Interim Progress Report for CDFA Agreement Number 12-0128-SA

Project Title: Evaluation of Pierce's resistance in transgenic *Vitis vinifera* grapevines expressing either grape thaumatin-like protein or a *Xylella fastidiosa* hemagglutinin protein

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Time period covered by the report: The results reported here are from work conducted 3/13/2013 to 9/2/2013

Introduction

Previous research in our lab identified two hypervirulent mutants of *Xylella fastidiosa* (Xf). These mutations were in large hemagglutinin (HA) adhesion genes that we named *Hxf*A and *Hxf*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture, indicating that Xf HAs play an important role in Xf attaching to itself as well as xylem cell walls and insect vector mouth parts, demonstrated by the Alemedia lab at UC Berkeley. We hypothesize that if Hxf protein, or a portion of the Hxf protein that mediates adhesion, could be expressed in the xylem fluid of transgenic grapevines then perhaps insect-inoculated *Xf* cells would clump together and be less capable of colonizing grapevines. During the past 3 years we produced 9 transgenic HA-expressing tobacco and grapevine lines. Greenhouse inoculations of these transgenic tobacco and grape lines with Xf showed these vines produced less severe symptoms of Pierce's disease (PD) and Xf cells were retarded in there colonization of the transgenic grape vines compared to Xf-inoculated non-transgenic vines. With the assistance of PIPRA we secured all the necessary permits to plant these lines in the field at UC Davis on April 1, 2013. After they have grown sufficiently, the transgenic, as well as non-transgenic (control) grapevines will be mechanically inoculated with Xf in April 2014, the same time of the year that other transgenic grapevines produced by other researcher inoculated their vines. PD symptom severity will be compared between the HA-

expressing transgenic lines and non-transgenic, Xf-inoculated control vines in September 2014 and September 2015.

In a 5 year project that sought to better understand the cold curing phenomenon of *Xf*- infected grapevines, we found that cold-treated vines had significantly elevated levels of phenolics compounds as well as a grapevine thaumatin-like protein (TLP). TLPs from other plant species have been shown to possess antimicrobial activity, and grapevine TLP that was cloned and expressed in *E. coli* possessed some anti-Xf activity *in vitro*. Even though additional funding was not recently allocated to evaluate the potential resistance of TLP-expressing transgenic grapevines, we had already submitted the appropriate TLP transformation constructs to the UC Davis Plant transformation facility. We recently received 15 TLP transgenic lines that were confirmed to be transformed by standard PCR. Analysis of TLP RNA expression by RT-PCR showed elevated levels of TLP mRNA in the transformed lines with some lines producing 30X as much grape TLP as non-transformed grapes. Thus we have secured very good levels of TLP expression in the transgenic vines. These TLP lines were propagated in the greenhouse were mechanically inoculated with Xf in August 2013. Disease ratings and Xf populations in the inoculated, TLP transgenic vines will be compared to disease and Xf populations in non-transformed grapevines in October 2013.

Objectives:

1. Complete the characterization of grape transgenic plants over-expressing Xf hemagglutinin (Hxf) protein.

2 Mechanically inoculate wild type *Xf* and evaluate the effect on Pierce's disease symptom expression, and the effect of Hxf expression on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR).

3. Secure permits to plant HA transgenic lines in the field at UCD. Plant transgenic vines in the field, compare disease severity and Xf movement in the transgenic vs control vines.

4. Following greenhouse testing, graft promising *Hxf* transgenic root stocks to untransformed scions.

Please Note: Objectives 5-7, described below, were not recommended for funding during the 2012 grant cycle. However, as previously noted, we had already submitted transformation constructs to the UC Davis Plant Transformation facility; they have since supplied us with 15 putative TLP-transgenic lines that we are now propagating and characterizing as time and plant materials permit.

5. Generate grape transgenic plants over-expressing the grape thaumatin-like protein (TLP).

6. Screen putative TLP-transgenic lines for quantitative gene expression by RT-PCR, protein expression by ELISA and western blot analysis testing both leaves and expressed

xylem fluid for the presence of TLP.

7. Mechanically inoculate greenhouse TLP-transgenics with wild type Xf and evaluate the effect on Pierce's disease symptom expression, and the effect of TLP expression on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR).

Summary of Accomplishments for Each Objective

Objective 1: Complete the characterization of grape transgenic plants over-expressing Xf hemagglutinin (Hxf) protein.

Twenty one transgenic Thompson seedless grape plants that potentially over-expressed the Hxf protein in the xylem using a binary plasmid with a polygalacturonase secretory leader sequence were obtained from the UCD Plant transformation facility in September 2010. These were initially obtained as small green 3" plants that needed to be grown in growth chambers and later in the greenhouse to produce hardened woody shoots that could be vegetatively propagated. It took approximately 4 months for each of the propagated shoots to grow up sufficiently to allow them to be further propagated or inoculated with Xf. By July 2011 we had propagated sufficient numbers of transgenic grapevines that we could begin analyzing them for HA expression using standard and qPCR. DNA from each of the 22 lines showed that 6 of 9 transgenic lines that contained Xf HA adhesion domains AD 1-3, labeled as SPAD1 and 3 of 12 transgenic lines of the full-length HA, labeled PGIP220 in Table 1 below, had the HA gene inserted into the grapevine chromosome.

TABLE 1 Results of PCR testing transgenic grapevines for the presence of full-length (PGIP 220) and AD1-3 fragment of *Xylella fastidiosa* hemagglutinin genes in grape chromosome

DNA ID#	genotype	Standard PCR	qPCR
1	PGIP 220-E	—	—
2	PGIP 220-5	—	—
3	PGIP 220-11	†	†
4	PGIP 220-1	—	†
5	PGIP 220-9	—	—
6	PGIP 220-14	—	—
7	PGIP 220-3	†	†
8	PGIP 220-13	—	—
9	PGIP 220-A	—	—
10	PGIP 220-D	—	—
11	SPAD1-4	NT	NT
12	SPAD1-10	†	†
13	SPAD1-6	—	†
14	SPAD1-7	†	†
15	PGIP 220-42A	†	—

16	**SPAD1-I**	†	†
17	**SPAD1-B**	†	†
18	**SPAD1-8**	†	—
19	**SPAD1-12**	†	†
20	**SPAD1-1A**	†	†
21	**PGIP 220-15**	—	—
22	**SPAD1-2**	—	—

Transgenic lines highlighted in tan color are the 3 full-length transgenic lines while lines highlighted in purple contain the AD1-3 HA fragment.

†= this line tested positive for a Xf hemagglutinin insert by standard and/or qPCR
— = this transgenic line tested negatively for a Xf hemagglutinin insert by PCR
NT = not tested by PCR for presence of hemagglutinin gene

The construct used to transform grapevines, which was recommended by the plant transformation facility, contained 2 copies of the 35S promoter flanking the HA construct. We hypothesize that recombination occurred within the Agrobacterium plasmid that allowed the HA insert to be deleted but the kanamycin selection marker was still inserted into the grape genome. This would explain why a number of the kanamycin resistant transgenics did not actually have the truncated or full-length form of Xf HA inserted into the grape chromosome.

RT-qPCR analysis on mRNA isolated from these lines confirmed the presence of AD1-3 or full-length HA mRNA in the lines that tested positive by standard or qPCR PCR, thus the HA inserted into the grape genome are being expressed (TABLE 2).

TABLE 2. RNA RT-qPCR of Thompson seedless HA transgenic lines	
LINE ID	**Relative transgenic *Hxf* RNA level**
SPAD1-B	28.9
SPAD1-10	28.1
PGIP 220-01	27.9
PGIP 220-11	26.6
SPAD1-07	25.8
PGIP 220-03	19.8
SPAD1-08	19
SPAD1-12	14.7
Untransformed Thompson seedless	0
Table 2. RNA analysis of HA expressing grapevines. Total RNA was isolated from leaves of transgenic grape plants, converted to cDNA by reverse transcriptase and quantified by qPCR with HA specific primers. SPAD1 lines express short constructs and PGIP220 lines express long constructs. The higher the number the higher the RNA level in the leaves.	

We have now propagated sufficient numbers of HA-transgenic grapevines to destructively sample and test for the presence of HA protein in foliar tissue as well as extracted xylem sap by ELISA and western blot analysis. This work should be completed within the next 3 months.

Objective 2. Mechanically inoculate transgenic and non-transgenic control grapevines growing in the greenhouse with wild type Xf cells. Compare disease progression and severity in transgenic grapevines with non-protected controls.

We have gone through multiple rounds of vegetatively propagating the lignified transgenic grapevine lines. We initially attempted to propagate green shoots but only 10-15% of the green shoots became established and for many months we only propagated lignified wood which was a much slower process than propagating from green shoots. However approximately 3 months ago a colleague suggested we try out a relatively new commercial plant cloning system called Turbo Klone. This system greatly increased the success of our propagation efforts and surprisingly we had the highest success with green shoot as compared to woody shoots, just the opposite of our previous results. The ability to propagate from green shoots has greatly accelerated our research and we can produce the needed number of vines for an experiment in a much shorter time period. Below is a picture of the Turbo Klone apparatus containing clones of our TLP transgenic lines;

We were very interested in determining whether any of the transgenic lines possessed PD resistance by testing the lines in the greenhouse as soon as we had sufficient plants, rather than waiting for the results of extensive ELISA and Western blot analysis of transgenics to determine if HA could be detected in grapevine xylem sap. In early 2012 we inoculated 10 reps of each of the 9 PCR-positive transgenic lines with 40ul of a 10^8 suspension of *Xf* Fetzer in PBS, typically done as 2 separate 20ul inoculations on each vine, an amount of inoculum that would be far greater than what a sharpshooter would inject into a vine.

We also inoculated untransformed Thompson seedless and 2 transgenic lines that did not contain HA inserts by PCR analysis, shown as Transformed Non-transgenic TS (Thompson seedless) in Figure 1, as positive controls. Figure 1 shows the results of disease severity in transgenic and non-transgenic control 16 weeks post inoculation with Xf. The TS control, inoculated at the same time as the transgenic vines had a mean disease rating of 3.65 while two of the lines, 1 containing the truncated HA fragment AD1-3 and 1 line containing the full-length native HA protein had the lowest disease ratings of 1.5. Most of the other lines had mean disease severity ratings below 2.0 and the average disease ratings for all of the lines representing the 2 HA constructs had disease ratings below 2.0. Considering the large amount of inoculum that was used, we are pleased with this promising preliminary result. While clearly some disease symptoms were evident, the severity was much less than the control and this could very well reflect lower Xf populations in the transgenic lines. If this does indeed turn out to be true then we might have produced a moderately resistant grapevine that could very well end up being like a Muscadine grapevine, i.e. they can be infected with Xf but populations are not high enough to compromise fruit quality or kill the vine. These initial greenhouse results with young vines certainly warranted further evaluations and as described below we now have 15 vines of each HA transgenic line, as well as non-transformed controls ready to plant in the field at UC Davis. .

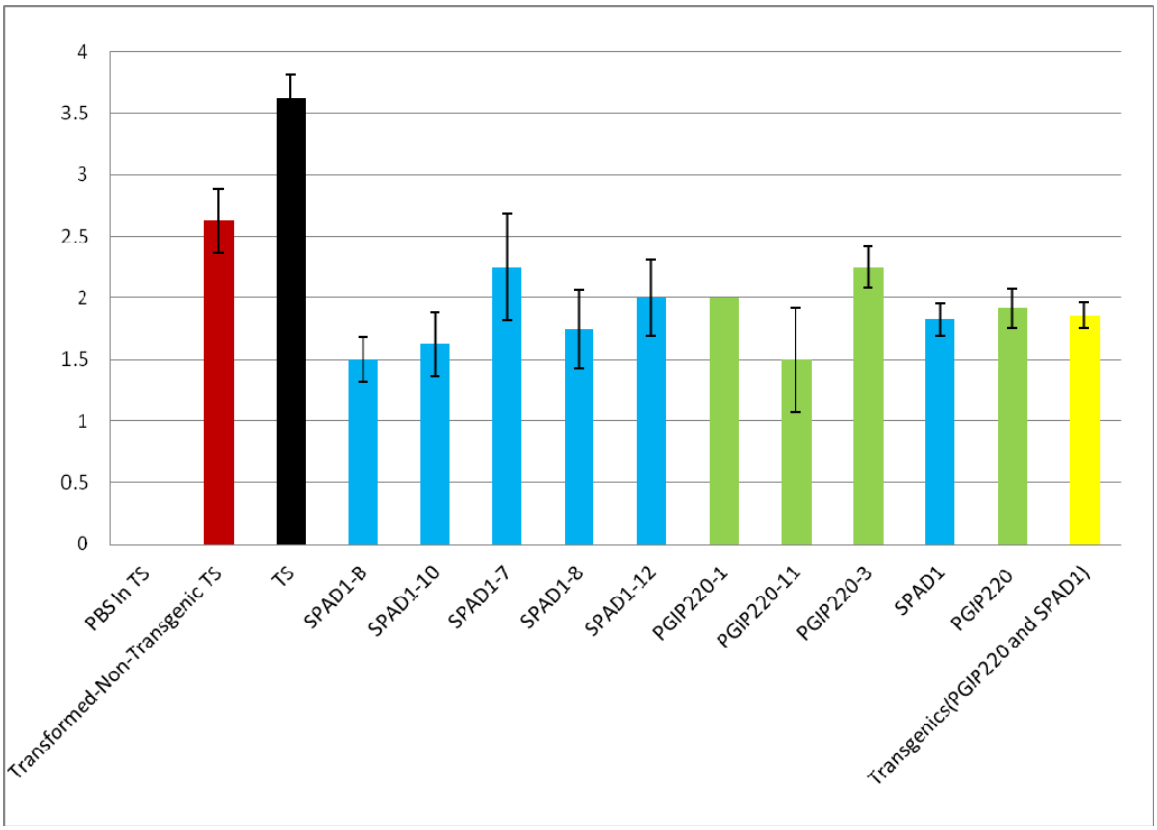


Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy 5 is dead) of PD symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *Xf* Fetzer at 16 weeks post inoculation, except for the Transformed-Non-Transgenic TS, which was inoculated 4 weeks later and its disease rating is for 12 weeks post inoculation, we anticipated these vines will have disease ratings similar to the TS control at 16 weeks post inoculation. The last three columns are the averages of all inoculated vines of the specified type of construct used, either transformed with AD1-3 (SPAD1) or the full length native HA (PGIP220). Error bars are the standard error of the 10 reps, all PGIP220-1 vines had the same disease rating.

We also performed Xf isolations from 3 individual plants from each transgenic line as well as the 2 non-transformed controls described above. Results of those isolations are shown in Figure 2 below. There was no statistically significant difference in the number of CFUs between the Thompson Seedless (control) lines and any of the transgenic lines. However at 25 cm above the point of inoculation there was up to 1,000 less Xf cells in some lines, such as SPAD1B, than either of the non-transformed controls. This result suggests that Xf movement in the HA-transgenics was retarded compared to the controls, a result that we were originally hoping for.

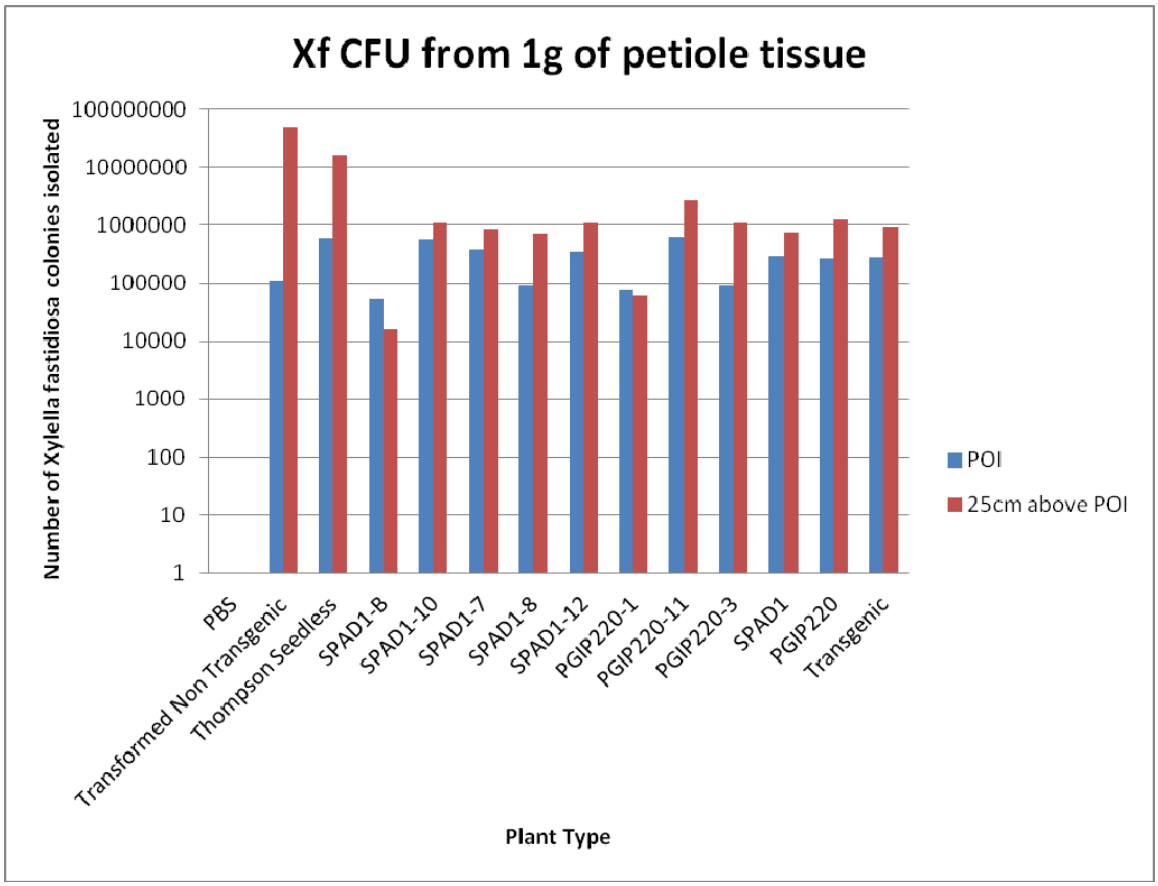


Figure 2: The number of colony forming units (CFU) that were isolated from one gram of petiole tissue from our various HA-transgenic plant lines. The "transformed non-transgenic" line is plants that were antibiotic resistant, but which we were not able to detect a HA insert via PCR.

Objective 3:

a. Secure permits to plant HA transgenic lines in the field at UCD.
This objective was completed with the assistance of PIPRA.

b. Plant transgenic vines in the field and inoculate with Xf.
Andy Walker and other viticulture experts strongly suggested we not plant our trial until this spring because of the danger of frost killing the young, newly transplanted vines. Considering the time and effort it took to produce the 140 vines that will be planted in the field we kept the vines in a screen house where they underwent natural dormancy. The trellis and wire plot was established at UCD in fall 2012 and we planted a total of 130 transgenic and control vines in the field in April 2013. The planted vines represented all of the lines that were expressing HA as determined RT-PCR. The vines grew well, were trained up stakes and 2 shoots were selected as cordons and trained up on wires. Because we wanted to inoculate our vines in the same time frame as other PD researchers inoculated their vines we will mechanically inoculated all vines with Xf in April 2014. the vines will be rated for the resulting severity of PD symptoms and Xf isolations will be made at various distances from the point of inoculation and Xf populations determine by culturing on medium and by qPCR.

Objective 4: Following greenhouse testing, graft promising *Hxf* transgenic root stocks to untransformed scions.
Additional vines from all 9 transgenic lines are being propagated in the greenhouse and when they obtain sufficient size they will be top grafted with non-transgenic Thompson seedless scions.

As noted above funding was not provided for producing and characterizing thaumatin (TLP) transgenic grapevines however we had already submitted the TLP constructs to the UC Davis plant Transformation facility. I will briefly summarize the results on the TLP project below:

Objective 5: Generate grape transgenic plants over-expressing the grape thaumatin-like protein (TLP).

The Plant Transformation Facility provided us with 15 lines that were growing on antibiotic selective medium. These were grown for several months in a growth chamber and then portions of the stems were cut to be propagated. This process was repeated until we had 3 or 4 plants from each vine. During the propagation process line 10 and 14 grew poorly and eventually died. PCR, using kanamycin specific primers was used to determine if the lines were likely transformed with the construct. Using TLP-specific primers in a standard PCR would reveal little information as non-transformed grapevines contain a copy of the TLP gene in their genome. The results shown below indicated that all the lines that survived the propagation process contained the antibiotic marker, suggesting they were indeed transformed with our construct.

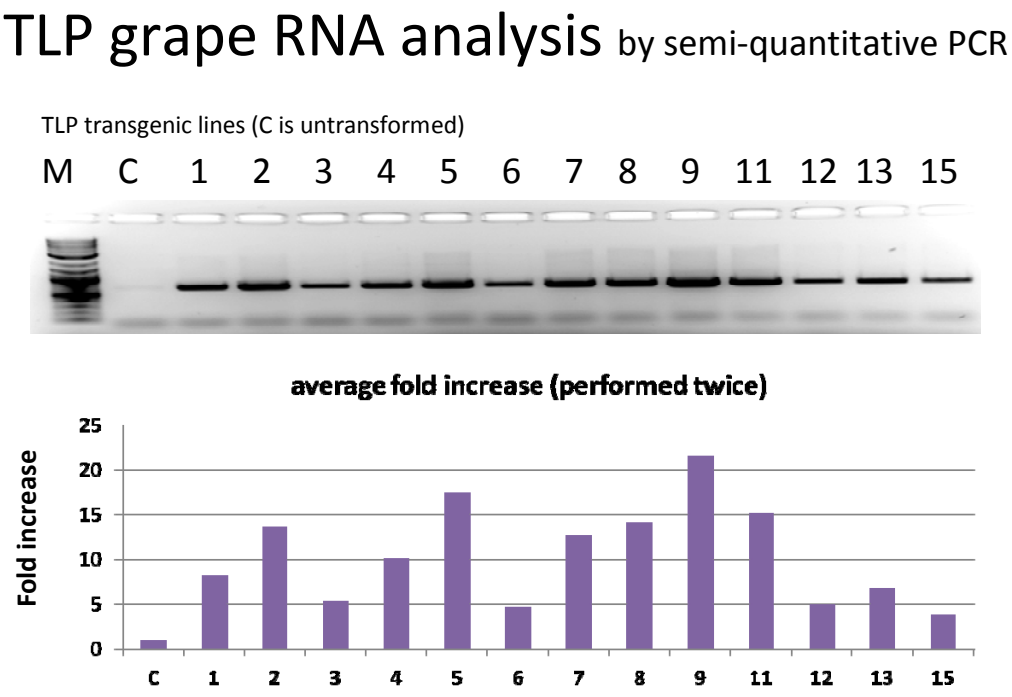
TLP transgenic grapes

line #	PCR	comment
1	P	
2	P	
3	P	
4	P	
5	P	
6	P	
7	P	
8	P	
9	P	
10	nd	non-vigorous; weak growth; died
11	P	
12	P	
13	P	
14	nd	non-vigorous; weak growth; died
15	P	

P= positive PCR
N=negative PCR
nd= not done

Objective 6: Screen putative TLP-transgenic lines for quantitative gene expression by RT-PCR, protein expression by ELISA and western blot analysis testing both leaves and expressed xylem fluid for the presence of TLP.

Definitive proof that the grapevine had been transform with the over-expressing TLP construct was obtained by analyzing RNA expression level by RT PCR. Results showed that all of the surviving 13 lines expressed TLP at higher levels than non-transformed controls; with some lines expressing 15-22X higher levels than the non-transformed control.



We are now in the process of propagating additional TLP transgenics so we can try to detect/quantify by ELISA and western blot analysis the amount of TLP in xylem sap of the transformed lines.

Objective 7: Mechanically inoculate greenhouse TLP-transgenics with wild type Xf and evaluate the effect on Pierce's disease symptom expression, and the effect of TLP expression on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR). TLP-expressing transgenic lines as well as non-transformed grapevines were mechanically inoculated with Xf in August 2013. Disease severity and Xf populations in transgenic vines will be compared with non-transformed controls in mid-October 2013. If results are encouraging we will establish an outdoor plot at UCD in April 2014.

Publications and Presentations

Voegel, T.M., J.G. Warren, A. Matsumoto, M.M. Igo, and B.C. Kirkpatrick. 2010 Localization and characterization of *Xylella fastidiosa* hemagglutinin adhesins. Microbiology 156:2177-2179.

B. Kirkpatrick, J. Lincoln and G. Kasun. Evaluation of Pierce's resistance in transgenic *Vitis vinifera* grapevines expressing either grape thaumatin-like protein of *Xylella fastidiosa* hemagglutinin protein. 2012 proceedings of the PD/GWSS Research Project. pp. 130-136.

Research Relevance Statement

The development of PD resistant grapevines, either by traditional breeding approaches or through transgenic technologies, is the most environmentally desireable solution to the PD problem. We are evaluating two genes, a Xf hemagglutinin (HA) and a grapevine thaumatin –like protein (TLP) as possible candidates to produce PD-resistant grapevines. We are evaluating these specific genes because 8+ previous years of research suggest these genes are worth evaluating. If successful, the HA-expressing transgenics might retard the colonization of the grapevine in which the introduced Xf would later be pruned out when vines are dormant in the fall according to standard horticultural practices. The TLP-expressing vines might provide a xylem environment hostile to Xf survival either by directly acting on the cells or by increasing the levels of polyphenolic compounds in the xylem. We view our efforts as an additional approach to produce a transgenic PD-resistant vine based on solid data we have obtained in previous studies funded by CDFA and UC/ARS, it is a logical extension of the knowledge we gained in those studies to undertake the evaluation of the transgenic vines we now have in hand.

Layperson Summary

Our 7+ year research effort on the role hemagglutinins (HA), large proteins that mediate the attachment of bacteria to themselves and to various substrates, in Pierce's disease pathogenicity and insect transmission has been very fruitful. Our early work showed that HA mutants were hypervirulent, ie. they caused more severe symptoms and killed vines faster that vines inoculated with wild type (wt) *Xylella fastidiosa* cells. HA mutants no longer clumped together in liquid cultures like wt cells, nor did HA mutants attach to inert substrates like glass or polyethylene when grown in liquid culture. ALL of these properties show that HA are very important cell adhesion molecules. Research conducted in the Alemeida lab also showed that HA mutants were transmitted at lower efficiencies that wt cells and they were compromised in binding to chitin and sharpshooter tissues compared to wt cells. Thus they have a very important role in insect transmission. Lindow's lab showed that DSF mutants, which are also hypervirulent, produced much less HAs that wt type cells, thus providing another line of evidence regarding the importance of these proteins in Xf pathogenesis and insect transmission.

We are now evaluating our hypothesis that HAs expressed in transgenic grapevines xylem sap may act as a "molecular glue" that would aggregate and thus slow the movement

of wt Xf cells introduced into grapevines by an infectious insect vector. If this happens then it is possible that HA-aggregated Xf cells would remain close to the site of inoculation and if that sight is in the terminal portion of a cane, which is where Xf is introduced by our native blue-green, green and red-headed sharpshooters, then that cane would likely be pruned off in the winter and the infection removed from the vine. Our most optimistic hope is that HAs could be expressed in transgenic rootstocks and the HAs would be translocated into a non-GMO fruiting scion and afford similar levels of functional PD resistance. Our first PD disease severity screening of the 9 HA transgenic lines showed that all of the HA-trangenic lines had much lower disease ratings than non-transgenic controls. Although we found that Xf populations in the transgenic lines were similar to the non-transgenic control at the point of inoculation, the populations levels at 25cm from the point of inoculation were much lower. If Xf populations are suppressed below levels that induce fruit raisining or cordon dieback the HA lines might provide some level of functional resistance/tolerance against PD. The evaluation of HA-expressing transgenic grapevines acting as rootstocks is an objective of a recent proposal that was submitted in 2012. We are now in the process of propagating sufficient numbers of HA transgenics that will serve as rootstocks grafted with Non-transgenic Thompson seedless vines. Once these grafted vines are of sufficient size they will be mechanically inoculated with Xf and disease symptoms and Xf movement will be determined as before.

Although not funded for additional research we are continuing analysis of TLP-over expressing transgenic grapevine for reasons outlined in this report. TLP was shown to have some deleterious effect on Xf *in vitro* and TLP, as well as phenolic compound concentrations, were greatly elevated in cold-exposed grapevines which were subsequently cured of active Xf infection. We now have TLP over expressing lines that will enable us to determine whether TLP expression at non-freezing temperatures also increases levels of phenolic compounds in the xylem fluid. Despite the lack of specific funds for the TLP research we feel it is important to finish this line of inquiry considering the time and money that has been previously spent to investigate the potential of TLP over-expressing transgenic vines might possess some level of PD resistance.

Status of funds:

Because this project was funded for two years we have chosen to spend other PD funds that are expiring in June 2013 to support the transgenic research. For that reason we have approximately $100,000 remaining of the original $120,000 that was allocated for this project.

Intellectual Property

Several years ago, Professor Alan Bennet presented an excellent talk on the intellectual property issues associated with transgenic plants at the PD/GWSS Conference. However, we believe it is important to evaluate the efficacy of this transgenic approach to mitigating PD using the same vectors that the Dandekar lab used in their work with PGIPs (Aguero et al., 2005). If the HA transgenic grapevines show some protection against Xf infection then the same genes can be subcloned into other plant transformation vectors if commercial

application is desired. A provisional UC patent, Case No. 2004-572, "Engineering resistance to Pierce's disease by expression of a *Xylella fastidiosa* HecA-like hemagglutinin gene" was submitted and accepted in April 2005. I view the submission of this patent as a mechanism to protect California grape growers from a having to compete with other national or international interests from patenting a similar approach for developing resistance to PD. The research proposed in this grant will provide the necessary data for assessing the feasibility of this novel approach for developing PD resistance.

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