CHIMERIC ANTIMICROBIAL PROTEIN AND POLYGALACTURONASE-INHIBITING PROTEIN TRANSGENIC GRAPEVINES FIELD TRIAL

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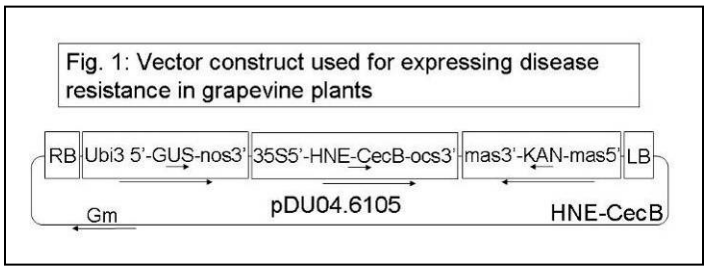
ABSTRACT
We have successfully established two field plantings to investigate two greenhouse-tested strategies to control the movement and clear *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease (PD) in grapevine. A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that connect adjoining xylem elements, enhancing long distance movement and vector transmission. The first strategy tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to inhibit *Xf* polygalacturonase activity necessary for long distance movement. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues via the expression of a chimeric antimicrobial protein, CAP. The expectation is that expressing these two proteins will prevent *Xf* movement and reduce *Xf* inoculum, curbing the spread of PD in California vineyards. Transgenic grapevine plants expressing either PGIP or CAP 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. At the Riverside County site, the plants have been naturally infected. At the Solano County site, plants on their own root were mechanically infected with *Xf* on 06/27/2011 to validate resistance to PD under field conditions. Two hundred and twenty four transgenic or untransformed control vines, own-rooted or grafted with untransformed TS, were planted in Riverside County on 05/08/2010. In Solano County, 112 own-rooted transgenic and untransformed control vines were planted on 08/02/2010 and 112 untransformed TS scions grafted onto transgenic or untransformed rootstocks were planted on 06/27/2011. At the Riverside County site, *Xf* infection has been confirmed and PD symptoms will be scored as they become apparent to validate resistance to PD under field conditions. At the Solano County site, non-grafted plants have been mechanically inoculated with the *Xf* type strain (Temecula 1), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been evaluated phenotypically; no differences were found between transgenic and untransformed. The DNA of CAP- and PGIP-expressing transgenic grapevine lines in Solano County has been checked to confirm the presence of the transgene.

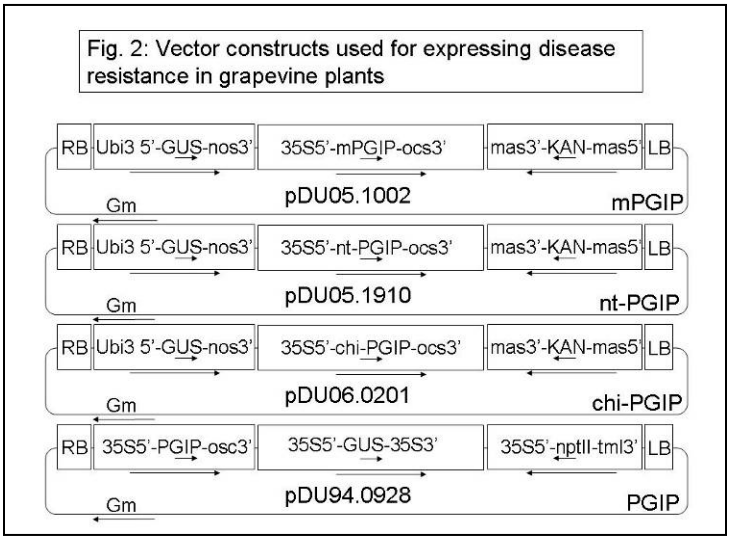
LAYPERSON SUMMARY
Transgenic grapevines are being evaluated as rootstocks to demonstrate the field efficacy of two strategies to control Pierce's disease (PD) in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa (Xf)* in 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. At the Riverside County site, *Xf* infection has been confirmed and PD symptoms will be scored as symptoms become more apparent 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), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been evaluated phenotypically; no visible differences were seen between transgenic and untransformed vines. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been also been tested to confirm the presence of the transgene.

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 (CAP, 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 and reduce its inoculum, reducing 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 tobacum*. 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, will serve as controls. The construct pDU94.0928 (event TS50), which uses the pear PGIP's own endogenous peptide, will 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.





The objective described here directly addresses the first 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.

OBJECTIVES

1. Validate the efficacy of *in planta*-expressed CAP 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.

The goals of this project are to field-test four CAP- and four PGIP-expressing transgenic Thompson Seedless grapevine lines to evaluate their horticultural characteristics and resistance to 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 PD pressure and plants have been naturally infected with *Xf*. In the location with no PD pressure, grapevines have been mechanically inoculated with *Xf*.

RESULTS AND DISCUSSION

Propagation, field planting, and grafting of CAP and PGIP transgenic grapevines.

Four selected transgenic grapevine lines expressing CAP 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 **(Figure 3, 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 (**Figure 3, Table 2**).

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

Table 1. Riverside Field Evaluation planted on May 18, 2010 and March 6th 2011.

Non-grafted		Grafted	
Event ID	**# Planted**	**Event ID**	**# Planted**
CAP 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**
CAP 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

CAP- 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 PCRed using ActinF (TACAATGAGCTTCGGGTTGC) and ActinR (GCTCTTTGCAGTTTCCAGCT) to determine DNA quality. Elastase primers were HNE5' (GCAGTTCAGAGGATCTTCGAGGATGG) and HNE3'(TTACTAGAGTGCTTTTGCTTCTCCCAG). 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
CAP 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

Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
CAP- and PGIP-expressing transgenic grapevine lines in Solano County were evaluated phenotypically in September 2011 to verify that horticultural and varietal characteristics of the parental genotype TS were unchanged. 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 2) density of prostrate hairs on young shoot tips, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on young 4th leaves, 5) shape of mature leaf blades, 6) number of lobes on mature leaves, 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 County CAP- and PGIP-expressing transgenic grapevines lines will also be phenotypically evaluated this year.

Evaluate PD resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.
Thirty-four pooled (Row samples A-I, Lines 1-25) 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 the EFTU and 16s primers. The ELISA (**Figure 4**) and PCR assay (**Figure 5**) results confirmed *Xf* infection in Riversida County. *Xf* cell counts will be done when we get enough *Xf*-infected vines. Since infection was confirmed in pooled samples at Riverside, individual grapevine lines will be sampled and tested for *Xf* infection. PD symptoms in Riverside County will be scored on each infected plant as they appear using a standardized score based on percentage of leaf area scorching, a characteristic of PD (Krivanek et al., 2005a, 2005b). As PD symptoms develop in Riverside County, we can evaluate PD resistance in CAP and PGIP grapevines. Non-grafted petiole samples planted in Solano County that were mechanically inoculated with *Xf* (Almeida and Purcell, 2003) in July of 2011 show no *Xf* infection to date.

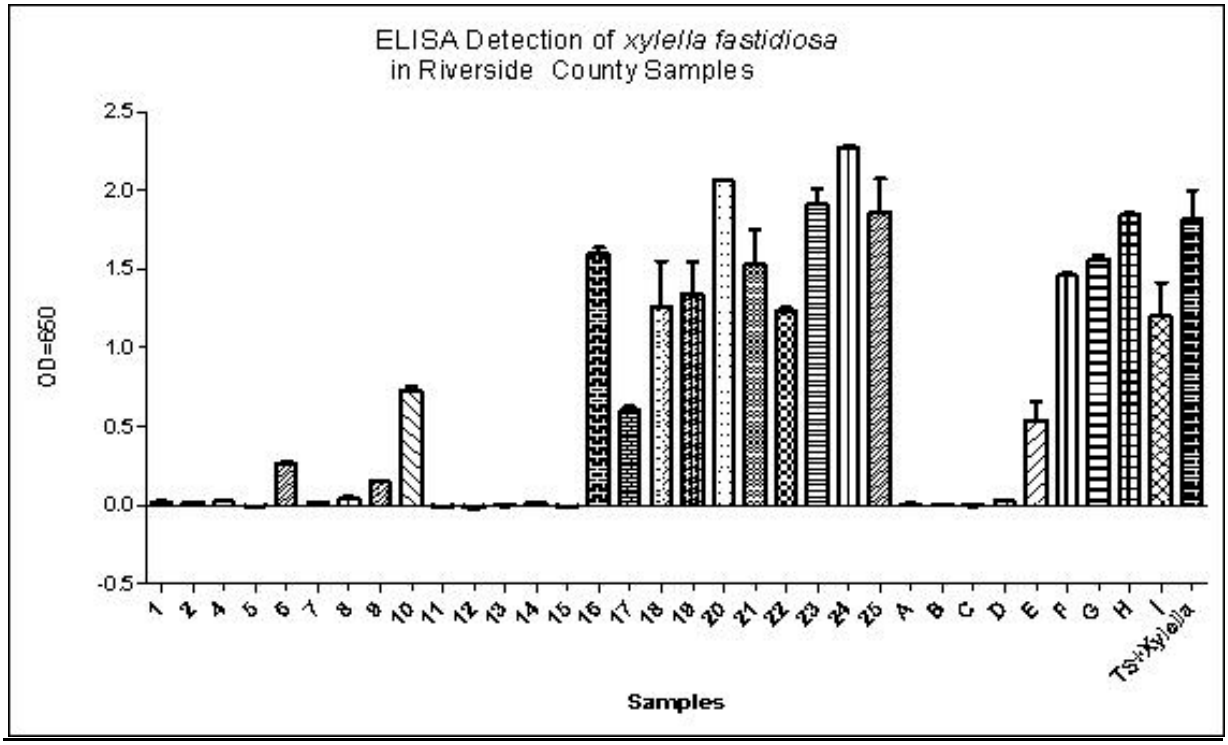


Figure 4. *Xf* detection in Riverside County pooled samples using ELISA.

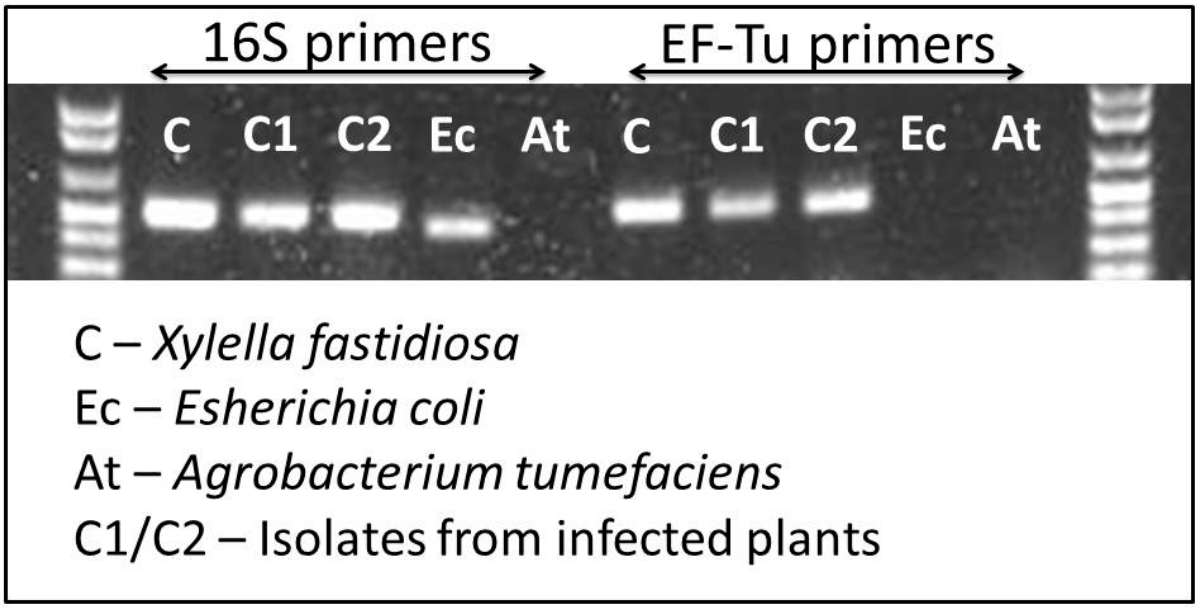


Figure 5. *Xf* detection in Riverside County samples using PCR.

CONCLUSIONS

We have successfully initiated two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xf*, a xylem-limited, Gram-negative bacterium that is the causative agent of 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, CAP. 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 CAP 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 06/27/2011. These transgenic grapevines will be 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 glassy-winged sharpshooter and *Xf* presence was confirmed by ELISA and PCR assays. PD symptoms will be scored as they appear 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), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in 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. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been also been genotyped, confirming the presence of the inserted transgene in all lines.

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