

ISOLATION, CHARACTERIZATION, AND GENETIC MANIPULATION OF *XYLELLA FASTIDIOSA* HEMAGGLUTININ GENES

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ABSTRACT

Xylella fastidiosa (*Xf*) possesses genes for hemagglutinins (HAs), large adhesion proteins involved in cell-cell aggregation and biofilm formation. Mutations in either one of the functional HAs, Hx/A (PD2118) or Hx/B (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. We generated antibodies against portions of the HA proteins, used them in Western blot analyses and showed that HA proteins are secreted into the supernatant as soluble proteins, associated with membrane vesicles, and inserted into the outer membrane of *Xf*. Native HA proteins are processed from a predicted size of 360 kD to 220 kD. We identified two N-terminal portions of the HA proteins that will be expressed in transgenic tobacco and grapevines where we hope the protein will act as a “molecular glue” to aggregate insect-inoculated *Xf* cells, retard their ability to systemically colonize plants and potentially provide a unique form of resistance against PD.

INTRODUCTION

Xylella fastidiosa (*Xf*) HAs are large secreted proteins that play important roles in mediating cell-cell aggregation and plant pathogenicity. Mutations were made in both *Xf* HA genes, Hx/A (PD2118) and Hx/B (PD1792), by transposon mutagenesis. The resulting mutants did not aggregate in liquid culture and they had reduced biofilm formation *in vitro* and *in planta* (1). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (1). The premise of this research is to determine if by expressing *Xf* HA adhesion domains in the xylem of transformed grapevines, the HA can act as a “molecular glue” to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce’s disease (PD).

Because of the large size of the HA genes (10 kb), it is difficult to transform grapevines with the whole HA gene. Therefore we have been trying to identify the active adhesion domains (ADs) responsible for cell-cell aggregation by dividing the HA genes into several smaller fragments that should contain the cell-cell AD. Recombinant proteins derived from these fragments were expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera were used in ELISA, Western blot analysis, immunolocalization studies and cell-cell clumping experiments to determine which of the HA fragment(s) contain functional ADs that could be transformed into plants.

OBJECTIVES

- 1a. 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 Hx/A and B specific-antibodies. Determine if Hx/A and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.
- 2a. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of Hx/A and B.
 - b. Prepare rabbit polyclonal antibodies against each Hx/A/B domain fusion protein. Use antibodies to determine native size and location of *Xf* HAs in cultured cells.
 - c. Determine if antibodies against various Hx/A/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.

- 3a.** Transform Thompson seedless grapevines and 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 and grapevine xylem fluid.
- b.** Mechanically inoculate HA-transgenic grapevines and tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in transgenic plants with non-protected controls.

RESULTS

Objectives 1a-d, 2a

The results of these objectives have been reported in the Proceedings, 2007 Pierce's Disease Research Symposium, CDFA, Sacramento, CA. Because of the low quality of the AD2-antibodies, we repeated Objectives 1a-d with high quality antibodies that were generated against AD1-3 and AD4 in Objective 2a (2).

Objective 2b

Determination of native size and location of *Xf*HAs in cultured cells.

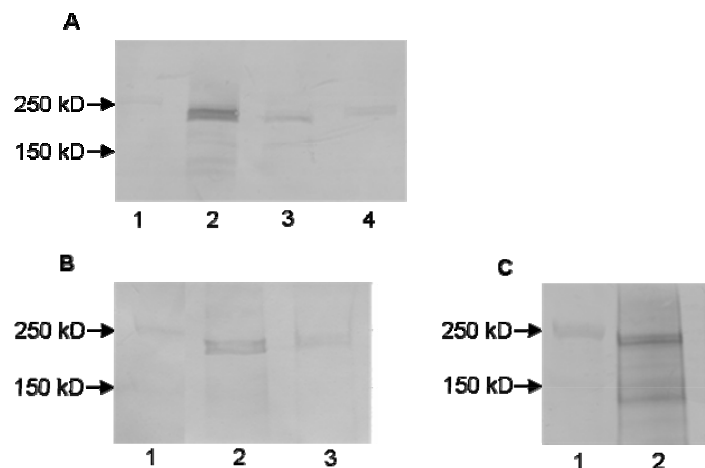


Figure 1 A. Western blot analysis of soluble proteins extracted *Xf* media supernatant and probed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula; lane 3: HxfA-, lane 4: HxfB-. A doublet of bands in the wild type Temecula is detectable. The larger protein band corresponding to HxfA is missing in the HxfA- mutant, the slightly smaller protein band corresponding to HxfB is missing in the HxfB- mutant strain.

Figure 1 B. Western blot analysis of *Xf* vesicle preparation developed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula vesicle preparation; lane 3: TCA-precipitated supernatant after isolation of vesicles, proteins represent soluble HA proteins. **C:** Western blot analysis of an *Xf* outer membrane protein preparation. Lane 1: Molecular weight standards; Lane 2: outer membrane preparation of wild type Temecula.

The antibodies that were raised against AD1-3 and AD4 of the HA proteins (2), were used in Western blot analysis of isolated *Xf* soluble, secreted proteins (**Figure 1A**), HA proteins associated with secreted vesicles (**Figure 1B**) and HAs present in the outer membrane (**Figure 1C**); HA proteins were detected in all three fractions.

A doublet of proteins at approximately 220 kD that corresponds to HxfA and HxfB was observed in wild type supernatant (**Figure 1A**, lane 2), and only one protein band was detected in the HxfA- and HxfB- mutant supernatant samples (**Figure 1A**, lanes 3 and 4). The large protein corresponding to HxfA is missing in the HxfA- mutant, the slightly smaller protein band corresponding to HxfB is missing in the HxfB- mutant strain (**Figure 1A**).

To distinguish between secreted soluble HA proteins, and HA proteins that are associated with membrane vesicles, the vesicles were isolated by centrifugation and proteins remaining in the supernatant were precipitated using trichloroacetic acid (TCA). Western blot analysis of the precipitate confirmed the presence of HA proteins as secreted soluble proteins (**Figure 1B**). Interestingly, 220 kD proteins in the vesicle fraction were also detected by the anti-AD4 antibody (**Figure 1B**). Vesicles that are released from the envelope of growing bacteria may contain virulence factors in many Gram-negative bacteria (3).

Western blot analysis of isolated outer membrane proteins of *Xf* wild type Temecula also revealed a doublet of bands at 220 kD corresponding to both HA proteins (**Figure 1C**). To ensure that the outer membrane were indeed being isolated, Western blots were also probed with anti-MopB antibodies (kindly provided by the Bruening lab). MopB is the major outer membrane protein in *Xf* (4), and a 38.5 kD protein corresponding to MopB was detected in the membrane fraction thus confirming that *Xf*HAs are inserted into the outer membrane (data not shown).

The size of the mature protein detected by Western blot analysis (220 kD) was smaller than the predicted size based on the amino acid sequence of the protein (360 kD). To identify the processing site of the mature HA proteins, we isolated secreted

HA proteins by size exclusion chromatography and subjected the native HA proteins to LC MS/MS mass spectrometry (Genome Center Proteomics Core, University of California, Davis). Identified peptides were associated only with the N-terminal portion of the HA protein, suggesting that the C-terminal portion is cleaved off in the mature protein and does not play a role in cell-cell aggregation(**Figure 2**).

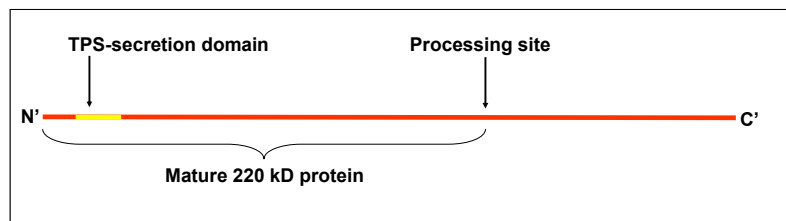


Figure 2. Secreted HA proteins were isolated by size exclusion chromatography and analyzed by LC MS/MS to estimate the location of processing site to produce the mature 220kD protein.

Objectives 2c

The results of these objectives were reported in the 2007 Pierce's Disease Research Symposium Proceedings.

Objectives 3

AD1-3 and the entire processed 220kD HA protein (protein220) will be used for transformation of tobacco SR-1 and Thompson seedless grapevines. These constructs were both cloned into the vector pCR-2.1 resulting in pCR2.1-AD1-3 and pCR2.1-220. Several clones of both constructs were sequenced to confirm the integrity of the cloned genes. We obtained suitable clones for both pCR-2.1-AD1-3 and pCR-2.1-220 that can be used for plant transformation. We obtained the pGIP-signal sequence, which directs the secretion of proteins into the plant apoplast and xylem, fused to the N-terminal portion of AD1-3 from a biotech company (DNA2.0) in a plasmid called pJ202:21008. This construct can be used to insert the pGIP-signal sequence (5) in front of AD1-3 as well as for protein220 to direct the secretion of the expressed proteins outside the plant cell. This secretion signal sequence has been successfully inserted into pCR2.1-220 and we are in the process of putting the secretion signal on pCR-2.1-AD1-3. After successful fusion of the pGIP-signal peptide to the HA proteins, the constructs will be cloned into a *Agrobacterium* binary vector that is available in the Dandekar lab (5) and used to transform *Agrobacterium tumefaciens*. The *Agrobacterium* cultures will then be used by the UC Davis Plant Transformation facility to transform tobacco SR-1 and Thompson seedless grapevine with the two HA protein constructs. Xylem sap will be expressed from transgenic tobacco and grapevines and analyzed by ELISA and Western blots to determine if *Xf* HA proteins are present in the sap of transgenic plants. The transgenic plants will be mechanically and insect inoculated with *Xf* and the plants will be evaluated for the presence of *Xf* and the severity of PD symptoms.

CONCLUSION

Our data suggests that HA proteins are needed for efficient aggregation of *Xf* cells because *Xf* cells that have a mutation in either HxfA or HxfB lose the ability to aggregate and to form biofilms. Also, *Xf* cell cultures that were incubated with Fab fragments against AD1-3 and AD4 of HxfB were inhibited in their ability to aggregate (2). We showed that HA proteins are secreted and processed to a mature 220 kD protein and that contain N-terminal hemagglutination domains. Taken together, this suggests that the secreted N-terminal portion of the HA proteins is responsible for cell-cell aggregation and biofilm formation. We hope that free *Xf* HA protein in the plant xylem may mediate increased cell-cell aggregation of insect inoculated *Xf* cells and increase the agglutination of *Xf* cells in the plant xylem, thereby retarding the systemic colonization of grapevines and possibly providing a novel resistance to Pierce's disease.

REFERENCES CITED

- Guillabert, M.R. and Kirkpatrick, B.C. (2005). Identification of *Xylella fastidiosa* Antivirulence Genes: Hemagglutinin Adhesins Contribute to *Xf* Biofilm Maturation and Colonization and Attenuate Virulence. MPMI Vol.18, No.8, 856-868.
- Kirkpatrick, B. C. and Voegel, T. M. (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.
- Kuehn M.J. and Kesty N.C. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev. 19:2645-2655.
- Bruening, G., Civerolo E., Lee, Y., Buzayan, J., Feldstein, P., and Re, E (2005). A major outer membrane protein of *Xylella fastidiosa* induces chlorosis in *Chenopodium quinoa*. Phytopathology 95 S14-S14.
- Agüero C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., Dandekar, A.M. (2005). Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol Plant Pathol 6:43-51.

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