Progress Report for CDFA contract number: 08-0175

Project Title: *In Planta* Testing of Signal peptides and Anti-Microbial Proteins for Rapid Clearance of *Xylella*.

Principal Investigator (PI)
Abhaya M. Dandekar, Plant Science Department; UC Davis, CA 95616
Phone: 530-752-7784; fax: 530-752-8502; Email: amdandekar@ucdavis.edu

Cooperators
John Labavitch, Plant Science Department; UC Davis, CA 95616
Phone: 530-752-0920; fax: 530-752-8502; Email: jmlabavitch@ucdavis.edu

Rodrigo Almeida, Dept of Environmental Science, Policy and Management; UC Berkeley, CA 94720
Phone: 510-642-1603; fax: 510-643-5438; Email: Rodrigo@nature.berkeley.edu

Ana Maria Ibanez, Plant Science Department; UC Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: amibanez@ucdavis.edu

Sandie L. Uratsu, Plant Science Department; UC Davis, CA 95616
Phone: 530-752-7784; fax: 530-752-8502; Email: sluratsu@ucdavis.edu

Cecilia Aguero, Plant Science Department; UC Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: cbaguero@ucdavis.edu

Reporting period: The results reported here are from work conducted October 2008 to February 2009.

ABSTRACT

Xylella fastidiosa (X. fastidiosa), a Gram-negative bacterium, is the causative agent of Pierces's disease. Because *X. fastidiosa* is xylem-limited, any potential anti-*X. fastidiosa* gene product must be present in xylem at an effective concentration to provide disease control. Understanding how existing proteins are transported to xylem is necessary to target delivery of therapeutic proteins to this organ. We collected xylem exudate from *Vitis vinifera* cv. Chardonnay and analyzed its protein composition by two-dimensional gel electrophoresis, then purified and sequenced some of the abundant proteins to identify corresponding genes in the grapevine EST database. We identified the signal sequences present in these gene sequences and made vectors where these signals were fused to mature polygalcturonase inhibiting protein (mPGIP), which is secretion competent. Five different vectors were successfully constructed to test four signal sequences. These vectors were incorporated into *Agrobacterium* and used to transform grapevine. Callus and embryos were successfully selected and regenerated to give transformed grapevine lines for each construct. Plants have been obtained for all five constructs and protein inhibiting activity for four constructs has been tested. 8 of 11 Chi, 5 of 5 Nt and 1 of 12 mPGIP *in vitro* lines have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to micropropagate. The remaining construct containing the XSP signal peptide is unusually slow to micropropagate. We also found that Chi signal peptide mobilized PGIP from the Chi transgenic plant used as rootstock to the TS wild plant used as scion. Next we will validate that other signal peptides are essential and sufficient to mobilize proteins into grapevine xylem. Such transgenic proteins, if synthesized in a rootstock, could confer resistance to xylem-specific infections such as Pierce's Disease and assist in control of *X. fastidiosa*.

As an alternative to various signal peptides fused to PGIP we designed a chimeric anti-microbial protein with two functional domains. One domain (the surface recognition domain, SRD) specifically binds to the bacterium outer-membrane and the other domain lyses the membrane and kills *X. fastidiosa*. In this chimera, human neutrophil elastase (HNE) is the SRD that recognizes MopB, the major outer membrane protein of *X. fastidiosa*. The second domain is cecropin B (CECB), a lytic peptide that targets and lyses gram-negative bacterial membranes. We have combined HNE and CECB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized in two versions, one with a mammalian signal peptide sequence designated HNE-CECB and the other with a signal peptide sequence from a plant *pgip* gene designated pgip-HNE-CECB, incorporated into binary vectors, and transformed into grapevine (*Vitis vinifera* var 'Thompson Seedless') and SR1 tobacco using *Agrobacterium.* Plant transformation experiments with both HNE-CECB and pgip-HNE-CECB were successful and plants have been obtained. Using PCR, the presence of HNE-CECB or pgipHNE-CECB was confirmed in 36 and 7 plants, respectively. 29 of 36 HNE-Cec and five of 11 pgip HNE-Cec *in vitro* lines have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to micropropagate.

OBJECTIVES

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

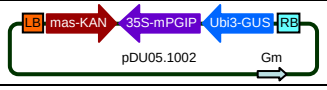
2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*X. fastidiosa* activity *in planta,* and test for graft transmissibility.

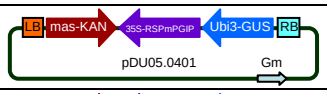
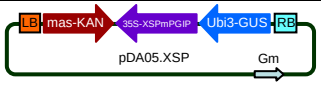
RESULTS AND DISCUSSION

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

In previous research, peptide spectrum and BLAST analysis showed that the proteins found in grape xylem exudates are secreted and share function similarities with proteins found in xylem exudates of other species (Buhtz et al. 2004). cDNA sequences matching 2 of them found in the TGI *Vitis vinifera* gene index (http:compbio.dfci.harvard.edu/tgi/plant.html) were used to design primers that were used to amplify the predicted fragments from genomic DNA of 'Chardonnay' and 'Cabernet Sauvignon' (Aguero et al 2008). These fragments were annotated as Chi1b and NtPRp27 (Table 1). These fragments were then fused to DNA sequences that contained the mature polygalacturonase inhibiting protein (mPGIP) gene through gene splicing using a PCR-based overlap extension method (SOE) (Horton et al. 1990) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). These two chimeric genes were then ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator and the resultant expression cassettes were then ligated into the binary vector pDU99.2215 (Escobar, et al. 2001) which contains an *nptII*-selectable marker gene and a *uidA* (β-glucuronidase, GUS) scorable marker gene. The mature PGIP sequences without any signal peptide sequences was also incorporated into pDU99.2215 to serve as a control and this vector is designated pDU05.1002 (Table 1). We also incorporated signal peptides from the xylem sap protein XSP30 and the rice amylase protein Ramy3D that we have described in earlier reports. These binary vectors are designated pDA05.XSP and pDU05.0401, respectively (Table 1).

Table 1: Construction of vectors for the expression of mature PGIP with various signal peptide sequences

No	Signal Peptide	Binary Plasmid Map	Reporter Gene	Promoter	Marker Genes	Vector
1	None		Mature PGIP	CaMV35S	GUS and Kan	pDU05.1002

2	Rice amylase-Ramy3Dsp		Mature PGIP	CaMV35S	GUS and Kan	pDU05.0401
3	Xylem sap protein 30-XSP30sp		Mature PGIP	CaMV35S	GUS and Kan	pDA05.XSP
4	Chi1b signal peptide		Mature PGIP	CaMV35S	GUS and Kan	pDU06.0201
5	NtPRp27 signal peptide		Mature PGIP	CaMV35S	GUS and Kan	pDU05.1910

Binary vector # 1 is the control and should be immobile although PGIP with its native signal peptide is secretion competent in grape. In binary vector #2, mature PGIP has been fused to the signal sequence of rice amylase 3 (Ramy3D), which has been very effective in secretion of human α_1-antitrypsin in rice cell cultures (Trexler et al. 2002). In binary vector # 3 mature PGIP has been fused to the signal sequence of cucumber XSP30, which is a xylem-specific protein. Constructs 4 and 5 have been described above.

As a result of the above transformation plants have been obtained for all 5 vectors (Table 1). Plants from the above vectors except the one containing the XSP signal peptide have been analyzed using PCR (Table 2). DNA was isolated from *in vitro* leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primers for detection of PGIP were 5' Mature PGIP: 5' ATGGATCTCTGCAACCCCGACGAC and 3'PGIP: 5' TTACTTGCAGCTTGGGAGTG.

Tissue from most of these plants has been tested for PGIP activity using the zone inhibition assay with PG (Table 2) (Taylor and Secor 1988). PG preparations were obtained from *Botrytis cinerea* strain Del 11 isolated from grape (Aguero et al. 2005). Protein from leaf tissue (~100mg) was extracted in extraction buffer (Dandekar, et al. 1998) at a ratio of 1 ul/mg. Tissue was ground in a 2 ml tube containing a 5mm stainless steel bead in a TissueLyzer (Qiagen). The homogenate was centrifuged at 16000 xg for 5 minutes and the supernatant was used for testing.

The 11 ChiPGIP, 10 NtPGIP, 10 mPGIP and 5 Ramy plants assayed for polygalacturonase inhibiting activity had a range of inhibition from 6-62 %, 0-45 % , 0-22% and 0-44%, respectively . The ChiPGIP plants had a greater number of lines with strong inhibition than the other lines and all lines assayed showed polygalacturonase inhibiting activity. Also, there were more, 3 NtPGIP, 5 mPGIP and 3 Ramy vs 0 ChiPGIP, that had no inhibition activity. The 6 ChiPGIP plants with strong , 5 NtPGIP with strong to medium and 1 mPGIP with medium polygalacturonase inhibiting activity are have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to propagate to obtain 30 clones of each line including TS wild type as a negative control and TS50 as a positive control.

Table 2: Current status of testing transgenic lines *Vitis vinifera* var Thompson Seedlees grapevines with different signal peptides and PGIP gene

No.	Signal peptide	Plasmid	Plant Lines	(+) PCR for PGIP	(+) PGIP Activity	Lines grafted	Moved to Greenhouse
1	Mature	pDU05.1002	12	10	4		1
2	Ramy	pDU05.0401	5	To be tested	2		none
3	XSP	pDA05.XSP	11	9	To be tested		none
4	Chi	pDU06.0201	11	11	10	3	8
5	Nt	pDU05.1910	10	10	5		5

Once they have been micropropagted they can be used for *Xylella* experiments to determine efficacy of the PGIP protein. The vines will be allowed to grow up to 6"-12" (about 10 nodes long), then inoculated with *X.*

fastidiosa by hand and by insect and evaluated for symptoms of Pierce's disease (PD) after 3 months. Since we found in earlier research that pPGIP with its endogenous signal peptide is xylem competent we are using a grapevine successfully transformed with this construct and highly expressed as a positive control in the inoculation experiments (Aguero et al. 2005).

We have also initiated grafting experiments where selected transformed lines were grafted with wild type TS scion (Fig 1). 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 xylem sap from Chi45-35 non grafted and grafted showed 18% of inhibiting activity for both, indicating that the PGIP is moving from the rootstock to scion. Also xylem sap from Chi 45-15 grafted showed a 14% inhibiting activity (Fig.2),

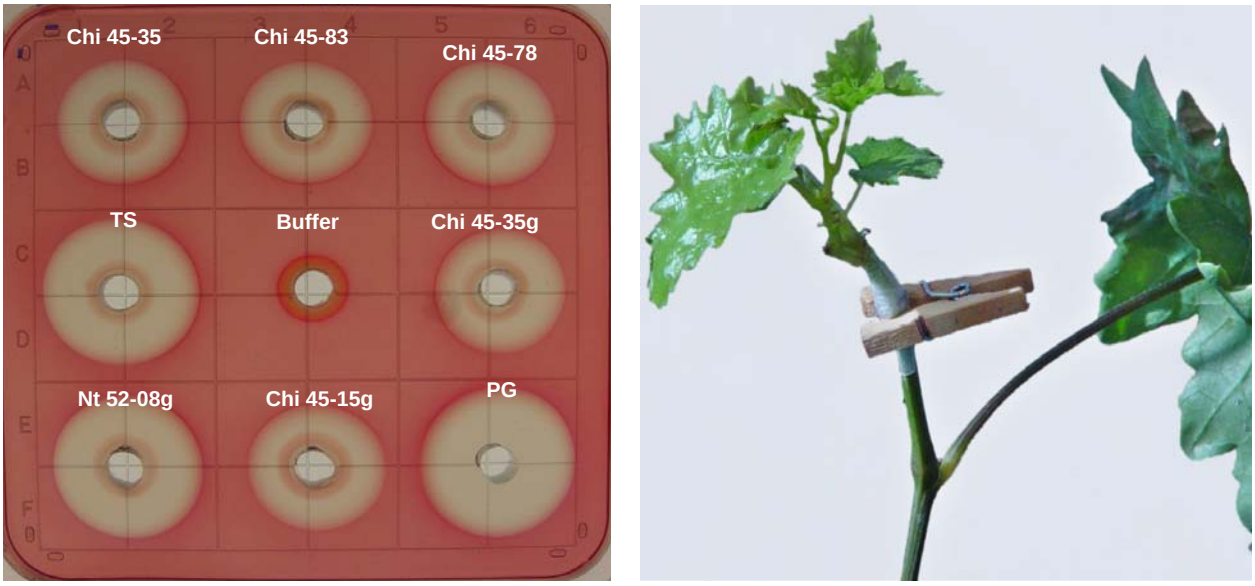


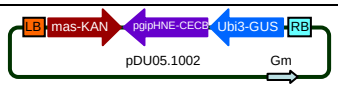
Figure 1. PGIP assay of xylem sap from wild type plants grafted to transgenic plants rootstocks expressing mPGIP with various signal peptides. Names ending with 'g' were grafted; the rest were not grafted.

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

In vitro grape plants transformed with the constructs, pDU04.6105 (Elastase-Cecropin = HNE-CECB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CECB) (Table 3), have been received from the Parsons Plant Transformation Facility. 69 HNE-CECB plants and 18 pgipSP-HNE-CECB plants have been screened by PCR to verify the individual transformation events. PCR was performed on DNA isolated from leaves using the Qiagen DNeasy Plant Mini Kit. Primers used for detection of elastase were: CaMV355-2: 5' GACGTAAGGGATGACGCACAAT and 3HNEb: 5' TTACTAGAGTGCTTTTGCTTCTCCCAG. The resulting 36 HNE-CECB plants (Table 4) are being micropropagated for transfer to the greenhouse and for RNA and protein analysis.

Table 4: Construction of vectors for the expression of HNE-CECB and pgipHNE-CECB

No	Signal Peptide	Binary Plasmid Map	Reporter Gene	Promoter	Marker Genes	Vector
1	HNE-CECB	LB mas-KAN HNE-CECB Ubi3-GUS RB pDU05.6105 Gm	HNE-CECB	CaMV35S	GUSand KAN	pDU04.6105

2	pgipHNE-CEPB		HNE-CECB	CaMV35S	GUSand KAN	pDA05.0525

29 of 36 HNE-Cec *in vitro* lines have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to micropropagate 30 plants each including the TS wild type as a control. Six lines have been analyzed via RT-PCR for elastase expression yielding results between 0.61-0.89 % of actin. RT-PCR was performed on RNA isolated from greenhouse grown grape leaves (fourth fully opened leaf) using Qiagen RNeasy Plant Mini kit (Valencia, CA). 50 ng of RNA was/were analyzed using Promega Access RT-PCR kit (Madison, WI). Primers used for amplification of elastase were HNE: HGCb-1, CGTCTCGCTTGCCTTTTCCTAGCCTGTGTGTTGC and HGCb-2, GGCTTTAACTATTCCGTTTCGAATATTCC. Actin primers were used as internal standards used to normalize the elastase expression levels. ActinF sequence was: TACAATGAGCTTCGGGTTGC and for ActinR: GCTCTTTGCAGTTTCCAGCT.

Five of 10 pgip HNE-Cec *in vitro* lines have been transferred to the greenhouse and are in the process of acclimating and growing enough tissue to micropropagate (Table 4). This will be followed up with a more significant evaluation disease susceptibility using both needle (Almeida and Purcell 2003a) and insect (Almedia and Purcell 2003b) inoculations in the greenhouse.

Table 4: Current status of testing transgenic lines *Vitis vinifera* var Thompson Seedlees grapevines with HNE-CECB and pgipHNE-CECB genes

No.	Signal peptide	Plasmid	Plants Lines	(+) PCR	(-) PCR	Moved to Greenhouse
1	HNE	pDU04.6105	36	36	0	29
2	PGIP	pDA05.0525	10	7	3	5

CONCLUSIONS

The main objective of this project is to develop a potent therapy against Xf by utilizing the principles of innate immunity by which plants counteract virulence factors like PG with PGIP or that recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. Because Xf is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and the antimicrobial chimeric proteins, may be used to prevent and control PD. Five different vectors were successfully constructed to test four signal sequences to target PGIP to the xylem of grapevine. Plants have been obtained for all five constructs and PG inhibiting activity for four constructs has been tested. Transgenic lines expressing Chi-PGIP, Nt-PGIP and mPGIP are in the process of acclimating and growing enough tissue to micropropagate We also found that Chi signal peptide mobilized PGIP from the Chi transgenic plant used as rootstock to the TS wild plant used as scion. We are testing two constructs containing two versions of the chimeric protein Elastase-Cecropin and have transformed tobacco and grapevine. Expression in tobacco indicates that protection against Xf looks promising. Transgenic grapevines expressing these two constructs have been obtained and are being screened and propagated for greenhouse testing. Next we will validate the efficacy of these two types of proteins to ensure that our signal sequences are essential and sufficient to mobilize proteins into grapevine xylem and that the targeted chimeric proteins control Xf in grapevine tissues. Such transgenic proteins, if synthesized in a rootstock, could confer resistance to xylem-specific infections such as Pierce's Disease and assist in control of *X. fastidiosa* infestations.

STATUS OF INTELECTUAL PROPERTY

No disclosures have been made.

PUBLICATIONS RESULTING FORM THIS WORK

Dandekar, A.M., Labavitch, J., Ibanez A.M., Aguero, Cecilia., and McFarland, S. 2008. : *In Planta* Testing of Signal peptides and Anti-Microbial Proteins for Rapid Clearance of *Xylella*. Syposium Proceedings for Pierce Disease Research Symposium. San Diego, CA USA,

Aguero, C.B., Thorne, E.T., Ibanez, A.M., Goubler, W.D., and Dandekar, A.M. 2008 Xylem Sap proteins from Vitis vinifera L. Chardonnay. A, J. Enol. Vitiv. 59:306-311.

LITERATURE CITED

Aguero, C.B., E.T. Thorne, A.M. Ibanez, W.D. Goubler, and A.M. Dandekar. 2008. Xylem sap proteins from Vitis vinifera L. Chardonnay. A. J. Enol. Vitiv. 59:306-311.

Aguero, C.B., S.L. Uratsu, C. Greve, A.L.T. Powell, J.M. Labavitch, and A.M. Dandekar. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pat. 6:43-51.

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.

Buhtz, A., A. Kolasa, K. Arlt, C. Walz,, and J. Kehr. 2004. Xylem sap protein composition is conserved among different plant species. Planta 219:610-618.

Dandekar, A.M., G.H. McGranahan, P.V. Vail, S.L. Uratsu, C.A. Leslie, and J.S. Tebbets. 1998. High levels of expression of full-length *cry*IA(c) gene from *Bacillus thuringiensis* in transgenic somatic walnut embryos. Plant Science 131:181-193.

Escobar, M.A., Civerolo, E. L., Summerfelt, K.R., Dandekar, A.M., 2001. RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis, Proc. Natl. Acad. Sci. USA 98:13437-13442.

Horton, R.M., Z.L. Cai, S.N. Ho, and L.R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques 8:528-35.

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.

Roper, M.C., Greve C.L., Warren JG., Labavitch J.M., and Kirkpatrick B.C. 2007. *X. fastidiosa* requires polygalacturonase for colonization and pathogenecity in *Vitis Vinifera* grapevines. MPMI 20:411-419

Taylor R.J. and G.A. Secor. 1988. An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytophathology 78:1101-1103.

Trexler, M.M., K.A. McDonald, and A.P. Jackman. 2002. Bioreactor production of human α_1-antitrypsin using metabolically regulated plant cell cultures. Biotechnol Prog 18: 501-508.

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

Yoshida K., T. Matsui, and A. Shinmyo. 2004. The plant vesicular transport engineering for production of useful recombinant proteins. J. Mol. Catal. B: Enzym. 28:167–171.

FUNDING AGENCY: Funding for this project was provided for the California Department of Food and Agriculture, Pierce's Disease Control Program (CDFA-PD).

RESEARCH RELEVANCE

X. fastidiosa, a gram-negative bacterium, is the causative agent of Pierce's Disease (PD) in grapevines. Because *X. fastidiosa* is xylem-limited, it is essential that any anti-Xylella gene product be targeted to the xylem and have the ability to accumulate in this compartment to an effective concentration. Work on understanding the mechanism of how proteins are targeted to this plant compartment is relevant for the delivery of therapeutic and or effector proteins like PGIP to the xylem. In addition, targeting proteins to the xylem could also be used to influence or disrupt the *Xylella* and glassy-wing sharpshooter (GWSS) interaction as GWSS feeds on the xylem fluid.

Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes. In eukaryotes like plants they comprise the N-terminal part of the amino acid chain and are cleaved off while the protein is translocated through the membrane of the endoplasmic reticulum (Nielsen et al. 1997). Generally, signal peptides are interchangeable and secretion of non-secreted proteins becomes possible by the attachment of a signal peptide at the N-terminus of the mature protein (Vitale and Denecke 1999). We have previously demonstrated that expression of a secretory protein was sufficient to allow its entry into the vesicular transport system (Aguero et al., 2005). Numerous reports of successful recombinant protein production using signal peptides in transgenic plants have been reported; however, changing the signal sequence of recombinant proteins can affect the degree of protein production. For example, the efficiency of secretion of heterologous proteins in transgenic tobacco was improved by replacing the heterologous endogenous signal peptide with a signal peptide from a tobacco protein (Yoshida et al. 2004). Clearly the choice of the type of signal peptide could influence the efficiency of secretion and protein accumulation in the xylem.

In previous research we have found that the product of the pear PGIP encoding gene, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al 2005). Pear PGIP has a peptide sequence that directs its secretion to the apoplast and its presence in xylem vessels may represent protein secreted into the vessels through pit membranes that serve as transfer pathways from neighboring parenchyma cells. Polygalacturonase (PG) is an enzyme required for X. *fastidiosa* to successfully infect grapevines and is a critical virulence factor for *X. fastidiosa* pathogenesis in grapevines (Roper et al. 2007). The pear PGIP would neutralize Xf PG activity, is secretion competent in grapes and can be further enhanced by optimizing its targeting mechanism to the xylem. We have used the mature pPGIP, as a secretion competent product, fusing it to various signal peptides that can be used to determine the one that is most efficient.

The work described in this report corresponds to research priorities developed by the National Academies in their publication, "California Agriculture Research Priorities: Pierce's Disease" as outlined in Chapter 4, Recommendations 4.3, 4.4 and 4.5 and Chapter 3, Recommendation 3.3. Additionally, the objectives of this research project are relevant to the research recommendations from the CDFA PD/GWSS research scientific review final report from August 2007 as described on page 21 section F.1 by the CDFA Research Scientific Advisory Panel, specifically Inhibition of X. fastidiosa Polygalacturonase (PG) enzyme. The results of this research will not only be applied in projects that test anti-*Xylella* gene products that should be delivered into the xylem but also in functional studies of proteins that are intended to target the *X. fastidiosa* and GWSS interaction.