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

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Reporting period: The results reported here are from work conducted July 2009 to February 2010 for the performance period July 1, 2009 to June 30, 2010.

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.

SUMMARY

Xylella fastidiosa (*X. fastidiosa*), a xylem-limited Gram-negative bacterium, is the causative agent of Pierces's disease (PD). A key feature of *X. fastidiosa* 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 *X. fastidiosa*. The expectation is that expression of these proteins will prevent *X. fastidiosa* movement and reduce its innoculum 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 5 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 8 of 10 mPGIP, 2 of 5 Ramy, 3 of 11 XPS, 8 of 11 ChiPGIP and 6 of 10 NtPGIP *in vitro* lines have been transferred to the greenhouse. Fifteen of 27 PGIP transgenic lines (1 mPGIP, 8 ChiPGIP and 6 NtPGIP) have been manually inoculated with *X. fastidiosa* and are in early stages of evaluation for tolerance to Pierce's Disease and movement of PGIP protein. The remaining mPGIP, Ramy, XPS and Chi lines are in the process of multiplication for future *Xylella* inoculation 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 *X. fastidiosa* outer-membrane protein MopB and the other domain inserts into the membrane causing pore formation that results in the lyses of *X. fastidiosa* causing its mortality. Twenty-one of 36 HNE-CECB transgenic grapevine lines have been manually inoculated with *X. fastidiosa* in the greenhouse**.** Observations from the first two rounds are very promising - 5 of these transgenic lines had low, and 6 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. Xylem sap from HNE-CecB transgenic lines showing higher phenotypic resistance, showed higher mortality effect against *Xf* when compared to non-transformed control grapevine plants. Interestingly, DNA extracted from the same HNE-CecB transgenic lines showed lower pathogen load than control plants. The remaining HNE-CECB and pgip-HNE-CECB are in the process of greenhouse propagation to conduct future *Xylella* infection tests.

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 *X. fastidiosa*:

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. 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, 3 Ramy, 5 NtPGIP vs 1 ChiPGIP, which had barely detectable inhibitory activity. Based on PGIP activity 8 mPGIP lines with none (expected) to medium, 2 Ramy with strong, 3 XPS with medium, 6 ChiPGIP with medium to strong and 8 NtPGIP with medium to strong PGIP activity have been transferred to the greenhouse and acclimated (Fig1 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**	***X. fastidiosa* 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

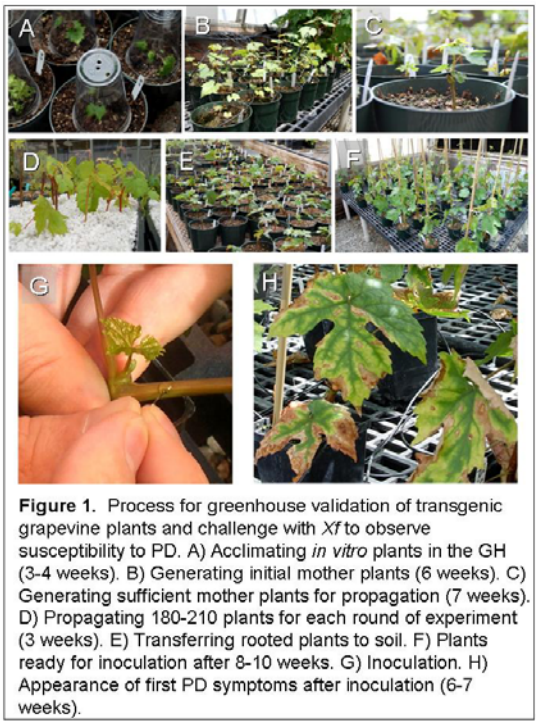

Figure 1. Process for greenhouse validation of transgenic grapevine plants and challenge with *Xf* to observe susceptibility to PD. A) Acclimating *in vitro* plants in the GH (3-4 weeks). B) Generating initial mother plants (6 weeks). C) Generating sufficient mother plants for propagation (7 weeks). 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 4-6 plants (Fig 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 (Fig 1D-F). *Xylella infection* experiments are done in multiple rounds. Each round consists of 5-6 transgenic lines and 2 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 (Almeida and Purcell 2003) 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 *X. fastidiosa* 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 3-4 inches above the soil using an insect pin number zero as shown in the Fig. 1G. Plants are pruned regularly and kept approximately 90-100cm tall until PD symptoms

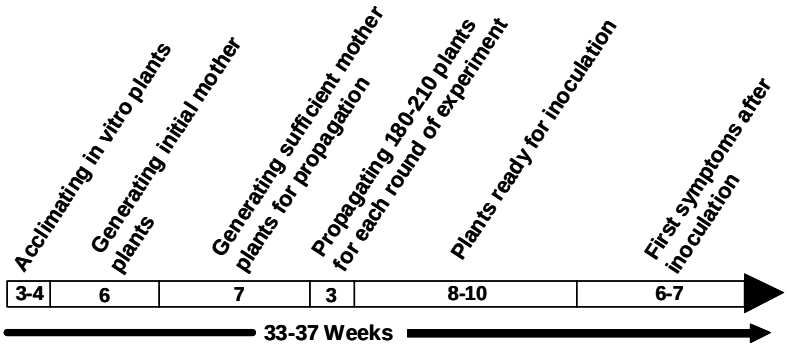

Figure. 2 *Xylella* Infection Experiment Timeline (Weeks)

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 (Fig. 2).

Fifteen of 27 PGIP transgenic lines (1 mPGIP, 8 ChiPGIP and 6 NtPGIP) have been manually inoculated (Almeida and Purcell 2003) with *X. fastidiosa* and they are in early stages of evaluation for tolerance against Pierce's Disease. Inoculated grapevines will be evaluated for symptoms of Pierce's disease (PD) after 3 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 Pierce's disease symptoms after manual inoculation will be tested by insect inoculation of *Xylella*. Transgenic grapevines after inoculation with *X. fastidiosa* are scored for Pierces's Disease 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).

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 (Fig.3).

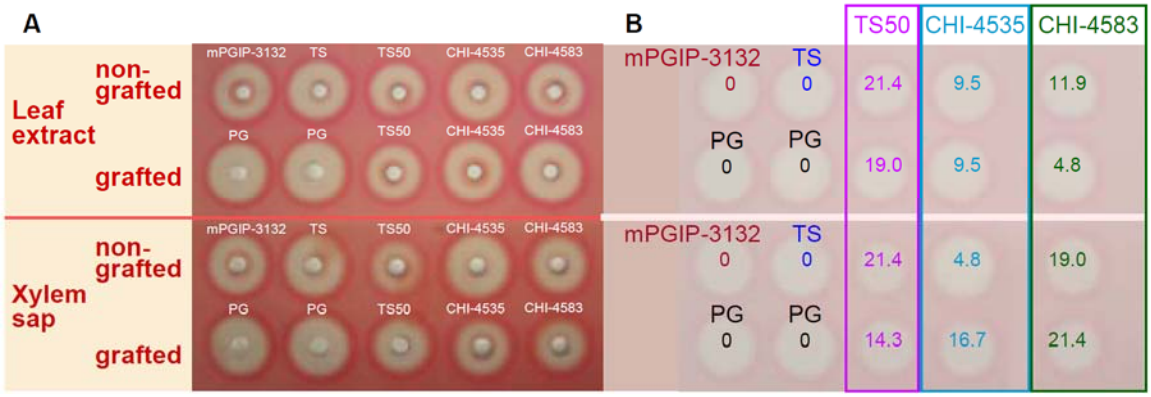

Figure 3. Zone inhibition assay to evaluate PG inhibition activity. **A,** Assay plate image; **B,** percent inhibition measured in the assay. Leaf extracts and xylem sap from non-grafted and grafted transformed TS50, CHI 45-35 and CHI 45-83 lines were positive for PGIP activity. Transgenic mPGIP 31-32 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-*X. fastidiosa* 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 Pierce's Disease. 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 6-7 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 5 lines were substantially more resistant than the rest (Fig. 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 *Xylella fastidiosa*

	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]
41-168	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 base 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 (Fig. 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.

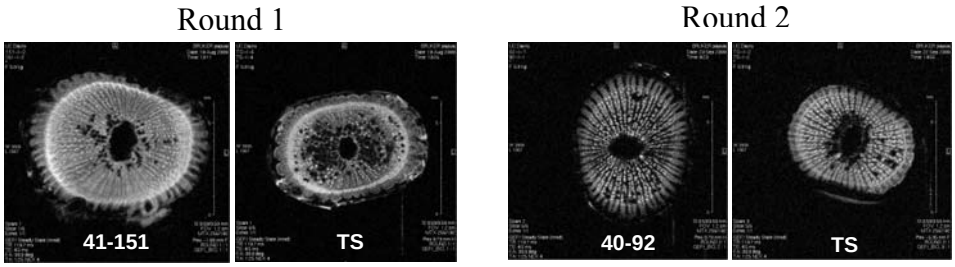

Fig. 5 MRI images from experimental and non-transgenic control (TS)

Xylem sap extracted from grape plants expressing HNE-CecB exhibits bacterial mortality. *Xf* was incubated with xylem sap extracted from transgenic lines at 28°C shaker. For each sample, three different dilutions of *Xf*-xylem sap mixture were plated on PD3 media at one hour time points for five hours. This experiment reveals the mortality effect of sap from transgenic lines containing antimicrobial protein (Fig. 6). In this experiment, transgenic lines expressing higher phenotypic resistance, also express higher *Xylella* mortality rate compared to untransformed control and buffer control (Fig. 6).

Semi Quantitative-PCR analysis of accumulation of Xf DNA has been done for groups of 3 stems sections collected approximately 10-15cm above point of inoculation. For each transgenic line DNA was extracted from 3 stem samples representing 3 individual plants, each bar in Fig. 7 is derived from 2-4 PCR reactions, with uncertainty bar representing the standard deviation. Based on this experiment, samples from plants expressing HNE-CecB contain less bacterial DNA which means less pathogen in the plant tissue.

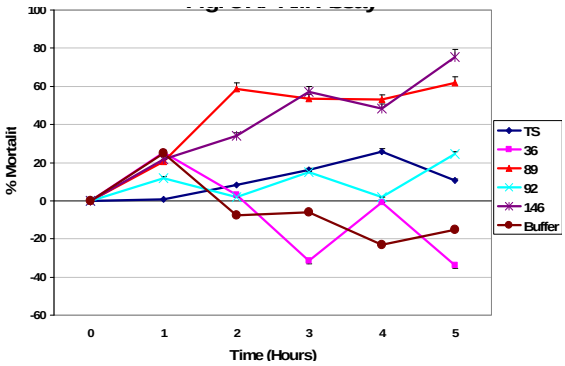

Figure 6. *Xylella* mortality curves obtained using xylem sap from Round 2 transgenic lines.

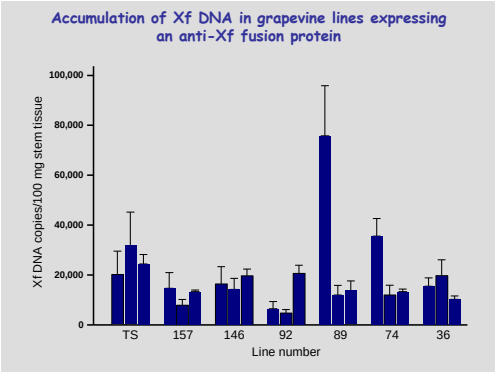

Figure 7. Sami-Quantitative PCR using DNA extracted form stem samples for Round 2 transgenic

PUBLICATIONS AND REPORTS RESULTING FROM THIS PROJECT

Dandekar, A.M., J. Labavitch, R. Almeida, A.M. Ibáñez, S.L. Uratsu, H. Gouran and C. Aguero, 2009. In planta testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*. Proceedings of the Pierce's Disease Research Symposium. Dec 9-11. Sacramento CA. pp. 117-122.

PRESENTATION ON RESEARCH

Dandekar, A.M. 2009. Improved transformation system for California grapevines rootstocks. Pierce's Disease Research Symposium. Dec 9-11. Sacramento CA.

Dandekar, A.M. 2009. Upcoming field trials of promising resistance technologies. Pierce's Disease Research Symposium. Dec 9-11. Sacramento CA. pp. 117-122.

The objective described in this proposal directly address the number 1 RSAP priority outlined in the, "Enabling tools- Development of grape regeneration and transformation systems for commercially important rootstocks" handout released in the December 2009 that outline the "Top 5 to 10 Project Objectives to Accelerate Research to Practice". 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.

LAYPERSON SUMMARY

Transgenic grapevines are being evaluated as rootstocks to mobilize two types of effector proteins to control Pierce's Disease in wild type scion cultivars grafted to such rootstocks. The growth and productivity of grapevines is compromised by growth and movement of *Xylella fastidiosa* (*X. fastidiosa*), 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 *X. fastidiosa* across xylem elements reducing its pathogenicity and the latter to clear *X. fastidiosa* preventing its ability to colonize. Plants expressing PGIP have 5 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 *X. fastidiosa*. We have obtained good evidence that at least 5 of the 11 evaluated lines show good tolerance to *X. fastidiosa* 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. Xylem sap from HNE-CecB transgenic lines showing higher phenotypic resistance, showed higher mortality effect against *Xf* when compared to control grapevine plants. Interestingly, DNA extracted from the same HNE-CecB transgenic 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.

STATUS OF FUNDS

The performance period for the first year of funding for this 2 year project is from July 1, 2009 to June 30, 2010 so as of March 1, 2010 the date of the report we are 8 month into spending on this grant. We have spent $70,235 on salaries and $11,099 on supplies as of March 1, 2010. The remainder $35,117 in salaries and $5,549 in supplies will be spent in the remaining 4 months March 1, 2010 to June 30 2010 that coincide with the completion of the 1st years funding.

STATUS OF INTELECTUAL PROPERTY

The discovery of the chimeric strategy to build antimicrobials is an LANL invention and UC Davis will work closely with LANL to further evaluate its commercial potential. The transgenic plants represent a unique contribution that further stimulates this idea and possibly creates a unique strategy for protein-based therapy of grapevines. The secretion sequences will be patented should they efficiently mobilize the chimeric protein into the xylem. Disclosures will be made to the UC office of technology transfer, which could develop these further as a US patent.

LITERATURE CITED

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

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

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