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, HxfA (PD2118) or HxfB (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. In previous work, we showed that *Xf* HA proteins are secreted into the supernatant as soluble proteins, inserted into the outer membrane, and associated with membrane vesicles. Furthermore, the HA proteins are secreted via a two-partner secretion pathway and processed from a predicted molecular weight of 360 kD to a 220 kD mature protein. Based on this information we generated HA-expressing transgenic *Nicotiana tabacum* SR-1 (Petite Havanah) tobacco and *Vitis vinifera* cv. Thompson seedless grapevines. We show that the T-DNA is successfully integrated into the tobacco genome and that HA mRNA is transcribed in the transformed plants. The T_2 tobacco generation is being evaluated for resistance against Pierce's disease (PD). We hope that the expressed HA protein will act as "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.

LAYPERSON SUMMARY
Hemagglutinin (HA) proteins play an important role in adhesion and biofilm formation of *Xylella fastidiosa* (*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 (1). 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 (2). Research conducted in our lab showed that HA proteins are present in the outer membrane of *Xf* cells and that these proteins are also secreted into culture medium and in vesicles at low concentrations. The 10.5 kb HA genes should theoretically encode a protein of approximately 360 kD, however we showed that the native size of the HA proteins in the outer membranes, culture supernatants and membrane of vesicles is approximately 220 kD (3). The cleavage site lies downstream of the N-terminal 2300aa and approximately one third of the C-terminal part is cleaved off (3). Based on this information, we created two different binary plasmids that we used for transformation of tobacco and grapevines. The first, smaller construct codes for the N-terminal hemagglutination domain that is proposed to mediate clumping of *Xf*. The second, larger construct codes for the mature 220 kD protein that we found to be secreted. Here, we show that the transformation was successful and that the tobacco plants express the HA gene. We are now evaluating the second transgenic tobacco generation for resistance against PD. We believe that the expressed HA proteins may mediate clumping of insect inoculated *Xf* cells. Therefore, the *Xf* infection may stay local at the point of introduction in form of cell clumps, and spreading of *Xf* cells through the plant can potentially be minimized. Regular pruning of grapevines in fall might eliminate the infections, leaving healthy plants behind.

INTRODUCTION
Xylella fastidiosa (*Xf*) hemagglutinins (HAs) are large secreted proteins that play important roles in mediating cell-cell aggregation and plant pathogenicity. Mutations made in either HA gene HxfA (PD2118) or HxfB (PD1792) resulted in strains that did not aggregate in liquid culture and 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 proteins in the xylem of transformed grapevines, the HAs can act as a "molecular glue" to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce's disease (PD). In our previous work we showed that *Xf* HA proteins contain an N-terminal hemagglutination domain that is putatively responsible for the aggregation of *Xf* cells (3). We also showed that the HA proteins are secreted into the outer membrane and into the supernatant, and that they are processed from a predicted 365 kD pre-protein to a mature 220 kD protein (3).

Here we describe the generation of transformed tobacco and grapevine plants by using two constructs for agrobacterium mediated plant transformation; firstly, plants were transformed to express the N-terminal portion of HxfB protein containing the hemagglutination domains, and secondly, plants were transformed to express the portion of HxfB corresponding to the mature 220 kD protein.

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 Hxf 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 HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.
3. a. 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 1 and 2

The results of these objectives have been reported in the Proceedings, 2008 Pierce's Disease Research Symposium. CDFA, Sacramento, CA.

Objective 3

Transformation of tobacco and grapevines

The 5' part of the HA gene coding for the hemagglutination domain (AD1-3), and the longer part coding for the 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 sequence 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. Upon verification 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 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 T0 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 with primers that bind to the HA gene was positive for 10 out of 11 tobacco plants for each construct. Untransformed wild type plants were used as negative control **(Figure 1).**

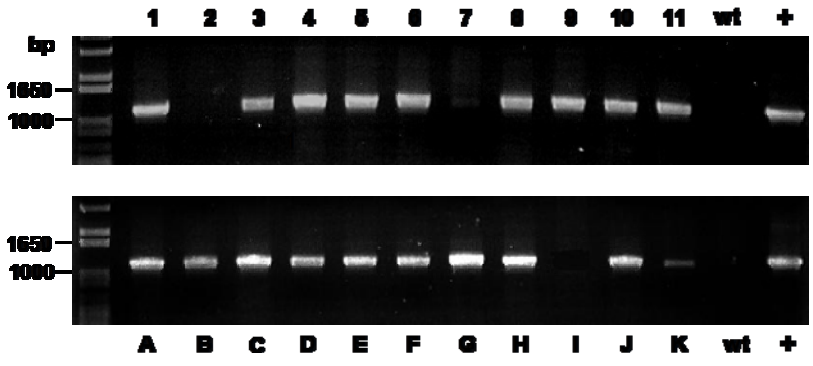

Figure 1: Confirmation of T-DNA insertion into the genome of tobacco SR-1 by PCR analysis of genomic tobacco DNA. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3, letters A-K indicate transgenic lines that were transformed with pCAMBIA-pGIP-220. Lines 2 and I do not have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

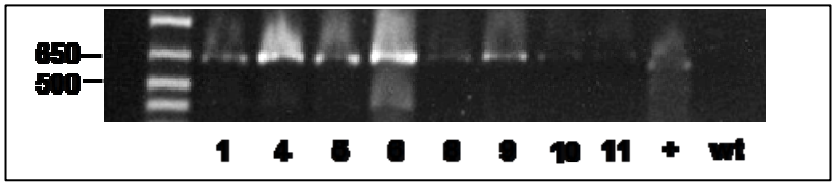

Figure 2. RT-PCR of transformed tobacco SR-1 using primer pair pGIP-HAfor and HArev. Numbers indicate transgenic lines that were transformed with pDU-pGIP-AD1-3. Wild type plants were used as negative controls and isolated plasmid pDU-pGIP-AD1-3 was used as positive control (+).

After three months, seed pods of the HA expressing T0 plants were harvested, the seeds sterilized and plated for germination of the T1-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 according to Mendel 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.

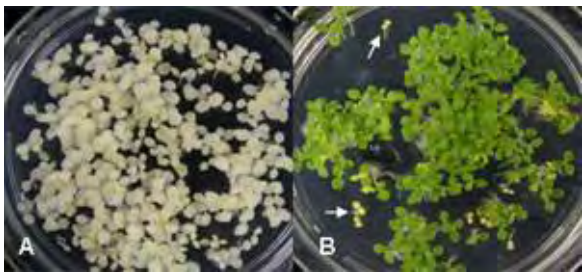

Figure 3. Tobacco seeds germinated on MSO medium supplemented with kanamycin. **A:** wild type SR-1 is not resistant. **B:** Transgenic line shows a 1:3 segregation pattern according to Mendel. Arrow indicate examples of the 25% azygous seedlings, the remaining 75% are homo- or heterozygous.

The germinated T1 generation plantlets were transferred into single pots and kept in a mist chamber (**Figure 4**). After 10 days, the plantlets were transferred to a greenhouse and grown for an additional three months until the production of T2 seeds (**Figure 5**). Plating and analysis of the germination pattern on selective medium will be repeated for the T2 seeds. T2 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 expect to obtain transformed grapevine plants in spring of 2010.

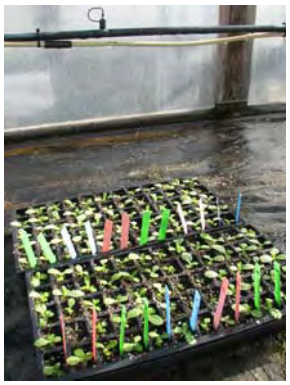

Figure 4. Germinated tobacco seedlings were transplanted from selective MSO plates to soil. Plantlets are kept in a mist chamber at the environmental horticulture facility (UC Davis, CA).

Figure 5. T_1 generation of HA-expressing tobacco. Plants are kept at the environmental horticulture facility (UC Davis, CA) until production of T_2 seeds.

CONCLUSIONS

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 (1). We showed that HA proteins are secreted and processed to a mature 220 kD protein and that they contain N-terminal hemagglutination domains (3). Taken together, this suggests that the secreted N-terminal portion of the HA proteins is responsible for cell-cell aggregation and biofilm formation. We transformed tobacco plants with the N-terminal portion of the HA proteins and could show that the genes are integrated in the tobacco genome and that HA mRNA is transcribed. We are in the process of generating T2 homozygous plants and will evaluate them for expression of HA proteins. 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 PD.

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