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 October 2008 to March 2009

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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 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 (CDFA progress report March 2008; Proceedings, 2007 Pierce's Disease Research Symposium. CDFA, Sacramento, CA; manuscript submitted)

Objective 3.

To overcome problems that might occur trying to transforma 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 (Proceedings, 2007 Pierce's Disease Research Symposium. CDFA, Sacramento, CA; manuscript submitted). 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 containing 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 their sequence. The obtained sequences were aligned to 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 recently received our first AD1-3 transformed tobacco plant and we expect to receive more plants in the immediate future. We will check for insertion of the expression cassette into the plant genome and analyze the expression of the transgenes by rtPCR and Western blot analysis using the antibodies we generated in objective 2b. Seeds of positive plants will be produced and the F1 generation challenged with *Xf* to determine potential resistance against PD.

Unfortunately, transformation of grapevines takes considerably more time than tobacco and we expect to obtain transformed grapevines at the end of 2009.

Publications:

Voegel T. M. Kirkpatrick B. C. (2006). Isolation and Characterization and Genetic Manipulation of *Xylella fastidiosa* Hemagglutinin Genes. Proceedings, 2006 Pierce's Disease Research Symposium. California Department of Food an Agriculture, Sacramento, CA

Tanja M. Voegel and Bruce C. Kirkpatrick. 2006. Characterization of a putative Two-Partner-Secretion pathway protein in *Xylella fastidiosa*. *Phytopathology 96:S119*

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 Disease Research Symposium. California Department of Food an Agriculture, Sacramento, CA

Voegel T. M. and Kirkpatrick B. C. (2008). Isolation, Characterization and Genetic Manipulation of *Xylella fastidiosa* Hemagglutinin Genes. Proceedings, 2007 Pierce's Disease Research Symposium. California Department of Food an Agriculture, Sacramento, CA

Tanja M. Voegel and Bruce C. Kirkpatrick (2008). A novel approach for generating *Xylella fastidiosa* resistant grapevines. Proceedings of the 2nd annual national viticulture research conference, Davis, CA.

Voegel TM, Warren JG, and Kirkpatrick BC (2009). Genetic and Biochemical Characterization of *Xylella fastidiosa* Hemagglutinin Adhesins. (Submitted)

Voegel TM and Kirkpatrick BC (2009). Identification of a novel two-partner secretion system in *X. fastidiosa*. (In preparation)

Presentations on research:

Annual APS meeting, Quebec City, Canada, 2006
Pierce's disease research symposium, San Diego, 2006
Annual APS meeting, San Diego, 2007
Pierce's disease research symposium, San Diego, 2007
National viticulture research conference, Davis, 2008
Pierce's disease research symposium, San Diego, 2008
Annual APS meeting, Portland, Oregon

Research relevance:

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 clumps 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. 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 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 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 HA-expressing transgenic tobacco and grapevines. We also identified another *Xf* gene, PD1933, that is responsible for directing the HAs into the outer membrane and secreting HAs into the medium (Voegel and Kirkpatrick, 2006; manuscript in preparation).

Status of funds: To date, approximately 2/3 of the first year's funding has been spent.

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:

Aguero, C.B., Uratsu S.L.,Greve C., Powell A.L.T., Labavitch J.M., Meredith C.P. and Dandekar A.M., 2005. Evaluation of tolerance to Pierce's disease and Boytrytis in transgenic plants of *Vitis vinifera* expressing the pear PGIP gene. Molecular Plant Pathology 6: 43-51.

Francis, M., E. Civerolo and Bruening G., 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* Cultivar SR1. Plant Disease 92:14-20.

Guilhabert, M.R. and Kirpatrick B.C., 2005. Identification of *Xylella fastidiosa* avirulence genes: hemagglutinin adhesions contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. Molecular Plant Microbe Interactions 18:856-868.

Killiny, N and Almeida R.P.P.,2009. *Xylella fastidiosa* Afimbrial Adhesins Mediate Cell Transmission to Plants by Leafhopper Vectors. Applied and Environmental Microbiology 75:521-528.

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