Progress report for CDFA contract number: 06-0223

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

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Reporting Period: This report largely presents research that was conducted from April 2009 to October 2010 and was presented in the PD/GWSS Symposium Proceedings**.**

Please Note:
Progress since October 2010 has been largely confined to growing and propagating the 23 transgenic lines that were produced from the 2 hemagglutinin transformation constructs. I will summarize what we are <u>now</u> in the process of doing on this project in the body of this report in BOLD type.

Abstract and Introduction:
Xf cell-cell attachment is an important virulence determinate in Pierce's disease. Our previous research has shown that if 2 HA genes which we 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 wt *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 essentially 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. Recent work reported by the Almeida lab has also shown the importance of HAs in vector transmission (Killiny and Almeida, 2009). The knowledge we gained here about the basic biology of *Xf* HA proteins provided the foundation for completing the last step of this project, where we transformed tobacco and grapevine plants with HA genes. Hopefully, these genes will be expressed in the xylem of transgenic plants and potentially act as a 'molecular glue' to retard systemic movement of inoculated *Xf* cells through grapevine xylem. If successful, this approach might provide a novel form of resistance against Pierce's disease.

Layperson Summary:

HA proteins play an important role in adhesion and biofilm formation of *Xf*. Previous studies by Guilhabert and Kirkpatrick, 2005 showed that mutants in the identified HA genes no longer

formed clumps in liquid medium like wt *Xf* cells. Clearly the HA proteins play an important role in mediating cell-cell interactions. Research in the Almeida lab has also shown that HA proteins play important roles in attachment processes during vector transmission (Killiny and Almeida 2009). Research conducted in our lab has shown that HA proteins are present in the outer membranes of *Xf* cells and that these proteins are also secreted into culture medium at low concentrations. Interestingly, we also showed that HAs are embedded in vesicles as it has been reported for some pathogenic Gram-negative bacteria (Kuehn and Kesty, 2005). The 10.5 kb HA genes should theoretically encode a protein of approximately 360 kD, however we have shown that the native size of the HA proteins in the outer membranes, culture supernatants and membrane of vesicles is approximately 220 kD. To identify the cleavage site where the processing occurs, we isolated native secreted HA proteins from culture supernatant. These proteins were analyzed by mass spectrometry and we determined that the cleavage site lies 2300aa downstream from the N-terminus of the gene such that approximately one third of the C-terminal part is cleaved off forming the full-length native HA . We used this information to create binary plasmids containing the identified portions of the HA proteins that mediate attachment and used them to generate putatively HA-expressing transgenic tobacco and grapevines. Transgenic tobacco were evaluated by PCR, RT-PCR and serological assays to detect expressed Xf HA. We verified that the transformed tobaccos produced HA mRNA but were unable to detect HA in the xylem sap by ELISA and western blot analysis. **Ten transgenic grapevines lines were produced using a HA construct that should contain the cell-cell binding domains and 13 independent grapevine lines have been produced using the full-length native 220kD HA protein. The transgenic grapevines are now being grown and propagated to produce plants that will be further evaluated using molecular and Xf pathogenicity assays.**

Project Objectives:

1 a. Use antibodies we have prepared against a conserved, putative adhesion domain (AD2) that is present in both *Xf* hemagglutinins (HA) to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
b. Determine if these antibodies (Fab fragments) 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 native HAs and HA domain fusion proteins 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 HxfA/B domain fusion protein. Determine the native size and location of *Xf* HA in *Xf* cultured cells using AD1-3 and AD4 antibodies.
c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.
3. **a.** Transform grapevines and tobacco, an experimental host of *Xf* and an easily transformable plant (Francis et al., 2008), with *Xf* HA binding domains. **Use RT-PCR and southern blot analysis to determine gene copy number and gene expression in transgenic grapevines. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.**

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

Results:

Objectives 1 and **2** are completed and results were reported previously (Pierce's Disease Research Symposium Proceedings 2007-2009 and in the manuscript: Voegel, et al., 2010. Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins, Microbiology 156: 2172 – 2179.

Objective 3.

To overcome problems that might occur trying to transform plants with a full-length hemagglutinin (HA) gene, we first determined the size of native Xf HA proteins. We found that both native HxfA and HxfB were processed from their potential size of 360 kD to 220 kD native size. Furthermore, we identified the cleavage site in the HA proteins. We also identified domains in the N-terminal portion of the HA proteins that mediate cell-cell attachment and showed that the active protein is secreted (Voegel, et al, 2010). Based on these results we prepared 2 different constructs for transformating grapevines and tobacco; one construct contained the N-terminal hemagglutination domains (AD1-3) and one contained the entire native 220 kD protein (220).

AD1-3 and 220 were PCR amplified from the gene HxfB (PD1792) using proof reading polymerase and wt Temecula genomic DNA as template. The resulting 4000 bp and 6300 bp PCR products were cloned into pCR-2.1-TOPO and fully sequenced using primers generated every 600 bp along the cloned HA fragments. The obtained sequences were aligned into a contig using the program Sequencher to verify amplification of the correct sequences. To enable secretion of the bacterial HAs outside the eukaryotic cells of tobacco and grape, a signal peptide pGIP (Aguero et al., 2005) was synthesized by 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 its integrity by sequencing, pDE00.0113-pGIP-AD1-3 was digested with AscI and the resulting cassette cloned into the binary vector pDU97.1005 (Dandekar lab) creating pDU08.2407. The plasmid was transformed into *Agrobacterium tumefaciens* strain EHA101 and the culture given to the Ralph M. Parsons foundation transformation facility on the UC Davis campus who performed the transformation of SR-1 tobacco.

Unfortunately, the pDU97.1005 marker gene nptII confers resistance to kanamycin. In transformation experiments with grapes using binary plasmids containing the nptII gene many escapes were observed by the transformation facility which prolonged the time needed to identify and generate transformed grapevines. Therefore we digested pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220 with EcoRI and ligated the resulting cassette into the binary plasmid pCAMBIA1300 (Canberra, Australia) which 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 cultures given to the Ralph M. Parsons foundation transformation facility who will transform Thompson seedless grapevines and SR-1 tobacco with pCAMBIA-pGIP-220 and pCAMBIA-PGIP-AD1-3.

We received our first AD1-3 transformed tobacco plants in 2009. DNA was extracted from the T0 tobacco plants and tested by PCR for the presence of T-DNA and 9/11 and 10/11 tested positively as shown in Figure 1. The PCR positive lines were tested by RT-PCR to determine whether the constructs were expressed; all of the T-DNA positive lines tested positively by RT-PCR indicating the HA constructs were being transcribed. Attempts were made to detect hemagglutinin in both tobacco xylem sap and in total leaf proteins using both ELISA and Western blot analysis. Unfortunately we were not able to detect measurable amount of HAs using either serological technique.

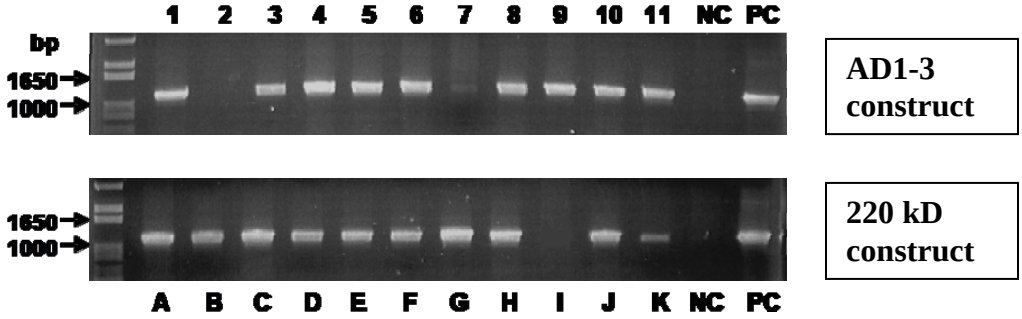


Figure 1. PCR analysis to confirm T-DNA insertion in 11 tobacco transformed lines T0 for AD1-3 and 220.

Six transgenic tobacco plants of lines 1 and 5, which were transformed with the AD1-3 construct and 6 plants from lines G and J, which were transformed with the 220kD construct were mechanically inoculated with wild type Fetzer Xf. Eight weeks after inoculation Xf was isolated from leaves that were 25cm above the point of inoculation. The results are shown in Table 1 below:

Tobacco Plant inoculated with Xf	**CFU/g of tissue 25 cm above P.O.I.**
Wild type SR1 tobacco	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) x 10^2
Line J	2.5 (+/- 3.8) x 10^3

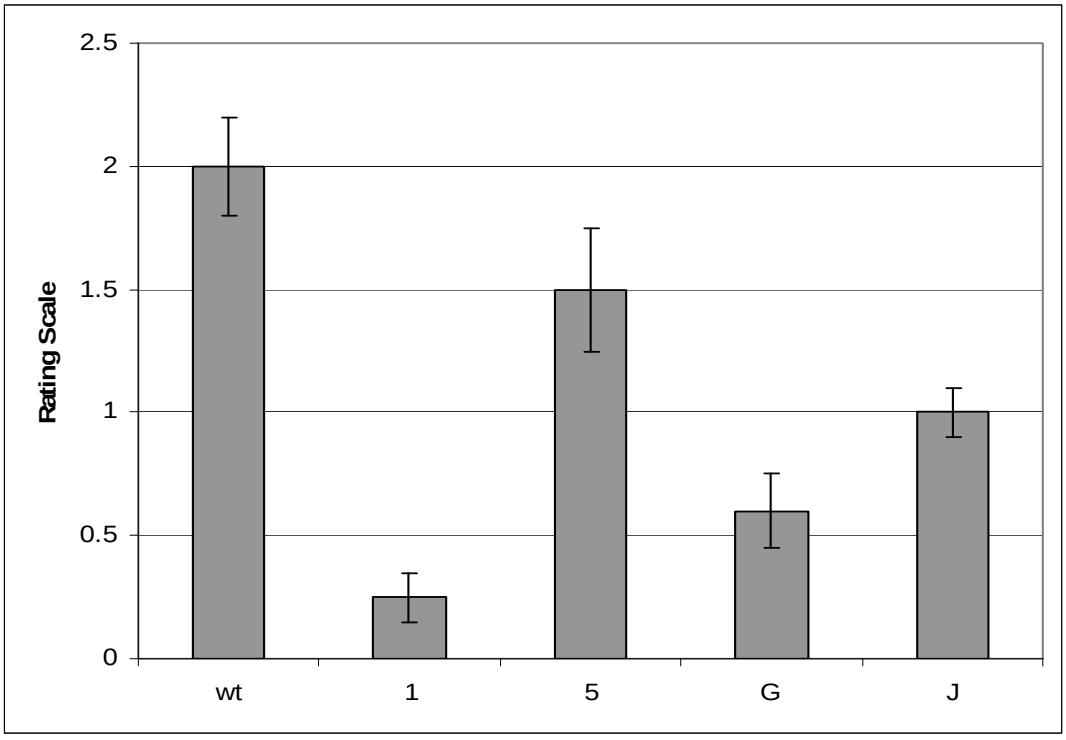
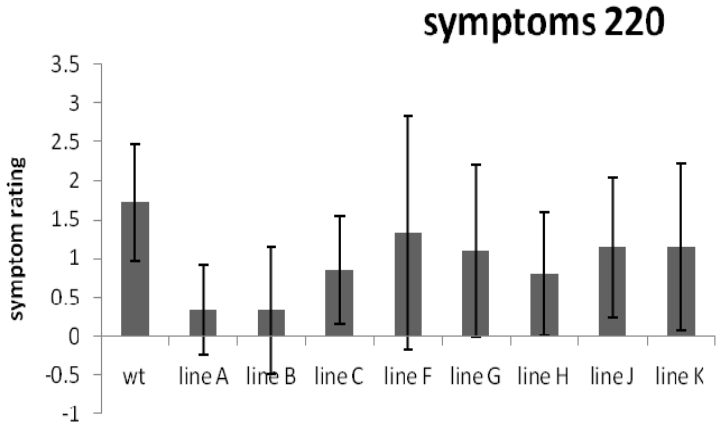


Figure 2: Disease severity ratings (0=healthy to 5 = severe symptoms) showed Lines 1, G and J developed significantly less severe disease symptoms that the non-transformed Sr1 tobacco 8 weeks following inoculation with *Xylella fastidiosa*.

There was a good correlation between the number of Xf CFUs that could be isolated 25cm above the point of inoculation and the severity of disease symptoms 8 weeks after inoculation with Xf (Figure 2). Lines 1, G and J yielded fewer Xf bacteria upon isolation and the severity of symptoms in those lines was less than Xf-inoculated non-transformed SR1 tobacco.

Unfortunately similar analyses of T2 generation transgenic tobacco were not as encouraging because the amount of variation in the plant reps rendered no statistically significant differences in disease severity between the T2 lines and the wild type SR1 tobacco (Figure 3).



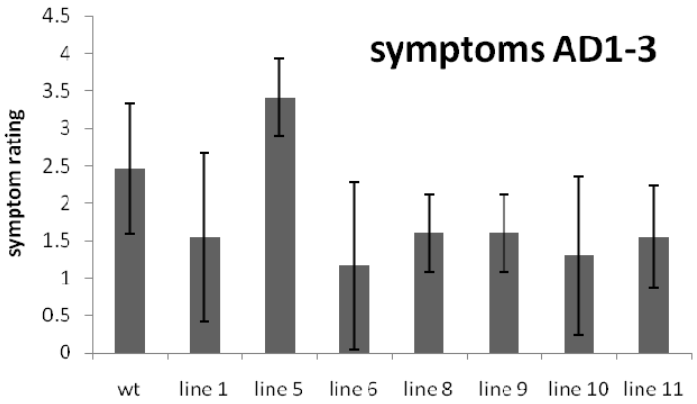


Figure 3. Disease severity in T2 HA-transgenic SR1 tobacco based on a 0 (healthy) to 5 (dead) 8 weeks after inoculation with wild type Fetzer Xf.

line	wt	1	5	6	8	9	10	11	
%	14	36	16	25	10	40	60	18	
line	wt	A	B	C	F	G	H	J	K
%	33	33	60	42	33	60	50	28	14

Table 2. Percentage of HA-transgenic and wild type SR1 tobacco plants in which NO Xf was isolated at 25cm above the point of inoculation. Lines 1, 5, 6, 8, 9, 10 and 11 are SR1 tobacco transformed with the AD1-3 hemagglutinin construct while lines A-K were transformed with the 220kD hemagglutinin construct.

While there was no statistically significant differences in disease severity of transformed versus non-transformed SR1 tobacco, we did observe differences in the number of inoculated plants in which Xf could be isolated 25cm from the point of inoculation (Table 2). For example, for the AD1-3 construct Xf was not isolated in only 145 of the wild type SR1 tobacco, while 60% of the Line 10 transgenic plants were devoid of Xf at 25cm above the POI, suggesting that the HA transgenic line maybe slowing the systemic movement of Xf compared to the movement in non-transformed tobacco. There 2 lines (B and G) whose numbers of Xf-free plant reps were less than non-transformed tobacco but a larger number of the control plants were Xf-free at 25cm compared to the Ad1-3 experiment. Because we now have transgenic grapevines in hand to evaluate we felt that it wasn't worth additional effort to repeat testing of the T2 tobacco lines.

In mid-August 2010 we began receiving Thompson seedless grapevines from the UCD Plant transformation facility. In mid-October we received the final transgenic lines: 10 lines transformed with the AD1-3 HA construct and 13 lines transformed with the 220kD construct. These were grown in a growth chamber until they were hardened off enough to

be moved into a greenhouse where they are being raised under supplemental lighting. The original mother plants grew sufficiently such that 7 cuttings were taken from each mother plant and successfully rooted and propagated in the green house. As per April 2011 these propagated cutting were approximately 10" tall. The original mother line plants are now approximately 12" tall and when they reach 18", we will produce another 7 to 10 cuttings off the original mother line plants. With the assistance of Jim Lincoln in the Gilchrist lab we are now in the process of performing RT-PCR on leaf RNA extracted from the original mother line plants to determine levels of HA gene expression. Southern blot analysis will be done to determine if each transgenic line is genetically unique and determine that indeed the HA structural gene is present in the transgenic line. When the first round of propagated plants get bigger we will test them by ELISA and western blot analyses to see if we can detect Xf HA proteins. We will first sample small amount of leaf tissue for the HA proteins and later when they get approximately 18-24" tall xylem sap will be extracted from 1 or 2 cuttings, the proteins concentrated and then subjected to the same serological tests to determine if HA protein is indeed in the transgenic grapevine xylem sap.

Reps of each line will be mechanically, and hopefully with the assistance of the Almeida lab at UC Berkley, sharpshooter inoculated with Xf in the summer of 2011. To date the production of the transgenic grapevines has taken 20 months and likely another 4 months will be required to produce sufficient numbers of transgenic plants to assess their potential resistance or susceptibility to Pierce's disease.

References:

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