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

EVALUATION OF PIERCE'S DISEASE RESISTANCE IN TRANSGENIC *VITIS VINIFERA* GRAPEVINES EXPRESSING *XYLELLA FASTIDIOSA* HEMAGGLUTININ PROTEIN

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INTRODUCTION

Xf cell-cell attachment is an important virulence determinate in Pierce's disease. Our previous research has shown that if 2 secreted hemagglutinin (HA) genes which we have named HxfA and HxfB are mutated, Xf cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both of these mutants are hypervirulent when mechanically inoculated into grapevines, i.e. they colonize faster, cause more severe disease symptoms and kill vines faster than wild type Xf. If either HxfA OR HxfB is individually knocked out there is no cell-cell attachment, which suggests that BOTH HA genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in Xf/plant interactions. Research by others PD researchers have shown that *Hxf*s were regulated by an *Xf*-produced compound DSF (Newman et al. 2004) and that they were important factors in insect transmission (Killiny and Almeida, 2009). The Xf HAs essential acts as a "molecular glue" that are essential for cell-cell attachment and likely plays a role in Xf attachment to xylem cell walls and contribute to the formation of Xf biofilms.

Our initial objectives proposed to further characterize these HAs using some of the techniques that were used to identify active HA binding domains in *Bordetella pertussis*, the bacterial pathogen that causes whooping cough in humans. *B. pertussis* HA was shown to be the most important protein that mediates cell attachment of this pathogen to epithelial host cells (Liu, et al., 1997; Keil, et al., 2000). In the first two years of research we identified the specific HA domain (s) that mediate Xf cell-cell

attachment and determined the native size and cellular location of Xf HAs. In the third year we identified a two component transport system that mediates the secretion of Xf HAs. In the final years of the initial project we expended consider time and effort in constructing transgenic tobacco and grapevines that expressed HA. We conducted pathogenicity evaluations of our 9 HA-transgenic lines. Disease severity ratings in greenhouse grown vines were considerably less in the transgenic lines than the non-transgenic controls.

This project was approved for additional funding to evaluate the level of PD resistance in field-grown HA-transgenic grapevines. Permits to establish a field planting of the HA vines were obtained with the assistance of PIPRA and a field trial was established in April 2013. The vines were trained as bilateral cordonds and the shoots were pruned back to 2 buds in February 2014. The vines will be inoculated with Xf in spring 2014 and PD symptoms of HA-transgenics will be compared to non-transgenic, Xf-inoculated control in September 2014. Vine will then be pruned back to 2 buds and allowed to go through the winter; symptoms on the vines will again be rated in September 2015.

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.

4. Inoculate HA-transgenic lines and non-transgenic controls with Xf in spring 2014 and compare PD symptom severity in September 2014 and 2015.

Methods and Summary of Accomplishments and Results

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. Analysis by **standard and qPCR** for the presence of the hemagglutinin transgene in genomic grapevine DNA from each of the 22 lines showed that 6 of 9 transgenic lines of containing 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 Presence of full-length (PGIP 220) of SPAD1-3 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 1. 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 HA constructs and PGIP 220 lines express long constructs. The higher the number, the higher the RNA level in the leaves.

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

We have gone through 5 rounds of vegetatively propagating the lignified transgenic grapevine lines. We attempted to propagate green shoots but only 10-15% of the green shoots became established, thus we are now propagating only lignified wood.

We were very interested in determining whether any of these 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 protein could be detected in grapevine xylem sap. On December 8th and 9th of 2011 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 injects 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 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. We will soon be quantifying by culture and qPCR the amount of Xf in each of these lines. 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. The original hypothesis was that transgenic vines producing HA in the xylem sap might facilitate clumping of Xf cells and slow their ability to colonize a mature vine during a growing season such that the incipient infection might very well be pruned off in the dormant season. It will take a couple of years to plant and train to a cordon system that would be then mechanically inoculated with Xf. These initial greenhouse results with young vines certainly warrant further evaluations.

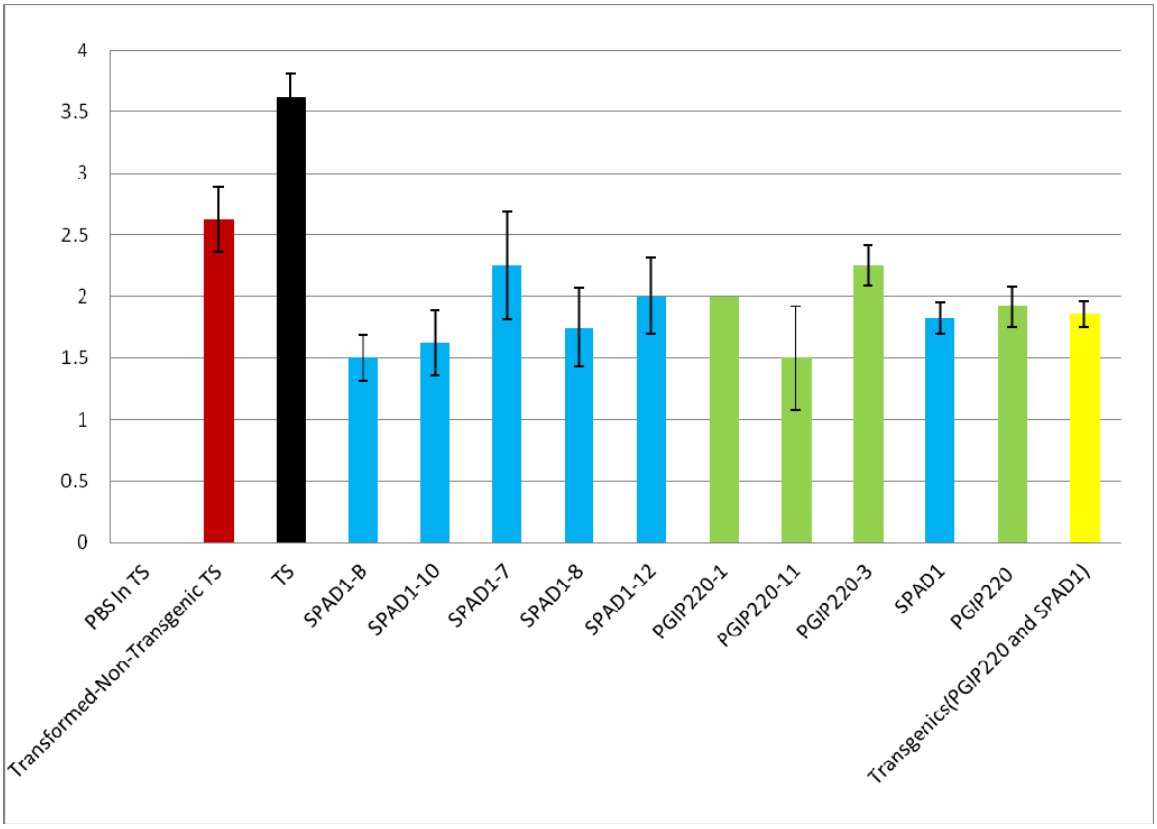

Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy 5 is dead) of PD symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *Xf* Fetzer at 16 weeks post inoculation, except for the Transformed-Non-Transgenic TS, which was inoculated 4 weeks 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.

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.**

Approximately 120 HA-transgenic vines representing all the transgenic lines that were produced were planted in the field in April 2013 and trained as bilateral cordons (Figures 2)

Figure 2 HA-transgenic and non-transgenic control vines planted in the field

Objective 4: Inoculate HA-transgenic lines and non-transgenic controls with Xf in spring 2014 and compare PD symptom severity in September 2014 and 2015.

This will be done in the coming months of 2014.

REPORTS AND PUBLICATIONS RELEVANT TO REPORTING PERIOD

Kirkpatrick, B.C. and J. Lincoln. 2012 . Evaluation of pierce's disease resistance in transgenic *Vitis vinifera* grapevines expressing either grape thaumatin-like protein or *Xylella fastidiosa* hemagglutinin protein. California Department of Food and Agriculture, Pierce's disease Research Progress Reports. pp.130-136.

Kirkpatrick, B.C. and J. Lincoln. 2013 . Evaluation of pierce's disease resistance in transgenic *Vitis vinifera* grapevines expressing either grape thaumatin-like protein or *Xylella fastidiosa* hemagglutinin protein. California Department of Food and Agriculture, Pierce's disease Research Progress Reports. pp.112-120.

RESEARCH RELEVANCE

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 *Hfx*A and *Hfx*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. 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 transgenic HA-expressing tobacco and grapevine lines; these transgenic lines exhibited less severe symptoms of Pierce's disease (PD) following mechanical inoculation of Xf cells. With the assistance of PIPRA we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines. We will cut back the shoots to 2 buds and then inoculated the shoots with Xf next spring at the same time that other PD workers inoculated their transgenic vines a couple of years ago. Symptoms will be rated in September on the inoculated shoots and we will score whether adjacent non-inoculated shoots develop PD symptoms. In January, 2015 the shoots were trimmed to 2 buds and the emerging shoots will be rated for PD symptoms in August 2015.

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, play 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* (Xf) 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 comprised 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. We finished a greenhouse PD disease severity screening of the 9 HA transgenic lines that were produced . The results were very encouraging with all of the HA-trangenic lines having much lower disease ratings than non-transgenic control. In spring 2013 12 reps of each HA-transgenic line were planted in the field. These vines grew well and were established as bilateral cordons. Four shoots on each vine will be inoculated with Xf in April 2014 and PD symptoms will be evaluated every 2 week after 8 weeks post inoculation.

CONCLUSIONS

Ten HA-transgenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the 9 HA-transgenic Thompson seedless grapes with cultured Xf cell showed all lines expressed less severe symptoms of PD than inoculated, non-transgenic controls. All transgenic lines as well as non-transgenic Thompson seedless vines that will be used as positive and negatives controls were planted in the field in spring 2013; the vines grew well and were trained as bilateral cordons. Two to 4 shoots on each vine will be mechanically inoculated with wt Xf in April 2014. PD disease symptoms on inoculated and non-inoculated shoots will be evaluated in September 2014. All shoots will be pruned back to 2 buds and allowed to push during the 2015 growing season; final PD symptoms will be recorded in September 2015. If Xf populations in HA-transgenic lines are low enough to prevent fruit symptoms and vine dieback we may have produced transgenic vines that are functionally tolerant of Xf infection. Their possible use as rootstocks grafted with non-transgenic scions will be evaluated in the coming years.

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REFERENCES CITED:

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5. Newman, K.L., R.P.P. Almeida, A.H. Purcell, and S.E. Lindow. (2004). Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. Proceedings of the National Academy of Sciences of the United States of America 101: 1737-1742.

Intellectual property associated with this project.

In 2010 a preliminary UC patent covering the use of *X. fastidiosa* hemagglutinin genes expressed in transgenic *V. vinifera* grapevines as adhesion proteins that could slow the movement of Xf was completed several years ago. It will be imperative to demonstrate the effficacy of this strategy before the technology could be commercialized.

Status of Funds

As per April 1, 2014, $48,607 remained in this grant. A no cost extension until 6/30/2015 was submitted approximately 2 months ago.