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:
3 months (03/15/2013 to 06/30/2013)

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 and insect CB domains with plant/grapevine orthologs, and to validate the efficacy the new CAP components in providing resistance to Pierce's Disease in grapevines. We are now

following the goals, objectives and activities as proposed in the revised proposal submitted earlier this year (2013) and now approved for 2 years.

Goal: Redesign the NE-CB chimeric antimicrobial with a plant elastase and plant-derived lytic domain and test its efficacy to combat Pierce's Disease in transgenic tobacco and grapevines.

Objective 1: Redesign the chimeric antimicrobial protein by substituting a plant counterpart (plant elastase or PE) 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.

Activity 3: *In planta* efficacy testing: construct binary vectors (PE-CB), transform grapevine and tobacco, and test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's Disease symptoms.

Objective 2: Redesign the chimeric antimicrobial protein by substituting a plant/grapevine counterpart (plant lytic domain or PLD) for the insect-derived Cecropin B (CB) component in the lytic domain and demonstrate its efficacy for bacterial clearance.

Activity 4: Identify a suitable PLD candidate that is comparable to insect-derived Cecropin B in primary and secondary structure using CLASP and other computational tools.

Activity 5: Test synthetic PLD protein for efficacy in killing *Xf* in culture.

Activity 6: *In planta* testing of the efficacy of grape-derived CAP components using transient expression of *Vs*P14-LPD and *Vs*P14m-LPD.

Objective 3: Construct and test a fully plant-derived CAP (PE-PLD) and test its ability to confer resistance to Pierce's Disease in grapevine rootstocks.

Activity 7: Construct a PE-PLD binary vector, transform grapevine and tobacco, and evaluate *Xf* resistance and Pierce's Disease development.

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 (plant elastase or PE) for the human neutrophil elastase (NE) component and demonstrate its efficacy for bacterial clearance.

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.

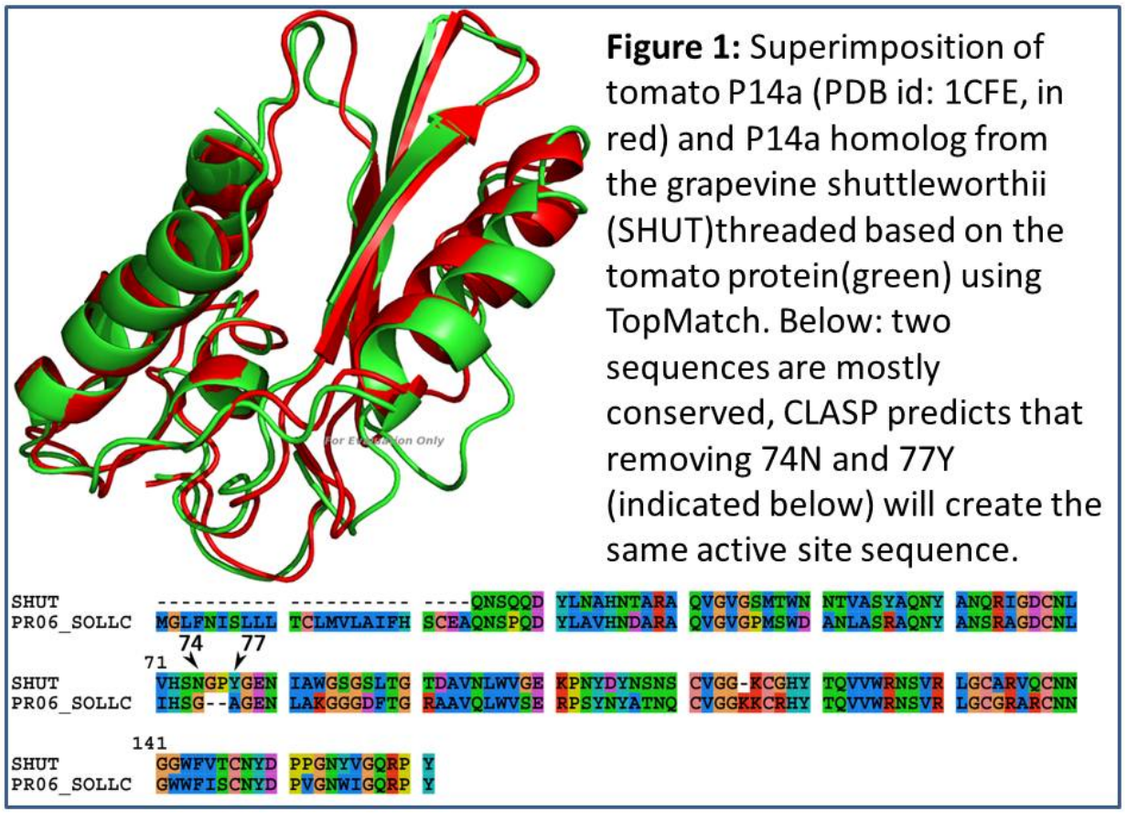
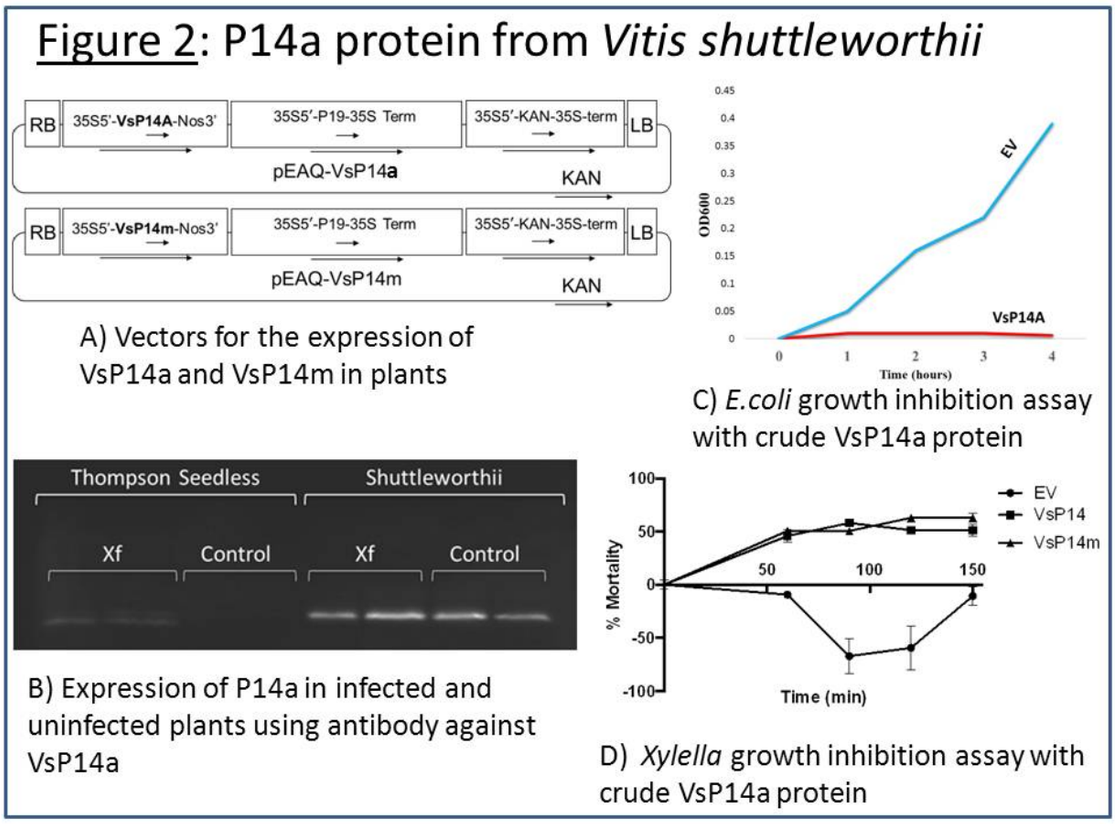


Figure 1: Superimposition of tomato P14a (PDB id: 1CFE, in red) and P14a homolog from the grapevine shuttleworthii (SHUT)threaded based on the tomato protein(green) using TopMatch. Below: two sequences are mostly conserved, CLASP predicts that removing 74N and 77Y (indicated below) will create the same active site sequence.

This activity has been successfully accomplished in our last report. A plant elastase (PE) candidate protein was identified from grapevine that has a similar but not identical 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. 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). 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 1).

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

To test the efficacy of *Vs*P14a and *Vs*P14m proteins in clearing *Xf,* we codon-optimized and chemically synthesized the two versions of VsP14a after addition of a 3xFlag purification tag (Sigma Aldrich). VsP14a-3xFlag and VsP14m-3xFlag genes were cloned into pEAQ-HT, a binary vector specific for transient protein expression system in the tobacco species *N. benthamiana* (Sainsbury et al., 2009), a system with which we have experience (Fig 2A). The constructed binary vectors were transformed into a super-virulent *Agrobacterium* strain (EHA105). As a negative control, an empty vector (pEAQ-HT) was also transformed into 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 optimized as described earlier (Huang et al., 2009). The infiltrated leaves were harvested six days post-infiltration and total protein was extracted using an apoplastic wash method to extract secreted proteins present in the leaf apoplast. Using Anti-Flag M2 antibody (Sigma), we detected *Vs*P14a on the first run. We tested the ability of the crude leaf protein extract from plants transformed with both constructs to inhibit growth of *E.coli* (Fig 2C) and Xylella (Fig 2D). After four hours, the *E.coli* with the extract from the plant expressing the empty vector showed growth while growth of those exposed to VsP14A was completely inhibited (Fig 2C). In the case of Xylella both proteins VsP14a and VsP14m inhibited the growth whereas protein obtained from the empty vector did not, by 50 mins 50% of the population was killed, however the mortality does plateau out around 60% mortality (Fig 2D). These results are encouraging and indicate that we may have our replacement for NE, but it must first be tested *in planta* to protect against Xf infection. We also developed antibodies against VsP14a and used these to detect the protein in plants using western blots. Proteins isolated from Thompson seedless and *Vitis shuttleworthii* infected and not infected with Xylella showed that the P14a in shuttleworthii was expressed in both infected and uninfected tissues but in TS it was only expressed in infected tissues un-infected tissues expressed no protein corresponding to P14a (Fig 2B).



Activity 3: *In planta* efficacy testing: construct binary vectors (PE-CB), transform grapevine and tobacco, and test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's Disease symptoms.

We will be initiating construction of binary vectors for the expression of VsP14a shortly. We are in the process of doing the vector design.

Objective 2: Redesign the chimeric antimicrobial protein by substituting a plant/grapevine counterpart (plant lytic domain or PLD) for the insect-derived Cecropin B (CB) component in the lytic domain and demonstrate its efficacy for bacterial clearance.

Activity 4: Identify a suitable PLD candidate that is comparable to insect-derived Cecropin B in primary and secondary structure using CLASP and other computational tools.

We are currently using a similar approach to Activity 1 to identify a replacement component PE for NE. Our focus here is to identify a plant/grapevine version of the Cecropin B lytic domain. We chose the structural motif Lys10, Lys11, Lys16, and Lys29 from Cecropin B (PDBid:2IGR) (Fig 3). Our preliminary analysis has identified some good candidates. It is noteworthy that a few defense-like proteins feature in the list, allowing us to speculate that another peptide might be a good replacement for Cecropin B in the chimera. We are currently evaluating the 8 protein candidates listed below in Fig 4. We are focusing of the 52 aa segment of the plasma membrane ATPase and should have this identified by the next report.

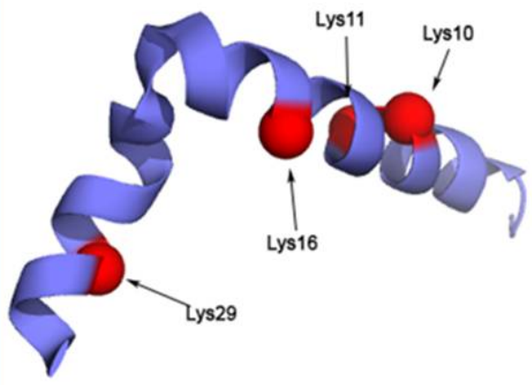


Figure 3: CecropinB structure (CB; PDBid: 2IGR) showing chosen motif (Lys10, Lys11, Lys16 and Lys29). The table below indicate the best matches in a set of approximately 200 proteins identified when queried using the motif (Lys10, Lys11, Lys16 and Lys29) from cecropinB (PDBid:2IGR).

PDB	Chain	Length	Description
1GPT	A	47	GAMMA-1-H THIONIN
2KGQ	A	53	Brazzein
1N4N	A	47	floral defensin-like protein 1
2O98	P	52	Plasma membrane H+ ATPase
1MR4	A	47	Nicotiana alata plant defensin 1 (NaD1)
1KRL	A	44	MONELLIN
2DS2	A	33	Sweet protein mabinlin-2 chain A
2HUG	A	57	Signal recognition particle 43 kDa protein

Activity 5: Test synthetic PLD protein for efficacy in killing *Xf* in culture.

Activity 6: *In planta* testing of the efficacy of grape-derived CAP components using transient expression of *Vs*P14-LPD and *Vs*P14m-LPD.

Objective 3: Construct and test a fully plant-derived CAP (PE-PLD) and test its ability to confer resistance to Pierce's Disease in grapevine rootstocks.

Activity 7: Construct a PE-PLD binary vector, transform grapevine and tobacco, and evaluate *Xf* resistance and Pierce's Disease development.

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.

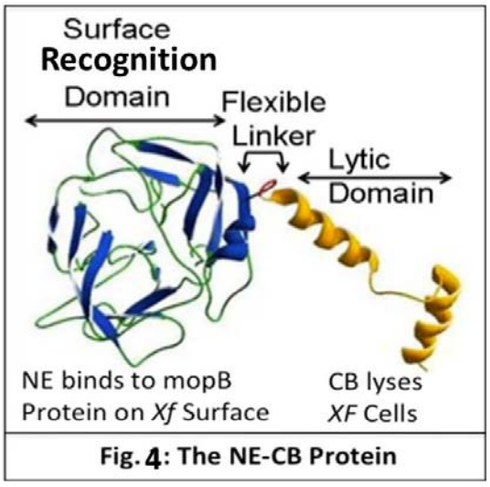


Fig. 4: The NE-CB Protein

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. Using the recently described computational tool (CLASP, Chakraborty et al., 2011) we identified the VsP14a protein from grapevine *Vitis shuttleworthii* based on its structural and conformational similarity with the active site of the 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 and successfully test this protein to evaluate its ability to inhibit the growth of *E.coli* a gamma-proteobacteria. We found that addition of the VsP14a protein to the media inhibits the growth of E.coli. Preliminary results with Xylella show that it is able to clear Xylella cells when viewed under a microscope. We have come with a grapevine candidate to replace the CB component using CLASP.

Status of funds.
All of the funds for the first year have been expended. We have been funded for years 2 and three for which we have begun spending on July 1, 2013.

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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