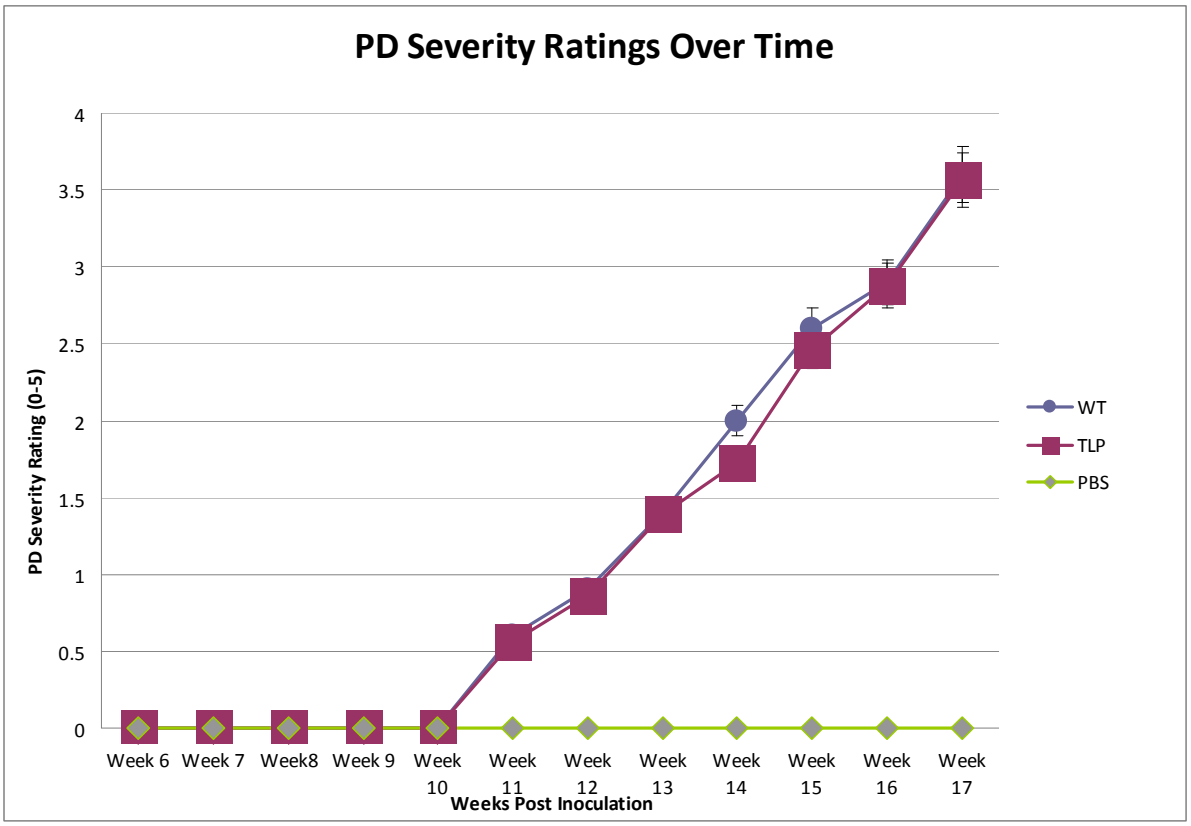


Figure 5: PD symptom severity following mechanical inoculation with wt Fetzer *X. fastidiosa.*

Total phenolic compounds were extracted and quantified from TLP-trangenic and non-transgenic controls and there were no significant differences in phenolic concentrations between TLP- and control grapes (data not shown).

Due to our past observations with TLP in non-transgenic grapes during the winter of 2013/14 we will over-wintering both the Thompson Seedless and TLP transgenic vines in an outdoor screen house at UCD to determine if elevated TLP levels could possibly encourage cold curing at temperatures that are not sufficient to produce cold curing in non transgenic grapes.

Objective 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.*

Results for this Objective was previously presented in two PD/GWSS Symposium Reports.

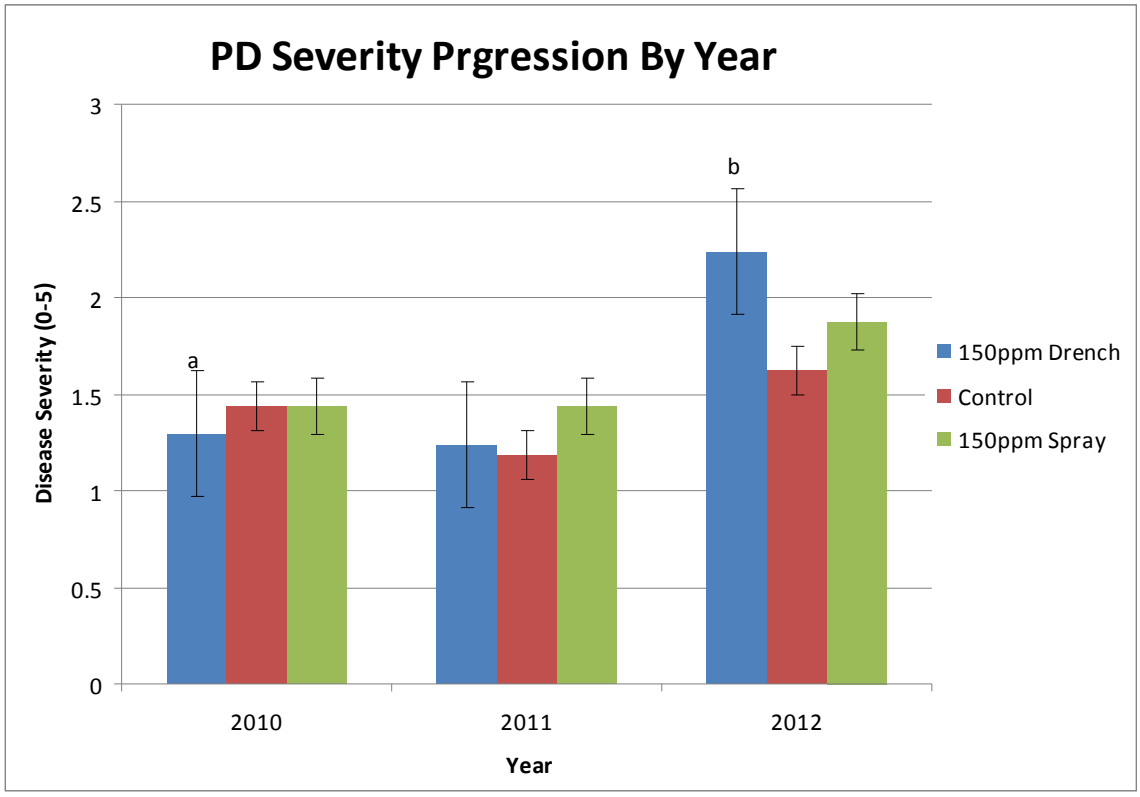
Objective 4) Determine if foliar or drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

In August 2010 we mapped and rated PD disease symptom severity of 45 Reisling vines, approximately 4 years old, that were growing in a vineyard near the Napa river. The majority of these vines had mild symptoms of PD, rated as a disease severity of 1 on a scale of 0 (healthy) to 5 (dead); approximate 3 to 4 vines in each group of 15 treatment and control vines were rated as a symptom category of 2.

15 of those vines were sprayed to run off with a 150ppm ABA solution prepared from a commercially available ABA product (Protone, marketed and kindly provided by Valent Chemical company). Approximately 2.5 L of ABA solution was sprayed with a backpack sprayer to each vine. A separate group of 15 PD-affected vines were drenched with 3 gallons each of a 150ppm solution ABA. The drench solution was applied into a shallow depression made on either side of the trunk of the vine. 15 diseased vines were left as untreated controls.

In August 2011 all of the vines were rated for PD symptom severity and the spray and drench applications were repeated on the same vines that were treated in 2010. In 2012 all of the vines were rated for PD symptoms and ABA applications were not repeated as analysis of PD disease severity showed no significant differences between the spray or drench treatment and the non-treated PD-affected control vines as shown in Figure 2 below. Actually the only significant difference that occurred was an INCREASE in the severity on the drench treatment rated in 2012.

FIGURE 2



It is likely that the significantly greater size of the foliar and root zones in the field-plant vines, compared to the 1 gallon potted vines that did respond to ABA in our previous research, did not permit sufficient ABA to be systemically acquired and cause a beneficial effect on the severity of PD symptoms.

Publications:

Lieth, J.H., M.M. Meyer, K.-H. Keo, and B.C. Kirkpatrick. 2011. Modeling cold curing of Pierce's disease in *Vitis vinifera* grapevines in California. Phytopathology 101:1494-1500.

Kirkpatrick, B. 2012. Evaluation of Pierce's disease resistance in transgenic *Vitis vinifera* grapevines expressing either grape thaumatin-like protein of *Xylella fastidiosa* hemagglutinin protein. Pierce's disease Research progress Reports, California Department of Food and Agriculture. pp. 130-136.

Kirkpatrick, B. 2012 identification and utilization of cold temperature induced metabolites to manage Pierce's disease. Pierce's disease Research progress Reports, California Department of Food and Agriculture. pp. 137-141

Research Relevance Statement:

We have verified and further expanded observations made in the Purcell lab that PD-affected vines exposed to cold winter temperatures emerge pathogen-free in the spring. We found that total concentrations of phenolic compounds in grapevine xylem sap were much higher in cold exposed vines than vines that overwintered in Davis. Earlier work by ourselves and other researcher showed that many of these phenolic compounds are toxic to Xf. We also observed that xylem sap extracted from cold exposed vines had much higher levels of grapevine thaumatinin-like protein (TLP) than sap from warm exposed vines. TLP has been shown to possess antimicrobial activity as well as regulate the synthesis of other antimicrobial compounds. We hypothesize that over expression of TLP under warm temperature conditions might result in vines that possess some resistance to PD. To that end we produced, in collaboration with the UC Davis Plant Transformation facility, 13 independent lines of transgenic grapevines that expressed TLP mRNA at much higher levels than non-transformed grapevines growing in the greenhouse. Pathogenicity assays are now under way to evaluate the potential resistance of these transgenic lines to PD. If successful we will determine if the TLP transformed vines can be used as rootstocks to impart PD resistance to non-transgenic grapevine scion varieties.

Layperson Summary of Accomplishments:

Research conducted in this project demonstrated a correlation between increased levels of phenolic compounds in the xylem sap of cold-exposed grapevines compared to phenolic levels in sap extracted from vines exposed to moderate winter temperatures. Several of these phenolic compounds were shown to be toxic to Xf in *in vitro* studies. However it is difficult to envision a method of elevating phenolic compound levels to sufficient levels to be toxic to Xf without potentially changing the characteristic of juice extracted from berries to make wine Similarly, higher levels of antimicrobial thaumatin-like protein (TLP) were found in the xylem sap of cold exposed vines as compared to xylem sap extracted from vines exposed to more moderate winter temperatures. Given the correlation between elevated levels of TLP in cold-exposed vines and higher rates of cold-curing we produced transgenic grapevines that over express TLP as compared to non-transgenic vines. All of the 13 TLP-transgenic lines produce significantly higher levels of TLP mRNA compared to non-transgenic vines. Twelve reps of each of the 13 TLP transgenic lines were inoculated with wild type X. fastidiosa in the greenhouse. The vines, as well as inoculated non-transgenic controls are being rated for PD symptom expression and in late September 2013 population levels of Xf will be determined by culture and qPCR. Total concentration of phenolic compounds in grapevine xylem sap will also be determined in September.

Field applications of ABA to vines with mild symptoms of PD, showed that vines treated by spraying or drench 150ppm of ABA did not result in any reduction in PD symptoms which we previous observed in experiments using 1 gallon potted grapevines. This is most likely due to the much larger mass of roots and canopy in the field vines compared to potted vines. Pressure injections of ABA into field vines may be one option for increasing the efficacy of ABA application, but it is unlikely that a grower would be willing to go to the extra cost of vine trunk injections.

Status of Funds:

As per 7/24/2013 there remained $22,043 in this grant which was granted a no cost extension until 6/30/2014.