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 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. The Xf HAs essential act 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 are currently in the process of evaluating potential pierce's disease resistance in the HA-expressing transgenic grapevines. We anticipate that initial stages of this evaluation will be completed in June 2012 and the pathogenesis evaluations will be repeated a second time with final evaluation in October 2012.

List of Objectives for all years, beginning with the <u>2006-08 proposal</u>:

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, additional 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.

<u>2008-2010 Objectives:</u>

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).

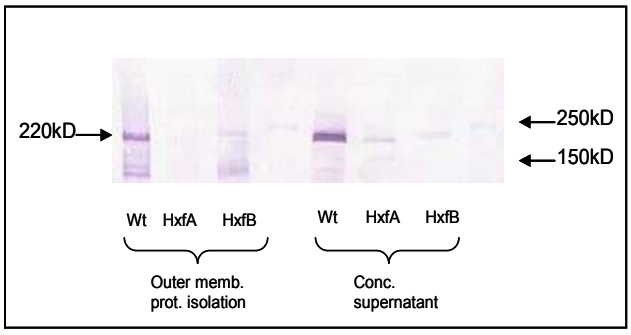


Figure. 1 Western blot analysis of membrane protein preparations (left) and culture supernatant (right) of wt and HA mutants revealed that HAs are secreted as well as inserted into the outer membrane and possess a size of 220kDa.

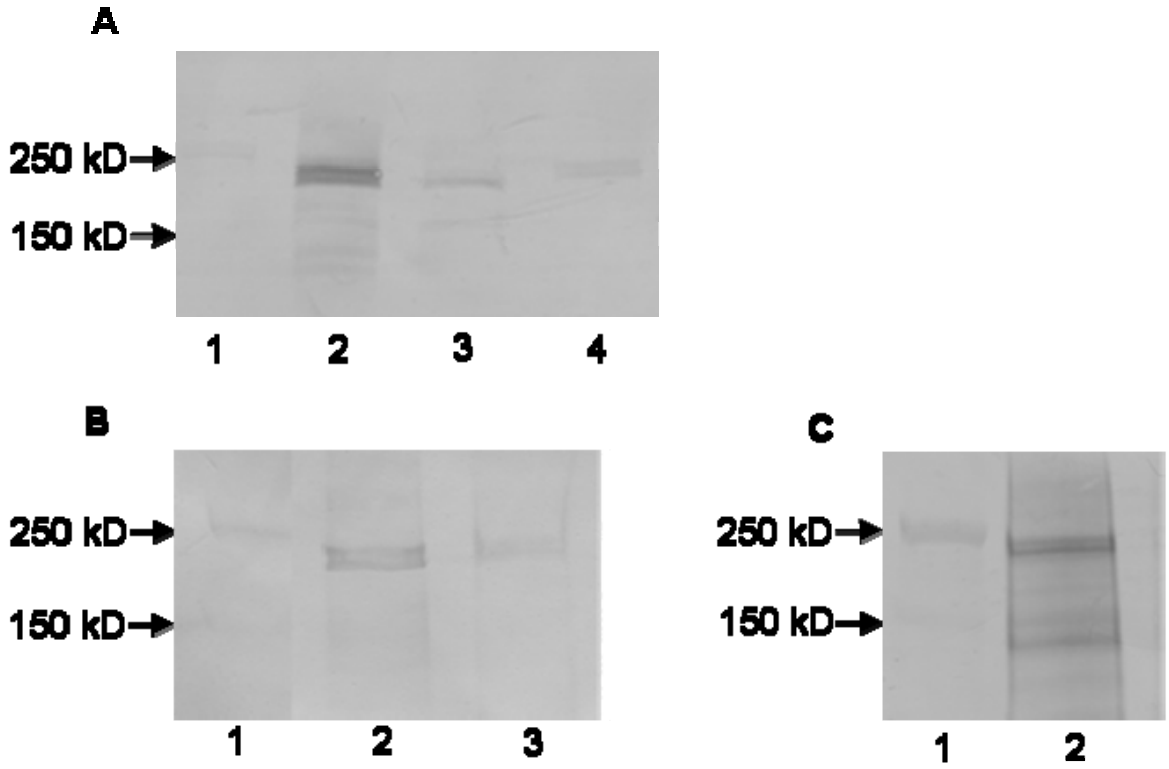


Fig.2: Western blot analysis of *X. fastidiosa* supernatant developed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula; lane 3: HxfA, lane 4: HxfB. A doublet of bands in the wild type Temecula is detectable, the single band in the HxfA mutant strain correlates 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.

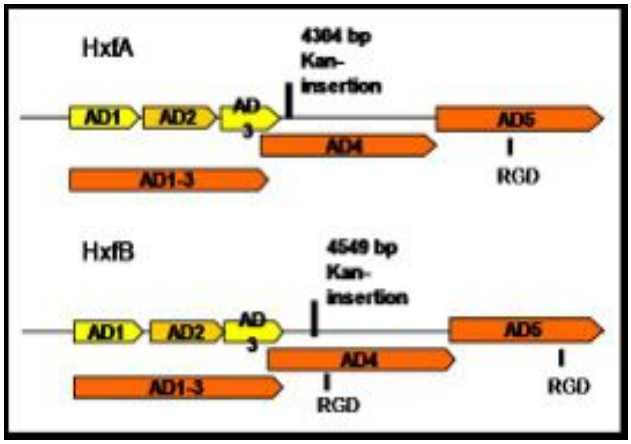
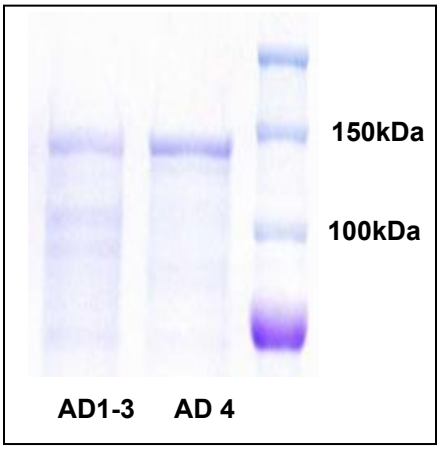


Figure. 3.
Identification of possible adhesion domains (ADs) and RGD motifs

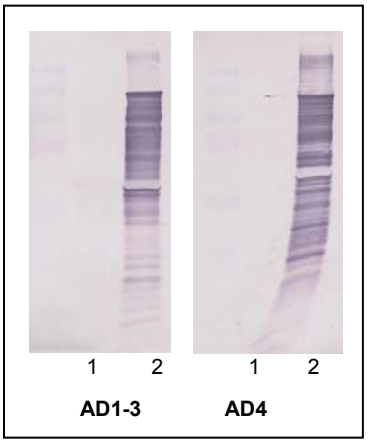
e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of Xf grown in liquid medium.

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 silco* 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.

Purified AD1-3 (predicted to have "hemagglutinin activity domains" (http://smart.embl-heidelberg.de) and AD4 (highest antigenic region according to hydrophobicity plots) 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 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.



A



B

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

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.

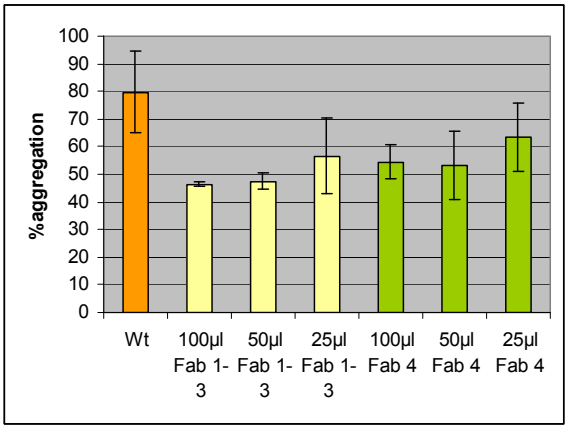


Figure 5. Blocking of cell-cell aggregation by addition of Fab fragments against AD1-3 and AD4 suggests that cell-cell binding domains are contained in AD1-3 and AD4.

1d. Determine if HA domain fusion proteins 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 AD 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 might contain cell-cell attachment domains and based on these results we propose that both these HA fragments need to be evaluated in transgenic plants.

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.

Knowing where the full-length HA protein is cleaved we then cloned the full-length 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.

2006-08 Objectives, continued:

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*.

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 us 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 might deliver Xf HAs into grapevine xylem. For this reason, this objective was abandoned.

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.

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 HxfB (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 is 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.

Analysis of transformed tobacco plants

Four months after submission of the constructs to the transformation facility, 11 transgenic tobacco plants T_0 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).

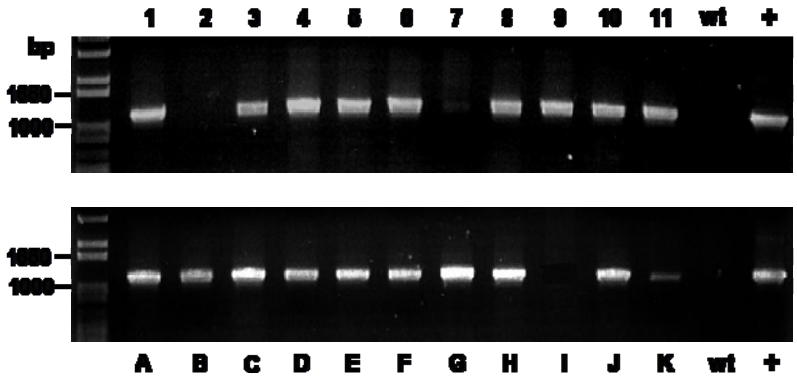


Figure 6: Confirmation of T-DNA insertion into the genome of tobacco SR-1 by PCR analysis of genomic tobacco DNA. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3, letters A-K indicate transgenic lines that were transformed with pCAMBIA-pGIP-220. Lines 2 and I do not have 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).

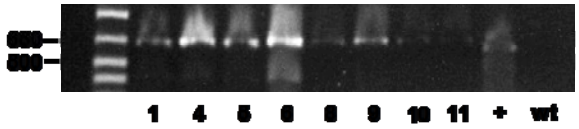


Figure 7: RT-PCR of transformed tobacco SR-1 using primer pair pGIP-HAfor and HArev. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3. Wild type plants were used as negative controls and isolated plasmid pDU-pGIP-AD1-3 was used as positive control (+).

After 3 months, seed pods of the HA expressing T_0 plants were harvested, the seeds sterilized and plated for germination of the T_1-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.

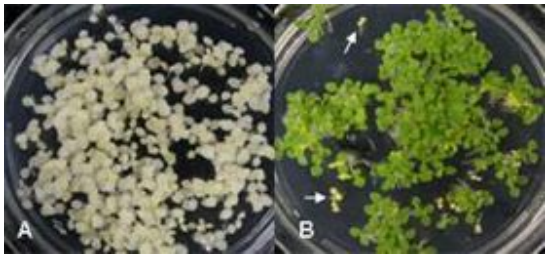


Figure 3: Tobacco seeds germinated on MSO medium supplemented with kanamycin. **A:** wild type SR-1 is not resistant. **B:** Transgenic line shows a 1:3 segregation pattern according to Mendel. Arrow indicate examples of the 25% azygous seedlings, the remaining 75% are homo- or heterozygous.

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 show a germination rate of 100% are homozygous and will be used for ELISA and Western blot analysis using the anti-HA antibodies we generated in our earlier work to test for expression of HA protein. Positive plants will be challenged with *Xf* to determine if movement of the bacteria is inhibited or delayed in the HA-expressing tobacco plants. Unfortunately, transformation of grapevines takes considerably more time than transformation of tobacco and we eventually obtained transformed grapevine plants in September 2010.

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.

Analysis of T_1 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 10^5
line 1	9.1 (+/- 16.2) x 10^2
line 5	1.3 (+/- 1.5) x 10^6
line G	3.3 (+/- 8)
Line J	2.5 (+/- 3.8) x 10^3

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).

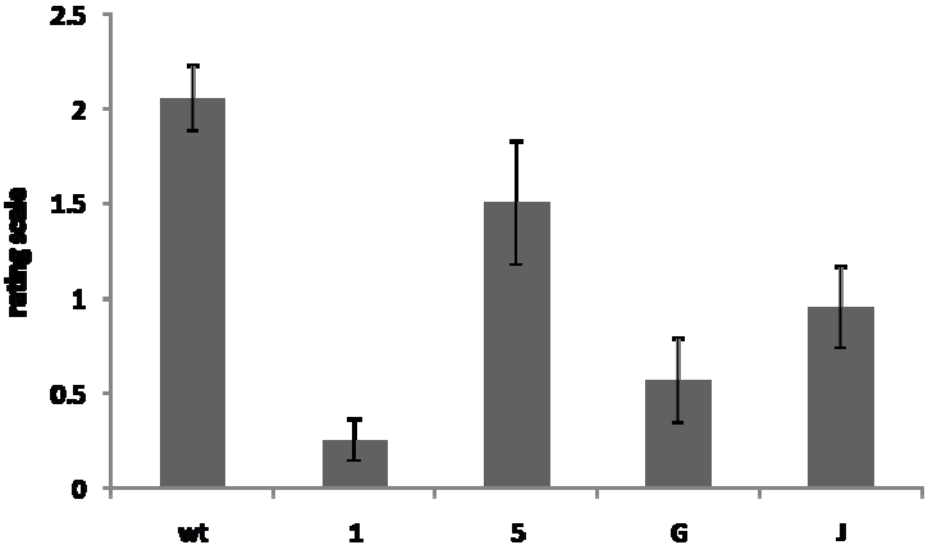


Figure 5a: Ratings of *X. fastidiosa*-inoculated tobacco lines wt, 1, 5, G and J on a scale of 0-5, ten weeks after inoculation. Line 5 shows symptom severity similar to wild type plants, whereas symptoms are reduced in lines 1, G, and J.

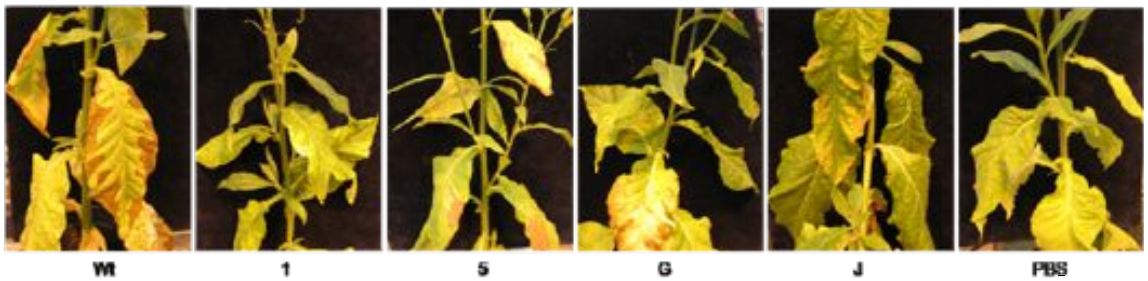


Figure 5b: Foliar symptoms of HA-transgenic tobacco lines 1, 5, G and J, as well as wild type Xf Fetzer and PBS controls 16 weeks following mechanical. Inoculation with *X. fastidiosa*.

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 propogated. 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 21 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

DNA ID#	genotype	Standard PCR	qPCR
1C (7/6)	PGIP 220-15	—	—
2C (7/6)	SPAD1-2	—	—
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	†	†

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).

TABLE 3. 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 constructs and PGIP220 lines express long constructs. The higher the number the higher the RNA level in the leaves.	

2010-2011

Objective 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.

We have gone through 3 rounds of vegetatively propagating the lignified transgenic grapevine lines. We tried our hand at propagating green shoots but only 10-15% of the green shoots became established.

We were very interested in determining whether any of these lines possessed PD resistance. So rather than waiting for the results of extensive ELISA and Western blot analysis of transgenic to determine if HA could be detected in grapevine xylem sap, we decided to take 10 reps of each of the 9 PCR-positive transgenic lines and mechanically inoculate them with Xf in mid-December, 2011. We also inoculated untransformed Thompson seedless and 2 transgenic lines that did not contain HA by PCR analysis as positive controls. We will be assessing disease symptoms in these vines within a month. The whole pathogenicity evaluation will be repeated this summer and we will determine whether HA can be detected by ELISA and western blot analysis in the coming months.

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).

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

Voegel., T. M., and B.C. Kirkpatrick. 2007. *Xylella fastidiosa* hemagglutinins: Identification of cell-cell binding domains and evaluation of their potential for producing *X. fastidiosa* resistant transgenic plants. Phytopathology 97:S118.

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 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 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 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. 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 final stages of assessing whether the 10 HA transgenic lines we produced with funding from this project will 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 Bennett 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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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. 2006. Characterization of a putative Two-Partner secretion pathway protein in *Xylella fastidiosa*. Phytopathology 96:S119.