IN PLANTA TESTING OF SIGNAL PEPTIDES AND ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

Principal Investigator:
Abhaya M. Dandekar
Dept. of Plant Sciences
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Cooperators:
John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Rodrigo Almeida
Dept of ESPM
University of California
Berkeley, CA 94720
Rodrigo@nature.berkeley.edu

Ana María Ibáñez
Dept. of Plant Sciences
University of California
Davis, CA 95616
amibanez@ucdavis.edu

Sandie L. Uratsu
Dept. of Plant Sciences
University of California
Davis, CA 95616
sluratsu@ucdavis.edu

Hossein Gouran
Dept. of Plant Sciences
University of California
Davis, CA 95616
hgouran@ucdavis.edu

Cecilia Aguero
Dept. of Plant Sciences
University of California
Davis, CA 95616
cbaguero@ucdavis.edu

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ABSTRACT

Xylella fastidiosa (*Xf*), a xylem-limited, Gram-negative bacterium, is the causative agent of Pierces's disease (PD). A key feature of *Xf* is its ability to digest pectin containing pit pore membranes inside the host plant's xylem elements, permitting long distance movement and enhancing virulence and vector transmission. In this project, we are evaluating the ability of xylem-targeted effector proteins polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein to restrict the movement of *Xf* and clear infection. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum, curbing the spread of PD. Transgenic grapevine plants expressing either PGIP (six versions) or the human neutrophil elastase-cecropin B (HNE-CecB) chimeric antimicrobial protein (two versions), have been obtained and the first plants have been tested to validate their susceptibility to PD.

Plants expressing pear PGIP have six different modified sequences to target gene expression. Four constructs contain 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. A fifth construct has the original pear signal peptide and the sixth lacks a signal peptide as a control. Forty-four PGIP *in vitro* transgenic lines have been transferred to the greenhouse. Sixteen were manually inoculated with *Xf* and scored for tolerance to PD and movement of PGIP protein. Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression among different transgenic lines and untransformed control lines have been initiated. The remaining lines are being multiplied for future *Xylella* inoculation experiments.

Transgenic Thompson Seedless grapevines expressing the chimeric anti-microbial protein HNE-CecB expressed with its own signal peptide or with that from pear PGIP (pgip-HNE-CecB) have been obtained. The expressed chimeric anti-microbial protein has two functional domains. The surface recognition domain, SRD, specifically binds to the *Xf* outer-membrane protein MopB. The other domain inserts into the membrane, forming pores that lyse and kill *Xf.* Twenty-one of 47 HNE-CecB transgenic grapevine lines have been manually inoculated with *Xf* in the greenhouse. Preliminary observations show that most transgenic HNE-CecB-expressing lines showed limited or delayed disease symptoms compared to the severe PD symptoms of untransformed control plants. Five lines were substantially more resistant than the rest. Magnetic resonance imaging (MRI) of stem sections revealed fewer clogged vessels in these transgenic lines. Xylem sap from HNE-CecB transgenic lines with the resistant phenotype killed *Xf* more efficiently than that from untransformed controls. Interestingly, DNA extracted from the same HNE-CecB transgenic lines showed lower pathogen load than control plants. HNE protein expressed in transgenic plants was detected using enzyme-linked immunosorbent assay (ELISA). The remaining HNE-CecB and pgip-HNE-CecB lines are in the process of greenhouse propagation to conduct future *Xf* infection tests.

In addition to ongoing mechanical inoculation, we are in the initial preparation steps of a new and more precise experiment which is using blue-green sharpshooters (BGSS) (transmitting insect vector) to infect our more promising transgenic lines determined from previous results (**Figure 1**).

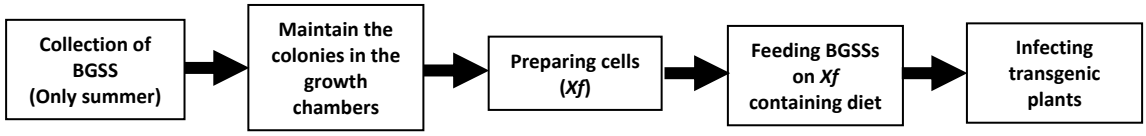

Figure 1: Steps involved in infection experiment by BGSS.

LAYPERSON SUMMARY

Transgenic grapevines are being evaluated as rootstocks that produce two types of effector proteins that may control Pierce's disease (PD) in untransformed scion cultivars grafted to such rootstocks. PD is caused by growth and movement of the bacterium *Xylella fastidiosa* (*Xf*) in the water-conducting xylem of the vine. As individual xylem elements are invaded and blocked, the vine is stressed, reducing vigor and productivity and eventually killing the vine. In this project, we are examining the ability of the xylem-targeted effector proteins polygalacturonase inhibiting protein (PGIP) to reduce disease severity by restricting the movement *Xf* across xylem elements and of a chimeric antimicrobial protein (HNE-CecB) to clear *Xf,* preventing its ability to colonize. We have created 44 vines expressing six different modifications of PGIP to better understand its ability to restrict disease spread. Sixteen of these plants are being evaluated in the greenhouse for resistance to PD and five lines show increased tolerance to PD over untransformed controls. Untransformed scions have been grafted movement of PGIP across the graft union. We have also evaluated 21of 47 HNE-CecB/pgipHNE-CecB lines in the greenhouse for clearance of *Xf*. At least five of the 21 evaluated lines show good tolerance to *Xf* infection and magnetic resonance imaging (MRI) of infected stem sections revealed fewer clogged vessels in the transgenic vines than in the controls, indicating clearance of the bacteria. Xylem sap from HNE-CecB transgenic lines inhibited growth of *Xf* more than sap from control vines. Interestingly, DNA extracted from the same HNE-CecB lines showed lower pathogen load than control plants. Further experiments with these transgenic lines will confirm the efficacy of these two effector proteins in controlling this important disease of grapevines.

OBJECTIVES

1. Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *Xf*.
2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta,* and test for graft transmissibility.

RESULTS AND DISCUSSION

1. <u>Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *Xf*</u>

Transgenic vines were created that express pear PGIP with six different signal peptide options (pDU06.0201 used chiPGIP; pDU05.1910, ntPGIP; pDA05.XSP, xspPGIP; pDU05.0401, ramyPGIP; pDU05.1002, mPGIP; and pDU94.0928, pPGIP). Four constructs contained 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. One construct used the pear signal peptide and the sixth was a control with no signal peptide. PGIP transgenic tissue extracts were assayed to validate transgene expression, which was highly variable. Based on polygalacturonase inhibiting activity, 44 PGIP *in vitro* transgenic lines (1 pPGIP, 6 ntPGIP, 8 chiPGIP, 12 mPGIP, 8 ramyPGIP, and 9 xspPGIP) have been transferred to the greenhouse.

Each acclimated transgenic line was propagated to obtain four to six mother plants that were further propagated to provide cuttings for *Xylella fastidiosa (Xf)* infection and grafting experiments. From each line, 25 to 35 plants were propagated from cuttings at the same time. *Xf infection* experiments were done in multiple rounds. Each round consisted of five to six transgenic lines with wild type Thompson Seedles s(TS) and TS50 as negative and positive control, respectively. Each round of experiments included 30 plants from each transgenic line. Fifteen were inoculated (Almeida and Purcell 2003) and the remaining 15 were non-inoculated controls. The positive control, TS50, is a transgenic PGIP-expressing grapevine previously described (Aguero et al. 2005).

Transgenic and control plants were inoculated with 20µL of a GFP-expressing *Xf* 3A2 culture (Newman et al. 2003) containing ~20,000,000 cells. The plants were inoculated with 10 µL the first day and re-inoculated with 10µL the second day; for each inoculation an independently grown *Xf* culture was used. The *Xf* was introduced into each plant three to four inches above the soil using an insect pin. Plants were pruned regularly and kept approximately 90-100cm tall until Pierce's Disease (PD) symptoms appeared. The time required to conduct each round of *Xf* challenge was 33 to 37 weeks, starting from *in vitro* plants transferred to greenhouse until the appearance of the first PD symptoms.

Twenty-two out of 44 PGIP transgenic lines are currently being evaluated for resistance to PD. Three rounds of infection have been completed on 16 PGIP transgenic lines manually inoculated with *Xf*. PD symptoms were 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). TS50 and five PGIP transgenic lines showed more tolerance to PD than the untransformed control (**Table 1**). Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression in PGIP transgenic lines and the untransformed control have been initiated. RNA was isolated from bottom and top stem sections of each infected and uninfected grapevine. Preliminary qRT-PCR data (Ct value, **Figure 2**) confirm that transgenic lines with more tolerance to PD had higher *pgip* gene expression. The remaining ntPGIP, chiPGIP, mPGIP, ramyPGIP, and xspPGIP lines are being propagated for future *Xf* challenge in the greenhouse. Those lines with low or moderate PD symptoms after manual inoculation will be tested by insect inoculation of *Xf*.

Table 1. PGIP transgenic lines status and results

Lines with yellow highlight are included in the field trial at Riverside and Solano counties. Siganl peptides: Chitinase (chi) and pathogen related protein 27 (PRp27)-like protein from *Nicotiana tobacum* (nt) from *Vitis vinifera*, polygalacturonase inhibiting protein from pear (pPGIP), rice amylase from *Oryza sativa* (Ramy), xylem sap protein from *Cucumis sativus* (XSP)

Line ID #	Gene	Construct	Status	Result
061045-015	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-056	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-070	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-077	chiPGIP	pDU06.0201	Tested-Round3	Low Resistance
061045-078	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-083	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061034-007	ntPGIP	pDU05.1910	Tested-Round4	No Resistance
061034-010	ntPGIP	pDU05.1910	Tested-Round4	No Resistance
061052-008	ntPGIP	pDU05.1910	Tested-Round4	High Resistance
061052-016	ntPGIP	pDU05.1910	Tested-Round4	High Resistance
061036-27	ntPGIP	pDU05.1910	Tested-Round5	No Resistance
061131-22	mPGIP	pDU05.1002	Tested-Round5	High resistance
061131-25	mPGIP	pDU05.1002	Tested-Round5	Low Resistant
061131-29	mPGIP	pDU05.1002	Tested-Round5	Low Resistance
061131-32	mPGIP	pDU05.1002	Tested-Round5	No Resistance
TS50	pPGIP	pDU94.0928	Tested-Round3-4	High resistance
061131-17	mPGIP	pDU05.1002	Tested-Round9	Exp. in progress
061131-35	mPGIP	pDU05.1002	Tested-Round9	Exp. in progress
061132-30	Ramy	pDU05.0401	Tested-Round9	Exp. in progress
061132-32	Ramy	pDU05.0401	Tested-Round9	Exp. in progress
061133-21	XSP	pDA05.XSP	Tested-Round9	Exp. in progress
061133-24	XSP	pDA05.XSP	Tested-Round9	Exp. in progress
061036-30	ntPGIP	pDU05.1910	To be tested	
061045-032	chiPGIP	pDU06.0201	To be tested	
061045-035	chiPGIP	pDU06.0201	To be tested	
061131-19	mPGIP	pDU05.1002	To be tested	
061131-21	mPGIP	pDU05.1002	To be tested	
061131-24	mPGIP	pDU05.1002	To be tested	
061131-27	mPGIP	pDU05.1002	To be tested	
061131-28	mPGIP	pDU05.1002	To be tested	
061131-31	mPGIP	pDU05.1002	To be tested	
061132-19	Ramy	pDU05.0401	To be tested	
061132-24	Ramy	pDU05.0401	To be tested	
061132-28	Ramy	pDU05.0401	To be tested	
071073-33	Ramy	pDU05.0401	To be tested	
071073-40	Ramy	pDU05.0401	To be tested	
071073-83	Ramy	pDU05.0401	To be tested	
061133-2	XSP	pDA05.XSP	To be tested	
061133-22	XSP	pDA05.XSP	To be tested	
061133-23	XSP	pDA05.XSP	To be tested	
061133-25	XSP	pDA05.XSP	To be tested	
061133-27	XSP	pDA05.XSP	To be tested	
061133-28	XSP	pDA05.XSP	To be tested	
061133-29	XSP	pDA05.XSP	To be tested	
061133-32	XSP	pDA05.XSP	To be tested	

To evaluate the efficiency of secretion, each transgenic line expressing each of the signal sequences fused to PGIP will be used as transgenic rootstocks grafted to wild type scions. After growth xylem sap will be extracted from the stem and leaves of the wild type scion to evaluate the amount of PGIP that is translocated via the xylem into the wild type tissues. The movement of the PGIP protein from the rootstock up into the xylem of the wild type scion was evaluated using a radial diffusion assay (Aguero et al. 2005). Preliminary testing of PGIP activity using leaf extracts and xylem sap from non-grafted TS50 (positive control), ChiPGIP 45-35 and ChiPGIP 45-83 showed PG inhibiting activity. The same lines when grafted

also showed inhibiting activity from leaf extract and xylem sap. TS50 showed the highest activity in grafted and non-grafted leaf and non-grafted xylem sap. Interestingly, xylem sap from Chi45-35 and Chi45-83 showed a greater inhibition when they were grafted with wild type TS as compared to non-grafted, indicating that the PGIP was moving quite efficiently from the rootstock to the scion with these particular signal peptides.

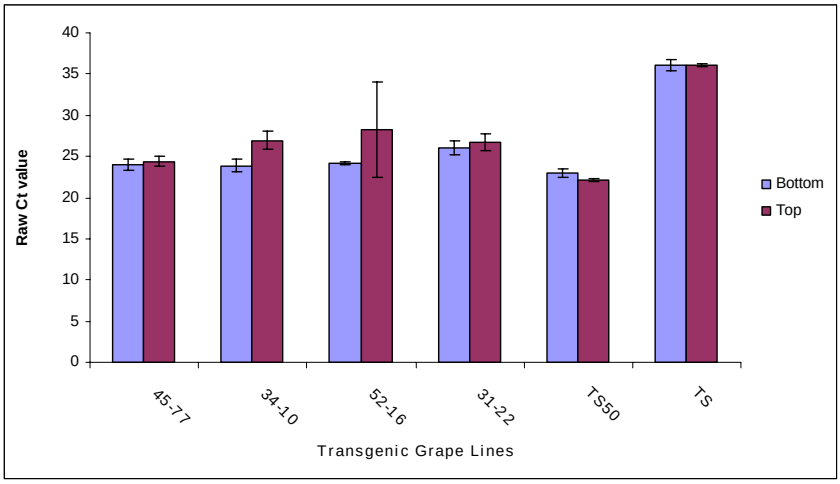

Figure 2: Expression of *pgip* gene on top and bottom stem section of transgenic grapevines inoculated with *Xf*. Lower Ct values represent higher pgip expression.

2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta*, and test for graft transmissibility

Transgenic grapevine plants were obtained with the two constructs pDU04.6105 (Elastase-Cecropin = HNE-CecB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CecB) as described in earlier reports. Twenty-six of 47 HNE-CecB lines are currently being evaluated for resistance/tolerance to PD. The first four rounds of infection have been completed for 21 transgenic lines. Within six to seven weeks post inoculation, the first PD-associated leaf scorch symptoms appeared: formation of green islands on the cane and scorching around outer edges of the lower leaves were visible on control grapevines (**Figure 3**). Most transgenic HNE-CecB-expressing lines showed less or delayed disease symptoms than controls and five lines were substantially more resistant than the rest. PD symptoms on each infected plant were numerically scored based on percentage leaf area scorch (**Table 2**).

Figure 3: Transgenic line (40-60) in the right showing less disease symptom compared to non-transgenic TS on the left.

Table 2. HNE-CecB and pgipHNE-CecB Transgenic Lines Status and results
Lines with yellow highlight are included in the field trial at Riverside and Solano counties.

Line ID Num.	Gene	Construct	Status	Result
0999040-39	HNE-CecB	pDU04.6105	Tested-Round1	Low Resistance
0999040-41	HNE-CecB	pDU04.6105	Tested-Round1	Moderately Resistance
0999041-151	HNE-CecB	pDU04.6105	Tested-Round1	High Resistance
0999041-166	HNE-CecB	pDU04.6105	Tested-Round1	Moderately Resistance
0999041-179	HNE-CecB	pDU04.6105	Tested-Round1	Low Resistance
0999040-36	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-74	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-89	HNE-CecB	pDU04.6105	Tested-Round2	High Resistance
0999040-92	HNE-CecB	pDU04.6105	Tested-Round2	High Resistance
0999041-146	HNE-CecB	pDU04.6105	Tested-Round2	Highly Resistance
0999041-157	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-87	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999040-96	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999040-97	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999041-164	HNE-CecB	pDU04.6105	Tested-Round6	Moderately Resistance
0999041-181	HNE-CecB	pDU04.6105	Tested-Round6	Low Resistance
0999040-60	HNE-CecB	pDU04.6105	Tested-Round7	Moderately Resistance
0999040-80	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
0999040-85	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
0999041-180	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
099946-019	pgip HNE-CecB	pDA05.0525	Tested-Round7	Not Resistance
099944-007	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
099946-002	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
099946-014	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
0999040-4	HNE-CecB	pDU04.6105	Tested-Round8	Exp. in progress
099944-026	pgip HNE-CecB	pDA05.0525	To be tested	
099946-018	pgip HNE-CecB	pDA05.0525	To be tested	
099946-020	pgip HNE-CecB	pDA05.0525	To be tested	
061073-016	pgip HNE-CecB	pDA05.0525	To be tested	
061073-028	pgip HNE-CecB	pDA05.0525	To be tested	
061073-036	pgip HNE-CecB	pDA05.0525	To be tested	
0999040-11	HNE-CecB	pDU04.6105	To be tested	
0999040-42	HNE-CecB	pDU04.6105	To be tested	
0999040-51	HNE-CecB	pDU04.6105	To be tested	
0999040-62	HNE-CecB	pDU04.6105	To be tested	
0999040-69	HNE-CecB	pDU04.6105	To be tested	
0999040-78	HNE-CecB	pDU04.6105	To be tested	
0999040-79	HNE-CecB	pDU04.6105	To be tested	
0999040-81	HNE-CecB	pDU04.6105	To be tested	
0999040-106	HNE-CecB	pDU04.6105	To be tested	
0999040-112	HNE-CecB	pDU04.6105	To be tested	
0999041-125	HNE-CecB	pDU04.6105	To be tested	
0999041-132	HNE-CecB	pDU04.6105	To be tested	
0999041-155	HNE-CecB	pDU04.6105	To be tested	
0999041-168	HNE-CecB	pDU04.6105	To be tested	
0999041-169	HNE-CecB	pDU04.6105	To be tested	
0999041-171	HNE-CecB	pDU04.6105	To be tested	
0999041-174	HNE-CecB	pDU04.6105	To be tested	

MRI images from stem sections approximately 15 to 20 cm above the inoculation point revealed that clearance of bacterial inoculums in transgenic lines expressing fewer PD symptoms correlated to fewer clogged vessels than in the control lines. To obtain MRI xylem vessel cross section images, an Avance 400 instrument was used. Instrument settings were: TR: 110.7, TE: 4.5ms, FA: 30.0deg, TA: 1:25NEx4, FOV: 1.2cm, MTX 256/192, Pos-0.80mmF.

Xylem sap extracted from grape plants expressing HNE-CecB kills bacteria. *Xf* was incubated with xylem sap extracted from transgenic lines at 28°C on a shaker. For each sample, three different dilutions of the *Xf*-xylem sap mixture were plated on PD3 media each hour for five hours. This experiment reveals the antimicrobial activity of sap from transgenic lines expressing HNE-CecB antimicrobial protein. Transgenic lines expressing high phenotypic resistance (**Table 2**) also displayed higher *Xf* mortality rates than untransformed and buffer controls.

Semi-Quantitative PCR analysis of Xf DNA accumulation was performed on groups of three stems sections collected approximately 10 to15 cm above the point of inoculation. For each transgenic line, DNA was extracted from groups of three stem samples representing three individual plants. Plants expressing HNE-CecB contained less bacterial DNA, and thus a lower pathogen load in the plant tissue, indicating more clearance.

We detected the HNE protein in transgenic plants using enzyme-linked immunosorbent assay (ELISA). Commercially available polyclonal antibody generated against elastase was used to create an ELISA sandwich assay; however, for greater precision we must generate a more specific monoclonal antibody against the chimeric protein (**Figure 4**). Total protein was extracted from transgenic plant tissue using a sodium bicarbonate extraction buffer (0.1M NaHCO3, pH 8.6). A 96-well MaxisorpTM microtiter plate (NUNC, NY, USA) was coated with 100 µL of the transgenic or control crude protein extracts overnight at 4ºC. The wells were washed one time with PBS-T 0.1% and blocked for one hour at 37ºC with PBS-BSA 3%. After blocking, wells were washed twice with PBS-T 0.1% followed by incubation with anti-elastase antibody (1:1000) at 37ºC for 1 hour. The plate was washed three times with PBS-T 0.1% followed by incubation with AP-conjugated anti-rabbit diluted (1:5000) in PBS-BSA 3% for 1 h at 37ºC. The plate was washed four times in PBS-T 0.1%, developed at 405 nm.

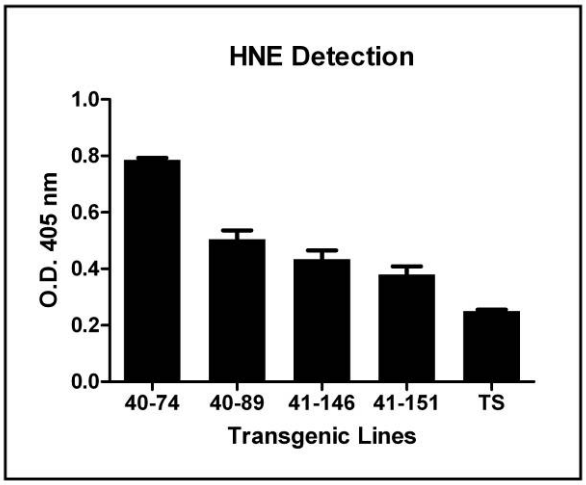

Figure 4. HNE detection by ELISA

CONCLUSIONS

The main objective of this project is to validate the efficacy of the xylem targeted effector proteins polygalacturonase inhibiting protein (PGIP) to limit movement and of a chimeric antimicrobial protein HNE-CecB to clear *Xf*.

Transgenic grapevines lines expressing PGIP with four different signal peptides are being evaluated for their improved ability to secrete PGIP long distance through the graft union. Sixteen of these plants are being evaluated in the greenhouse for resistance to PD and five lines show increased tolerance to PD over untransformed controls. Untransformed scions have been grafted onto PGIP transgenic lines to evaluate the long distance movement of PGIP. Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression among different transgenic lines and untransformed control lines have been initiated.

Twenty one HNE-CECB transgenic grapevine lines have been evaluated for PD resistance by inoculating with *Xf*. Several promising transgenic lines showed low or moderate symptoms of PD. MRI stem sectioned images revealed a variation in number of vessels clogged between negative control and HNE-CECB transgenic lines, indicating that clearance of the bacteria may be occurring in some of the transgenic lines (Dandekar et al. 2009). Further protein assays are required to detect and quantify the expression of transgenic protein.

Insect inoculation of transgenic plants via bluegreen sharpshooter has been initiated to test a more natural mode of disease transmission and better predict field resistance levels, since these vines will be inoculated with a more realistic number of bacteria.

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