Interim Progress Report for CDFA Contract Number: 04-0486

Project Title: Identification and utilization of cold temperature induced grapevine metabolites to manage Pierce's disease.

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Time Period: January 2010-February 2012

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 decreased the viability of the Xf cells compared to incubating the Xf cells with other appropriate controls. In February 2012 we cloned the TLP gene in an Agrobacterium binary vector with the intention of over-expressing the TLP in transgenic grapevines. The construct was given to the UCD Plant transformation facility that is now in the process of producing TLP-expressing Thompson seedless grapevines. Recent technical advancements have decreased the production time of transgenic grapevines from 14 to 8 months, so we should receive the 12 independent transformed lines sometime in late 2012. Once TLP-transgenic grapevines are vegetatively propagated they with be screened by RT-PCR for TLP gene expression and xylem saps will be screened for TLP by ELISA using anti-TLP antibodies. Following the expression studies, 10 vines from each line will be inoculated with Xf using mechanical and insect inoculation (courtesy of the Alemida lab at UC Berkeley). . 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 foliar and drench applications in the fall to PD-infected vines growing in a vineyard in Napa. The severity of PD symptoms in the ABA-treated vines was rated in October 2011; unfortunately there were no significant differences in disease severity of treated versus non-treated controls. The ABA applications were again applied to the same vines in October 2011 and they will be rated for disease severity in October 2012.

List of 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.

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.

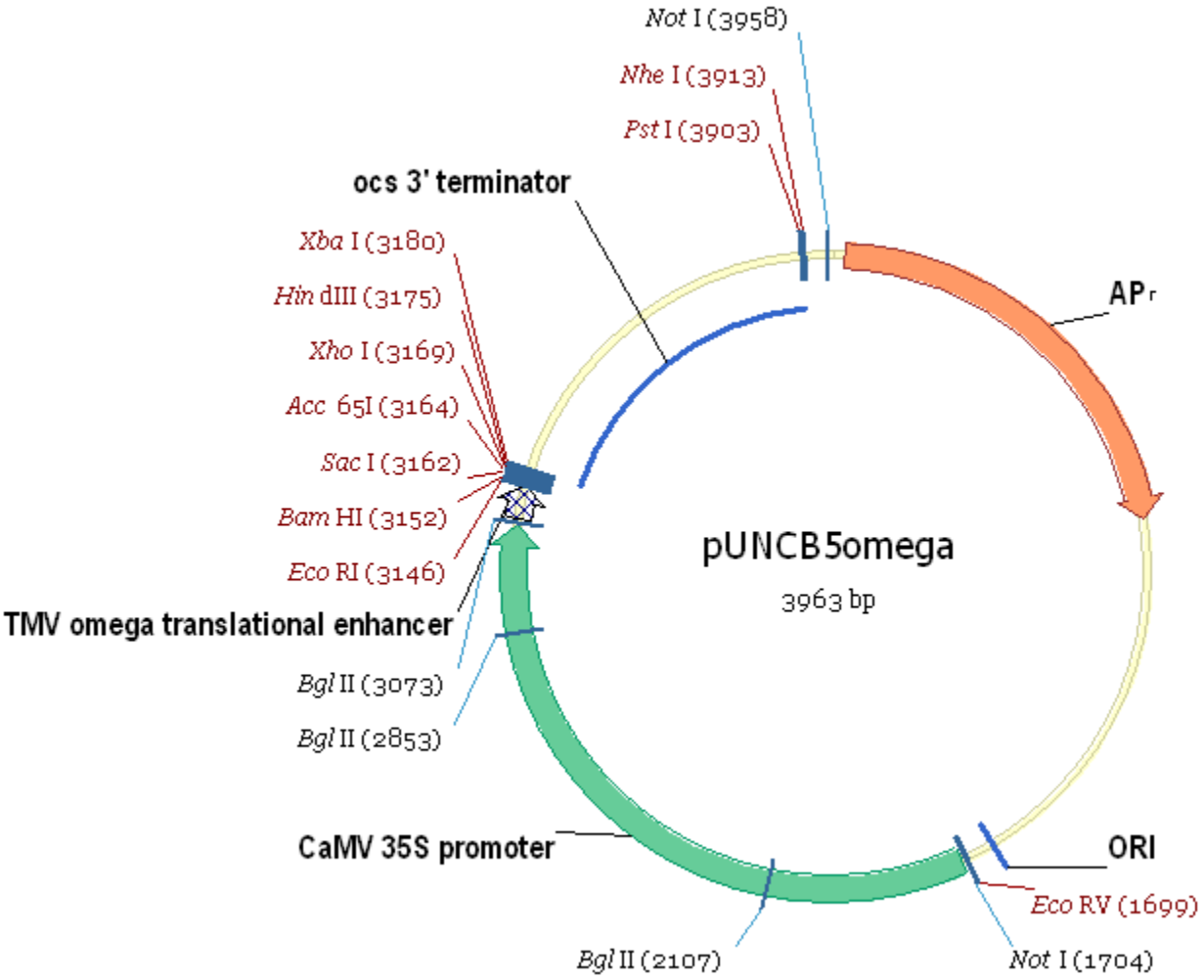
Procedures and Results to Date for each Objective

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.

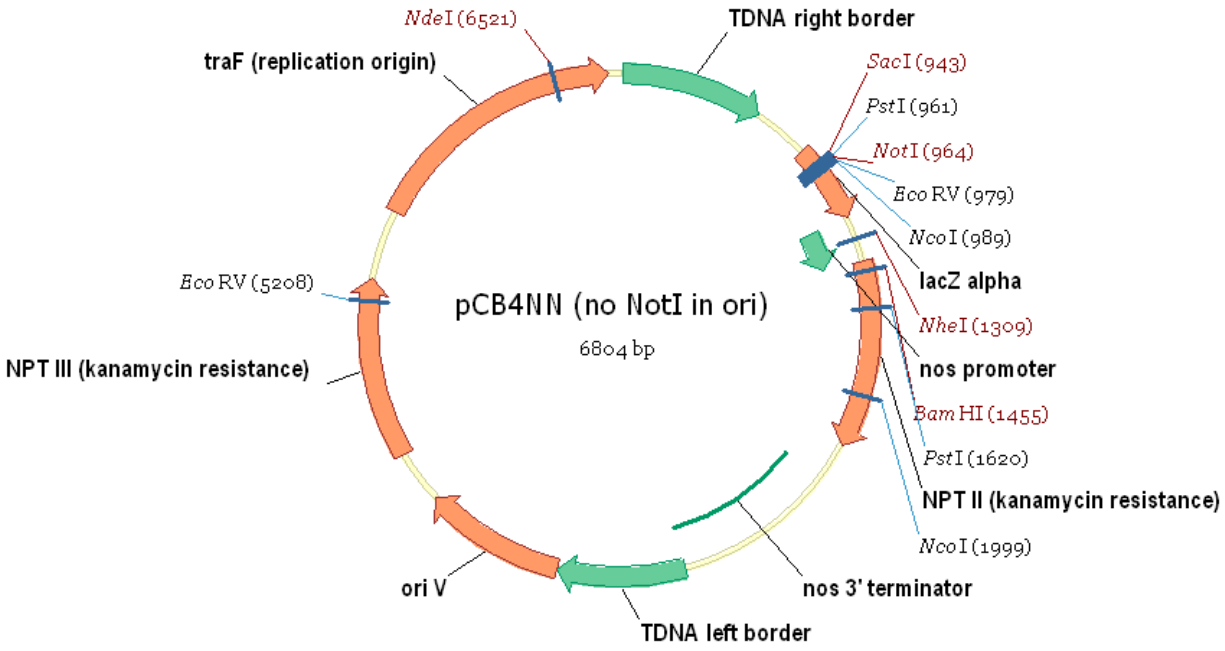
In February 2012 we cloned the TLP gene in an Agrobacterium binary vector with the intention of over-expressing the TLP in transgenic grapevines. The construct was given to the UCD Plant transformation facility that is now in the process of producing TLP-expressing Thompson seedless grapevines. Recent technical advancements have decreased the production time of transgenic grapevines from 14 to 8 months, so we should receive the 12 independent transformed lines sometime in late 2012. Details of the cloning strategy and vectors used are shown below:

The TLP construct was made by PCR amplification of *Vitis vinifera* "Chardonnay" genomic DNA using primers designed from the sequence found in Genbank AF227324. This works because TLP has no introns. A BamHI site was added to the 5' end and a XhoI site was added to the 3' end during the PCR. The two oligos used were:
5'GCGGATCCATGCGCTTCACCACCACCCTCCCAATTC3' and
5'GCGCTCGAGTCATCAAGGGCAAAACGTGACCTTGTAGTTG3' This amplified a 666bp fragment that was cloned into BamH1 and Xho1 cut plasmid pUNCB5omega. This will express

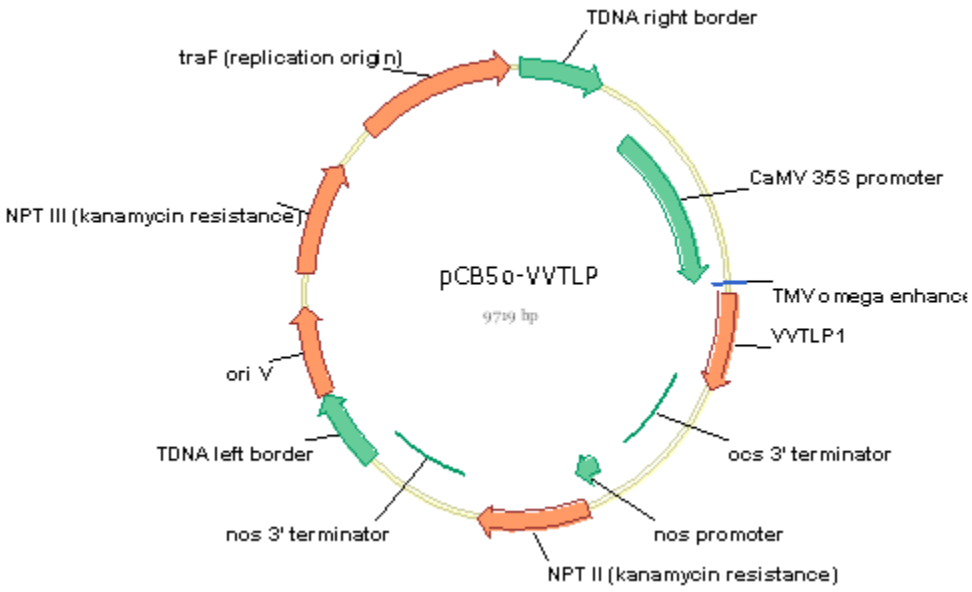
the VVTLP from a strong constitutive promoter (CaMV35S) and uses a translational enhancer from TMV (omega) for strong translation.



After sequence verification of the new plasmid pUNCB5omega-VVTLP, it was cut with Not1 and the TLP containing fragment was ligated to the binary vector pCB4NN which had been cut with Not1.



This completes the binary and it looks like:



Agrobacterium tumefaciens strain LBA4404 was transformed and selected on kanamycin. This agro strain was given to David Tricoli at the UCD Plant Transformation facility.

We are also in the process of making a larger quantity of pure recombinant TLP to be given to the Comparative Pathology Laboratory at UC Davis for polyclonal antibody production. TLP-specific antibodies will subsequently be used for quantification of TLP in the transgenic grapevines.

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

This is dependent on first acquiring grapevines transformed with TLP, which is a goal for the coming year.

3) a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.

In 2010 and 2011we 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 are further analyzing the MS/HPLC data after two winters of collecting sap, and hope to construct an accurate picture of what happens with regards to polyphenolic concentrations during the winter.

b. Compare the phenolic content of xylem sap of field grown grapevines treated with ABA under non-freezing conditions to phenolics in cold-exposed xylem sap.

If the applications of ABA to field grown vines shows promise as a means of inducing the cold curing process under non-freezing conditions then we will collect sap from ABA-treated field vines at the time of application in early November and bi-monthly collections thru the winter. Initial analysis of PD-affected vines growing in a Napa vineyard that were treated with foliar and drench applications of a commercially available, and registered ABA compound (Protone SG, Valent BioSciences) showed no significant disease severity symptom differences between treated versus non-treated PD-affected vines. If subsequent applications of ABA produce significant differences in disease severity then xylem saps will be extracted using a pressure bomb and analyzed by the Waterhouse lab for presence and relative quantities of phenolics.

c. Determine if these compounds affect Xf growth/survival *in vitro*.

The paper by Maddox et. al. published in October of 2009 in the journal Current Microbiology examined the effect that different polyphenolic compounds on *Xf* growth *in vitro*. One of the compounds they tested was trans-resveratrol. They found that this compound inhibited the growth of *Xf in vitro*, which is interesting because in our analysis of "cold sap" we found that resveratrol was present, while it was absent in "warm sap. However the concentrations used in

the Maddox study may not reflect the actual concentrations of phenolics that are present in grapevine xylem sap. The determination of actual concentrations of various phenolic compounds in sap is a time-consuming endeavor which the Waterhouse lab is now in the process of undertaking.

We also added purified trans-resveratrol to solid media used to grow *Xylella fastidiosa* 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 vines 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.

In the coming months we will incubate cultured Xf cells with xylem sap extracted from grapevines exposed to "cold" and "warm" winter temperatures. Following a defined incubation period the cells will be plated out on PD3 medium and viable CFUs exposed to warm and cold saps will be compared.

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

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 with relatively mild winter temperatures that do not typically induce PD cold curing. Our previous work also showed that these vines had higher levels of polyphenolics than did vines growing in Davis which did not receive ABA applications (Meyer and Kirkpatrick, 2011).

In 2010 we applied ABA foliar and drench applications in the fall to PD-infected Riesling vines growing in a vineyard in Napa. There were a total of 45 vines used in the study; all vines were initially rated for PD disease severity using a 0 to 4 scale, where 0=healthy and 4 =dead. Only vines rated as 1 or 2 were used as test vines. Fifteen vines were left as untreated controls. Fifteen vines were sprayed to run with a 100ppm commercial formulation of ABA (Protone SG, #30054, kindly provided by Valent BioSciences Company) off using a backpack sprayer. Fifteen vines were drenched with 3 gallons of a 10ppm solution of the same ABA. The severity of PD symptoms in the ABA-treated vines was rated in October 2011; unfortunately there were no significant differences in disease severity of treated versus non-treated controls. The ABA applications were again applied to the same vines in October 2011 and they will be rated for disease severity in October 2012.

Publications:

1) Meyer, M. and B.C. Kirkpatrick. 2011. Exogenous applications of abscisic acid increase curing of Pierce's disease-affected grapevines growing in pots. Plant Disease 95: 173-177.

2) We have completed the first draft of a paper describing the work we have done cloning TLP in *E. coli*, and the results of toxicity screens that were performed with recombinant TLP.

Research Presentations:
We presented this work at the 2010 and 2011 Pierce's Disease Research Symposium both in the Symposium Proceedings and in poster form.

Kirkpatrick, B. 2010. Identification and utilization of cold temperature induced grapevine metabolites to manage Pierce's disease. PD/GWSS Research Symposium Proceedings, San Diego, California December 15-17. pgs 191-195

Kirkpatrick, B. 2011. Identification and utilization of cold temperature induced grapevine metabolites to manage Pierce's disease. PD/GWSS Research Symposium Proceedings, Sacramento, California December 13-15. pgs 132-135.

Research Relevance:

Both the TLP and polyphenolic aspects of this project have shown promise as agents that can inhibit the growth of *Xf*. We are hopeful that further evaluating these results in grapevines growing in the field may lead to a novel approach to managing PD. Furthermore, because TLP is naturally found in grapevines, the public may be more open to the idea of over-producing TLP in modified grapevines as opposed to the introduction of an exogenous, non-grapevine gene product. Similarly, if the application of the plant hormone ABA shows an increase in anti- *Xf* activity the general public may accept this more readily than a genetically modified grapevine. However, there are significant differences between treating Xf-infected grapevines growing in 1 gallon pots in the lath house compared to treating large PD-affected vines growing in the field. Even if the application of ABA to field infected vines shows promise we will have to determine the costs involved with purchasing and applying ABA in the field. ABA applications on grapevines growing in California have been registered, largely for increasing the color of table grapes.

Layperson Summary:

We have succeeded in producing recombinant TLP in *E.* coli. W*e* have also observed that grapevine TLP produced by *E. coli* has a deleterious effect on *Xylella fastidiosa* 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 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 *Xylella fastidiosa* 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.

Status of Funds: As per March, 2012 approximately 1/3 of the appropriated funds were unspent. This was largely because the P.I. elected to spend his "19900" funds that he was able to save for 20+ years on salary and benefits of individuals working on this project. This decision was made because in July, 2012 19900 funds will no longer cover the cost of benefits, as they have done in the past. This was the most appropriate use of these funds for my research on PD. A no cost extension request has been submitted that will allow the CDFA funds associated with this project to be spent through June, 2013.

Intellectual property associated with this project

There is no specific intellectual property associated with this project. The TLP Agrobacterium constructs are the same as those used by the Dandekar lab. We chose to use these constructs because they have been successfully used to transform grapevines in the past.

References cited:

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Kuwabara, C., D. Takezawa, T. Shimada, and K. Arakawa. 2002. "Abscisic acid and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mold, *Microdochium nivali"*. Physiolgia Plantarum 115: 101-110.

Langcake, P amd Pryce, RJ. "The production of resveratrol by Vitis vinifera and other members of the Vitaceae as a response to infection or injury Physiological Plant Pathology" p. 77-86 (1976).

Maddox, C. E., L. M. Laur, and L. Tian "Antibacterial Activity of Phenolic Compounds Against the Phytopathogen *Xylella fastidiosa"* Curr Microbiol. 2010 January; 60(1): 53–58.

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