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

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

Cooperators:

John Labavitch
Department of Plant Sciences
University of California
Davis CA 95616
jmlabavitch@ucdavis.edu

Rodrigo Almeida
Dept. Environ. Sci., Policy, & Mgmt.
University of California
Berkeley, CA 94720
Rodrigo@nature.berkeley.edu

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

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

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

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

Reporting Period: The results reported here are from work conducted July 2009 to September 2009.

ABSTRACT

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 containing pit pore membranes inside the xylem elements permitting its long distance movement enhancing its virulence and vector transmission. In this project we are analyzing the efficacy of xylem targeted effector proteins like polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein to restrict the movement and to clear *Xf*. The expectation is that expression of these proteins will prevent *Xf* movement and reduce its inoculum leading to a reduction of the spread of PD. Transgenic grapevine plants expressing either PGIP, the human neutrophil elastase-cecropin B (HNE-CECB) chimeric antimicrobial protein and pgip-HNE-CECB have been obtained and the first batches have been tested to validate their efficacy against PD.

Plants expressing pear PGIP have five different modifications to better understand its ability to restrict disease spread. Four of the PGIPs 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) and one without a signal peptide which serves as a control. Based on PGIP activity eight of 10 mPGIP, two of five Ramy, three of 11 XPS, eight of 11 ChiPGIP and six of 10 NtPGIP *in vitro* lines have been transferred to the greenhouse. Fifteen of 27 PGIP transgenic lines (one mPGIP, eight ChiPGIP and six NtPGIP) have been manually inoculated with *Xf* and are in early stages of evaluation for tolerance to PD and movement of PGIP protein. The remaining mPGIP, Ramy, XPS and Chi lines are in the process of multiplication for future *Xylella* infection challenge experiments.

Transgenic grapevine plants expressing a chimeric anti-microbial protein HNE-CECB with its own signal peptide and pgip-HNE-CECB expressed with the signal peptide from pear PGIP have been obtained. The expressed chimeric anti-microbial protein has two functional domains, one (the surface recognition domain, SRD) that specifically binds to the *Xf* outer-membrane protein MopB and the other domain inserts into the membrane causing pore formation that results in the lyses of *Xf* causing its mortality. Twenty-one of 36 HNE-CECB transgenic grapevine lines have been manually inoculated with *Xf* in the greenhouse**.** Observations from the first two rounds are very promising - five of these transgenic lines had low, and six lines had moderate symptoms when compared with wild type Thompson seedless control plants whose symptoms were severe. Magnetic resonance imaging (MRI) of stem sections revealed a variation in number of vessels clogged between negative control and transgenic lines. The remaining HNE-CECB and pgip-HNE-CECB are in the process of greenhouse multiplication to conduct future *Xylella* challenge infections.

LAYPERSON SUMMARY

Transgenic grapevines are being evaluated as rootstocks to mobilize two types of effector proteins to control Pierce's disease (PD) in wild type scion cultivars grafted to such rootstocks. The growth and productivity of grapevines is compromised by growth and movement of *Xylella fastidiosa (Xf)*, its invasion of individual xylem elements and its ability to colonize and occlude the water-conducting vessels which stresses the plant leading to its death. In this project we are analyzing the efficacy of xylem targeted effector proteins like polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein, the former to restrict the movement *Xf* across xylem elements reducing its pathogenicity and the latter to clear *Xf*

preventing its ability to colonize. Plants expressing PGIP have five different modifications to better understand its ability to restrict disease spread. These plants are being evaluated in the greenhouse for resistance to PD and in grafted plants to evaluate the long distance movement of PGIP. We have also evaluated 21of 36 HNE-CECB that we have in the greenhouse for clearance of *Xf*. We have obtained good evidence that at least five of the 11 evaluated lines show good tolerance to *Xf* infection and magnetic resonance imaging (MRI) of infected stem sections further revealed less number of vessels clogged in the transgenic as compared to control grapevine plants indicating clearance of the infected bacteria. Further experiments with these transgenic lines will confirm the efficacy of these two effector proteins in controlling this important disease of grapevines.

INTRODUCTION

Pierce's disease (PD) in grapevines is a vector transmitted disease where the causative agent a Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is deposited into the xylem tissue by the feeding action of the glassy-winged sharpshooter (GWSS), the insect vector that efficiently transmits the disease and is of greatest concern to growers in California. The virulence of the bacterium is associated with its ability to colonize xylem and its ability to move through pit pore membranes into adjacent water conducting elements (Roper et al. 2007). The growth and productivity of grapevines is compromised by growth and movement of this bacteria that limits itself to xylem tissues and its ability to occlude the water-conducting vessels. The University of California reported that the disease destroyed over 1,000 acres of California grapevines between 1994 and 2000, causing $30 million in damages. Globally, one-fifth of potential crop yields are lost to plant diseases primarily of bacterial origin. Our strategy is based on developing and testing proteins that will limit movement of *Xf* and proteins that will clear the bacteria. Xylem, the target tissue for this organism, is composed of nonliving cells (tracheids and vessel elements) which join end to end to form water-conducting "pipes" from roots to the leaves and fruits. We are developing a transgenic rootstocks that will produce proteins that can migrate through the graft union into the xylem of the scion to immobilize and clear infecting *Xf* bacteria (Aguero et al. 2006). We have previously shown that grapevine plants expressing a polygalacturonase inhibitory protein (PGIP) are able to protect the plant presumably by limiting the movement of *Xf* (Aguero et al. 2005). Recently, it has been shown that *Xf* expresses a polygalacturonase (PG) a virulence factor that it uses to degrade the pectin containing pit pore membranes in grapevines allowing it to move from one xylem vessel to another (Roper 2007). We also showed that expression of PGIP in grapevine rootstocks is associated with secretion of this protein into the xylem and its movement through the graft union and its presence in xylem sap of the grafted wild type scion grapevine (Aguero et al. 2005). Because *Xf* is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and potential antimicrobial proteins needs to occur to prevent and control PD infestations. Signal peptides control entry of virtually all proteins to the secretory pathway in both eukaryotes and prokaryotes. The N-terminal part of the amino acid chain is cleaved off when the protein is translocated through the endoplasmatic recticulum membrane (Nielsen 1997). Signal peptides are generally interchangeable, so proteins that are not usually secreted can become secretion-competent through attachment of a signal peptide to the N-terminus of the mature protein, allowing its entry into the vesicular transport system (Vitale and Denecke 1999). We and others have characterized proteins naturally secreted to the xylem of grapevines as they are excellent sources of potential signal peptides (Aguero et al. 2008; Jain and Basha 2003). Our final goal is to use signal sequences from grapevine xylem proteins to deliver therapeutic proteins into the xylem of transgenic rootstocks, thus conferring resistance to PD in the entire plant without modifying the scion or affecting the fruit. Additionally we have taken a structure-based approach to develop a chimeric anti-microbial protein for rapid destruction of *Xf* (Kunkel et al. 2007). The designed chimeric anti-microbial protein has two functional domains, one a surface recognition domain, SRD that specifically targets the bacterium's outer membrane and the other domain contains a lytic protein to lyse the membrane and kill *Xf*. In this chimera, human neutrophil elastase (HNE) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (Bruening et al. 2002). The second domain is cecropin B, a lytic peptide that targets and lyses Gram-negative bacteria (Kunkel et al. 2007). We have combined HNE and cecropinB using a flexible linker so both components can bind simultaneously to their respective targets. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several immediate and long term impacts. The strategy is based upon the fundamental principle of innate immunity in which pathogen clearance occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction (Pieters 2001, Baquero and Blazquez 1997).

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

12 mPGIP, 5 Ramy, 11 XSP, 11 ChiPGIP and 10 NtPGIP, plants were assayed for polygalacturonase inhibiting activity in transgenic tissue extracts to validate the introduced transgene expression and were found to display a range of PG inhibitory activity from 0-22%, 0-44%, 0-28%, 0-57 % and 0-45 %, respectively corresponding to the source of the indicated signal

peptide (**Table 1**). The ChiPGIP expressing plants displayed the greater number of lines with strong inhibition than the other lines and all lines assayed showed some level of polygalacturonase inhibiting activity. Also, compared to ChiPGIP there were more lines, three Ramy, five NtPGIP vs one ChiPGIP, which had barely detectable inhibitory activity (**Table 1**). Based on PGIP activity eight mPGIP lines with none (expected) to medium, two Ramy with strong, three XPS with medium, six ChiPGIP with medium to strong and eight NtPGIP with medium to strong PGIP activity have been transferred to the greenhouse and acclimated (**Figure 1 A, B**).

Table 1. Current status of testing of transgenic *Vitis vinifera* var Thompson Seedless grapevines lines expressing PGIP fused with different signal peptides

No.	Signal peptide	Binary Vector	Plant Lines	(+) PCR for PGIP	(+) PGIP Activity	Moved to Greenhouse	*Xf* inoculated	Lines grafted
1	none	pDU05.1002	12	10	9	8	1	1
2	Ramy	pDU05.0401	5	5	4	2	0	0
3	XSP	pDA05.XSP	11	11	5	3	0	0
4	Chi	pDU06.0201	11	11	10	8	8	1
5	Nt	pDU05.1910	10	10	5	6	6	1

Figure 1. Process for greenhouse validation of transgenic grapevine plants and challenge with *Xf* to observe susceptibility to PD. A) Acclimating *in vitro* plants in the GH (3-4 weeks). B) Generating initial mother plants (6 weeks). C) Generating sufficient mother plants for propagation (7 weeks). D) Propagating 180-210 plants for each round of experiment (3 weeks). E) Transferring rooted plants to soil. F) Plants ready for inoculation after 8-10 weeks. G) Inoculation. H) Appearance of first PD symptoms after inoculation (6-7 weeks).

Each acclimated transgenic line was propagated to obtain four-six plants (**Figure 1C**) that are used as mother plants for further propagation to provide cuttings for *Xylella* infection and grafting experiments. From each line, 25-35 plants are propagated (from cuttings) at the same time (**Figure 1D-F**). *Xylella infection* experiments are done in multiple rounds. Each round consists of five-six transgenic lines and two controls, wild type Thompson (TS) and TS50 as negative and positive control, respectively. Each round of experiments includes 30 plants from each transgenic line, 15 of these are inoculated and the remaining 15 are non-inoculated controls. The positive control, T50 is a transgenic PGIP expressing grapevine previously described (Aguero et al. 2005).

Transgenic TS and controls (wild type TS and TS50) plants are inoculated with 20µl of the GFP expressing *Xf* 3A2 (Newman et al. 2003) containing ~20,000,000 cells. The plants are inoculated with 10 µl the first day and re-inoculated with 10µl the second day; for each inoculation an independently grown *Xylella* culture was used. The *Xylella* is introduced to each plant approximately three-four inches above the soil using an insect pin number zero as shown in the **Figure 1G**. Plants are pruned regularly and kept approximately 90-100cm tall until PD symptoms appear. The time required to conduct each round of *Xylella* challenge is 33 to 37 weeks, starting from *in vitro* plants transferred to greenhouse until the appearance of the first PD symptoms (**Figure 2**).

Fifteen of 27 PGIP transgenic lines (one mPGIP, eight ChiPGIP and six NtPGIP) have been manually inoculated (Almeida and Purcell 2003a) with *Xf* and they are in early stages of evaluation for tolerance against PD. Inoculated grapevines will be evaluated for symptoms of PD after three months. The remaining mPGIP, Ramy, XPS and Chi lines are in the process of multiplication for *Xylella* challenge in the greenhouse. Those lines that show low or moderate PD symptoms after manual inoculation will be tested by insect inoculation of *Xylella* (Almeida and Purcell 2003b). Transgenic grapevines after inoculation with *Xf* are scored for PD symptoms at regular intervals after infection using a standardized score based on percentage of leaf area scorching , a characteristic of PD (Krivanek et al. 2005a, 2005b).

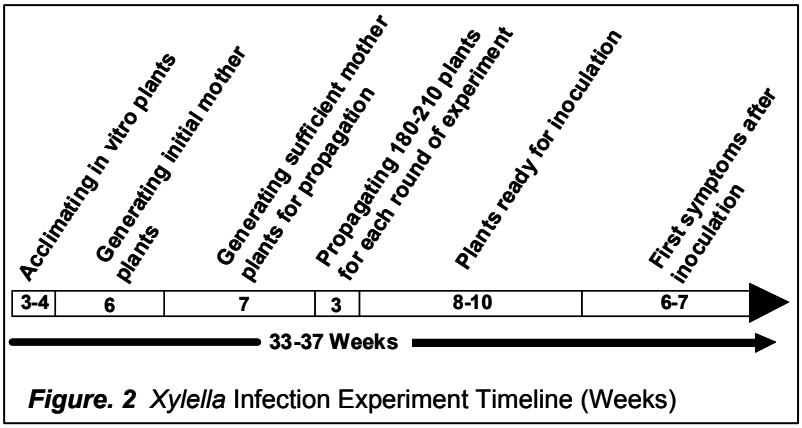

Figure. 2 *Xylella* Infection Experiment Timeline (Weeks)

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 scion. 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. We have initiated grafting experiments where selected transformed lines (rootstocks) were grafted with wild type TS (scion). The movement of the PGIP protein from the rootstock up into the xylem of the wild type scion was evaluated using the radial 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 3**).

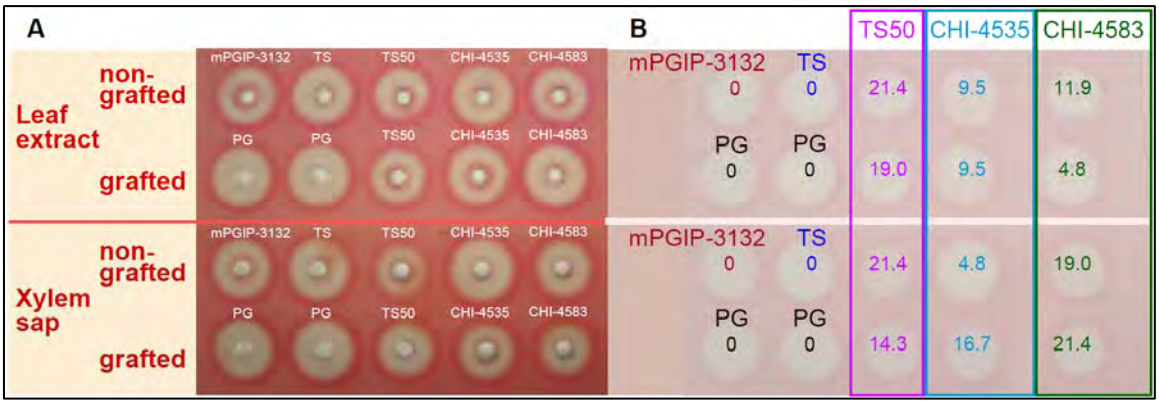

Figure 3. Zone inhibition assay to evaluate PG inhibition activity. **A,** assay plate image; **B,** percent inhibition measured in the assay. Leaf extracts and xylem sap from non-grafted and grafted transformed TS50, CHI 45-35 and CHI 45-83 lines were positive for PGIP activity. Transgenic mPGIP 31-32 that has no signal peptide and wild type TS that has no PGIP show no inhibitory activity. PG is the negative control and TS50 is a positive control.

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 as described in earlier reports with the two constructs, pDU04.6105 (Elastase-Cecropin = HNE-CECB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CECB). Sixteen of 21 HNE-CECB lines are currently being evaluated for resistance/tolerance to PD. The first two rounds of infection have been completed for the testing of 11 transgenic lines. First PD associated leaf scorch symptoms were visible on control TS grapevines within six-seven weeks post inoculation which consists of formation of green islands on the cane and scorching around outer edge of the lower leaves. Most transgenic HNE-CECB expressing lines showed less or delayed disease symptom compared to non-transgenic control and five lines were substantially more resistant than the rest (**Figure 4**). PD symptoms on each of the infected plants were numerically scored based on percentage scorch (**Table 2**).

Figure 4. Leaf number 8 above point of inoculation harvested 10 -11 weeks post-inoculation

Table 2. Disease phenotypic scoring[a] for transgenic grapevines infected with *Xf*.

	Mean	Mean		Mean	Mean
Round 1	**7 weeks post-inoculation**	**11 weeks post-inoculation**	**Round 2**	**10 weeks post-inoculation**	**14 weeks post-inoculation**
TS	0.73	4.90	TS	4.15	4.40
40-39	0.80	4.18	40-36	2.30	3.00
40-41	0.80	3.34[b]	40-74	2.80	3.10
41-151	0.14[b]	2.70[b]	40-89	2.00[b]	2.50[b]
41-166	0.50	3.74	40-92	1.50[b]	2.50[b]
41-179	0.60	4.78	41-146	2.12[b]	2.50[b]
[a]Scoring system is based on scale of 0 to 5, 0 = 0% and 5 = 100% scorch (leaf dropped). [b]P value is less than 0.001.			41-157	2.30	3.10

MRI images from stem sections from approximately 15-20cm above point of inoculation reveal clearance of bacterial inoculums in transgenic lines expressing less PD symptoms correlated to a variation in number of vessels clogged between negative control and transgenic lines (**Figure 5**). To obtain MRI xylem vessel cross section images an Avance 400 instrument was used. Instrument setting was: TR: 110.7, TE: 4.5ms, FA: 30.0deg, TA: 1:25NEx4, FOV: 1.2cm, MTX 256/192, Pos-0.80mmF.

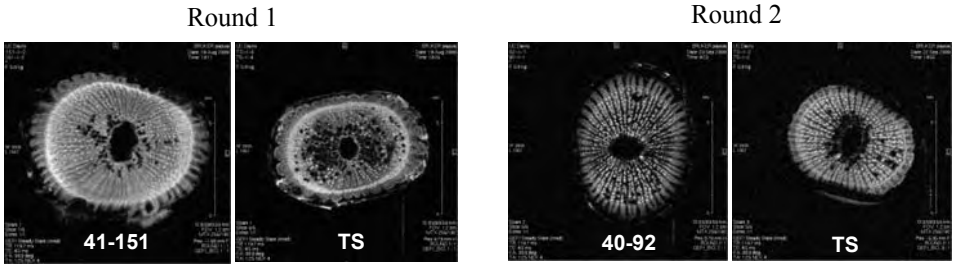

Figure 5. MRI images from experimental and non-transgenic control (TS).

CONCLUSIONS

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

PGIP transgenic grapevines lines that are secreted with four different signal peptides are being evaluated for their improved ability to secrete PGIP long distance through the graft union. These plants are also being manually inoculated with *Xf* to evaluate increased tolerance against PD associated with increased secretion efficiency associated with specific signal peptide sequence. Initial grafting experiments showed that Chi signal peptide mobilized PGIP efficiently when Chi transgenic plant was used as rootstock to the TS wild plant used as scion.

Eleven 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.

REFERENCES CITED

Aguero, C.B., S.L. Uratsu, C. Greve, A.L.T. Powell, J.M. Labavitch, and A.M. Dandekar. 2005. Evaluation of tolerance to PD and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pat. 6:43-51.

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.

Aguero, C.B., E.T. Thorne, A.M. Ibanez, W.D. Gubler and A.M. Dandekar. (2008). Xylem sap proteins from *Vitis vinefera* L. Chardonnay. Am. J. Enol. Vitic. 59(3): 306-311.

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

Almeida, R.P.P., and A.H. Purcell. 2003b. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). J. Econ. Entomol. 96: 264-271.

Baquero, F., and J. Blazquez. 1997. Evolution of antibiotic resistance. Trends Ecol. Evol. 12:482-487.

Bruening, G., E. Civerelo, B. Kirkpatrick, and D. Gilchrist. 2002. Virulence analysis of the Pierces Disease agent *Xf*. PD research symposium Dec 15-18 2002, San Diego, CA.

Jain, A.K., and S.M. Basha. 2003. A capillary electrophoretic method for isolation and characterization of grape xylem proteins. African J. Biotechnol. 2: 66-70.

Krivanek, A.F., Stevenson, J.F., and Walker, M.A. 2005a. Development and comparison of symptom indices for quantifying grapevine resistance to PD. Phytopathology 95:36-43.

Krivanek, A.F., Stevenson, J.F., and Walker, M.A. 2005b. *Vitis* resistance to PD is characterized by differential *Xylella fastidiosa* population in stems and leaves. Phytopathology 95:44-52.

Kunkel, M., M. Vuyisich, G. Gnanakaran, G.E. Bruening, A.M. Dandekar, E. Civerolo, J.J. Marchalonis and G. Gupta. 2007. Rapid clearance of bacteria and their toxins; development of therapeutic proteins. Crit. Rev. Immunol. 27(3): 233-245.

Newman, K.I., Almeida, R.P.P., Purcell, A.H., and Lindow, S.E. 2003. Use of green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Appl. Env. Microbiol. 69: 7319-7327.

Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10: 1-6.

Pieters, J. 2001. Evasion of host cell defense by pathogenic bacteria. Curr. Opin. Immunol. 13:37-44.

Roper, M.C., Greve, L.C., Warren, J.G., Labavitch , and J.M., Kirkpatrick, B.C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. MPMI 20:411-419.

Vitale, A., and J. Denecke. 1999. The endoplasmic reticulum: Gateway of the secretory pathway. Plant Cell 11: 615-628.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.