Final Report for CDFA Agreement Number 12-0128-SA

Project Title:
Evaluation of Pierce's Disease Resistance in Transgenic *Vitis vinifera* Grapevines Expressing *Xylella fastidiosa* Hemagglutinin Protein

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Reporting Period:
The results reported here are from work conducted July 1, 2012 to June 30, 2016.

INTRODUCTION

Note: Professor Bruce Kirkpatrick was the original Principal Investigator of record on this project. This final report is being prepared by David Gilchrist who accepted the responsibility to complete the data collection on this project following the recent death of Dr. Kirkpatrick and to submit the final report after June 30, 2016.

The bacterium *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease (PD) of grapes, is confined to the xylem, and is spread from plant to plant by xylem feeding insects. *Xf* cell-cell attachment is an important virulence determinate in Pierce's disease as shown by previous research. Two secreted hemagglutinin (HA) genes named *Hxf*A and *Hxf*B are required for adhesion and if either is 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 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 *Hxf*A or *Hxf*B 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 other PD researchers have shown that *Hxf*s were regulated by an *Xf*-produced compound diffusible signal factor (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 is essential for cell-cell attachment and likely plays a role in *Xf* attachment to xylem cell walls and contributes to the formation of *Xf* biofilms.

The field evaluation experiments described herein follow a series of greenhouse pathogenicity evaluations of two versions of HA-transgenic lines. In the preceding greenhouse studies, the results indicated that eight independent lines had disease severity ratings that were considerably less in the transgenic lines compared to the non-transgenic controls. Three are full length HA transgenes (PGIP220-) and five are just adhesion domains 1 through 3 transgenes (SPAD1-). The field planting of the HA transgenic vines occurred in April 2013 in the same location where other PD-related transgenic grapes are being grown under an APHIS permit had been established previously for transgenic grapes.

OBJECTIVES

1. Plant transgenic vines in the APHIS Permitted field in Solano County and train them into traditional bilateral cordon arrangement.
2. Inoculate four canes on each HA-transgenic field vine with wild-type infectious *Xf* in Spring 2014. Rate PD symptoms in September 2014 on inoculated canes. Take samples for qPCR to confirm bacterial presence.
3. Cut back all canes to two buds and rate cane growth in Spring 2015 and for PD symptoms in September 2015 and Spring of 2016 to determine if expression of *Xf* HA in the transgenic vines affected the movement of the inoculated *Xf* into the cordons, resulting in systemic protection against PD.

ACTIVITIES AND SUMMARY OF RESULTS TO ACCOMPLISH OBJECTIVES

Objective 1

Forty HA-transgenic vines representing all the transgenic lines previously evaluated in the greenhouse were planted in the field in April 2013 and trained as bilateral cordons (**Figure 1**). The vines were inoculated with *Xf* in summer of 2014 (Objective 2). PD symptoms were observed on the non-transgenic, *Xf*-inoculated control plants and HA-transgenic plants in September 2014. The vines were then pruned to two buds and PD symptoms on the vines were evaluated in the Spring of 2015 (**Table 1**).

Figure 1. HA-transgenic and non-transgenic control vines planted in the field. Photo August 2014.

Objective 2

A combination of *Xf* Temecula and Stags Leap strains were grown on solid PD3 medium and the harvested cells, and suspended in phosphate buffered saline to a concentration of 10^8 cells/ml. Four canes on replicates of each transgenic line were labelled and then mechanically inoculated by the standard needle prick method with a 20 ul drop of *Xf* cell suspension containing $2x10^6$ bacterial cells. Inoculations were done in mid-May 2014 and inoculum droplets were quickly taken up by the transpiring canes under negative pressure. The inoculations were successful in establishing infection of the plants as evidenced by the PD symptoms and by PCR assessment of isolated cane DNA from the inoculated canes using *Xf* specific probes.

Objective 3

Canes were cut back to 2 buds once vines were completely dormant in January/February 2015. The vines were rated for PD symptoms in late August 2015 (**Table 1**). 95% of the inoculated canes had some level of leaf scorching which indicated our inoculation procedure was successful as shown in **Figures 2A, 2B, and 2C**.

Table 1. PD symptom ratings of HA-transgenic grapevines in August 2015.

Transgenic Lines	**# of Inoculated Vines**	**# of PD Rated Canes**	**Mean Plant Disease Rating** (cane ratings)
HA Adhesion Domain only			
SPAD 1- 6	3	10	**0.7** (5as0; 4as1; 2as2)
SPAD 1-7	4	15	**0.9** (7as0; 2as1; 6as2)
SPAD 1-8	5	20	**1.6** (2as0; 5as1; 12as2; 1as3)
SPAD 1-10	3	10	**1.7** (1as0; 2as1; 6as2; 3as1)
SPAD 1- 12	5	19	**1.2** (5as0; 5as1;9as2)
HA Gene full coding sequence			
PGIP 220-1	4	10	**1.6** (4as0; 1as1; 6as2; 1as3)
PGIP 220-3	3	12	**1.3** (4as0; 1as1; 6as2; 1as3)
PGIP 220-11	3	10	**0.3** (8as0; 1as1; 1as2)
Control	3	12	(13as5; 3as4; 2as3)

Note: PD symptoms of inoculated transgenic canes were rated August 2015. Symptom ratings of individual canes were as follows:

0 is no symptoms of PD, i.e. no scorched leaves on cane;
1 is 2 to <10% scorched leaves on cane;
2 is >10% to <75% scorched leaves on cane;
3 is all leaves showing PD scorch symptoms, no cane dieback observed;
4 is cane dieback, cane still alive; and
5 is dead cane.

Cane ratings are of the form [# of canes]as[rating].

In 2015, three out of the five lines expressing the HA adhesion domains only showed no PD symptoms. In the two other adhesion domain lines the majority of the inoculated canes were dead or had severe PD symptoms. In the three full length HA gene construct lines the majority of all the canes were healthy with no PD symptoms. These initial results were encouraging and were consistent with the greenhouse results in terms of occurrence of PD symptoms in relation to inoculation.

These results were similar to what was observed in the greenhouse inoculations. However, it is also clear from the field inoculations that none of the transgenic lines completely prevented the onset of PD symptoms in inoculated canes.

Figure 2A. HA Adhesion Domain transgenic Thompson Seedless grapevine 11 months following inoculation with *Xylella fastidiosa* in 2014 showing the state of the vine, which was defoliated and dead from Pierce's disease in June 2015.

Figure 2B. Typical Pierce's disease symptoms on HA transgenic Thompson Seedless grapevines 13 months following inoculation with *Xylella fastidiosa*. Image recorded in August 2015.

Figure 2C. Healthy appearing full length transgenic vine, August 2015.

The results obtained in the spring of 2016 are summarized in **Figure 3**. None of the transgenic plants were free of PD symptoms, although all were slightly less than the non-transgenic control plants. Furthermore, there was no indication that the bacteria were suppressed in movement from the site of inoculation.

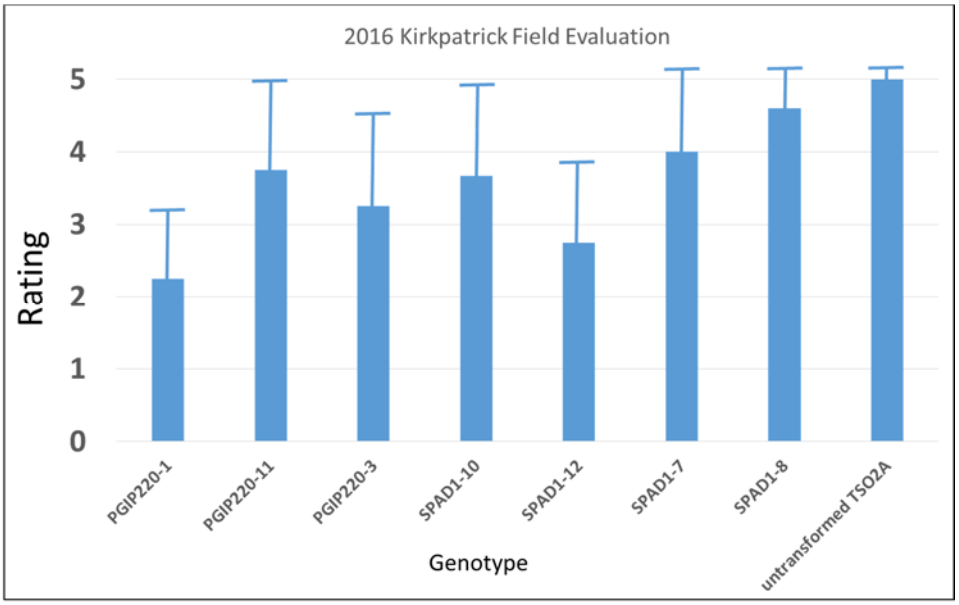

Figure 3. Solano field ratings of transgenic Thompson Seedless grapevines expressing two versions of the Hemagglutinin gene from *Xylella fastidiosa* (*Xf*). Graph shows the mean disease ratings from 1 to 5 (1 is healthy and 5 is dead) of PD symptoms in June of 2016. Plants were inoculated with *Xf* in June of 2014. PGIP220 (full HA) and SPAD (HA subdomain) transgenics are compared to TSO2A untransformed controls. Data are the average and standard deviation from four plants for each genotype.

SUMMARY OF ACCOMPLISHMENTS

Eight HA-transgenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the eight 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 were used as controls were planted in the field in spring 2013. The vines grew well and were trained as bilateral cordons. Four shoots on each vine were mechanically inoculated with wild-type *Xf* in May 2014. PD disease symptoms on inoculated and non-inoculated shoots were evaluated in September 2014. A high percentage of the inoculated shoots developed scorched leaves typical of PD symptoms indicating our needle inoculation technique was successful. PD symptom severity ratings were lower among HA-transgenic lines than inoculated non-transgenic grapevine controls in the first year following inoculation. Canes from transgenic and non-transgenic vines were collected to determine the presence of the bacteria by qPCR. All shoots were pruned back to two buds in January / February 2015 and allowed to push during the 2015 growing season. Spring shoot growth and PD symptoms were recorded in September 2015 to determine if the *Xf* infections overwintered and formed systemically-infected vines. Most of the adhesion domain vines and full length HA gene transformants had some vines that appeared PD free. However, with other replicates of the transgenic lines some replicates were either dead or had PD symptoms on inoculated canes that varied in severity.

Evaluation of the inoculated vines in June 2016 (**Figure 2**) indicated the bacteria had now gone systemic and nearly all the transgenic plants were dead or clearly dying. There were no significant differences in disease severity between the transgenic plants and the non-transgenic controls two years after inoculation.

RESEARCH RELEVANCE

Previous research in Dr. Kirkpatrick's lab identified two hypervirulent mutants of *Xylella fastidiosa* (*Xf*). These mutations were in large hemagglutinin (HA) adhesion genes called *Hfx*A and *Hfx*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. He hypothesized 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 *Xf* cells would clump together at the site of insect delivery and be less capable of colonizing grapevines. Transgenic HA-expressing grapevine line were produced, and mechanically inoculated with *Xf* cells in the greenhouse. These transgenic lines were transplanted to the APHIS permitted field site for transgenic plants coordinated by Dr. Gilchrist in Solano County in spring 2013. These vines grew well and were trained up to the wire and established as conventional bilateral cordon vines. The shoots were cut back to two buds and then four shoots/vine were mechanically inoculated with a mixture of Temecula and Stag's Leap *Xf* strains in April 2014. Over 95% of the inoculated canes showed scorch symptoms typical of PD in September 2014, indicating that the inoculations were successful, with at least two PD-symptomatic canes being present on all inoculated vines. In January 2015 the shoots were trimmed to two buds, and the emerging shoots and the entire vines were rated for PD symptoms in August 2015. In three out of the five lines expressing the HA adhesion domain the majority of the vines showed no PD symptoms. However, PD symptoms were evident on the canes of other adhesion domain transgenes. The HA transgenes appeared to retard the progression of the PD symptoms initially, but eventually all plants expressed PD symptoms; the effect of the transgenes was no longer evident. The final conclusion is that the genes expressed as HA transgenes do not provide long-term protection.

LAYPERSON SUMMARY

Dr. Kirkpatrick invested more than 10 years investigating the role of hemagglutinins (HA), large proteins that mediate the attachment of bacteria to themselves and to various substrates, how these proteins may play in Pierce's disease (PD) pathogenicity and insect transmission. Early work showed that HA mutants were hyper-virulent: they caused more severe symptoms and killed vines faster than vines inoculated with wild-type (wt) *Xylella fastidiosa* (*Xf*) cells (Guilhabert and Kirkpatrick, 2005). HA mutants no longer clumped together in liquid cultures like wt cells, indicating that cell adhesion molecules were important in

establishing a pathogenic population of bacteria in the grape xylem. **This information is of fundamental importance in understanding a genetic mechanism regulating spread of *Xylella fastidiosa* in grape.**

The next logical step was to try to block this behavior transgenically as is reported herein. The current project tested the hypothesis that HAs expressed in xylem sap of transgenic grapevines may act as a "molecular glue" that would aggregate and thus slow the movement of *Xf* cells introduced into grapevines. Transgenic lines expressing various HA constructs were moved to the field in Spring 2013. The vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines with two bud spurs and then inoculated four shoots/vine with *Xf* in April 2014. PD symptoms were rated in September 2014 on the inoculated shoots, including whether the bacteria had moved to adjacent non-inoculated shoots and were expressing PD symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of PD in September 2014 indicating that the inoculations were successful. There was no evidence of plant-to-plant spread. Uninoculated controls remained disease free throughout the experiment. The summary observation is that PD symptom severity was lower in the inoculated HA-transgenic grapevines than the *Xf*-inoculated non-transgenic controls in the first year following inoculation and establishment of infection.

The pruning and inoculation programs were repeated beginning in January 2015 and the shoots were rated for PD symptoms in August 2015.The results continued to be encouraging in three out of the five independently transformed lines expressing the HA adhesion domain wherein the majority of the vines showed no PD symptoms. In the three full length HA gene construct lines the majority of all the vines were healthy with no PD symptoms. These initially encouraging results, however, were not borne out by the evaluations conducted in 2016. The final conclusion is that the HA transgenes appeared to retard the progression of the PD symptoms initially but eventually all plants expressed PD symptoms and the effect of the transgenes was no longer evident and the genes expressed as transgenes do not provide long-term protection.

STATUS OF FUNDS

All funds budgeted for this project were expended at the end of the current funding cycle (June 30, 2016).

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY

Record of invention disclosures were submitted to the UC Office of Technology Transfer but did not proceed beyond the disclosure. The field research results did not indicate that the expression of the HA gene in either conformation substantially reduced the movement of *Xf* or expression of PD symptoms.

LITERATURE CITED

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