EXPLOITING XYLELLA FASTIDIOSA PROTEINS FOR PIERCE'S DISEASE CONTROL

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ABSTRACT

The principal objective of this project is to construct and express in test plants, and then in grapevine rootstock, a protein or protein chimera capable of inactivating *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine. Prior results from this project identified MopB as a, or possibly the, major outer membrane protein of *Xf*. We have shown that MopB is accessible on the *Xf* cell exterior and is a member of the OmpA family of outer membrane proteins of Gram-negative bacteria. The abundance of MopB in *Xf* cell extracts, known packing density of OmpA in a crystal, and *Xf* cell dimensions allowed us to estimate that MopB probably accounts for at least 10% of the *Xf* cell exterior. Thus, MopB is a highly suitable target for inactivation of *Xf* cells. Previous results suggested that some portion of the intact MopB gene from *Xf* is sickening to *E. coli*. Nevertheless, two *E. coli* strains were generated by substitution into the endogenous *OmpA* gene, one expressing mature MopB and the other a MopB-OmpA chimera with the amino-terminal half from MopB. Cells of both strains display MopB antigen on their surface, though accumulation is to a level much lower than MopB achieves in *Xf*. The strains are immune to bacteriophage K3, for which OmpA is the receptor. We modified and randomly mutated the OmpA-binding gp38 adhesin protein of bacteriophage K3 and will use *Xf* cells and the MopB-surface *E. coli* strains described above to select bacteriophage K3 variants that use MopB as the receptor. The selected gp38 gene will form the core of an anti-*Xf* protein. A readily transformed and regenerated tobacco line, SR-1, was identified as being susceptible to *Xf* and producing PD-like symptoms and cytology. SR-1 will be a used to test anti-*Xf* proteins and optimize constructions for grapevine transformation. High level expression of a fragment of MopB has been achieved and the same technology will be used to obtain sufficient quantities of MopB to complete its biological characterization.

INTRODUCTION

Resistant grapevine cultivars present the best approach to long term, effective, economical and sustainable control of Pierce's disease (PD). This project has developed data showing that the OmpA class protein MopB of *Xylella fastidiosa* (*Xf*) is a major outer membrane protein of the bacterium. The demonstrated accessibility of parts of the MopB molecule on the cell surface and its abundance identify MopB as a high priority potential target for inactivation of the *Xf* cell or interference with the *Xf* infection cycle. As background, results from our prior research are summarized below (Bruening et al. 2005):

a. *Xf* cells, fresh or heat-killed, when pressure-infiltrated into *Chenopodium quinoa* leaves, induce within two days chlorosis (chloroplast bleaching) that is limited to the infiltrated area of the leaf (CqC activity).
b. The CqC activity is protease sensitive and was associated with a gel electrophoresis band that was found, by mass spectrometry, to contain predominantly the putative (OmpA class) *Xf* outer membrane protein MopB.
c. The mature, 38.5K MopB protein was found to result from the release of a 22 amino acid leader peptide. The bulk of mature MopB molecules have a pyroglutaminyl amino end.
d. MopB was partially purified in soluble form using sodium dodecyl sulfate (SDS) solutions but reducing, at the last step, SDS to very low levels.
e. Application of anti-MopB antibody demonstrated that MopB is accessible on the *Xf* cell exterior and appears to be evenly distributed over the *Xf* cell surface.
f. MopB is an abundant protein of *Xf* and may be the major outer membrane protein of the bacterium.
g. *E. coli* did not tolerate plasmid constructions bearing the entire MopB gene from *Xf*. However, placing the MopB open reading frame under control of a bacteriophage-derived promoter allowed the production of low amounts of MopB in *E. coli.*
h. Both purified MopB and MopB still embedded in *Xf* cells showed a strong propensity to associate tightly with porous materials of a variety of chemical types. This result is reminiscent of the observed association of a *Pseudomonas fluorescens* OmpA protein, OprF, with root surfaces (De Mot and Vanderleyden 1991, Deflaun et al. 1994), MopB may be involved in the association of *Xf* cells with the interior of xylem elements in the inoculated plants.

Our principal objective is to construct and express in test plants, and then in grapevine rootstock, a MopB-binding protein (MBP) or protein chimera capable of inactivating *Xf*. We expect that a construction of suitable design will confer, on the

grapevine scion, resistance to *Xf*. If rootstock expression does not confer scion resistance to *Xf*, we will turn to transformation of the scion. To obtain a high affinity MBP, we are modifying a protein of a T2-like bacteriophage: the tail fiber adhesion gp38 (Riede et al. 1987). The gp38 protein of bacteriophages K3, M1 and OX2 recognizes and binds to *E. coli* OmpA, the receptor for bacteriophage infection. Based on the rapidity and irreversibility of bacteriophage association with *E. coli*, gp38 likely binds very tightly to OmpA. Bacteriophage mutants with "shifted allegiance" away from OmpA and to other *E. coli* surface proteins were selected using *E. coli* mutants with altered or missing OmpA. In one instance, the new receptor was a polysaccharide rather than an outer membrane protein (Drexler et al. 1991). The mutations controlling affinity for the new receptor mapped to four polypeptide loops of gp38 (Drexler et al. 1989). We believe a mutated gp38 could have a high affinity for MopB. OX2 apparently has been lost. M1 has been more readily adapted than has K3 to new receptors (Henning and Hashemolhosseini 1994). We obtained inocula of bacteriophage K3 and bacteriophage M1 from a former postdoctoral associate in the laboratory of Ulf Henning (deceased) in Germany.

The predominant conformation of a typical OmpA protein, as it resides in the outer membrane of *E. coli*, almost certainly has the polypeptide chain composed of amino acid residues 1-171 inserted into the outer membrane with 8 trans-membrane segments and four external loops (Pautsch and Schulz 1998, Singh et al. 2003). We have cast MopB into a similar conformation based on the crystallographic structure of OmpA (Pautsch and Schulz 1998) and computer predictions of folding for OmpA and MopB and have initiated research aimed at converting gp38 from a OmpA-binding protein to a MBP. That is, our initial aim is to select a version of gp38 that has been modified in its receptor-binding four loops to recognize and adhere tightly to the cell-external four loops of MopB.

OBJECTIVES

The goal of this project is to generate *Xf*-resistant grapevine rootstock and plants based on expression of a MBP.

Specific objectives:

1. Discover or develop low molecular weight MBPs with high affinity for portions of the MopB protein that are displayed on the *Xf* cell exterior.
2. Test MopB-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic grape expressing the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*.

RESULTS

Under Objective 1 (discover MBPs)

Expression of MopB on the E. coli cell surface

Obtaining *E. coli* cells that express MopB sequences and display MopB surface polypeptide loops, as is characteristic of MopB in *Xf*, is central to our selection procedure for MBPs. As was reported in the previous period, we created an *E. coli* strain that was designed to display MopB sequences on the cell exterior but to otherwise be compatible with expression in *E. coli*. Using a gene-replacement approach, a recombinational event replaced the amino terminal region, residues 1-171, of the chromosomal *OmpA* gene of *E. coli* with the corresponding region of MopB. This construction retains the OmpA signal peptide and the OmpA carboxyl half of the molecule, which includes the trans-periplasmic space sequences and the sequence that is inserted into the peptidoglycan layer. The replacement was confirmed by sequence analysis after PCR amplification of the chimeric gene region from chromosomal DNA. A similar approach has now produced an OmpA replacement which was designed to generate the entire mature MopB molecule. Cells of the new *E. coli* strains were found to be entirely resistant to bacteriophage K3 and to be agglutinated by beads displaying anti-MopB IgG, as expected. Immunoblots were prepared after SDS-PAGE of *E. coli* hot-SDS cell extracts for the two strains. Results (not shown) revealed the accumulation of MopB-like proteins of the expected mobility. However, the MopB-immunoreactive material from the *E. coli* strains amounted to no more than a few percent of the signal observed for similar amounts (total protein) of *Xf* cells. Expression of mature MopB and MopB-OmpA chimera proteins appears to be below the level of OmpA accumulation in wildtype *E. coli*. We suspect that there is a codon usage problem for the synthesis of MopB and MopB-OmpA chimera in *E. coli* and are taking steps to introduce the cognate tRNAs.

Modification of gp38 for adhesion to MopB.

We attempted to find bacteriophage K3 variants capable of infecting MopB-OmpA chimera-bearing *E. coli* cells. The cells were exposed to 10^{10} plaque forming units of untreated bacteriophage K3 and to bacteriophage K3 populations that had been treated with the mutagen hydroxylamine or that had been increased in cells exposed to the *in vivo* mutagen 2-aminopurine. No infecting K3 variant was found. Henning and Hashemolhosseini (1994) report that bacteriophage M1 is more suited than K3 to adaptation to receptors other than OmpA. We obtained bacteriophage M1 inoculum, but found it to behave similarly to K3 in our tests. PCR amplification of a gp38 sequence from the "M1" DNA revealed the sequence of K3 gp38, suggesting that bacteriophage M1, like bacteriophage OX2, may no longer be available. We are in the process of creating a library of mutated gp38 sequences based on the published M1 gp38 sequence. These will be introduced into bacterophage K3 to create

a library of mutants for selection of MopB-binding gp38. Selection will use *Xf* cells as well as the MopB-surface *E. coli* strains.

MopB and the CqC assay

Cell suspensions from the chimeric MopB-OmpA and mature MopB *E. coli* strains were pressure infiltrated into *C. quinoa* leaves. Both cell suspensions, as well as wildtype *E. coli* cell suspensions, behaved similarly. A CqC reaction was observed at the higher cell suspension densities, and the reaction was similar for all three suspensions. That is, *E. coli* appears to have an endogenous CqC-like activity. Given the demonstrated low accumulation of MopB-OmpA chimeric protein and MopB in the *E. coli* strains, CqC activity from the expressed proteins, if any, most likely was overshadowed by the endogenous CqC-like activity of *E. coli*.

We are preparing constructions for expression of intact MopB and specific MopB fragments using the high level expression (Dubendorff and Studier 1991) pET160 plasmid system. The first to be completed produced the carboxyl half of MopB, as indicated in Figure 1. The CqC assay of the purified protein preparation (analyzed at Figure 1, lane 5) failed to induce chlorosis when infiltrated into *C. quinoa* leaves, whereas control preparations of *Xf* cells induced the usual CqC reaction, suggesting that CqC activity does not reside in the carboxyl half of MopB alone.

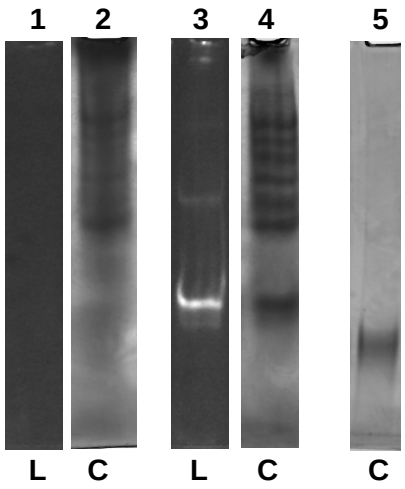

Figure 1. Production of microgram amounts of the carboxyl half of *Xf* outer membrane protein MopB in transformed *E. coli*. Sequence encoding the carboxyl half of the mature MopB protein was inserted into a pET160 plasmid vector on the 3'-side of tetracysteine- and hexahistidine-encoding sequences and under control of a bacteriophage T7 promoter and *lac* operator sequence. The construction was transformed into an *E. coli* strain that lacks a bacteriophage T7 RNA polymerase gene for characterization and propagation of the clone. This approach is intended to prevent even basal level expression of the insert sequences. For high level expression, purified plasmid is transformed into BL21 Star *E. coli* cells, which encode T7 RNA polymerase under control of a *lacUV5* promoter. Cultures were grown up directly without isolation of individual colonies and were exposed (lanes 3-5) or not exposed (lanes 1, 2) to the gratuitous inducer IPTG. Protein extracts were incubated with the tetracysteine-binding fluorescent lumio reagent prior to electrophoresis through 12% polyacrylamide in SDS. Detection was by lumio fluorescence (lanes 1 and 3, L) or by staining of the gel with coomassie brilliant blue (lanes 2, 4, and 5, C). Lanes 1-4 are from one gel. Material for lane 5 was purified from lane 3 material by nickel column chromatography in urea solution.

Under Objective 4 (transgenic plant expression of anti-Xf protein)

Test bed for analysis of constructions designed to express anti-Xf protein

To facilitate our goal of creating grape rootstock that can confer resistance to *Xf* on its grafted scion, we developed a plant model system for rapid transformation with anti-*Xf* constructions and rapid testing for phenotype (Francis et al. 2005), compared to grapevine. Constructions discovered to have promising anti-*Xf* activity will be used to transform a grapevine rootstock line. We have demonstrated that tobacco (*Nicotiana tabacum*) line SR-1, which is routinely transformed and regenerated at the UC Davis College of Agricultural and Environmental Sciences Plant Transformation Facility, is readily infected by needle inoculation into the petiole axil or stem. *Xf* was recovered from the petiole above inoculation points, whereas no bacteria were recovered from water-inoculated controls. Symptoms developed (Figure 2A) and *Xf* accumulated, as indicated by ELISA, quantitative PCR, and clogging of xylem vesicles (Figure 2B), providing unequivocal evidence of infection. Others have succeeded in infecting N. tabacum strains with *Xf* (Lopes et al. 2000, Alves et al. 2003). The symptoms we observe appear to be more dramatic than those reported. *Xf* isolated from SR-1 tobacco caused typical PD symptoms following artificial inoculation to grapevines (Figure 2C).

CONCLUSIONS

The goal of this project is to create genes encoding anti-*Xf* proteins for transformation of grape rootstock and protection of the grafted scion against PD. *E. coli* strains were created that display on the cell exterior portions of a *Xf* major outer membrane protein, MopB. These strains are expected to be suitable hosts for a bacteriophage that will accept a displayed portion of MopB as a receptor. A synthetic bacteriophage gp38 adhesin gene has been randomly mutated and will be incorporated into a population of bacteriophage K3 to produce a library from which bacteriophage strains that use MopB as a receptor will be selected. The selected gp38 gene will form the core of an anti-*Xf* protein. High level expression of a fragment of MopB has been achieved and will be applied to full length MopB to complete its biological characterization. A

readily transformed and regenerated tobacco line, SR-1, has been identified as a suitable platform for testing and optimizing anti-*Xf* protein gene constructions.

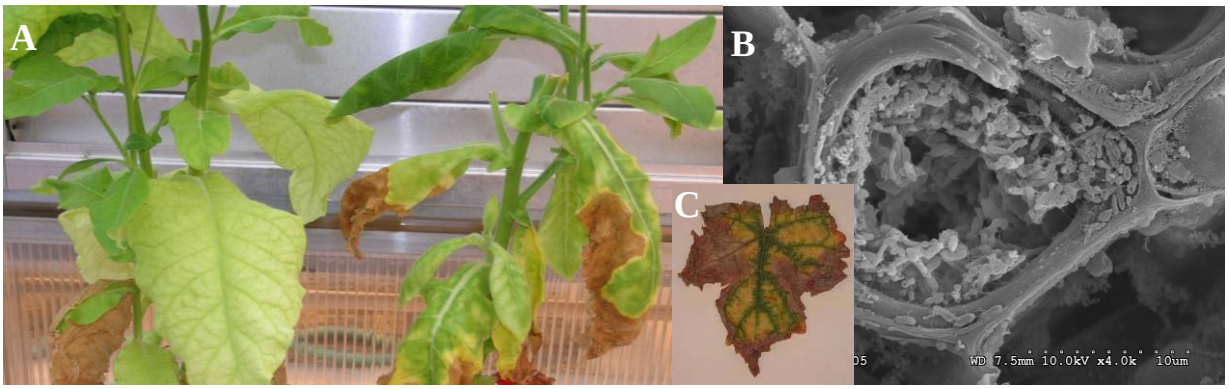

Figure 2. SR-1 tobacco as a host for *Xf*. A. Water-infiltrated (left) and *Xf*-inoculated plants 3 months after inoculation at the 6 leaf stage. Although leaves of control plants developed senescence, none developed the downward curvature, cupping and tip- and margin-necrosis with chlorotic halo that are characteristic of the *Xf*-inoculated plants. 4/4 leaves from two control plants were negative for *Xf* by ELISA and PCR. Extracts of 7/7 leaves from three *Xf*-inoculated plants generated ELISA signals averaging 4x the control level; quantitative PCR signals exceeded the threshold product accumulation at 19-31 cycles. B. Electron microscopy of SR-1 petiole sections at 10-12 nodes above the inoculated leaf revealing bacterial cells occluding a xylem element. C. Sap from Temecula-1 *Xf*-inoculated, symptomatic SR-1 tobacco was inoculated to grapevine cuttings, resulting in typical PD symptoms and accumulation of *Xf*.

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