

**Title of Report:** Final Report for CDFA Agreement Number 06-0223

**Title of Project:** Isolation, characterization and genetic manipulation of *Xylella fastidiosa* hemagglutinin genes.

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**Time Period Covered:** July 1, 2006 to 6/30/11

### **Introduction:**

Xf cell-cell attachment is an important virulence determinant 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. 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.

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 project we expended consider time and effort in constructing transgenic tobacco and grapevines that expressed HA. We recently completed our first pathogenicity evaluations of our 9 HA-transgenic lines. Disease severity ratings were considerably less in the transgenic lines than the non-transgenic controls. We are currently in the process of evaluating Xf population levels in all of the plants used in this trial. It is possible that Xf populations will be considerably less in the transgenic lines than in non-transgenic vines. If Xf levels are below the level where fruit raisining and cordon dieback don't occur, similar to what happens in muscadine grape vines infected with Xf, these vines might possess a tolerance to Xf. A second round of pathogenicity assays will performed in the greenhouse this summer and arrangements are being made through PIPRA to establish a field plot at UC Davis.

**List of Objectives for all years, beginning with the 2006-08 proposal:**

- 1 a.** Use antibodies we have prepared against a conserved, putative binding domain that is present in both Xf hemagglutinins (HA), which we have named HxfA and HxfB, to determine the native size and location of Xf HA in cultured Xf cells and PD-affected grapevines.
- b.** Determine if these antibodies can prevent cell-cell clumping in liquid Xf cultures.
- c.** Prepare an affinity column using HA domain antibodies and isolate native Xf HAs from culture cells. Establish the identity of affinity purified, putative HAs by n-terminal sequencing.
- d.** Determine if HA domain fusion proteins antibodies can bind to Xf cells.
- e.** Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of Xf grown in liquid medium.
- 2. a.** PCR-amplify, clone and express as fusion proteins, hypothetical adhesion domains of HxfA and B.
- b.** Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein.
- 3. a.** Transform bacterial grapevine endophytes with portion(s) of the Xf HA domains that mediate cell-cell clumping. Determine if transformed endophyte cells can bind Xf cells *in vitro*.
- b.** Transform tobacco, an experimental host of Xf and an easily transformable plant, with Xf HA binding domains. Use antibodies prepared in Objective 1 to determine if Xf HA proteins can be found in tobacco xylem fluid.
- 4. a.** Mechanically inoculate grapevines with Xf HA-expressing grapevine endophytes.

**b.** Mechanically inoculate endophyte colonized grapevines and HA-transgenic tobacco with wild type (wt) Xf cells. Compare disease progression and severity in endophyte colonized grapevines and transgenic tobacco with non-protected controls.

### **2008-2010 Objectives:**

**1a.** Identify C-terminal processing site where 330 kD pre-HA protein is processed into native 220kD HA.

**1b.** Clone that fragment of the HA gene that encode full-length, native HA into E. coli.

**1c.** Express, purify and prepare polyclonal antibodies against native, full-length HA protein.

**2.** Clone AD1-3, AD4 and full-length native HA DNAs in Agrobacterium transformation vectors. Provide constructs to the UCD Plant Transformation facility who will transform tobacco, an experimental host of Xf, and grapevines with AD1-3, AD4 and full-length native HA constructs.

**3.** Use RT-PCR and HA antibodies to verify and quantify expression of HA proteins in transgenic plants. Use antibodies prepared in Objective 1 to determine if Xf HA proteins can be found in tobacco and grapevine xylem fluids.

**4.** Mechanically inoculate transgenic grapevines and tobacco with wild type (wt) Xf cells. Compare disease progression and severity in transgenic tobacco and grapevines with non-protected controls.

### **2010-2011 Objectives:**

**1.** Use RT-PCR and HA antibodies to verify and quantify expression of HA mRNA and proteins in transgenic plants. Use anti-HA antibodies to determine if Xf HA proteins are present in tobacco and grapevine xylem fluids.

**2.** Mechanically inoculate transgenic grapevines and tobacco with wild type Xf cells. Compare disease progression and severity in transgenic tobacco and grapevines with non-protected controls.

### **Methods Used and Results Obtained for each Objective:**

#### **2006-2008**

**1 a.** Use antibodies we have prepared against a conserved, putative binding domain that is present in both Xf hemagglutinins (HA), which we have named HxfA and HxfB, to determine the native size and location of Xf HA in cultured Xf cells and PD-affected grapevines.

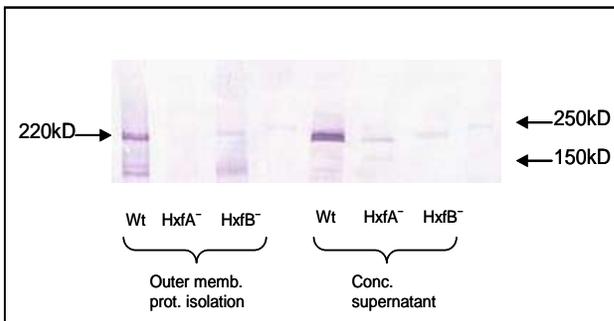
**Methods and results for this objective were published:**

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

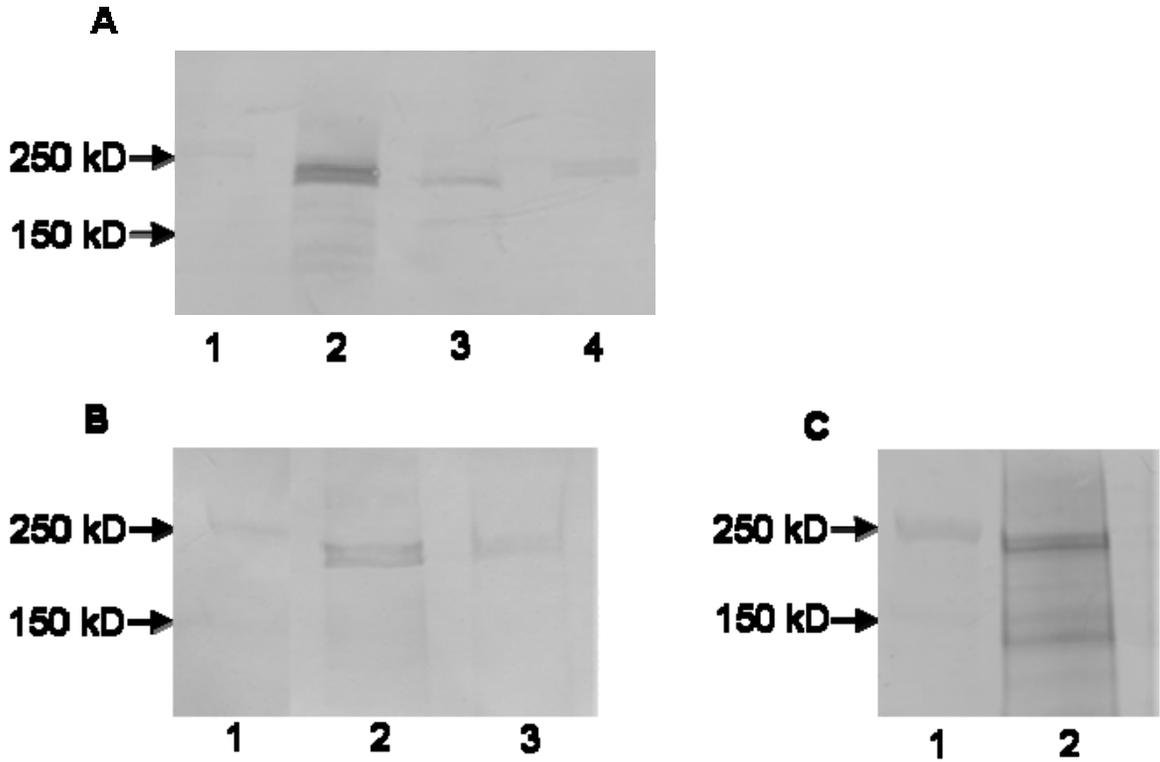
A summary of those results follows below:

Results showed that HxfA and B are present in the outer membranes of Xf cells, as proteins found in secreted vesicles and native proteins secreted into culture medium at low concentrations. The 10.5kb HA genes should theoretically encode a protein of approximately 330kD however we have shown that the native size of the HA proteins in both the outer membranes and culture supernatants is approximately 220kD. At the 2007 PD/GWSS Conference Steve Lindow suggested that we make sure that our “secreted” proteins are not really HA proteins embedded in a membrane microsomal fraction as has been reported for some *Xanthomonas* membrane proteins. Although other bacterial HAs are known to be secreted into the medium and we have purified good quality Xf HA from culture supernatants by size exclusion chromatography, we will verify that the HAs in the culture supernatant are indeed secreted by ultracentrifugation which should pellet microsomal associated HAs while leaving truly secreted HAs in the supernatant. We also identified another Xf gene that is responsible for directing the HAs in the outer membrane and secreting HAs into the medium (Voegel and Kirkpatrick, 2006).

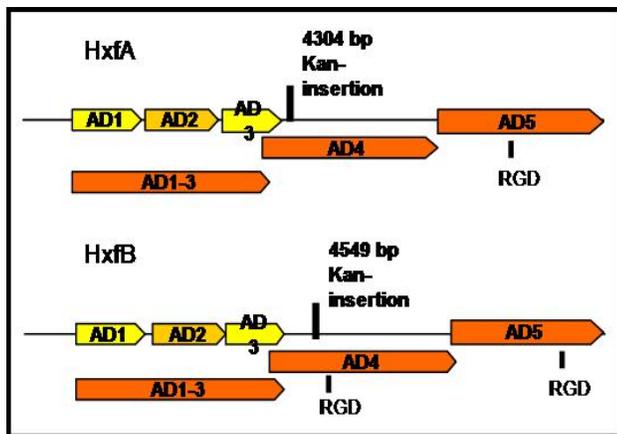
Research conducted during the first 4 years showed that HxfA and B are present in the outer membrane, in secretory vesicles produced by Xf cells and these proteins are also secreted into culture medium at low concentrations. The 10.5kb HA genes should theoretically encode a protein of approximately 330kD however we showed that the native size of HA proteins in outer membranes, vesicles and culture supernatants is approximately 220kD (Vogel, et al.,2010). We also identified another Xf gene that is responsible for directing the HAs into the outer membrane and secreting HAs into the medium (Voegel and Kirkpatrick, 2007).



**Figure. 1** Western blot analysis of membrane protein preparations (left) and culture supernatant (right) of wt and HA mutants revealed that HAs are secreted as well as inserted into the outer membrane and possess a size of 220kDa.



**Fig.2:** Western blot analysis of *X. fastidiosa* supernatant developed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula; lane 3: HxfA, lane 4: HxfB. A doublet of bands in the wild type Temecula is detectable, the single band in the HxfA mutant strain correlates to HxfB, the single band in HxfB mutant strain correlates to HxfA. HxfA is slightly larger than HxfB. **B:** Western blot analysis of *X. fastidiosa* vesicle preparation developed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula vesicle preparations; lane 3: TCA-precipitated supernatant after isolation of vesicles, proteins represent soluble HA proteins. **C:** Western blot analysis of outer membrane protein preparation. Lane 1: Molecular weight standards; lane 2: outer membrane preparation of wild type Temecula.



**Figure. 3.**  
Identification of possible  
adhesion domains (ADs)  
and RGD motifs

**2006-2008 Objective 1c.** Prepare an affinity column using HA domain antibodies and isolate native Xf HAs from culture cells. Establish the identity of affinity purified, putative HAs by n-terminal sequencing.

**2008-2010 Objective 1a.** Identify C-terminal processing site where 330 kD pre-HA protein is processed into native 220kD HA.

Because nearly 1/3 of the C terminus of the HA proteins are apparently processed away during localization and secretion we determined where the exact cleavage site lies on the precursor 330kD HA proteins. This was accomplished by concentrating Xf culture supernatant proteins from 10 liters of medium. With the assistance of the Labavitch lab the proteins were size fractionated on a Sepharose column and fractions containing HA protein were identified by Western blot analysis. We then ran a preparative PAGE gel and submit the HA proteins for mass spectrometer analysis at the UCD Protein Analysis facility. Protein sequencing revealed numerous peptides from the N terminal 2/3 of the HA protein while no peptides were found that matched with the C-terminal 1/3 of the protein as predicted by PAGE analysis. This means that the C-terminus of the pre-cursor HA protein is cleaved off and presumably degraded.

**2006-2008 Objective 1e.** Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies.

While we were able to isolate sufficient native Xf HxfA and HxfB by size exclusion chromatography to determine that the theoretical 330kD protein was processed to the native 220kD (as stated above in Objective 1a), we could not isolate sufficient amounts of native, full-length HAs to use as antigen for producing anti-HA antibodies. We also felt that *in silico* analysis showed that recombinant, his-tagged proteins produced from the three putative adhesion domains AD1-3, AD4 and AD5 shown in Figure 3 would likely produce high quality antibodies that would allow us to complete the localization and cell binding objectives.

**Objective 1e, second part:** Determine if HxfA and B specific antibodies can block cell-cell clumping of Xf grown in liquid medium. Results for this portion of the Objective are presented below on page 7:

**2006-2008 Objective 2. a.** PCR-amplify, clone and express as fusion proteins, hypothetical adhesion domains of HxfA and B.

**b.** Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein.

Because the 10.kb HA genes would likely be too large to express in transgenic plants one of the principle objectives of the previous research was to try and identify which domains(s) on the HA proteins were responsible for cell-cell binding, with the idea that if such domains were identified they could be more readily expressed in transgenic plants. *In silico* analyses identified possible adhesion domains, shown as AD1, AD2 and AD3 in Figure 3. RGD (Arg-Gly-Asp) motifs, which are known to mediate cell attachment to host cells for *Bordetella pertussis* (Manning, et al. 2004) were also found in HxfA and B. The *in silico* analyses (hydrophobicity plots) led us to divide HxfA and HxfB into 3 regions named AD1-3, AD4 and AD5, each for HxfA and HxfB (Fig 3). All 6 ADs were PCR-amplified and cloned into the *E. coli* expression plasmid pet30b. The identity and integrity of the amplified fragments were verified by DNA sequencing. Vectors containing AD5 of HxfA and AD1-3 and AD4 of HxfB and were transformed into a *E. coli* fusion protein strain (ArcticExpress DE3, Stratagene) that worked well in our previous Xf

polygalacturonase research. AD1-3 and AD4 of HxfB were expressed to high levels, the protein was purified by affinity column chromatography and the integrity of the purified protein was verified by sequencing.

Affinity purified recombinant AD1-3 (predicted to have “hemagglutinin activity domains” (<http://smart.embl-heidelberg.de>) and AD4 (highest antigenic region according to hydrophobicity plots) and recombinant AD5 were each injected into 2 rabbits and the sera obtained. The 4 serum bleeds were analyzed and compared to each other by indirect ELISA using the recombinant AD fusion proteins as target antigen. High titer antisera were obtained for both AD1-3 and AD4 in all injected rabbits. Western Blot analysis, using antibodies produced against recombinant AD1-3 and AD4, of *E. coli* expressing the antigens showed high specificity of the Abs and showed that *E. coli* produces truncated forms of the antigens (Figure 4) that is commonly observed with this *E. coli* expression system. All localization and cell binding assays were performed with anti-AD1-3 and AD4 antibodies.

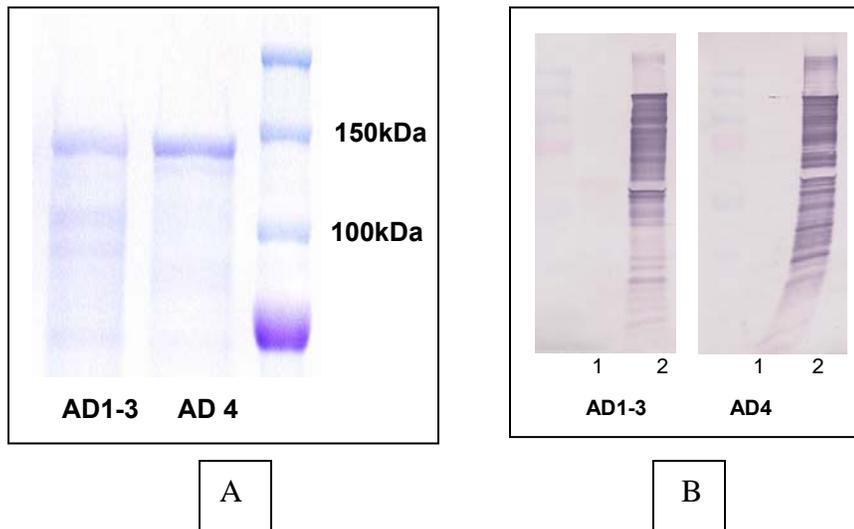
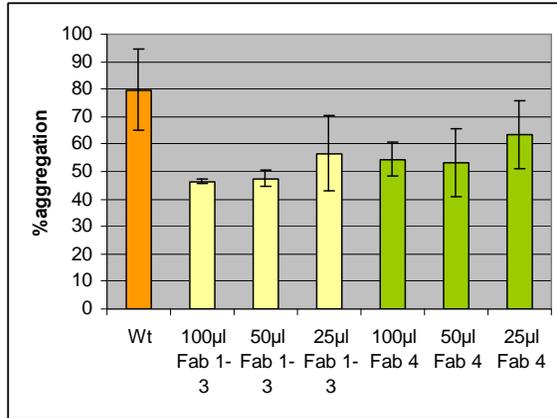


Figure 4. PAGE (A) and Western blot (B) analysis of AD1-3 and AD4 recombinant proteins using polyclonal rabbit antisera.

**2006-2008 Objective 1e (second portion).** Determine if HxfA and B specific antibodies can block cell-cell clumping of Xf grown in liquid medium.

IgG Fab fragments were prepared from anti-AD1-3 and AD4 antisera and used in cell-cell binding assays to determine if antibodies specific for these domains could affect Xf cell-cell binding. As shown in Figure 5, a decrease in cell-cell binding was achieved by treating Xf cells with both AD1-3 and AD4 Fab fragments.



**Figure 5.** Blocking of cell-cell aggregation by addition of Fab fragments against AD1-3 and AD4 suggests that cell-cell binding domains are contained in AD1-3 and AD4.

**2006-2008 Objective 1d.** Determine if HA domain fusion protein antibodies can bind to Xf cells.

Antibodies against AD1-3 and AD4 were also used in immunolocalization assays to try and determine which domain(s) might reside on the outer membrane of Xf cells. Despite repeated attempts and use of all appropriate negative and positive controls, we were not able to show attachment of either antibody to the surface of Xf cells. There are at least 2 potential explanations for this observation. First, the antibodies were produced against denatured HA proteins that were recovered from a PAGE gel, and it's possible that native domains on the HA proteins were not recognized by antibodies produced against the denatured proteins. The second possible explanation is that the exposed HA protein was masked by other surface proteins or EPS. Dean Gabriel reported at the 2007 PD Conference that they had similar results when they tried to localize TolC of the Type I secretion system using anti-TolC antibodies. Despite the negative immunolocalization results the Fab blocking results would suggest that both AD1-3 and AD4 contain cell-cell attachment domains and based on these results we propose that both these HA fragments need to be evaluated in transgenic plants.

**2006-08 Objectives 3a.** Transform bacterial grapevine endophytes with portion(s) of the Xf HA domains that mediate cell-cell clumping. Determine if transformed endophyte cells can bind Xf cells *in vitro*.

We originally proposed this objective with the expectation that our separately funded PD project that isolated and characterized bacterial endophytes from healthy and PD-affected grapevines would yield bacterial endophytes that could systemically move through grapevines. Unfortunately neither our large collection of endophytes from that project, nor endophytes collected separately by the Lindow lab ever identified a suitable grapevine endophyte that could be used to deliver Xf HAs into grapevine xylem. For this reason, this objective was abandoned.

**2008-2010 Objective 1b.** Clone the HA gene that encodes full-length, native HA into *E. coli*.

Knowing where the precursor HA protein is cleaved, we then cloned the full-length, 220 kD HA protein in *E. coli*. That full-length, native processed HA construct, as well as the AD1-3 and AD4 fragments were then sub-cloned into *Agrobacterium* plant transformation vectors, in preparation for transforming tobacco and grapevine plants (see below).

**2008-2010 Objective 1c.** Express, purify and prepare polyclonal antibodies against native, full-length HA protein.

Identifying the cleavage site where the 330kD precursor HA protein is cleaved to 220kD native full-length HA, it was clear that the antibodies we prepared against AD1-3 and AD-4 would essentially cover the entire length of the 220 native HA protein. Thus there was no reason to prepare another antibody against the 220kD fusion protein, especially considering that expressing and purifying a protein that large would take considerable time and effort.

In addition, while a small amount of native HA protein we isolated from 10L of culture media was sufficient for mass spec analysis to identify the cleavage site, it was an insufficient amount to inject into rabbits and prepare antibodies.

**2006-2008 Objective 3b.** Transform tobacco, an experimental host of Xf and an easily transformable plant, with Xf HA binding domains. Use antibodies prepared in Objective 1 to determine if Xf HA proteins can be found in tobacco xylem fluid.

**2008-2010 Objective 2.** Clone AD1-3, AD4 and full-length native HA DNAs in *Agrobacterium* transformation vectors. Provide constructs to the UCD Plant Transformation facility who will transform tobacco, an experimental host of Xf, and grapevines with AD1-3, and full-length native HA constructs.

#### Transformation of tobacco and grapevines

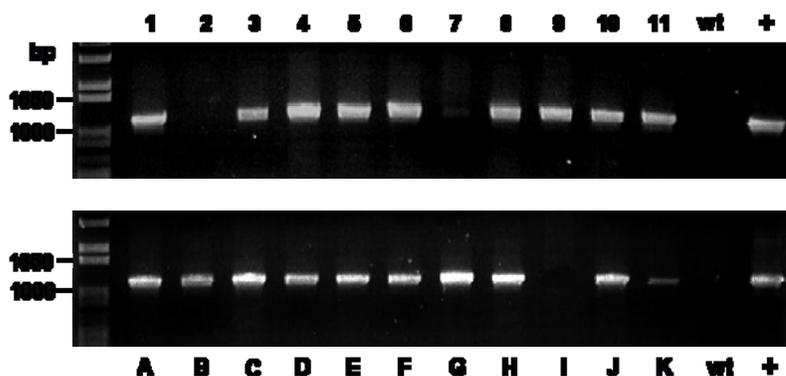
A 5' fragment of the HA gene coding for the hemagglutination domain AD1-3, and a longer fragment that codes for the full-length native 220 kD protein (220) were PCR amplified from the gene HxB (PD1792). The resulting 4000 bp and 6300 bp PCR products were cloned into pCR-2.1-TOPO and sequenced using primers generated every 600 bp on the gene to confirm the integrity of the cloned fragments. To enable secretion of the expressed HA proteins outside the eukaryotic cells of tobacco and grapevines, a signal peptide pGIP (4) was synthesized by the company DNA2.0 (Menlo Park, CA) and fused N-terminally to the cloned HA products. The codon usage of the synthesized signal peptide was optimized for expression in eukaryotes. The pGIP-HA fusions were cloned into vector pDE00.0113 (Dandekar lab) containing the 35S promoter and ocs3' terminator creating pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220. After verifying the integrity of the cloned insert by sequencing, pDE00.0113-pGIP-AD1-3 was digested with *AscI* and the resulting cassette cloned into the *Agrobacterium* binary vector pDU97.1005 (Dandekar lab) creating pDU08.2407. This plasmid was transformed into *Agrobacterium tumefaciens* strain EHA101 and the culture submitted to the Ralph M. Parsons foundation transformation facility on the UC Davis campus for transformation of tobacco SR-1. Unfortunately, the pDU97.1005 marker gene *nptII* confers resistance to kanamycin. In transformation experiments with grapevines using binary plasmids containing the *nptII* gene, many escapes were observed that prolonged the time needed to generate transformed grapevines. Therefore, we digested pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220 with *EcoRI* and ligated the obtained cassette into the binary plasmid pCAMBIA1300 (Canberra, Australia). Vector pCAMBIA confers resistance to hygromycin. This marker gene is more suitable for transformation of grapevines than *nptII* and is functional in grapevines as well as in tobacco. Binary plasmids pCAMBIA-pGIP-AD1-3 and pCAMBIA-pGIP-220 were transformed into *Agrobacterium tumefaciens* strain LBA4404 and the culture submitted

to the Ralph M. Parsons foundation transformation facility for transformation of Thompson seedless grapevines as well as transformation of tobacco SR-1 for pCAMBIA-pGIP-220.

**2008-2010 Objective 3.** Use RT-PCR and HA antibodies to verify and quantify expression of HA proteins in transgenic plants. Use antibodies prepared in Objective 1 to determine if Xf HA proteins can be found in tobacco and grapevine xylem fluids.

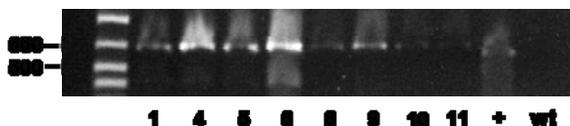
Analysis of transformed tobacco plants

Four months after submission of the constructs to the transformation facility, 11 transgenic tobacco plants T<sub>0</sub> representing single transformation events were obtained for both plasmids pDU-pGIP-AD1-3 (lines 1-11) and pCAMBIA-pGIP-220 (lines A-K). The lines were maintained in a growth chamber at the controlled environmental facility (CEF, UC Davis, CA) at 25°C with a photoperiod of 16 h and 50% relative humidity. Genomic DNA was isolated and PCR assay using primers that amplify the HA gene were positive for 10 out of 11 tobacco plants for each construct. Untransformed wild type plants were used as negative control (Figure 6).



**Figure 6:** Confirmation of T-DNA insertion into the genome of tobacco SR-1 by PCR analysis of genomic tobacco DNA. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3, letters A-K indicate transgenic lines that were transformed with pCAMBIA-pGIP-220. Lines 2 and I do not have the T-DNA insertion. Wild type plants were used as negative controls and isolated plasmids pDU-pGIP-AD1-3 and pCAMBIA-pGIP-220 were used as positive controls (+).

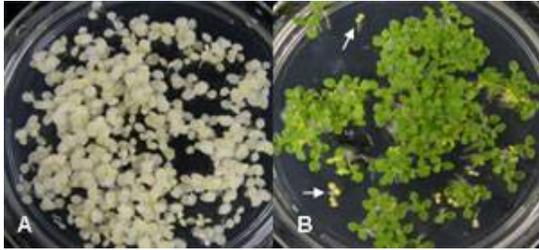
RNA was extracted from all PCR positive lines and cDNA generated by reverse transcription. PCR analysis using primer pair pGIP-HAfor and HArev confirmed that all plants were expressing the transgene coding for AD1-3 (Figure 7).



**Figure 7:** RT-PCR of transformed tobacco SR-1 using primer pair pGIP-HAfor and HArev. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3. Wild type plants were used as negative controls and isolated plasmid pDU-pGIP-AD1-3 was used as positive control (+).

After 3 months, seed pods of the HA expressing T<sub>0</sub> plants were harvested, the seeds sterilized and plated for germination of the T<sub>1</sub>-generation on MSO medium supplemented with kanamycin sulfate or

hygromycin B, according to the selectable marker gene present on the T-DNA. For plants transformed with pDU-pGIP-AD1-3, 8 out of 10 plants germinated in a 1:3 segregation pattern on MSO supplemented with kanamycin (Figure 3). The germinated 75% seedlings are either homo- or heterozygous regarding the transgene. The remaining 25% seedlings are azygous. Although PCR positive, lines 3 and 7 did not germinate on the selective medium, it is possible that the transgene is located in an area of the tobacco genome where expression is silenced; these lines were not further considered. For plants transformed with pCAMBIA-pGIP-220 all 10 PCR positive lines were germinating in 1:3 segregation pattern on MSO supplemented with hygromycin B.



**Figure 3:** Tobacco seeds germinated on MSO medium supplemented with kanamycin. **A:** wild type SR-1 is not resistant. **B:** Transgenic line shows a 1:3 segregation pattern according to Mendel. Arrow indicate examples of the 25% azygous seedlings, the remaining 75% are homo- or heterozygous.

The germinated  $T_1$  generation plantlets were transferred into single pots and kept in a mist chamber. After 10 days, the plantlets were transferred to a greenhouse and grown for an additional three months until the production of  $T_2$  seeds (Figure 4). Plating and analysis of the germination pattern on selective medium will be repeated for the  $T_2$  seeds.

$T_2$  lines that showed a germination rate of 100% are homozygous and were analyzed by ELISA and Western blot analysis using the anti-HA antibodies we generated in our earlier work for determining the size and location of native Xf HAs. Unfortunately we were not able to detect recombinant HAs in the transgenic tobacco plants. Analysis of HA-transgenic grapevines is currently underway (see below).



**Figure 4:**  $T_1$  generation of HA-expressing tobacco. Plants were kept at the environmental horticulture facility at UC Davis until the production of  $T_2$  seeds.

**2006-2008 Objectives 4a.** Mechanically inoculate grapevines with Xf HA-expressing grapevine endophytes.

As stated above, neither our lab or the Lindow lab has identified a bacterial endophyte of grapevine, despite years of work. Therefore the use of an endophyte to deliver transgenic Xf HAs into plant xylem could not be performed.

**2006-2008 Objectives 4b.**

**2008-2010 Objective 4:** Mechanically inoculate HA-transgenic tobacco with wild type (wt) Xf cells. Compare disease progression and severity in transgenic tobacco with non-protected controls.

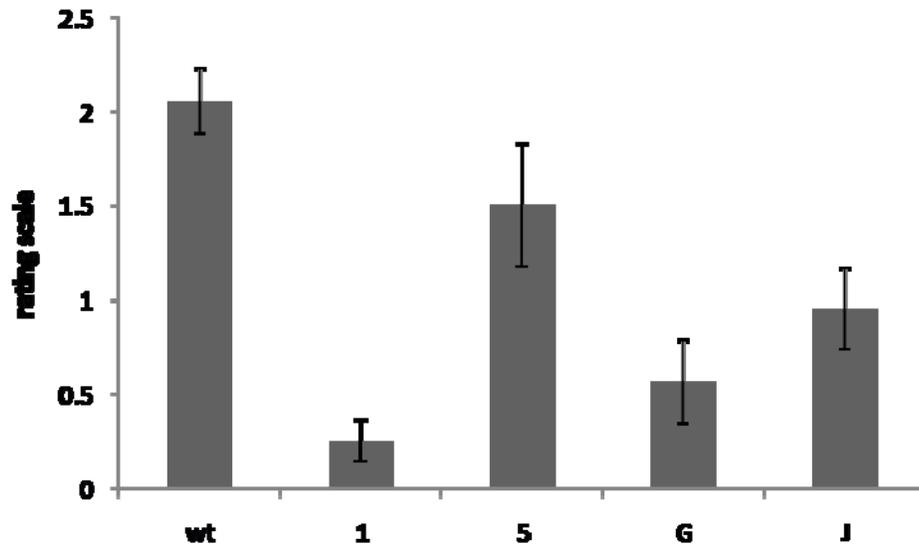
Analysis of T<sub>1</sub> generation tobacco plants for functional resistance against *X. fastidiosa*

Six tobacco plants for lines 1 and 5 (transformed with pDU-pGIP-AD1-3), 6 plants for lines G and J (transformed with pCAMBIA-pGIP-220), and 10 wild type plants were inoculated with *X. fastidiosa* wild type cells using a standard pin-prick method. Eight weeks after inoculation, leaves at 25 cm above the POI were harvested and the bacterial population per gram of leaf tissue was determined. Results are summarized in Table 1.

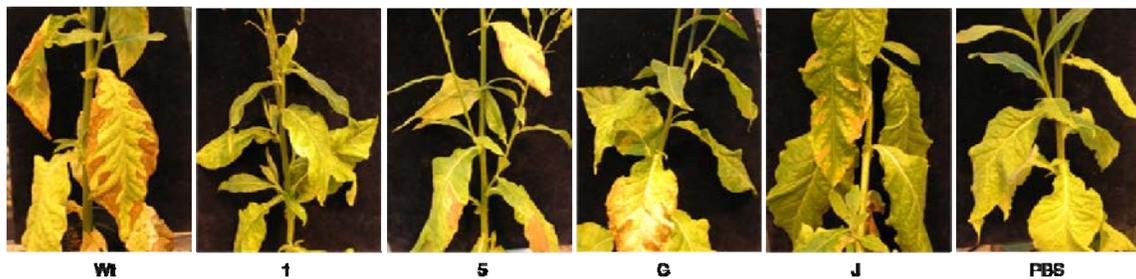
Strain	CFU/g of tissue 25 cm above POI
wild type	9.9 (+/- 7.3) x 10 <sup>5</sup>
line 1	9.1 (+/- 16.2) x 10 <sup>2</sup>
line 5	1.3 (+/- 1.5) x 10 <sup>6</sup>
line G	3.3 (+/- 8)
Line J	2.5 (+/- 3.8) x 10 <sup>3</sup>

**Table 1:** Bacterial populations at 25 cm above the POI of wild type and transformed tobacco lines 1,5, G, and J at 8 weeks after inoculation. Bacterial populations are similar for wild type and line 5, whereas lines 1, G, and J have reduced quantities of bacteria.

Ten weeks after inoculation, the tobacco plants were rated on a visual scale from 0 to 5 as previously described (Guilhabert and Kirkpatrick, 2005). Results are presented in Figure 5a and 5b below:



**Figure 5a:** Ratings of *X. fastidiosa*-inoculated tobacco lines wt, 1, 5, G and J on a scale of 0-5, ten weeks after inoculation. Line 5 shows symptom severity similar to wild type plants, whereas symptoms are reduced in lines 1, G, and J.



**Figure 5b:** Foliar symptoms of HA-transgenic tobacco lines 1, 5, G and J, as well as wild type Xf Fetzer and PBS controls 16 weeks following mechanical. Inoculation with *X. fastidiosa*.

A good correlation between the number of bacteria that were isolated from the inoculated tobacco plants and symptom severity was found. Lines 1, G, and J have reduced quantities of bacteria and also show fewer symptoms than wild type plants. These lines are very promising and may be considered moderately resistant to *X. fastidiosa*. Line 5 has lower ratings than wild type plants, but a similar bacterial population at 25 cm above the POI and will not be further considered.

### **2010-2011 Objectives:**

1. Use RT-PCR and HA antibodies to verify and quantify expression of HA mRNA and proteins in transgenic plants. Use anti-HA antibodies to determine if Xf HA proteins are present in tobacco and grapevine xylem fluids.

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 AD 1-3, labeled as SPAD1 and 3 of 12 transgenic lines of the full-length HA, labeled PGIP220 in Table 2 below, had the HA gene inserted into the grapevine chromosome.

**TABLE 2 Results of PCR testing transgenic grapevines for Presence of full-length (PGIP 220) of AD1-3 fragment of *Xylella fastidiosa* hemagglutinin genes in grape chromosome**

<u>DNA</u>			
<u>ID#</u>	<u>genotype</u>	<u>Standard PCR</u>	<u>qPCR</u>
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 3).

<b>TABLE 3. RNA RT-qPCR of Thompson seedless HA transgenic lines</b>	
<b>LINE ID</b>	<b>Relative transgenic <i>Hxf</i> RNA level</b>
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 constructs and PGIP220 lines express long constructs. The higher the number the higher the RNA level in the leaves.

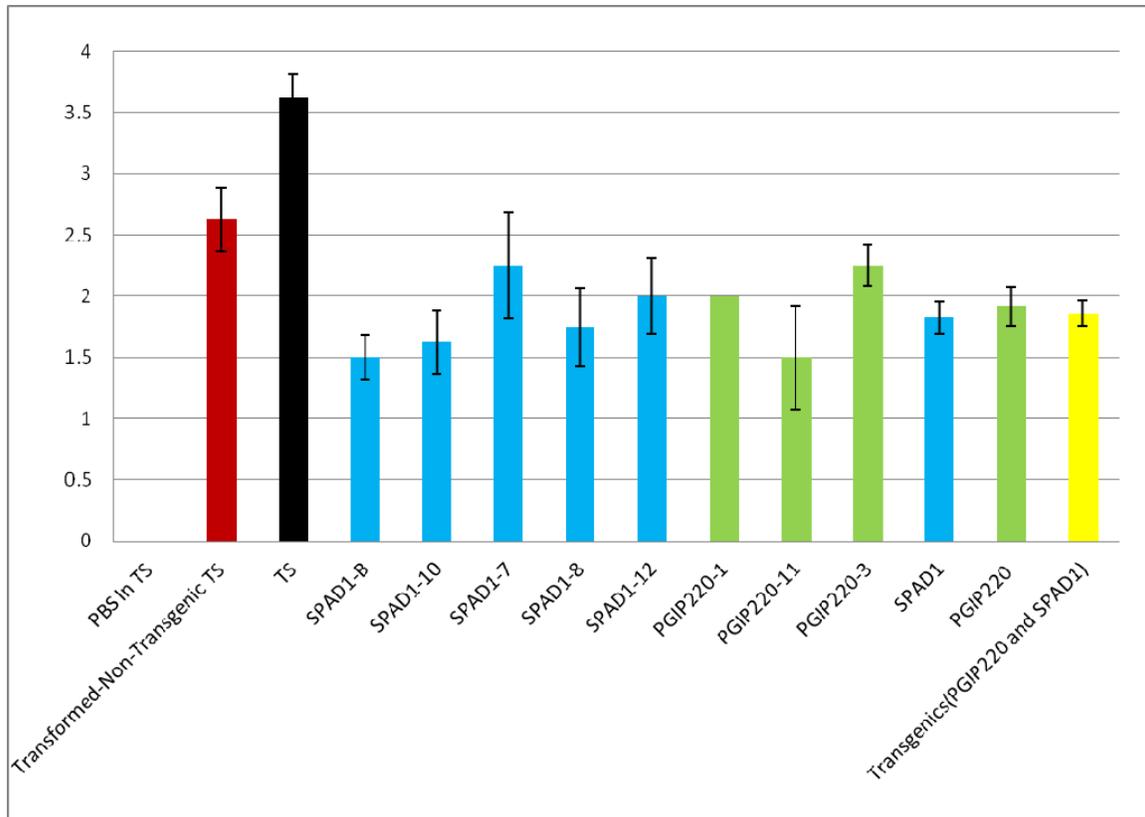
As per 4/12 we now have produced sufficient numbers of HA-transgenic grapevines to destructive sample and test for the presence of HA protein in foliar tissue as well as extracted xylem sap by ELISA and western blot analysis. This work should be completed within the next 2 months.

**2010-2011 Objective 2.** Mechanically inoculate transgenic grapevines with wild type *Xf* cells. Compare disease progression and severity in transgenic grapevines with non-protected controls.

We have gone through 3 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 could be detected in grapevine xylem sap. On December 8<sup>th</sup> and 9<sup>th</sup> of 2011 we inoculated 10 reps of each of the 9 PCR-positive transgenic lines with 40ul of a 10<sup>8</sup> 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 inject into a vine.

We also inoculated untransformed Thompson seedless and 2 transgenic lines that did not contain HA inserts by PCR analysis, shown Transformed Non-transgenic TS in Figure 6, as positive controls. Figure 6 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 quite pleased with this promising preliminary result. Another set of greenhouse inoculations will be performed this summer. 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. We have recently contacted PIPRA to ask for assistance in getting the needed permits to plant the HA transgenic lines in the field at UCD. 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 the the incipient infection might very well be prune 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, or hopefully with the assistance the Almeida lab insect inoculated with Xf. These initial results certain warrant further evaluations.



**Figure 6.** 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.

#### **Publications and Presentations Produced and Pending:**

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

Voegel, T.M and B.C Kirkpatrick. A two-partner secretion system in *Xylella fastidiosa* is responsible for the secretion of the hemagglutinin adhesins HxfA and HxfB. FEMS Letters (submitted).

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Kirkpatrick, B.C., T. Voegel, M. Igo and G. Bruening. 2007. Isolation, characterization and genetic manipulation of *Xylella fastidiosa* hemagglutinin genes. Pierce's disease Research Symposium Proceedings, California Department of Food and Agriculture, San Diego, pp. 204-208.

Kirkpatrick, B.C., T. Voegel, M. Igo and G. Bruening. 2008. Isolation, characterization and genetic manipulation of *Xylella fastidiosa* hemagglutinin genes. Pierce's disease Research Symposium Proceedings, California Department of Food and Agriculture, San Diego, pp. 176-179.

Kirkpatrick, B.C., T. Voegel, M. Igo and G. Bruening. 2009. Isolation, characterization and genetic manipulation of *Xylella fastidiosa* hemagglutinin genes. Pierce's disease Research Symposium Proceedings, California Department of Food and Agriculture, Sacramento, pp. 142-145.

Kirkpatrick, B.C., T. Voegel, M. Igo and G. Bruening. 2010. Isolation, characterization and genetic manipulation of *Xylella fastidiosa* hemagglutinin genes. Pierce's disease Research Symposium Proceedings, California Department of Food and Agriculture, San Diego, pp. 201-205.

## **Research Relevance Statement and Lay Person Summary**

Our 7+ year research effort on the role hemagglutinins, 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 than 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 Almeida lab also showed that HA mutants were transmitted at lower efficiencies than 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 research showed that DSF mutants, which are also hypervirulent, produced much less HAs than 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 site 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. The evaluation of HA-expressing transgenic grapevines acting as rootstocks is an objective of a recent proposal that was submitted in 2012. We have recently finished a first PD disease severity screening of the 9 HA transgenic lines we produced with funding for this project. The results were very encouraging with all of the HA-transgenic lines having much lower disease ratings than non-transgenic control. We will repeat the pathogenicity assays this summer and we are now in the process of determining the sizes of Xf populations in the transgenic lines versus the non-transgenic control. If we find that Xf populations are suppressed below levels that induce fruit raisining or cordon dieback the Ha lines might provide some level of functional resistance against PD. If this occurs, we will have obtained the goal we set 5 years ago.

### **Status of Funds**

All of the funding allocated to this project has been spent.

### **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 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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Voegel, T.M. and B.C. Kirkpatrick. 2006. Characterization of a putative Two-Partner secretion pathway protein in *Xylella fastidiosa*. *Phytopathology* 96:S119.