"Interim Progress Report"
Project Title: HNE-CecB and PGIP transgenic grapevines field trial.

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Introduction
'Thompson Seedless' (TS, *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from a neutrophil elastase (NE) that specifically binds to the *Xylella fastidiosa*(*Xf*) outer-membrane protein MopB and a lytic domain, Cecropin B (CB) that clears *Xf* the causative agent for Pierce's Disease (PD) in grapevines (Dandekar et al., 2012). We have also similarly transformed TS grapevines with a gene encoding polygalacturonase inhibitory protein (PGIP) that results in the expression of a PGIP that inhibits the action of a polygalacturonase (PG), a virulence factor expressed by *Xf,* to interfere with long distance movement of *Xf* that provides resistance to Pierce's Disease in grapevine (Aguero et al. 2005). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions and lines several lines that showed resistance to PD as compared to controls were identified by mechanically inoculating with *Xf* (Dandekar et al. 2012).

Selected transgenic grapevine plants expressing either NE-CB or PGIP, own-rooted or grafted with untransformed Thompson Seedless (TS), were planted in 2010-11 and are being tested for PD resistance under field conditions in two locations. At the Riverside County, a site with PD pressure, plants have been naturally infected, PD symptoms have been detected and *Xf* has been detected in petiole extracts and xylem sap by ELISA and plating. At Solano County site where plants were mechanically inoculated, PD symptoms have also been detected and *Xf* has been detected in petiole extracts using an ELISA assay.

List of objectives
The goals of this project are to finish the field test of 4 NE-CB and 4 PGIP transgenic grapevine clones, to evaluate their horticultural characteristics and their resistance to PD. Transgenic grapevines have been tested in two field locations as non-grafted plants and as transgenic rootstocks grafted with wild type grapevine scion. One field location has PD pressure and plants were naturally infected with *Xf* and in the location with no PD pressure, grapevines were mechanically inoculated with *Xf* .

Objective 1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *Xf* infection in xylem tissue and to pass through the graft union under field conditions.

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.
Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
Activity 3. Evaluate PD resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.

Description of activities conducted to accomplish each objective

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.
Four independent transgenic events expressing HNE-CecB (40-41, 40-89, 40-92 and 41-151) and four expressing different PGIP constructs (31-25, 45-77, 52-08, TS50) were planted in two experimental fields. Two hundred and ten transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, were planted in Riverside County on 5/18/10 and the remaining 10 were planted on 3/6/11, completing the planting at this location **(Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on 8/2/2010 and 110 vines grafted with untransformed TS scions on 6/27/11 in Solano County, completing the planting at this location (**Table 1**). HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been genotyped, confirming the presence of the inserted transgene in all lines.

Table 1. Transgenic and control grapevines planted at Riverside and Solano fields

Non-grafted		**Grafted**	
Event ID (Vector)	**# Planted**	**Event ID (Vector)**	**# Planted**
HNE-CecB lines			
40-41 (pDU04.6105)	12	40-41G (pDU04.6105)	12
40-89 (pDU04.6105)	12	40-89G (pDU04.6105)	12
40-92 (pDU04.6105)	12	40-92G (pDU04.6105)	12
41-151 (pDU04.6105)	12	41-151G (pDU04.6105)	12
PGIP Lines			
31-25 (pDU05.1002)	12	31-25G (pDU05.1002)	12
45-77 (pDU06-0201)	12	45-77G (pDU06-0201)	12
52-08 (pDU05.1910)	12	52-08G (pDU05.1910)	12
TS50 (pDU94.0928)	12	TS50G (pDU94.0928)	12
Control line			
TS	16	TS-G	12

Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
To verify that horticultural and varietal characteristics of the parental genotype TS were unchanged, HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano and Riverside Counties were evaluated phenotypically in September 2011 and November 2011, respectively. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV, 1983). The descriptors used were 1) aperture of young shoot tip/opening of young shoot tip, 2) density of prostrate hairs between main veins on 4th leaf lower side of blade, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on 4th young leaf, 5) shape of mature leaf blades, 6) number of lobes on mature leaf, 7) area of anthocyanin coloration

on main veins on upper side of mature leaf blades, 8) shape of teeth on mature leaves, 9) degree of opening of mature leaves/overlapping of petiole sinuses, 10) mature leaf petiole sinus bases limited by veins, 11) density of prostrate hairs between main veins on lower side of mature leaf blades, and 12) density of erect hairs on main veins on lower sides of mature leaf blades. Riverside and Solano Counties, HNE-CecB and PGIP-expressing transgenic grapevines lines were also phenotypically evaluated in the fall of 2012 and will be evaluated in the fall of 2013, the evaluation will include fruit shape, color and size. Up to date no difference between transgenic grapevines and parenteral genotype TS were observed.

Activity 3. Evaluate PD resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.

Grafted and non-grafted transgenic grapevine lines naturally infected in the field planting in Riverside were scored for Pierce's disease symptoms using a 0-4 scale, where 0 = healthy no PD symptoms, 1 = a few leaves on a few shoots that are symptomatic on cane(s), 2 = Many symptomatic leaves on multiple canes (in a mature bilateral cordon trained vine), 3 = dieback/death of canes/codons, and 4 = death of whole vine (Fig. 1). Stem samples from Riverside grapevines were harvested and number of *Xf* cells were determined using ELISA kit from Agdia, the standard curve was created using *Xf* from liquid culture (Fig. 2). Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in Riverside County field.

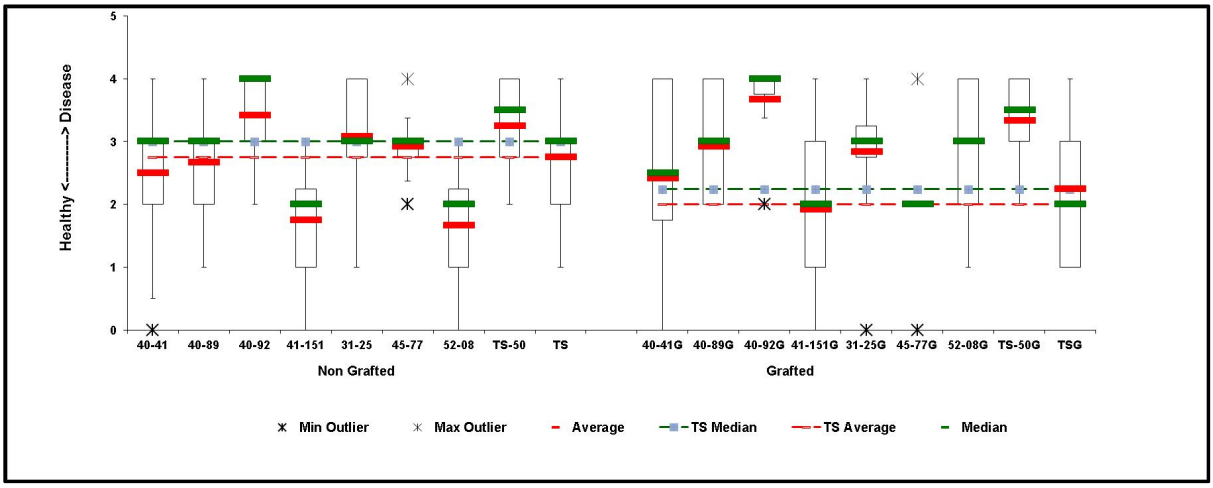

Figure 1: Riverside Pierce's Disease symptoms scoring. Spring of 2013

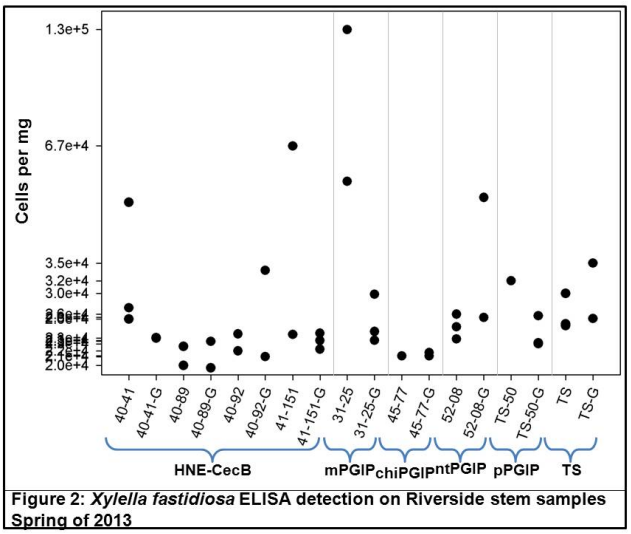

Figure 2: ***Xylella fastidiosa*** **ELISA detection on Riverside stem samples Spring of 2013**

Non-grafted transgenic grapevine lines in Solano County field were manually inoculated as described by Almeida et al., (2003) for the first time in July 2011, for a second time on May 2012 and for a third time on June 2013. The manually inoculated runners of grafted and non-grafted transgenic grapevine were scored for Pierce's disease symptoms using a 0-1 scale, where 0 = alive runner and 1 = dead runner (Fig. 3). Stem samples from runners in the Solano plot inoculated in 2011 or 2012 were harvested in spring 2013 and number of *Xf* cells were determined using ELISA kit from Agdia, the standard curve was created using *Xf* cells obtained from liquid culture (Fig. 4). Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in Solano County field. Solano non-grafted and grafted grapevines that were not inoculated in the previously two inoculations (2011 and 2012) were manually inoculated on June 17, 2013, completing the manual inoculation of all grapevines at this location.

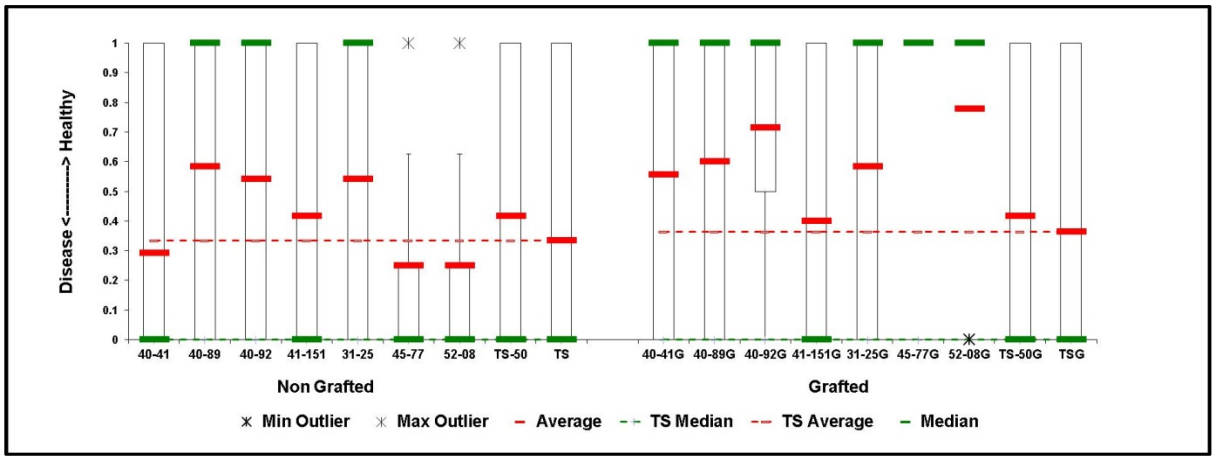

Figure 3: Solano Pierce's Disease symptoms scoring. Spring of 2013

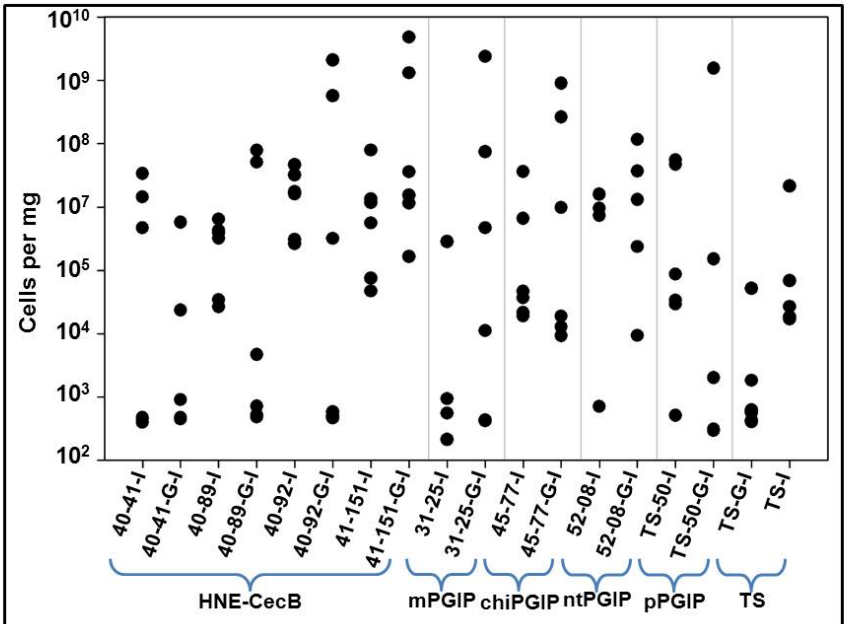

Figure 4: *Xylella fastidiosa* ELISA detection on Solano stem samples. Spring of 2013

Summary of accomplishments and results for each objective

We have successfully established two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's Disease (PD). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements, enhancing long distance movement and vector transmission. The first strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *Xf* PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, HNE-CecB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum size, curbing the spread of PD in California vineyards.

Transgenic grapevine plants expressing either PGIP or HNE-CecB along with untransformed controls have been successfully planted in two locations. In Riverside County, planting was completed with 220 vines in the ground: 210 planted on 05/18/2010 with the remaining 10 planted on 03/06/2011. In Solano County, where planting was also completed with all 220 vines in the ground, 110 were planted on 08/02/2010 and the remaining 110 on 6/27/2011. These transgenic grapevines have been evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Riverside and Solano County have been evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypical/horticultural differences were observed between transgenic and untransformed TS vines. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been genotyped, confirming the presence of the inserted transgene in all lines. At the Riverside County site, the plants have been naturally infected by wild populations of GWSS and *Xf* presence in petioles extracts was confirmed by ELISA, PCR, and plate cell count in fall 2011. *Xf* presence was also confirmed in Riverside xylem sap samples collected in spring 2012, in petiole's extracts collected in fall 2012 and in stem extracts collected in the spring 2013. PD symptoms were assessed using a standardized score based on percentage of leaf area scorching earlier now we are using a 0-1 and a 0-4 scale to validate resistance to PD under field conditions. At the Solano County site, non-grafted vines were mechanically inoculated with the *Xf* type strain (Temecula 1) in 2011 to validate resistance to PD under field conditions, *Xf* presence was confirmed by ELISA in fall 2011, but no *Xf* growth in plate or PD symptoms were detected. Solano County grafted plants were for the first time mechanically inoculated with *Xf* and non-grafted plants were re-inoculated on spring 2012. Leaf scorching the characteristic symptom of PD was observed in Solano Country for the first time in fall 2012 and *Xf* presence was confirmed by ELISA in petiole extracts collected in the same season and in stem samples collected on the spring of 2013. Solano non-grafted and grafted grapevines that were not inoculated previously were manually inoculated on June 2013, completing the manual inoculation of all grapevines.

For field trials, please include information on the status of the field trial, including planting and sampling activities, the condition of the plants, and any factors impacting the progress of the field trial. Also, please include photos of the field planting.

Riverside and Solano fields planting were completed on 03/06/2011 and 6/27/11, respectively. After each of the fields were planted completely no additional planting activities have been made. At Riverside field petioles and leaves were sampled, on 9/26/2011 and 10/17/2012, xylem sap was sampled on 4/2/2012 and stems samples were sampled on 5/10/13. At Solano Field petioles and leaves were sampled on 9/14/2011, 10/4-2011 and 10/22/2012, xylem sap was sampled on 4/30/2012 and stems were sampled on 4/26/13.

Figure 5. Transgenic grapevines planted in Riverside (left; Summer 2012) and Solano County (right; Spring 2013).

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

This research aims to provide a transgenic solution to the Pierce's Disease problem for the grower community of California. The objectives described in this proposal directly address the number 1 RSAP priority outlined in the, "Accelerate regulatory process". Establish and facilitate field trials of current PD control candidate vines / endophytes / compounds in multiple locations" handout released in the December 2009 Pierce's Disease Research symposium that outline the "Top 5 to 10 Project Objectives to Accelerate Research to Practice". This document updates the priority research recommendations provided in the report "PD/GWSS Research Scientific Review: Final Report" released in August 2007 by the CDFA's Pierce's Disease Research Scientific Advisory Panel.

Layperson summary of project accomplishments.

Four hundred and forty (440) transgenic grapevine plants expressing either polygalacturonase-inhibiting protein (PGIP; 192 plants) or a chimeric antimicrobial protein (HNE-CecB; 192) and 56 control untransformed vines have been planted in two locations, one in Riverside County (220 plants) and the other in Solano County (220 plants). Exactly half of these transgenic grapevines are being evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's Disease (PD) in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa (Xf)* in the water-conducting xylem of the vine through expression of PGIP. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissue by expressing HNE-CecB. At the Riverside County site, natural *Xf* infection has been confirmed in petioles and xylem sap by ELISA and appear to be uniform. At the Solano County site, about 25% of the plants were mechanically inoculated in 2011, another 25% in 2012 and the remainder 50% in 2013. The presence of *Xf* was confirmed in petiole and stem extracts of grapevines using the ELISA assay. We have used various phenotyping scoring techniques and while these are not perfect we can clearly see lines that consistently score better than the control for both strategies and those that do not. We also observe that those lines that show resistance are also able to transmit their resistance from the rootstock. However, the resistance transmitted from the rootstock is weaker when compared to that obtained with a transformed plant. Further observations in the next two years will give further confidence of the ability of the elite lines that we have identified to provide resistance to Pierce's Disease.

Status of funds.

We have expended all funds from Feb to June 2013 and have begun expending the July 2013 through June 2014 funding.

Summary and status of intellectual property associated with the project.

The intellectual properties issues connected with the specific constructs and approach have not been investigated in any formal investigation. However, this needs to be done when the elite lines are identified and need to be patent protected. Disclosures will be made at that point to the UC office of technology transfer, which could develop these further as a US patent variety.

Literature cited.

Aguero, C.B., C.P. Meredith, and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45:1-8.

Almeida, R.P.P., and A.H. Purcell. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. App. Env. Microbiol. 68:7447-7452.

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