Interim Progress Report for CDFA Agreement Number 12-0130-SA.

Title of Project:
Building a next generation chimeric antimicrobial protein to provide rootstock-mediated resistance to Pierce's Disease in grapevines

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Time period:
8 months (06/30/2012 to 03/15/2013)

Introduction
We demonstrated that the synergistic combination of two innate immune functions, 1) pathogen surface recognition and 2) pathogen lysis, combined in a single protein, provide a robust class of antimicrobial therapeutic (Dandekar et al., 2012). In support of this idea, we have demonstrated that expression of a chimeric anti-microbial protein (CAP) that links two bioactive protein domains one from human neutrophil elastase (NE; surface recognition domain; SRD) and Cecropin B (CB; lytic domain) linked by a flexible linker provides resistance to Pierce's Disease in grapevine (Dandekar et al., 2012). Transgenic grapevine lines expressing the NE-CB chimeric protein show very reduced or no PD symptoms: less xylem blockage and leaf scorching. Currently, some of these lines are bring field tested in two locations, one with natural PD pressure due to an abundant supply of Glassy Winged Sharp Shooters (GWSS) and one without GWSS but field inoculated with *Xf* bacteria. In this proposal, we seek to swap the human NE domain with an equivalent protein from a plant source and confirm whether it functions as effectively as the human protein. This addresses concerns about expressing a human protein in grapevines. Our second goal is to swap the CB lytic domain with a protein from grapevine that has similar activity.

List of objectives
The goal of this project is to redesign our existing therapeutic NE-CB CAP, replacing the human NE with a plant ortholog (PE), and validate the efficacy the new CAP to provide resistance to the transmission of Pierce's disease in grapevines.

Goal: Redesign the NE-CB chimeric antimicrobial with a plant elastase and test its efficacy in transgenic grapevines to combat *in planta Xf* movement and insect (GWSS) transmission of Pierce's Disease.

Objective 1: Redesign the chimeric antimicrobial protein by substituting a plant counterpart (PE – Plant Elastase) for the human neutrophil elastase (NE) component and demonstrate its efficacy for bacterial clearance.

Activity 1: Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the 'CLASP' computational tool.

Activity 2: Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.

Objective 2: Compare the efficacy of PE-CB with NE-CB in plants with *Xf* challenge.

Activity 3: Construct binary vectors and transform grapevine and tobacco.

Activity 4: Test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's Disease symptoms.

Objective 3: Compare the efficacy of PE-CB and NE-CB in *Xf* vector colonization and disease transmission.

Activity 5: Evaluate colonization of GWSS and transmission of *Xf* exposed to NE-CB in culture and in transgenic grapevines.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Objective 1: Redesign the chimeric antimicrobial protein by substituting a plant counterpart (PE – Plant Elastase) for the human neutrophil elastase (NE) component and demonstrate its efficacy for bacterial clearance.

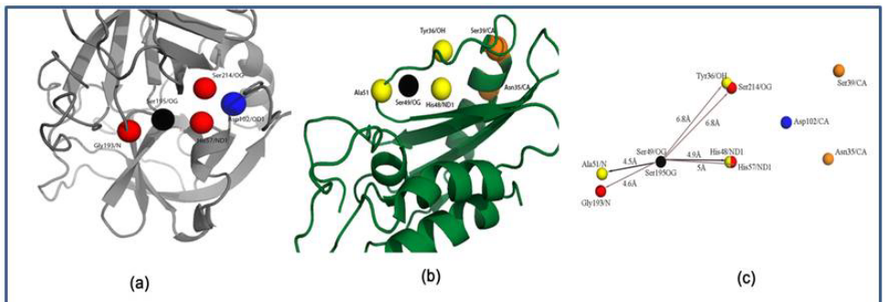

Figure 1: (a) Human neutrophil elastase (NE) (PDBid:1B0F) with active site atoms - Ser195/OG, His57/ND1, Ser214/OG, Gly193/N. (b) P14A protein (PDBid:1CFE) with predicted active site residues - Ser49/OG, His48/ND1, Tyr36/OH, Ala51/N. (c) Distance between pairs of residues in the partial matches. It can be seen that the Asp102 in PDBid:1B0F is close to Asn35 and Ser39 in PDBid:1CFE.

Since the CAP components work synergistically we will replace them one at a time maintaining the other original component. In this time period we focused our efforts in replacing the human neutrophil elastase (NE) with a plant/grapevine version of NE (PE). In order to do this we need to discover an appropriate protein in plants and possibly more appropriately in grapevine that has the same activity as NE.

Activity 1: Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the 'CLASP' computational tool.

This activity has been successfully accomplished in 2012-2013. A plant elastase (PE) candidate protein was identified from grapevine that has a similar active site configuration as NE. Working with Sandeep Chakraborty and B.J Rao in the Biosciences Department at the Tata Institute of Fundamental Research in Mumbai (India), who have utilized the computational tool CLASP (CataLytic Active Site Prediction) that they developed and used like BLAST to search among known proteins for a particular active site conformation (Chakraborty et al., 2011). For NE, the search for the precise active site motif was created from the human NE protein PDBid:1B0F (Fig 1 a). The active site residues comprised of the following residues - Ser195, His57, Asp102, Ser214 and Gly193 (Chakraborty et al., 2013). Our preliminary results yielded a significant match in a member of the PR-14A group of pathogenesis-related proteins in *Solanum lycopersicum* (tomato) (Fig 1 b and c). The CLASP analysis yielded a list of significant matches. We initially chose the pathogenesis-related protein P14A (PDB: 1CFE), a protease associated with the pathogenesis related proteins (Milne et al., 2003). Furthermore, there was a striking structural homology shared between P14A and a protein found in snake venom which has been demonstrated to be an elastase (Bernick and Simpson 1976). Since P14A is a protein from tomato, we decided to look for similar pathogenesis-related proteins in grape. Extensive BLAST searches resulted in a list of grape proteins with sequence similarity to P14A, including a pathogenesis-related protein (*Vs*P14A) from a wild variety of grape which is resistant to Pierce's disease. We then analyzed the active sites of *Vs*P14A using CLASP and observed that *Vs*P14A has a very similar structure and active site configuration as NE (Fig 2).

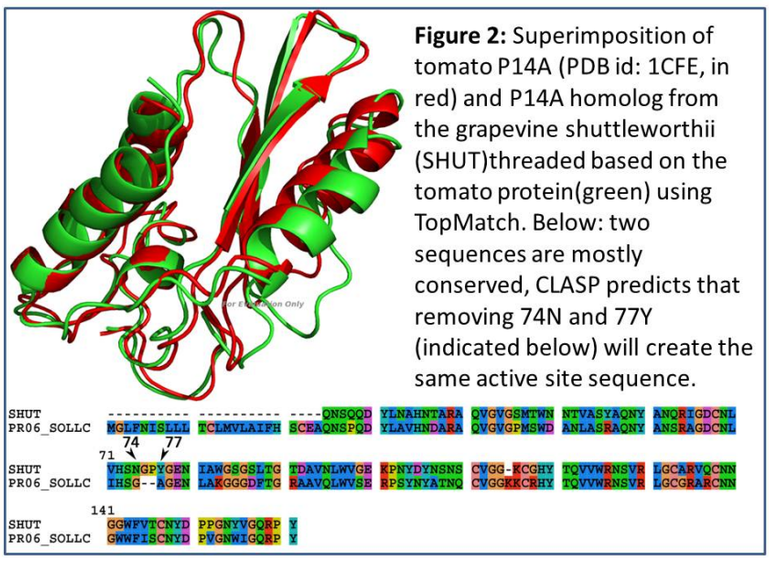

Figure 2: Superimposition of tomato P14A (PDB id: 1CFE, in red) and P14A homolog from the grapevine shuttleworthii (SHUT)threaded based on the tomato protein(green) using TopMatch. Below: two sequences are mostly conserved, CLASP predicts that removing 74N and 77Y (indicated below) will create the same active site sequence.

Activity 2: Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.

In order to test the efficacy of *Vs*P14A and *Vs*P14m proteins in clearing Xf we have codon optimized and chemically synthesized the two versions of VsP14 after addition of 3xFlag purification tag (Sigma Aldrich). VsP14A-3xFlag and VsP14m-3xFlag genes are cloned into pEAQ-HT a binary vector specific for transient protein expression system in the tobacco *N.benthamiana* (Sainsbury et al., 2009) which we have extensive experience using in our lab (Fig 3A). The constructed binary vectors were transformed into a super-virulent *Agrobacterium* strain (EHA105). As a negative control, an empty vector (pEAQ-HT) is also transformed in the same strain of *Agrobacterium*. Tobacco leaves were harvested from greenhouse-grown plants and vacuum-infiltrated with *Agrobacterium* suspensions containing *Vs*P14A and the empty vector. Agro-infiltration conditions were also optimized as described earlier (Huang et al., 2009). The infiltrated leaves were harvested after 6 days post infiltration and total protein was extracted using an apoplastic wash method to extract the secreted proteins present in the leaf apoplast.

Using Anti-Flag M2 antibody (Sigma) we were able to detect *Vs*P14A (Fig 3B). This was our first run with this protein. We tested the ability of the crude protein extract from leaves that we were able to detect the protein as shown in Fig 3A to inhibit the growth of *E.coli*. Our preliminary analysis of diluting an overnight culture of *E.coli* and adding a crude extract obtained from leaves infiltrated with the empty vector (Fig 3C) or infiltrated with the vector expressing VsP14A (Fig 3D). After 4 hours of growth the *E.coli* that contain the extract from the empty vector show a turbid growth while the *E.coli* with VsP14A are completely inhibited and did not grow after 4 hrs. These results are encouraging and indicate that we may have our replacement for NE, now we need to test it as a chimeric construct with CB and then find a replacement for CB as outlined in the below objectives and in our proposal.

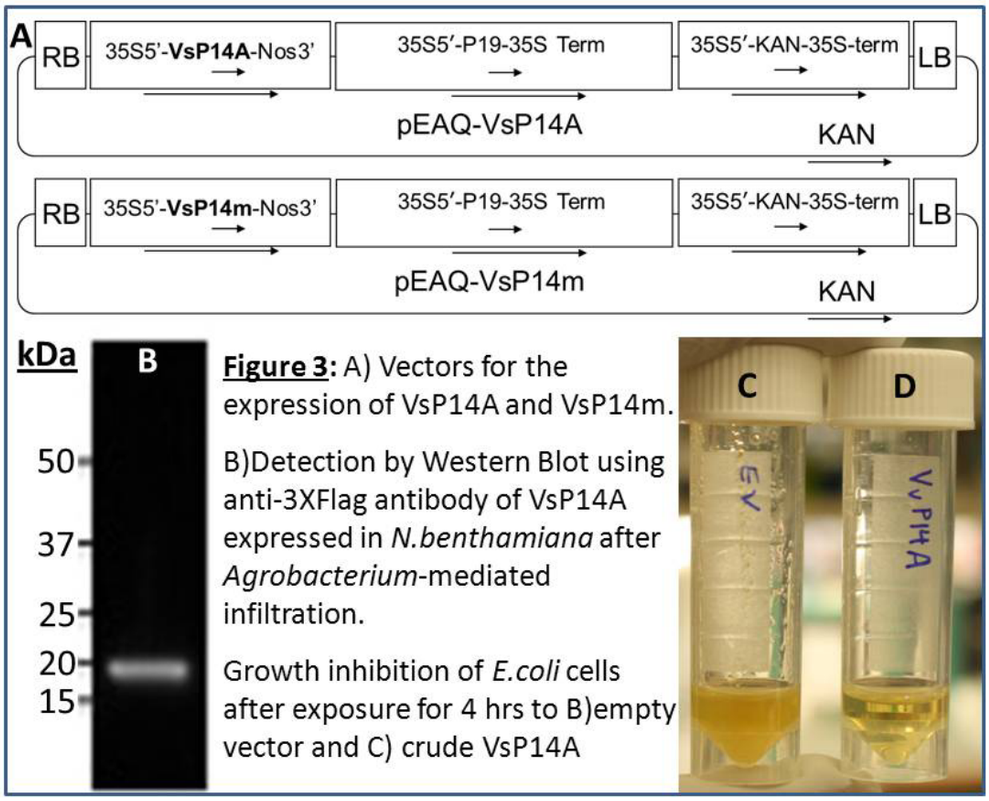

Figure 3: A) Vectors for the expression of VsP14A and VsP14m.

B)Detection by Western Blot using anti-3XFlag antibody of VsP14A expressed in *N.benthamiana* after *Agrobacterium*-mediated infiltration.

Growth inhibition of *E.coli* cells after exposure for 4 hrs to B)empty vector and C) crude VsP14A

Objective 2: Compare the efficacy of PE-CB with NE-CB in plants with *Xf* challenge.

We expect to address this objective in Year 2 of the project.

Activity 3: Construct binary vectors and transform grapevine and tobacco.

Activity 4: Test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's Disease symptoms.

Objective 3: Compare the efficacy of PE-CB and NE-CB in *Xf* vector colonization and disease transmission.

We expect to address this objective in year 2 and 3 of this project.

Activity 5: Evaluate colonization of GWSS and transmission of *Xf* exposed to NE-CB in culture and in transgenic grapevines.

Publications produced and pending, and presentations made that relate to the funded project.

Chakraborty S., R. Minda, L. Salaye, A.M. Dandekar, S.K. Bhattacharjee and B.J. Rao. 2013. Promiscuity-based enzyme selection for rational directed evolution experiments. In, J. Samuelson (ed.), "Enzyme Engineering: Methods and Protocols". Pub: Springer New York. Methods in Molecular Biology. 978: 74-78.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

Xylella fastidiosa (Xf), the causative agent of Pierce's Disease, has a complex lifestyle requiring colonization of plant and insect. Its growth and developmental stages include virulence responses that stimulate its movement *in planta* and its ability to cause disease in grapevines (Chatterjee et al., 2008). Thus, any control or resistance measure must by necessity be multifaceted to block this pathogen at different stages in its complex lifestyle. A key issue for the industry is the reservoir of bacterial inoculum already present in California that poses an immediate threat in the presence of a significant insect vector like the GWSS. Chemical pesticides are now used to suppress the GWSS population, which is effective but does not reduce this reservoir of bacterial inoculum. Resistance mechanisms must be directed to degrade this inoculum and prevent the further disease spread. It is critical to know whether any resistance mechanism under consideration can clear *Xf* and if so, by what mechanism. The resistance mechanism must limit spread and movement of the bacterium *in planta* and block transmission of the disease by insect vectors. We have previously shown that *Xf* exposed to xylem fluid from resistant lines expressing NE-CB shows significant mortality (Dandekar et al., 2012). Transgenic grapevine lines expressing the NE-CB chimeric protein show very reduced or no PD symptoms: less xylem blockage and leaf scorching. Currently, some of these lines are bring field tested in two locations, one with natural PD pressure due to an abundant supply of Glassy Winged Sharp Shooters (GWSS) and one without GWSS but field inoculated with *Xf* bacteria. In this proposal, we seek to swap the human NE domain with an equivalent protein from a plant source and confirm whether it functions as effectively as the human protein. This addresses concerns about expressing a human protein in grapevines.

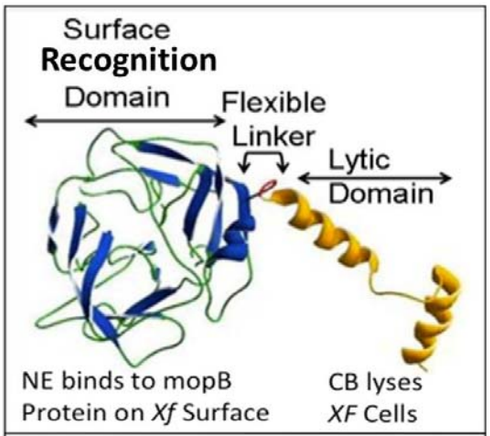

Fig. 4: The NE-CB Protein

Layperson summary of project accomplishments

We have successfully investigated the replacement of the surface recognition domain (SRD) of our NE-CB chimeric antimicrobial protein with a protein from grapevine. We used the recently described computational tool (CLASP, Chakraborty et al., 2011) to identify the VsP14A protein from grapevine based on its structural and conformational similarity of the active site's 3D (3 dimentional) signature of human neutrophil elastase (NE domain). In order to verify the biological activities of this protein we have constructed a gene cassette to express and produce this VsP14A protein in plants. This step has also been accomplished and we have been able to make this protein in tobacco leaf extracts. Initial testing of this protein was to evaluate for its ability to inhibit the growth of *E.coli* a gamma-proteobacteria as this could be determined quickly as E.coli grows much faster than Xylella.We found that addition of the VsP14A protein to the media inhibits the growth of E.coli. The next step is to test the ability of this protein to inhibit the growth or to clear Xylella cultures. We will then construct and test CAP constructs where the NE

component has been swapped with VsP14A while keeping the CB component constant to make sure that the new component is performing as anticipated.

Status of funds.
Sixty five percent of the funds have been expended the remainder will be expended in the remaining four months of our performance period.

Summary and status of intellectual property associated with the project
We have not made any intellectual property disclosures to date.

Literature cited

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