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 (Pieters, 2001). 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 have developed a novel protein-based therapy that circumvents the shortcomings of traditional antibiotics. We have designed a chimeric anti-microbial protein with two functional domains (Figure 1). 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 (HNE; 5-10) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (Bruening et al., 2002). The second domain is cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have combined HNE 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 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 being generated for the analysis of 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*.

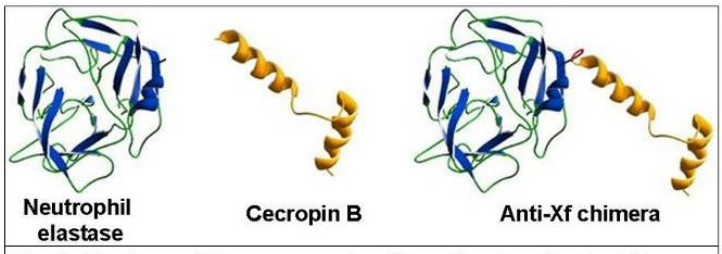


Fig. 1: Structures of the components of the chimeric anti-microbial proteins for the rapid clearance of *Xylella*.

INTRODUCTION

Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf)* is a devastating bacterial pathogen that causes Pierce's disease (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, are developing 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 (Pieters, 2001; Baquero and Blazquez, 1997). 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 immediate and long term impacts.

OBJECTIVES

Objective 1

a. Utilize literature data and computer modeling to identify an SRD that specifically targets MopB (HNE)
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 (HNE) and *Xyllela*-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of HNE and Cecropin B.

Objective 2:

a. Design and construction of synthetic gene encoding HNE-Linker-Cecropin B Chimeric protein.
b. Expression HNE-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.

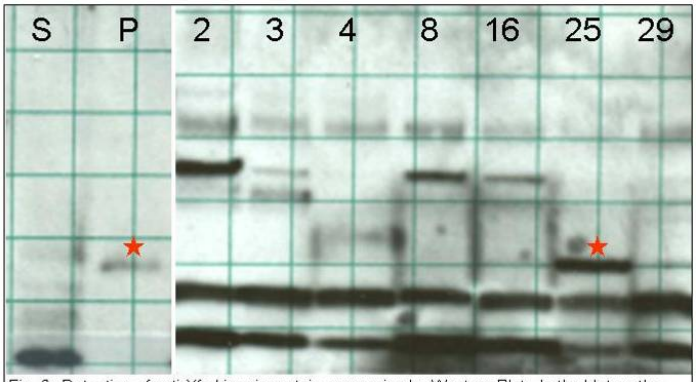


Fig. 2: Detection of anti-Xf chimeric protein expression by Western Blot. In the blot on the left, the first lane contains protein extracted from High Five cell pellet, 72 hours after infection with recombinant anti-Xf baculovirus. The second lane contains protein from the supernatant of that infection. In the blot on the right, 7 samples of callus transformed with anti-Xf chimera were probed for recombinant protein expression. Lanes containing recombinant protein of expected size (~28 kDa) are marked with a red star. The primary antibody used for detection is rabbit derived anti-cecropin B, and the secondary antibody is a peroxidase conjugated goat derived anti-rabbit IgG.

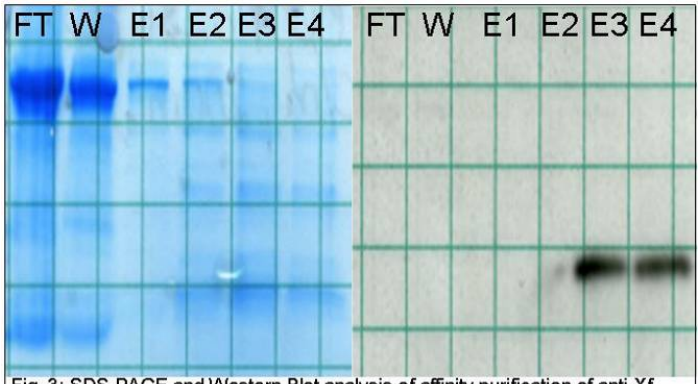


Fig. 3: SDS-PAGE and Western Blot analysis of affinity purification of anti-Xf chimeric protein. Fractions from batch chromatography on elastin-agarose were run on a denaturing gel (on left). FT = flow through, W = wash, E = elution. Recombinant anti-Xf protein is detected in elution fractions 3 and 4 only, by Western Blot (on right).

RESULTS

Following our successful accomplishment of Objectives 1a, b and c in the first year of our project, where functional activity of HNE (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 High Five 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 (Figure 2). The supernatant is collected, concentrated and dialyzed. Concentrated supernatant is then run on an elastin affinity column, chimeric protein containing fractions are pooled and dialyzed, and the dialyzed fractions are run on a weak cation exchange column (Figure 3). 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 µg active protein from 50mL supernatant. These conditions are being scaled up to produce the amounts required for testing against *Xf* (currently purifying liter size preps).

Currently recombinant anti-*Xf* protein is being purified and then it will be quantitated by UV spectroscopy, flash frozen in 50 mM Na-Acetate pH 5, 0.1M NaCl, 50% glycerol and shipped from LANL to UC Davis and the ARS for testing in *Xylella* cultures. We have also cloned the chimera into a plant vector (Fig. 4) 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' successfully.

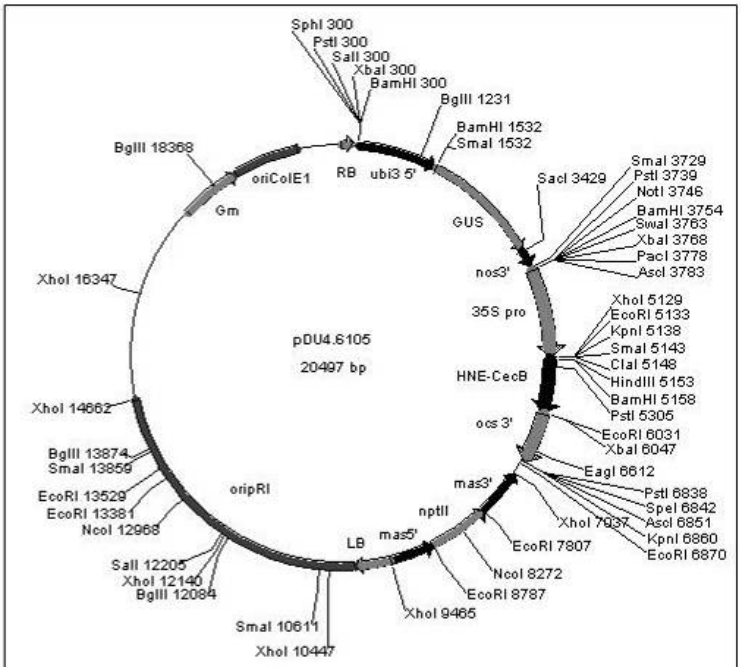
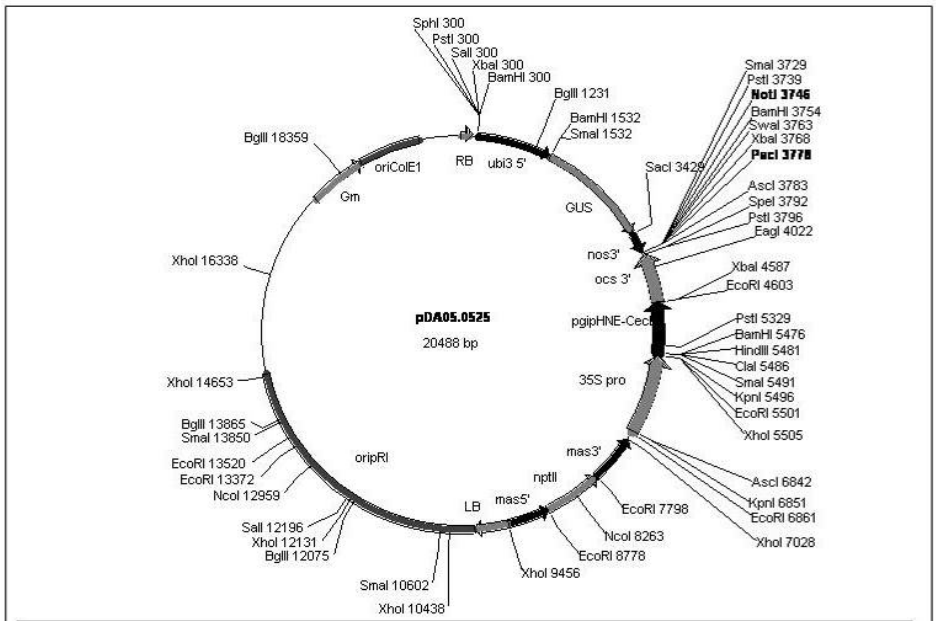


Fig. 4: Schematic representation of binary plasmid pDU04.6105

We have obtained transformed callus of 'Thompson Seedless (TS)' and have regenerated ~49 independent lines of TS. These are being propagated and transferred to the greenhouse. Transformation of the rootstocks 'St George' and '110' will be initiated. The binary vector has also been used to transform *Nicotiana tabaccum* 'benthamiana' and 13 independent transformed lines were selected, these were placed on callus inducing media to induce callus formation. These callai will be evaluated for HNE-CecropinB production. This construct will also be transformed into *Nicotiana tabaccum* 'SR1' to obtain plants. At the moment we have selected 18 shootlines and these will be rooted and then transferred to the greenhouse for seed production. Seedlings will then be tested for sensitivity to *Xf* inoculation and disease resistance.

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 amino acid sequence of this chimeric gene product is shown in Figure 5. 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 (Aguero et al., 2005).



*MELKFSTFLSLTLLFSSVLNPALS***IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHC VANVNVRAVRVVLGAHNLSRREPTRQVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQ LPAQGRRLGNGVQCLAMGWGLLGRNRGIASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFG DSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAPVAQFVNWIDSIIQ**GSTA*KWKVFKKIEKMGRNI RNGIVKAGPAIAVLGEAKAL***

Fig. 5: pPGIP-HNE-Cecropin B chimeric amino acid sequence (see above) and a schematic representation of a binary plasmid pDA05.0525 that contains the above sequence (see above). 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.

We have obtained transformed callus of 'Thompson Seedless (TS)' with the pPGIP-HNE-Cecropin B gene and have regenerated ~27 independent shoot lines of TS. These are being propagated and then will be transferred to the greenhouse. The pPGIP-HNE-Cecropin B binary vector has also been used to transform *Nicotiana tabaccum* 'benthamiana' and this transformation that was recently carried out is still in progress. This construct was also transformed into *Nicotiana tabaccum* 'SR1' to obtain plants. At the moment we have selected 29 shoot lines and these will be rooted and then transferred to the greenhouse for seed production. Seedlings will then be tested for sensitivity to *Xf* inoculation and disease resistance.

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 *Xylella* outer-membrane whereas the other will lyse the membrane and kill *Xylella*. 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 successfully made two vector constructs for expression in plants. Plant transformation experiments are ongoing in grapevine and tobacco to test chimeric protein production in plants. With the first construct transgenic *V.vinifera* L. 'Thompson Seedless' shoots have been obtained. Additional transformations of grapevine scion and rootstocks are in progress to obtain transgenic plants. Once obtained the transgenic plants will undergo acclimation in the greenhouse and then they will be inoculated with *Xf* and tested for PD tolerance/resistance.

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RESPONSE OF UNGRAFTED GRAPE ROOTSTOCKS TO *XYLELLA FASTIDIOSA* AT A PIERCE'S DISEASE SITE IN TEXAS.

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ABSTRACT

There were highly significant differences in year 2 among 12 commonly used grape rootstocks in TX for Pierce's disease (PD) symptoms and *Xylella fastidiosa*-serology. The rootstocks Salt Creek, Dog Ridge, and Champanel were the most PD resistant and 1616C, Freedom, and Harmony were most susceptible. Growers in areas at high risk for PD should consider PD reactions with other traits when selecting rootstocks for specific vineyard sites. There were no correlations in year two between rootstock vigor and either PD symptoms or serology results.

INTRODUCTION

Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa* (*Xf*), is the most limiting factor for growing grapes in much of TX and other U.S. gulf-coast states. Multiple management strategies are needed to improve PD control, including genetic resistance of scions and rootstocks, site selection, vegetation management, and vector management.

OBJECTIVES

1. Evaluate *Xf* reactions among commonly planted grape rootstocks in TX at a vineyard with a history of PD.

RESULTS

This report summarizes results midway through year two of a planned three-year rootstock study. Planting was initiated in 2005 in Llano County, TX at a site where two previous plantings of *V. vinifera* cultivars succumbed to PD. Planting continued as plants became available and to replace plants lost to transplant shock and PD (Table 1). Entries were 5BB, 5C, 110R, 1103P, 1613C, 1616C, Champanel, Dog Ridge, Freedom, Harmony, Salt Creek and SO4. There were five plants per plot and five replications. Leaves with PD symptoms in cv. Black Spanish (Le Noir) border rows consistently tested positive for *Xf* with ELISA and *Xf* was isolated. We anticipate collecting data on symptoms and ELISA reactions through 2007.

There were significant differences among entries for PD leaf scorch, proportion of plants positive with *Xf*-serology, mean optical density from *Xf*-ELISA, and plant vigor (Table 2). Harmony and 5C were least vigorous. SO4, 1613C, 1103P, 1616C and Salt Creek were most vigorous. PD symptoms were most prominent in 1616C, Freedom, and Harmony, but lowest in Salt Creek, Dog Ridge, Champanel and 110R. The proportion of plants positive for *Xf* with ELISA and the mean ELISA optical density were greatest for 1616C, Harmony, Freedom and SO4. Proportion of plants positive for *Xf* with ELISA and the mean ELISA optical density were lowest for Champanel, Salt Creek, and Dog Ridge. There were significant correlations between PD leaf scorch symptoms and both serology parameters (Table 3).

CONCLUSIONS

The rootstocks Salt Creek, Dog Ridge, and Champanel apparently have lower risk for PD than 1616C, Freedom, and Harmony. Growers in areas at high risk for PD should consider these PD reactions when selecting rootstocks for specific vineyard sites.

No rootstock entry in this trial was completely free of PD at mid-season of the second year of this study but there were highly significant differences among entries for symptoms and *Xf* serology. Serology (ELISA) is a general indicator of the concentration of *Xf* cells in grape tissue, and acquisition of *Xf* by vector insects is more efficient during feeding on plants with high populations (Hill and Purcell, 1997). The interaction of rootstock, scion and *Xf* has not been carefully studied, but our hypothesis is that vine mortality can be delayed or reduced if both scion and rootstock are not highly susceptible.

Genetic resistance in commercially-grown *Vitis* genotypes is useful in PD management in southern and southeastern U.S. (Hopkins and Thompson, 1984). Several rootstock cultivars derived from crosses with native *Vitis* species apparently have