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

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ABSTRACT
We have successfully initiated two field trials to investigate two greenhouse-tested strategies to control the movement of and to clear *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* is its ability to digest pectin-rich pit pore membranes inside the host plant's xylem elements, permitting long distance movement and potentially enhancing vector transmission. The first strategy being evaluated tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear as an effector protein to counter virulence associated with bacterial PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues via the expression of a chimeric antimicrobial protein, HNE-CecB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum, curbing the spread of PD in California vineyards. Transgenic grapevine plants expressing either PGIP or the HNE-CecB chimeric antimicrobial protein have been planted in two locations, one in Riverside County and the other in Solano County. These transgenic grapevines will be evaluated as plants on their own roots and as rootstocks grafted with wild type Thompson Seedless (TS) scions. At the Riverside County site, the plants will be naturally infected. At the Solano County site, plants will be mechanically infected with *Xf* to validate resistance to PD under field conditions. Two hundred and thirteen transgenic and wild type control vines, own rooted and grafted with wild type TS, were planted in Riverside County on 5/8/10. In Solano County, 112 transgenic and wild type controls on their own roots vines were planted on 8/2/2010. HNE-CecB- and PGIP-grafted plants for the Solano County site are being generated for planting in November 2010.

LAYPERSON SUMMARY
Transgenic grapevines are being evaluated as rootstocks to demonstrate the field efficacy of two strategies to control Pierce's disease 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 an HNE-CecB chimeric antimicrobial protein.

INTRODUCTION
Xylella fastidiosa (Xf), a xylem-limited Gram-negative bacterium, is the causative agent of Pierce's disease (PD). A key feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes inside the xylem element (Roper et al., 2007), permitting its long distance movement and enhancing its virulence and vector transmission. In this project, we are examining the ability of the xylem-targeted effector proteins 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 to 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 HNE-CecB 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 wild type 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 disease spread and virulence of *Xf*. 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, serve as controls. Vector pDU05.1002 (event 31-25) eliminates the endogenous signal peptide so the expressed PGIP cannot be secreted and should not limit *Xf* spread. 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.

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

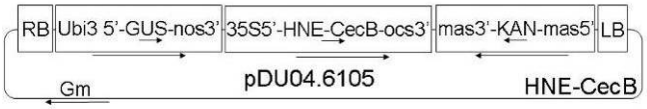

Fig. 2: Vector constructs used for expressing disease resistance in grapevine plants

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.

OBJECTIVES

The goals of this project are to field test four HNEC-CecB and four PGIP transgenic grapevine lines to evaluate their horticultural characteristics and their resistance to PD. Transgenic grapevines will be tested in two field locations as non-grafted plants and as transgenic rootstocks grafted with wild type scions. One field location has PD pressure and plants will be naturally infected with *Xf*. In the location with no PD pressure, grapevines will be mechanically inoculated with *Xf*.

Objective 1. Validate the efficacy of *in planta* expressed chimeric HNE-CecB and PGIP proteins containing different signal peptides to inhibit and clear *Xf* infection in xylem tissue and through the graft union in grapevines under field conditions.

RESULTS AND DISCUSSION

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. These lines showed resistance to PD under greenhouse conditions after mechanical *Xf* inoculation. After the root system was developed, the plants were transferred to 5.5-inch pots. Twenty-four clones were grafted with wild type TS scions **(Figure 3**). Once acclimatized, they were transferred to the lath house and finally planted in two experimental fields. Two hundred and thirteen transgenic and wild type controls, own rooted or grafted with wild type TS scions, were planted in Riverside County on 5/8/10, where they are exposed to glassy-winged sharpshooters (GWSS), the insect vector for *Xf*. We also planted 112 transgenic and wild type controls on their own roots in Solano County on 8/2/2010 (**Figure 4**). Additional HNE-CecB and PGIP grafted plants for Solano County field are being generated for planting in November 2010.

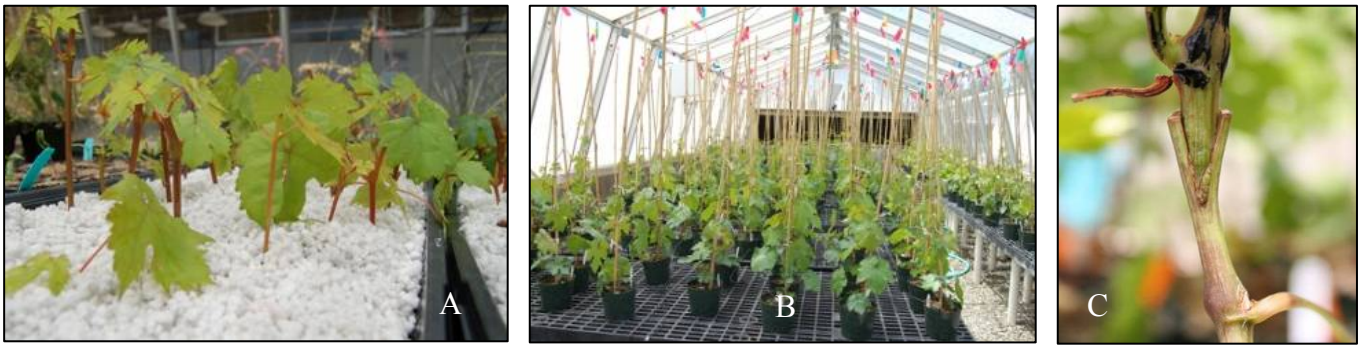

Figure 3. A) Propagated plants from vegetative tissue; B) Rooted plants and C) Grafted plant

Figure 4. Riverside and Solano County transgenic grape fields.

Evaluate preservation of varietal characteristics in transgenic grapevines grown as plants on their own roots or used as rootstocks.

HNE-CecB- and PGIP-expressing lines will be evaluated in the field in 2011. Grafted and non-grafted transgenic grapevine lines will be evaluated phenotypically for shoot growth and leaf shape to see if they are normal and have maintained the horticultural and varietal characteristics of the parental genotype TS. This examination will use the variables proposed by the International Organization of Vine and Wine (OIV, 1983).

Evaluate PD resistance of HNE-CecB and PGIP grapevines after inoculation with *Xf*.
Grafted and non-grafted transgenic and control grapevines planted in Riverside County will be naturally infected with *Xf* to validate resistance to PD under field conditions in 2011. Grafted and non-grafted grapevines planted in Solano County will be mechanically inoculated with *Xf* (Almeida and Purcell, 2003). PD symptoms will be scored on each infected plant using a standardized score based on percentage of leaf area scorching, a characteristic of PD (Krivanek et al., 2005a, 2005b). Expression of *hne* and *pgip* genes will be analyzed using Quantitative Real-Time PCR (qRT-PCR). PGIP movement from transformed rootstocks into wild type scions will be monitored using antibody detection and a radial diffusion assay that evaluates PGIP activity.

CONCLUSIONS
Selected HNE-CecB- and PGIP-expressing grapevines and wild type controls were successfully planted as non-grafted plants and as transgenic rootstocks in Riverside County. Presence of the GWSS at the Riverside County field is very promising, since natural insect inoculation of these plants is one of the main objectives of this trial. Non-grafted HNE-CecB- and PGIP-expressing grapevines and wild type controls were also successfully planted in the Solano County field. Grafted plants for the Solano County field are being generated to be planted in November 2010.

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