

Progress Report for CDFA agreement number 09-0729

Project Title: HNE-CecB and PGIP transgenic grapevines field trial.

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LAYPERSON SUMMARY

Transgenic grapevine plants expressing either polygalacturonase-inhibiting protein (PGIP) or a chimeric antimicrobial protein (HNE-CecB) have been planted in two locations, one in Riverside County and the other in Solano County. These transgenic grapevines are being evaluated both 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 the expression of polygalacturonase-inhibiting protein. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissues through the expression of a chimeric antimicrobial protein. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Riverside and Solano Counties have been evaluated phenotypically; no visible differences were seen between transgenic and untransformed vines. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have been also tested to confirm the presence of the transgene. At the Riverside County site, natural *Xf* infection has been confirmed and PD symptoms were scored using a standardized score based on percentage of leaf area scorching to validate resistance to PD under field conditions. At the Solano County site, non-grafted plants have been mechanically inoculated with *Xf* type strain (Temecula 1), the presence of *Xf* was confirmed in petiole extracts of mechanically inoculated grapevines by ELISA but no *Xf* growth was observed when petioles extracts were plated and no PD symptoms have been detected to date.

INTRODUCTION

Xylella fastidiosa (*Xf*), a xylem-limited Gram-negative bacterium, is the causative agent of Pierce's disease (PD). A key feature of *Xf* virulence is its ability to digest pectin-rich pit pore membranes that connect individual xylem elements (Roper et al., 2007), enhancing long distance movement and vector transmission. In this project, we are examining the ability of xylem-targeted polygalacturonase inhibiting protein (PGIP, Aguero et al., 2005, 2006) and a chimeric antimicrobial protein (HNE-CecB, Kunkel et al., 2007) to restrict bacterial movement and clear *Xf* under field conditions (Dandekar et al., 2009). The expectation is that expression of these proteins will prevent *Xf* movement reducing its inoculum and thus the spread of PD.

We are field-testing four independent transgenic lines (40-41, 40-89, 40-92, and 41-151) resulting from transforming grapevine plants with the vector pDU04.6105 expressing the chimeric antimicrobial protein (**Figure 1**). In each location, 24 plants are being field tested: 12 replicates of each line as non-grafted plants and 12 as transgenic rootstocks grafted with untransformed Thompson Seedless scions.

We have also planted vines carrying four different constructs of PGIP (**Figure 2**). The four different modifications allow us to better understand how to control/restrict *Xf* spread and thus disease virulence. Two versions have

different signal peptide sequences to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues. In vector pDU05.1910 (event 52-08), the pear PGIP signal peptide was replaced with a signal peptide from a grapevine xylem-secreted protein that is similar to the PRp27-like protein from *Nicotiana tabacum*. In vector pDU06.0201 (event 45-77), the pear PGIP protein was linked to a signal peptide from the Ch1b chitinase protein found in the xylem of grapevine (*Vitis vinifera*). The remaining two vectors, with and without the endogenous signal peptide, serve as controls. The construct pDU94.0928 (event TS50), which uses the pear PGIP's own endogenous peptide, serve as a control to evaluate the efficiency of exogenous signal peptides in targeting PGIP to the xylem tissue. Vector pDU05.1002 (event 31-25) eliminates the endogenous signal peptide; the expressed PGIP cannot be secreted and should not limit *Xf* spread.

Fig. 1: Vector construct used for expressing disease resistance in grapevine plants

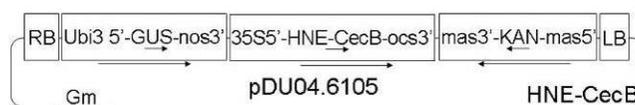
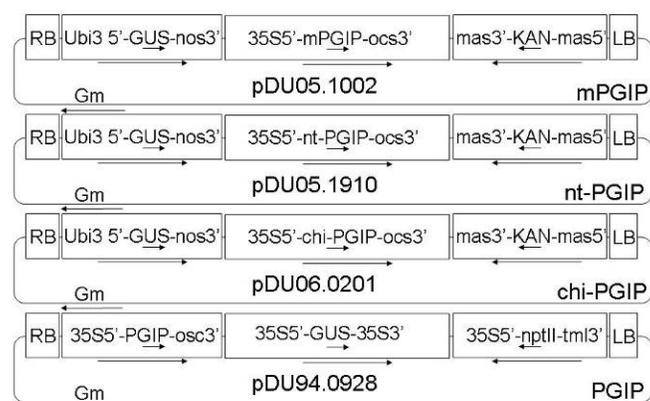


Fig. 2: Vector constructs used for expressing disease resistance in grapevine plants



OBJECTIVES

The goals of this project are to field-test four HNE-CecB and four PGIP expressing transgenic TS grapevine lines to evaluate their horticultural characteristics and resistance to Pierce's Disease (PD). Transgenic grapevines are being evaluated at two field locations as own-rooted plants and as transgenic rootstocks grafted with untransformed TS scions. One field location has endemic PD pressure and plants have been naturally infected with *Xf*. In the location with no PD pressure, grapevines have been mechanically inoculated with *Xf*.

Objective 1. Validate the efficacy of *in planta*-expressed chimeric HNE-CecB and PGIP containing 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*.

RESULTS AND DISCUSSION

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.

Four selected transgenic grapevine lines expressing HNE-CecB and four expressing different PGIP constructs were propagated from cuttings in the greenhouse to obtain 48 clones of each line. After the root system developed,

cuttings were transferred to 5.5-inch pots to develop into plants. Twenty-four clones were grafted with untransformed TS scions. Well-established plants were transferred to the lath house to acclimatize and then 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 5/8/10 and the remaining 10 were planted on 3/6/11, completing the planting at this location (**Fig 3, Fig 4, Table 1**). We also planted 110 transgenic and untransformed vines on their own roots on 8/2/10 and 110 vines grafted with untransformed TS scions on 6/27/11 in Solano County, completing the planting at this location (**Fig 3, Fig 4, Table 2**).



Figure 3. Riverside (left) and Solano County (right) transgenic grapevine plantings (Fall 2011).

Table 1. Riverside field evaluation planted on May 18, 2010 and March 6th 2011			
Non-grafted		Grafted	
Event ID	# Planted	Event ID	# Planted
HNE-CecB lines			
40-41	12	40-41G	12
40-89	12	40-89G	12
40-92	12	40-92G	12
41-151	12	41-151G	12
PGIP Lines			
31-25	12	31-25G	12
45-77	12	45-77G	12
52-08	12	52-08G	12
TS50	12	TS50G	12
Control lines			
TS	16	TS-G	12

Table 2. Solano County field evaluation planted on July 6th 2010 and July 27th 2011			
Non-grafted		Grafted	
Event ID	# Planted	Event ID	# Planted
HNE-CecB lines			
40-41	12	40-41G	12
40-89	12	40-89G	12
40-92	12	40-92G	12
41-151	12	41-151G	12
PGIP Lines			
31-25	12	31-25G	12
45-77	12	45-77G	12

52-08	12	52-08G	12
TS50	12	TS50G	12
Control lines			
TS	16	TS-G	12

HNE-CecB- and PGIP-expressing transgenic and untransformed grapevine lines in Solano County were randomly sampled and tested for the transgenes by PCR (**Table 3**). DNA was isolated from young leaves collected from the field using the Qiagen DNeasy Plant Mini kit according to manufacturer's instructions. DNA was PCR amplified using ActinF (TACAATGAGCTTCGGGTTGC) and ActinR (GCTCTTGCAGTTTCCAGCT) to determine DNA quality. Elastase primers were HNE5' (GCAGTTCAGAGGATCTTCGAGGATGG) and HNE3' (TTACTAGAGTGCTTTTGCTTCTCCAG). Primers for PGIP determination were CaMV 35S-2 (GACGTAAGGGATGACGCACAAT) and mPGIP-4 (CGGATCCTTACTTGCAGCTTGGGAGTGGAGCACCG).

Table 3. PCR genotyping of Solano County transgenic grapevine lines				
Event ID	Inserted Gene	ActinF/R	HNE3/5	CaMV35S/mPGIP4
HNE-CecB lines				
40-41	HNE	Positive	Positive	Negative
40-89	HNE	Positive	Positive	Negative
40-92	HNE	Positive	Positive	Negative
41-151	HNE	Positive	Positive	Negative
PGIP Lines				
31-25	PGIP	Positive	Negative	Positive
45-77	PGIP	Positive	Negative	Positive
52-08	PGIP	Positive	Negative	Positive
TS50	PGIP	Positive	Negative	Positive
Control				
TS	None	Positive	Negative	Negative



Figure 4. Riverside (left) and Solano County (right) transgenic grapevine plantings (Winter 2012).

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 (**Fig. 5**), 5) shape of mature leaf blades, 6) number of lobes on mature leaf (**Fig. 5**), 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 will also be phenotypically evaluated on 2012.

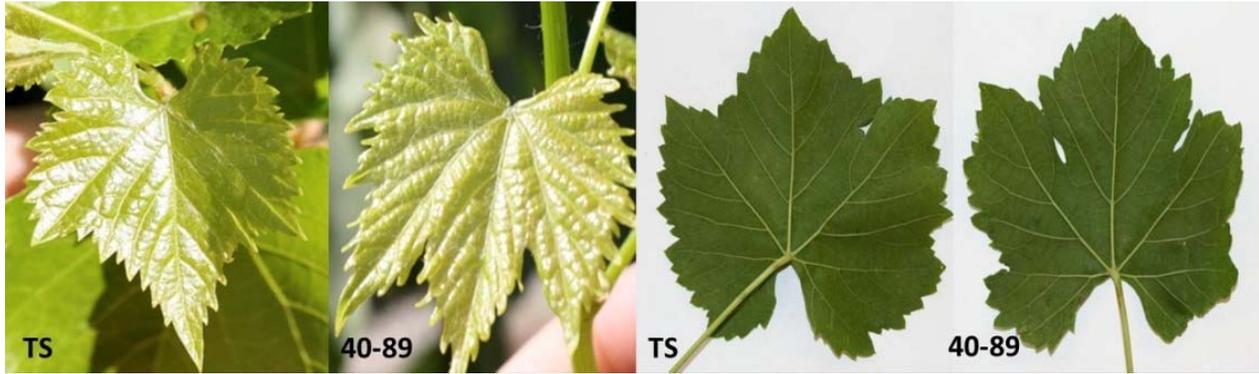


Figure 5. Color of upper side of blade on 4th young leaf (left) and number of lobes of mature leaf of TS and 40-89 transgenic line (right).

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

Two hundred and twenty four petiole samples from grafted and non-grafted transgenic and control grapevines planted in Riverside County, a positive infected control TS, and *Xf* were evaluated using a commercial ELISA kit for *Xf* detection (Agdia, Elkhart, IN). The assay is based on a mixture of *Xf* antibodies against eight grape *Xf* isolates. Sample extracts were also plated on PD3 medium and *Xf* growth was verified by PCR using EFTu and 16s primers. The ELISA cell count (**Fig. 6**), PCR assay (**Fig. 7**) and plate cell count (**Fig. 8**) results confirmed *Xf* infection in Riverside County.

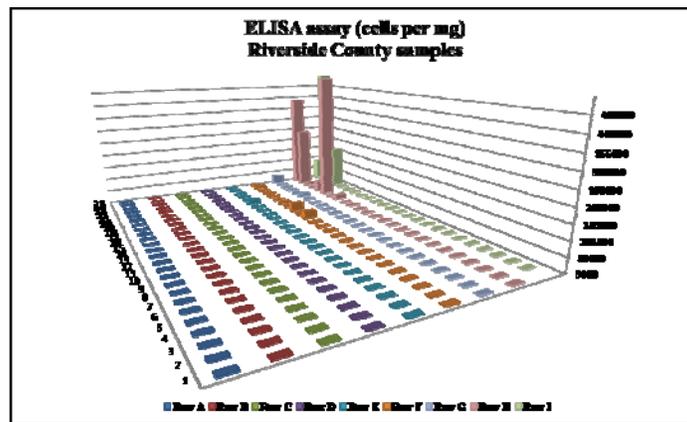


Figure 6. ELISA *Xylella fastidiosa* detection in 2011 Riverside County's petiole samples

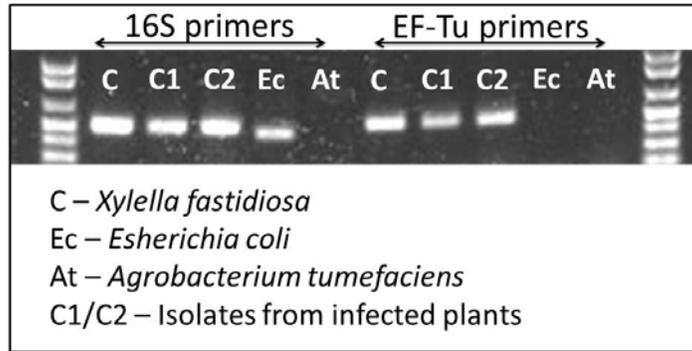


Figure 7. *Xylella fastidiosa* detection in Riverside County samples using PCR

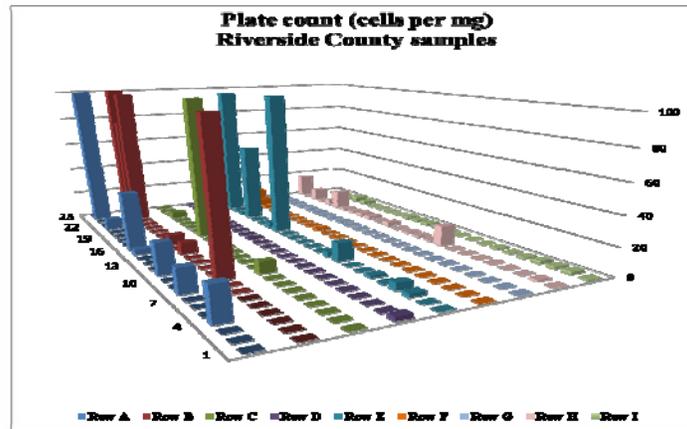


Figure 8. Plating *Xylella fastidiosa* cell counts from 2011 Riverside County’s petiole samples

At the Solano County site petioles from transgenic and non-transgenic plants that were mechanically inoculated with *Xf* (Almeida and Purcell, 2003) in July of 2011 and from TS and TS50 non-inoculated plants were evaluated for *Xf* detection using the commercial ELISA kit. Solano County sample extracts were also plated on PD3 medium. *Xf* was detected in petiole extracts by ELISA (Fig. 9, Table 4), but no growth was observed when petioles extracts were plated.

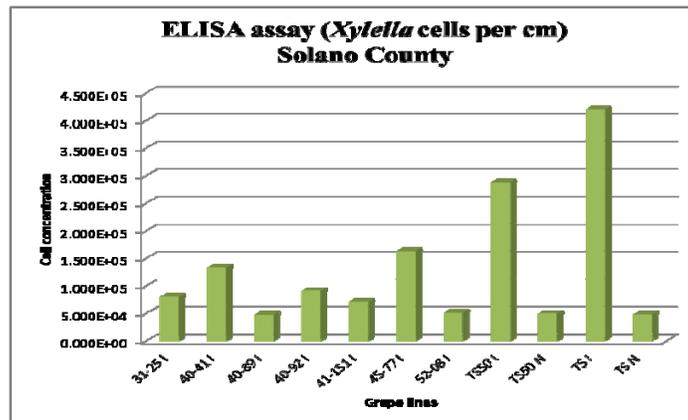


Figure 9. ELISA *Xylella fastidiosa* detection in 2011 Riverside County’s petiole samples

Table 4. Xylella concentration in petioles from Solano County grapevines (ELISA assay)		
Line	Gene	Cell/cm
31-25 inoculated	mPGIP	8.026E+04
40-41 inoculated	HNE	1.329E+05
40-89 inoculated	HNE	4.728E+04
40-92 inoculated	HNE	9.104E+04
41-155 inoculated	HNE	7.136E+04
45-77 inoculated	chiPGIP	1.625E+05
52-08 inoculated	ntPGIP	5.099E+04
TS50 inoculated	Control	2.877E+05
TS50 non-inoculated	Control	4.931E+04
TS inoculated	Wild type	4.199E+05
TS non-inoculated	Wild type	4.768E+04
TS non-inoculated + <i>Xf</i>	Positive control	3.675E+12

PD symptoms in each single Riverside County HNE-CecB- and PGIP-expressing grapevines were scored using a standardized score based on percentage of leaf area scorching (Fig. 9), a characteristic of PD (Krivanek et al., 2005a, 2005b). The following scoring system was used: 0 = no infection, 1 = potential infection, 2 = definitive infection (1-5 leaves infected, 3 = 5-1- leaves infected, 4 = more than 10 leaves infected, 5 = systematic infection on 1 runner, 6 = systematic infection in more than one runner, 7 = systematic infection in all runners, 8 = completely systematic with less than 50% leaf loss and 10 = dead plant. Riverside field average score was 3.25, the highest scoring line was 40-92 at 4.8 and the lowest was 40-41grafted at 1.7. PD symptoms in each single Solano County HNE-CecB- and PGIP-expressing grapevines will be scored in 2012

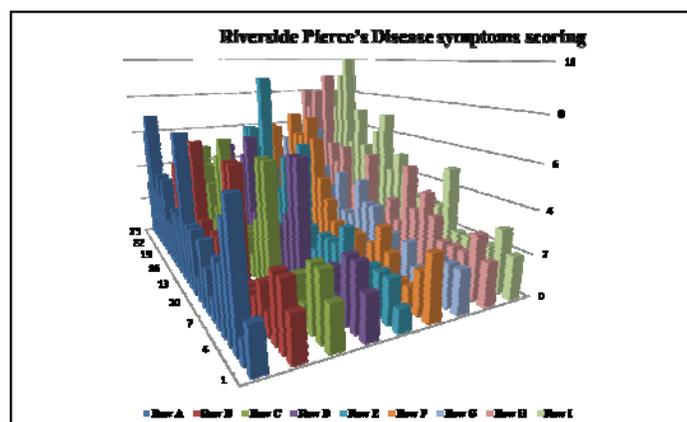


Figure 9. Pierce's Disease symptoms scoring in 2011 Riverside County's plants

SUMMARY OF MAJOR RESEARCH ACOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE

We have successfully initiated 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 being evaluated tests 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 is now complete with all 220 vines in the ground: 210 planted on 05/08/2010 with the remaining 10 planted on 03/06/2011. In Solano County, where planting is also completed with all 220 vines in the ground, 112 were planted on 08/02/2010 and the remaining 108 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. At the Riverside County site, the plants have been

naturally infected by wild populations of GWSS and *Xf* presence was confirmed by ELISA, PCR assays and plate cell count. PD symptoms were scored using a standardized score based on percentage of leaf area scorching to validate resistance to PD under field conditions. At the Solano County site, non-grafted vines have been mechanically inoculated with the *Xf* type strain (Temecula 1) to validate resistance to PD under field conditions, *Xf* presence was confirmed by ELISA, but no *Xf* growth in plate or PD symptoms have been detected to date. 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 been also been genotyped, confirming the presence of the inserted transgene in all lines.

PUBLICATIONS

Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Aguero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. PNAS Early Edition pp 1-5.

Dandekar, A.M., A.M. Ibáñez, H. Gouran, S.L. Uratsu, D. Gilchrist and T. Miller. 2011. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce’s Disease Research Symposium. Dec 13-15. Sacramento, CA. pp. 101-106.

RESEARCH RELEVANCE STATEMENT

The objective described in this report directly address the number 1 RSAP priority outlined in the “Top 5 to 10 Project Objectives to Accelerate Research to Practice” handout released at the December 2009 Pierce’s Disease Research Symposium: “Accelerate regulatory process: Establish and facilitate field trials of current PD control candidate vines / endophytes / compounds in multiple locations”. 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.

STATUS OF FUNDS: First and second year funding has been spent (100%).

INTELLECTUAL PROPERTY: No intellectual property was generated during this time period.

LITERATURE CITED

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