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

CDFA contract number: **06-0223**

Time period covered: This report presents research that was conducted from April to July 2008

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Objectives:

1 a. Use antibodies we have prepared against a conserved, putative binding 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 tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.
b. Mechanically inoculate HA-transgenic tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in transgenic tobacco with non-protected controls.

Results:

1 a. Use antibodies we have prepared against a conserved, putative binding 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.

The results of this objective have been reported in the Proceedings, 2007 Pierce's Disease Research Symposium. CDFA, Sacramento, CA

b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.

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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.

Due to the repeated negative results in isolating native HAs by using an affinity column, a different method to isolate native HAs was evaluated. Our former results showed that HAs are secreted into the *Xf* culture supernatant (objective 2 b) and therefore 10 liters of *Xf* wt culture were grown and the proteins in the supernatant precipitated by ammonium sulfate precipitation. With help of Carl Greve (Labavitch lab) the proteins in the precipitated supernatant were separated by molecular weight using size exclusion chromatography and the fractions containing high molecular weight proteins collected. The fractions were pooled, further concentrated and resolved by SDS-PAGE gels. The protein bands at 220 kD corresponding to HAs were submitted to the Genome Center Proteomics Core at the University of California, Davis, for Mass Spectrometry (LC MS/MS) protein sequence analysis. HxfA and HxfB could be identified. Although the quantity of HAs isolated was not sufficient for N-terminal sequencing we were able to establish the approximate site where the 330kD pre-protein is processed into the 220kD protein that is present on the Xf cell, in membrane vesicles and as free protein in the culture supernatant..

d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.

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2. a. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.

The results of this objective have been reported in the Proceedings, 2007 Pierce's Disease Research Symposium. CDFA, Sacramento, CA

b. Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein. Determine the native size and location of *Xf* HA in *Xf* cultured cells using AD1-3 and AD 4 antibodies.

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At the 2007 PD/GWSS Conference Steve Lindow suggested that we make sure that our "secreted" proteins are not really HA proteins embedded in membrane vesicles. Vesicles released from the envelope of growing bacteria serve as vehicles for proteins of gram-negative bacteria (Kuehn and Kesty, 2005).

Therefore we isolated vesicles by ultracentrifugation of cell free *Xf* wt supernatant for 2 hours at 36,000 rpm. Both, the pellet containing vesicles, as well as the remaining supernatant containing soluble proteins were shown by Western blot analysis to contain HA proteins. This confirms our earlier analyses that both HAs are secreted as processed proteins into the culture supernatant.

c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.

The results of this objective have been reported in the Proceedings, 2007 Pierce's Disease Research Symposium. CDFA, Sacramento, CA

3. **a.** Transform tobacco, an experimental host of *Xf* and an easily transformable plant (Francis et al., 2008) with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.

This is the portion of the project that has been pursued during the past four months of this reporting period (April to July, 2008).

It is difficult to transform tobacco or grapes with a gene the size of native HAs. Therefore we identified by mass spectroscopy analysis (described in objective 1 c) peptides that were present in the secreted, "biologically active" protein. We could determine for HxfA and B that the amino acids downstream of 2300 are not present in the active protein and therefore concluded that one third of the C-terminal part of the HA proteins is cleaved off during or after secretion through the outer membrane like it is described for *Bordetella* FHA (Domenighini et al., 1990).

We will use for plant transformation the N-terminal portion of the processed HA protein which is approximately 220 kD and the N-terminal fragment containing AD1-3 which is approximately 150 kD. In collaboration with the Dandekar lab, a pear signal peptide will be fused to the N-terminal part of the HA protein that directs the fusion protein to the xylem (Aguero et al, 2005). A synthetic peptide containing the xylem signaling leader sequences was commercially order and is now in our hands. In the past 4 months we PCR amplified AD1-3 and a DNA fragment approximately 6.3kb that should code for the entire 220kD native protein in TOPO cloning vectors. All of these contructs have been sequenced by the UCD Sequencing facility and the structural genes of both AD1-3 and the 220 fragment are error free. We are now in the process of ligating the signal peptide onto the HA structural genes. These contstructs will again be sequenced to verify their integrity. If all is well then they will be cut out and ligated into a 35S plant expression cassette and then finally cloned into an Agrobacterium binary vector used for plant transformation.

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

Transformation will be performed by the plant transformation facility on the UC Davis campus. Expression of HAs in transgenic plants will be assessed by RT-PCR and ELISA/Western blot analysis using anti-HA antibodies. Lines with the highest HA expression will then be needle inoculated with *Xf* cells and the severity of disease in the transgenics will be compared with non-transgenic controls.

Publications:

T. M. VOEGEL 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

Voegel T. M. and Kirkpatrick B. C. (2007). Isolation, Characterization and Genetic Manipulation of *Xylella fastidiosa* Hemagglutinin Genes. Proceedings, 2007 Pierce's DiseaseResearch Symposium. California Department of Food an Agriculture, Sacramento, CA

Voegel TM, Warren JG, Greve CL and Kirkpatrick BC (2008). Genetic and Biochemical Characterization of *Xylella fastidiosa* Hemagglutinin Adhesins. Biochemical and Biophysical Research Communications (To be submitted 9/4/08)

Research relevance:

Xf cell-cell attachment is an important virulence determinate in Pierce's disease. Our previous research showed that if 2 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 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 (Almeida, 2007) has also shown the importance of HAs in binding various substrates. The recent 2007 science advisory panel that reviewed the potential of various avenues of PD research, also identified HA and other attachment proteins as high priority areas for further research. The knowledge we gained here about the basic biology of *Xf* HA proteins will provide the foundation for completing the last step of this project, where we will transform tobacco and grapevine plants with HA genes. These genes will be expressed in the xylem of transgenic plants and hopefully act as a 'molecular glue' to retard systemic movement of inoculated *Xf* cells through the grapevine xylem. If successful, this approach might provide a novel form of resistance against Pierce's disease.

Summary in lay terms:

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 (Almeida 2007) has also shown that HA proteins play important roles in attachment processes. 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 of the native protein to the 220 kD proteins 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 downstream of the N-terminal 2300aa and that approximately one third of the C-terminal part is cleaved off. We also identified another *Xf* gene PD1933 that is responsible for directing the HAs in the outer membrane and secreting HAs into the medium (Voegel and Kirkpatrick, 2006).

Summary and status of intellectual property:

Although Professor Alan Bennet presented an excellent talk on the intellectual property issues associated with transgenic plants at the 2007 PD/GWSS Conference, we believe it is important to evaluate the efficacy of this transgenic approach to mitigating PD using the same vectors that Aguero et al., 2005 used in their work with PGIPs. 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.

References:

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Kuehn, M.J. and Kesty, N.C., 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes and Dev. 19:2645-2655

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Voegel T. M. and Kirkpatrick B. C. (2007). Isolation, Characterization and Genetic Manipulation of *Xylella fastidiosa* Hemagglutinin Genes. Proceedings, 2007 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA