DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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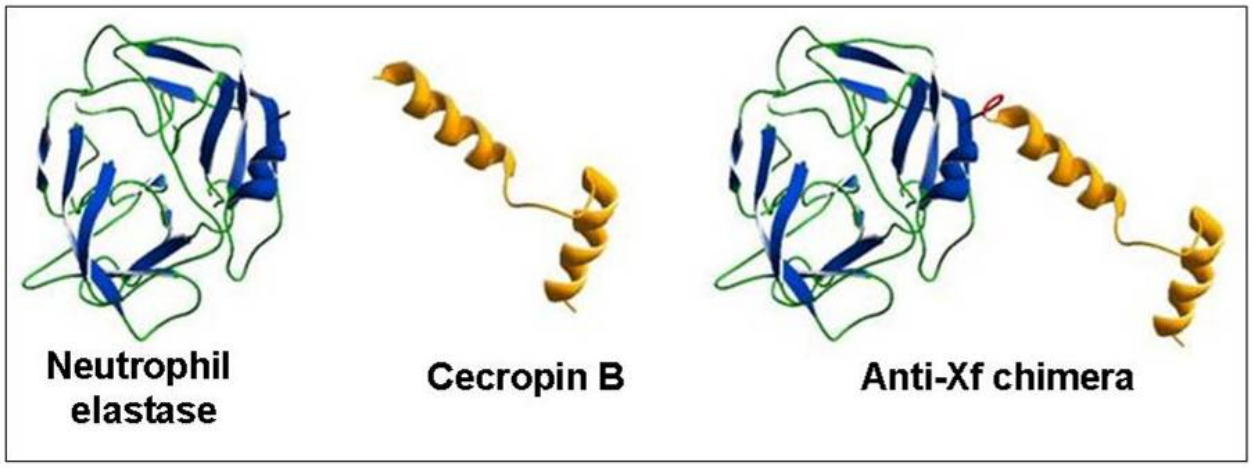
ABSTRACT
Xylella fastidiosa (*Xf*) is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (1). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, human neutrophil Elastase (5-10) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (11). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have combined Elastase and CecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized and cloned into different vectors for insect and plant transformation. Five transformed insect cell lines are being evaluated and production and processing of the protein is being optimized in in liter size preps. Plant transformation experiments have been completed and we have obtained plants of *Nicotiana tabacum* var *benthamiana* and plants of *Vitis vinifera* 'Thompson Seedless' transformed with this gene that are currently being analyzed for gene expression and protein production. The proteins obtained from the transgenic insect and plant cell lines will be used to test for antimicrobial activity against *Xf*.



INTRODUCTION
Globally, one-fifth of potential crop yields is lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf)* is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (http://danr.ucop.edu/news/speeches). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the

availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

OBJECTIVES

Objective 1:

a) Utilize literature data and computer modeling to identify an SRD that specifically targets MopB (Elastase)

b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)

c) *In vitro* testing of anti-*Xylella* activity of the MopB-specific SRD (Elastase) and Xyllela-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.

Objective 2:

a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein.

b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.

Objective 3:

a) Expression in transgenic plants

b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

RESULTS

Following our successful accomplishment of Objectives 1a, b & c in the first year of our project, where functional activity of Elastase (SRD for MopB) and Cecropin B (defensin) components were tested individually, we designed a chimeric protein of Cecropin B and HNE (Objective 2a). The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

The HNE-Cecropin B chimera gene was synthesized and cloned into pBacPAK8 baculovirus vector. The chimeric gene inserted into pBacPAK8 was co-transfected with BacPAK6 viral DNA into Sf21 cells. Recombinant viruses formed by homologous recombination were amplified, and the protein expression was optimized in High Five cells (Invitrogen, Carlsbad, CA), derived from *Trichoplusia ni* egg cell homogenates. High Five cells have been shown to be capable of expressing significantly higher levels of secreted recombinant proteins compared to Sf9 and Sf21 insect cells. Optimal conditions for the expression have been worked out in HighFive cells; suspension cells in logarithmic growth are infected with recombinant *Xf* chimera baculovirus, with a multiplicity of infection of 10, and grown for 72 hours. About 25-50% of the expressed chimeric protein is secreted into the supernatant and is detected on a Western Blot as a single band. The supernatant is collected, concentrated and dialyzed. Concentrated supernatant is then run on a weak cation exchange column, chimeric protein containing fractions are pooled and dialyzed, and the dialyzed fractions are run on an elastin affinity column. All chromatography steps are carried out by gravity flow. Chimeric protein containing fractions are pooled and dialyzed and tested for elastase activity. By these methods, we are able to purify ~250 □g active protein from 50mL supernatant. These conditions are being scaled up to produce the amounts required for testing against *Xylella fastidiosa* (currently purifying liter size preps).

We have also cloned the chimera into a plant vector (Figure 1) that was electroporated into disarmed *Agrobacterium tumefaciens* strain EHA 105 creating a functional plant transformation system that has been used to transform pre-embryogenic callus of *Vitis vinifera* 'Thompson Seedless' and the rootstock 'Freedom'.

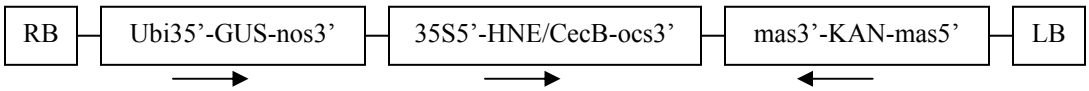


Figure 1. Schematic representation of binary plasmid pDU04.6105

We have obtained more than 40 seedlings of 'Thompson Seedless' from independent lines and expect that, based in our experience with grape transformation, the majority of them will develop into normal plants. Those plants will be micropropagated and acclimated in the greenhouse and analyzed for gene expression, PD tolerance and graft transmissibility.

In addition, the same experiments have been performed using a second construct in which the coding sequence of the signal peptide of HNE was replaced with that of the pear polygalacturonase inhibiting protein (pPGIP). The aminoacid sequence of this chimeric gene product is shown in Figure 2. Our hypothesis is that the pPGIP signal peptide will direct/improve the secretion of the chimeric protein and, as a consequence, increase its concentration in the xylem. This hypothesis in based in previous results that have shown that the product of the pPGIP encoding gene, heterologously expressed in transgenic

grapevines, is present in xylem exudates and moves through the graft union (14). Leaf discs of *Nicotiana tabaccum* 'benthamiana' and 'RT1' have also been transformed with HNE-Cecropin and pPGIP-HNE-Cecropin B genes. The plants obtained are currently being analyzed for gene expression.

*MELKFSTFLSLTLLFSSVLNPALS***IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAV RVVLGAHNLSRREPTRQVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQLPAQGRRLGNGVQCL AMGWGLLGRNRGIASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCA SGLYPDAFAPVAQFVNWIDSIIQ**GSTA*KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL***

Figure 2. pPGIP-HNE-Cecropin B chimeric amino acid sequence. The signal peptide of HNE (MTLGRRLACLFLACVLPALLLGGTALASE*)* has been replaced with the predicted signal peptide of pPGIP (italics) which has been fused to the N-terminal of the mature HNE (bold). HNE is attached to Cecropin B (bold italics) by the GSTA linker, which is underlined.

CONCLUSIONS

The main objective of this project is to develop a potent therapy against *Xf* by utilizing the principles of innate immunity by which plants recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. We have developed a chimeric anti-microbial protein containing two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the *Xyllela* outer-membrane whereas the other will lyse the membrane and kill *Xyllela*. In this chimera, elastase is the SRD that recognizes mopB, the major outer membrane protein of *Xf*. The second domain is cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (elastase and cecropin B) display activity against *Xf*, which is synergistic when both proteins are combined. We have tested the protease activity of elastase against the purified mopB and intact *Xf* cells to demonstrate that the *Xylella* protein is degraded and therefore, a target for elastase. We have successfully combined the elastase and cecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene has been synthesized, cloned into a pBacPAK8 baculovirus vector, and packaged into recombinant baculovirus in Sf21 insect cells. Optimization of chimeric protein production is ongoing. We have also transformed pre-embryogenic callus of *V.vinifera* L. 'Chardonnay' and 'Thompson Seedless' and the rootstock 'Freedom'. Transgenic callus will be cultured in bioreactors designed to optimize protein production by secretion into the medium. We plan to use this system as well as the insect bioreactors to validate the anti-*Xylella* properties of the chimeric protein. Transgenic plants will be obtained from transgenic callus cultured in germination medium. After acclimation in the greenhouse, they will be inoculated with *Xf* and tested for PD tolerance/resistance.

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