BUILDING A NEXT GENERATION CHIMERIC ANTIMICROBIAL PROTEIN TO PROVIDE ROOTSTOCK-MEDIATED RESISTANCE TO PIERCE'S DISEASE IN GRAPEVINES

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ABSTRACT

We have successfully identified grapevine-derived replacement components for both the surface recognition (SRD) and lytic (LD) domains of the NE-CB chimeric antimicrobial protein that perform identical functions as these individual protein components. We identified a P14a protein from *Vitis shuttleworthii* (Vs-P14a) as a grapevine-derived replacement for NE using CLASP (Chakraborty et al., 2011; 2013). To verify the efficacy of P14a, we expressed it in a plant system to make sufficient quantities that were then tested against *Xylella fastidiosa* (Xf) and *E. coli* (Ec) cells grown in culture, where we documented clearance of Xf and growth inhibition of Ec (Dandekar et al., 2013). Two plant expression vectors were successfully constructed to express VsP14a by itself and as a CAP with CB (VsP14a-CB). Also using CLASP, we identified a 52-amino acid structure, VsHAT52, of a portion of a protein from *Vitis vinifera* designated HAT that showed a 3-D structural similarity to CB (Dandekar et al., 2013). We also constructed a binary vector to express this protein as a CAP designated VsP14a-VvHAT52. We then developed two additional computational tools, PAGAL (Chakraborty et al., 2014a) and SCALPEL (Chakraborty et al., 2014b), that further refined the metrics used to search out and identify portions of *Vitis* proteins that could be used to replace the lytic peptide component CB. Using this methodology, we identified a 20-amino acid peptide from a *Vitis* protein designated VvPPC20 that can clear Xf at concentrations similar to that observed for CB (Chakraborty et al., 2014b). A binary vector for expressing VsP14a-VvPPC20 has been constructed. The four binary vectors that are required to express VsP14a, VsP14a-CB, VsP14a-VvHAT52 and VsP14a-VvPPC20 have been used to transform the grapevine rootstock 110-14 Mgt (110-14, Christensen, 2003), and are currently under selection to obtain transgenic rootstock plants.

LAYPERSON SUMMARY

We have engineered transgenic grapevines that can protect themselves from Pierce's Disease by making a chimeric antimicrobial protein, NE-CB, that kills the causative agent, *Xylella fastidiosa*. We build on that success in this project by identifying in grapevine, components that are similar in structure to the human neutrophil elastase (NE) or insect Cecropin B (CB) protein components. Since the 3D structural details of both NE and CB are known, we used recently developed computational tools (CLASP, PAGAL and SCALPEL) to identify structurally/functionally similar proteins from grapevine based upon specific structural features present in NE and CB. Using these tools, we found that the P14a protein from grapevine would be a good substitute for the NE component. The isolated protein P14a killed Xf at levels comparable to that observed for NE. Using an additional set of tools, we identified two grapevine candidates, HAT52 and PPC20, as replacements for the CB component. Both purified proteins killed Xf, but that PPC20 did so at levels comparable to that observed for CB. We have made the vectors to generate grapevine plants expressing these to grapevine proteins to confirm that they can provide resistance to Pierce's Disease using the methods reported previously for NE-CB constructs (Dandekar et al., 2012).

INTRODUCTION

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 foster its ability to cause disease in grapevines (Chatterjee et al., 2008). Resistance to this pathogen must be multifaceted to block the pathogen at different stages of its complex lifestyle. A key issue 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 still used to suppress the GWSS population, which is effective but does not reduce this inoculum reservoir. Resistance mechanisms capable of degrading the reservoir could prevent further spread of the disease. 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. Our group has successfully designed and tested a NE-CB chimeric protein that specifically targets *Xf* in plant xylem (the site of infection), rapidly clears the pathogen, and blocks infection (Dandekar et al., 2009, 2012; Kunkel et al., 2007). The protein contains two separate domains. A surface-binding domain recognizes outer membrane proteins; we previously showed that it recognizes and cleaves mopB, a major *Xf* outer membrane protein (Dandekar et al., 2012). This surface-recognition domain is encoded by a synthetic gene derived from the human innate defense protein neutrophil elastase (NE) (Dandekar et al., 2012; Kunkel et al., 2007). The second, CB domain is a clearance domain, connected with a flexible linker to the C-terminal of NE. This domain is a synthetic gene that encodes an antimicrobial peptide, cecropin B (CB), that specifically lyses Gram-negative bacteria like *Xf* (Andrès and Dimarcq, 2007). The two domains work in tandem to recognize and lyse *Xf*. Our current hypothesis for the mode of action is that NE binds to the surface of *Xf* via its mopB outer membrane protein, bringing the cecropin B peptide close to the bacterial surface where it can lyse and kill the pathogen. Transgenic expression of this protein in tobacco and grape has provided phenotypic evidence of bacterial clearance and biochemical evidence of mopB degradation by NE (Dandekar et al., 2012). A major concern is that the presence of a protein of human origin in grapevines is potentially controversial to groups opposed to GMOs. Therefore, substituting NE and CB proteins derived from plants, ideally from grapevine, would be less controversial.

OBJECTIVES

The goal of this project was to redesign our existing therapeutic NE-CB CAP, replacing the human NE and insect CB domains with plant/grapevine derived orthologs, and to validate the efficacy of the new CAP components in providing resistance to Pierce's Disease in grapevine.

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.

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.

RESULTS AND DISCUSSION

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). To do this we sought an appropriate protein in plants and possibly more appropriately in grapevine that had 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.

A plant PE candidate protein was identified using the CLASP (Chakraborty et al., 2011) and PROMISE packages (http://www.sanchak.com/clasp/). The potential differences between reactive atoms in the active site of the target proteins were calculated using the Adaptive Posson-Boltzmann Solver (APBS) and PDB2PQR packages (Baker et al., 2001 and Dolinsky et al., 2004). The NE structure (1B0F) was chosen from Protein Data Bank (PDB). The elastase scaffold residues Ser195, His57, Asp102, Ser214, and Gly193 were chosen based on their known interaction with the elastase enzymatic function. The best plant candidate based on this analysis was a pathogenesis related (PR) protein P14a from tomato (1CFE). Details of the protocol and workflow used to make this selection have been described in Chakraborty et al., (2013). The P14a from *Solanum lycopersicum* (tomato) is a promising choice since we found similar, highly conserved proteins from other plant species, including grapevine. Additionally, the PR superfamily is widely distributed in animals, plants, and fungi. Some of these related to CRISP have demonstrated, substrate-specific protease activity (Milne et al., 2003). Furthermore, a striking structural homology was observed between P14a and a protein found in snake venom that has been demonstrated to be an elastase (Bernick and Simpson 1976). The structure of the 135 amino acid mature P14a protein from tomato was also determined using NMR (Fernandez et al., 1997). Based on these considerations, we used the tomato P14a to find an appropriate P14a in *Vitis*. We chose, from several matching these criteria, the P14a from *Vitis shuttleworthii* (Vs), as this species is resistant to PD (Walker, personal communication). More recently, 21 different PR-1 genes from grapevine, including those from Vs, were characterized and shown to confer resistance to bacterial disease (Li et al., 2011). Further analysis of the predicted active site of *Vs*P14a using CLASP indicated that removal of amino acids 74 and 77 had the potential to improve elastase/protease activity. The sequence of VsP14a without the two amino acids is designated VsP14m.

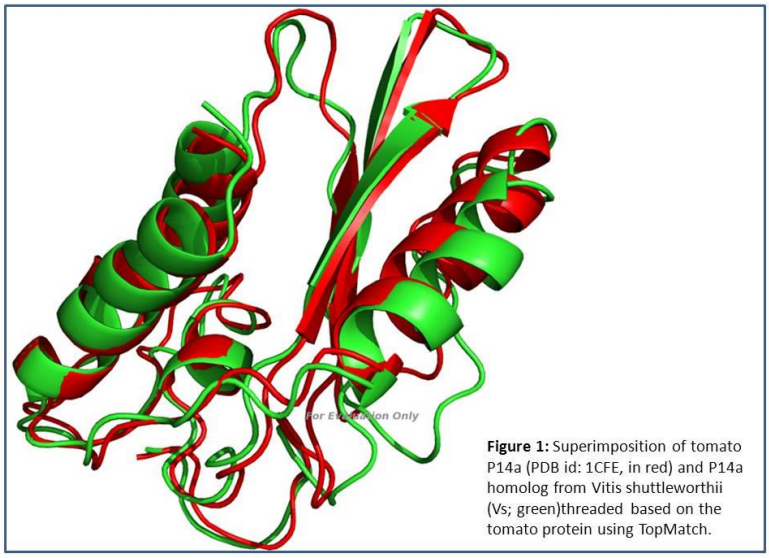

Figure 1: Superimposition of tomato P14a (PDB id: 1CFE, in red) and P14a homolog from Vitis shuttleworthii (Vs; green)threaded based on the tomato protein using TopMatch.

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 the clearing of *Xf,* we codon-optimized and chemically synthesized the two versions of VsP14a after adding 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) (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. 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). Proteins VsP14a and VsP14m also inhibited the growth of *Xylella* while protein obtained from the empty vector did not. By 50 minutes, 50% of the population was killed, but mortality reached a plateau at ~ 60% mortality (Fig 2D). These results are encouraging and indicate that we may have found the desired replacement for NE. Now it must be tested *in planta* for protection against *Xf* infection. We also developed antibodies against VsP14a and used these to detect the protein in the plants using Western blots. Proteins isolated from Thompson Seedless (TS) and *Vitis shuttleworthii* infected and not infected with *Xylella* showed that the P14a in *V. shuttleworthii* was expressed in both infected and uninfected tissues. In TS, it was only expressed in infected tissues and un-infected tissues expressed no protein corresponding to P14a (Fig 2B).

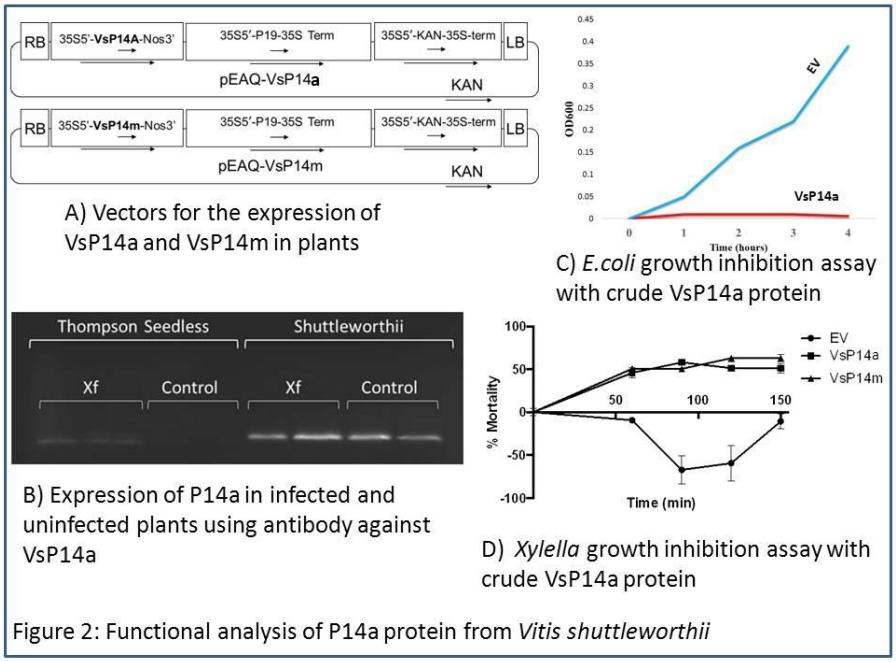

Figure 2: Functional analysis of P14a protein from *Vitis shuttleworthii*

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 have completed the construction of two binary vectors, one for expressing VsP14a by itself (Fig 3) and another which links the VsP14a sequence to CB. This recreates the original CAP protein, but with the SRD domain replaced by the P14a protein (Fig 3). In the first construct, the P14a coding sequence was fused to a signal peptide from the Ramy3D protein which is cleaved upon expression in the plant. Next to the signal peptide cleavage site and at the N-terminal of P14a is a 3XFLAG sequence to improve the immune detection of the P14a protein. In the second construct, the coding sequence is fused to the CB sequence via the four-amino acid flexible linker sequence used in the original CAP design (Dandekar et al., 2012). Both coding regions have a TMV omega sequence in the 5' non-coding region to improve translation efficiency. The regulatory sequence in both constructs is a double CaMV35S promoter sequence. The binary vectors were introduced into a disarmed *Agrobacterium* strain to reconstitute a functional plant transformation system. Both vectors are currently being used to transform grapevine rootstock and SR1 tobacco to evaluate the efficacy of these two constructs.

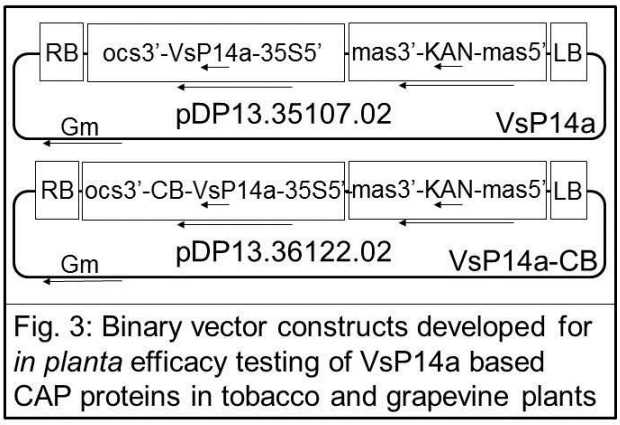

Fig. 3: Binary vector constructs developed for *in planta* efficacy testing of VsP14a based CAP proteins in tobacco and grapevine plants

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.

The goal of this objective is to identify a CB-like protein in plants to replace the lytic domain of CAP described earlier (Dandekar et al., 2012). Unlike the search for the HNE, where the focus was the congruence of the active site (spatial and electrostatic), a similar approach cannot be used for CB as it has no enzymatic function and thus no active site. The approach for finding a CB-like protein in plants focused instead on the highly structured nature of CB.

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 have used an approach similar to that described above in Activity 1 to identify a replacement component for CB. However, instead of comparing the reactive atoms as was done for the HNE matching algorithm, in this case we match for the Cα atoms of specific residues based on the overall shape of CB. Thus, we adopted a slightly different computational flow in this case. First, we did a keyword search, 'plants' in http://www.pdb.org/ that yielded about 1000 proteins. Each pdb was expanded on basis of each chain. For example, PDB X.pdb with chains A and B resulted in PDB files XA.pdb and XB.pdb. This list was filtered based on a 80% similarity redundancy. Then proteins larger than 60 aa in length were ignored. APBS was run on each pdb (the ones that failed electrostatic analyses, a few, were excluded). We were able to extract 200 proteins which we then analyzed further using CB motif. The CB structure is characterized by two helices (Fig 4). We chose specific residues from these two helices such that the residues were polar, and had stereochemical matches. For example, Lys can be replacing by either Arg or His, and thus constitutes a good candidate. We chose, Lys10, Lys11, Lys16 and Lys29 as the input motif from CB (PDBid:2IGR; Fig 4), allowing Lys to matched by Lys, Arg or His. Our analysis successfully identified good candidates (Fig 4 Table). It is noteworthy that several defense-like proteins feature in the list, allowing us to speculate that another peptide might be a good replacement for CB in the chimera. We have evaluated eight protein candidates and chose the 52 amino acid segment of the plasma membrane H^+ ATPase, whose structure matches very well with the CB structure (Fig 5).

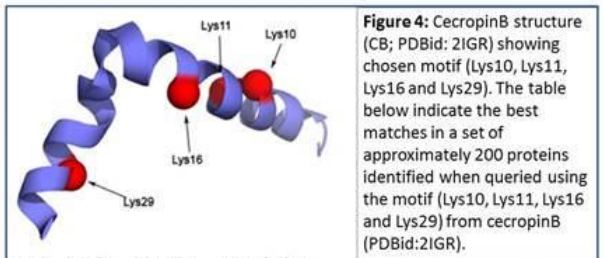

Figure 4: CecropinB structure (CB; PDBid: 2IGR) showing chosen motif (Lys10, Lys11, Lys16 and Lys29). The table below indicate the best matches in a set of approximately 200 proteins identified when queried using the motif (Lys10, Lys11, Lys16 and Lys29) from cecropinB (PDBid:2IGR).

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

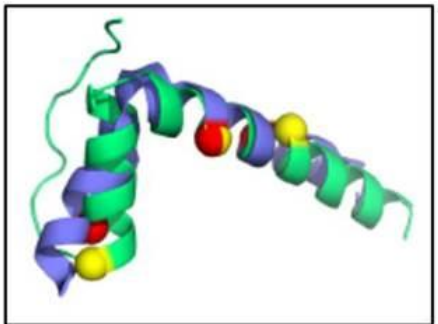

Fig 5: Superimposition of Cecropin B (PDBid: 2IGR, in blue) with Plasma membrane H+ ATPase (PDBid:2O98, in green) showing structural similarity

We refined our approach and focused instead on the alpha-helical structure itself to generate a greater diversity of candidates for our previously described CAP (Dandekar et al., 2012). To better understand the functionality of the alpha-helical domains of CB, we have developed two new computational tools, PAGAL (Chakraborty et al., 2014a) and SCALPEL, to better predict antimicrobial activities in portions of existing proteins. PAGAL (**P**roperties **a**nd corresponding **g**raphics of **al**pha helical structures in proteins) is open source software that implements previously known and established methods of evaluating the properties of alpha-helical structures, providing very useful information of the amphipathicity, hydrophobicity and charge moments

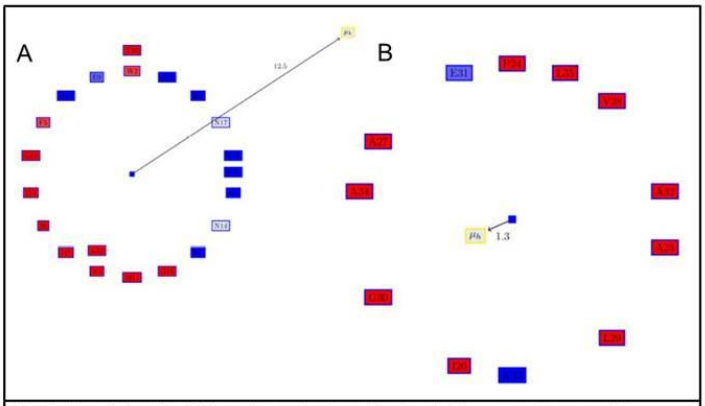

Fig. 6: Visualizing the Edmundson wheel and hydrophobic moment some alpha helices: The color coding is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored blue - dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides. (A) N-Terminal helix of CB. (B) C-Terminal helix of CB.

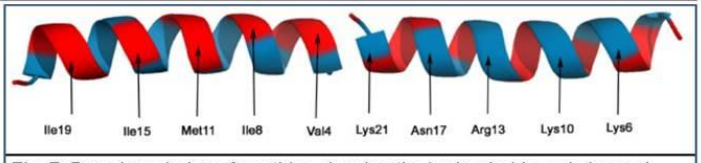

Fig. 7: Pymol rendering of peptides showing the hydrophobic and charged surfaces for the N-terminal helix of CB. All hydrophobic residues are colored red, while hydrophilic residues are colored blue.

within these structures. A key feature of lytic peptides is the distribution of hydrophobic and charged residues on the surface of the protein. To find proteins like cecropin that have an alpha-helical structure, we used PAGAL to evaluate CB. CB contains two alpha helixes (AHs) joined by a short stretch of random coil. Fig 6A and B show the Edmundson wheel and hydrophobic moment of the two AHs. The N-terminal AH has a large hydrophobic moment and a specific positive charge distribution. This can also be seen in a Pymol rendering of the peptide surface (Fig 7). The Pymol script for this rendering is automatically generated by PAGAL. On the other hand, the C-terminal AH of CB has neither of the above two properties.

We then developed the second program, SCALPEL to search for alpha-helical structures of a particular type. We searched for the smallest peptide with a large hydrophobic moment and a high proportion of positively charged residues on the hydrophilic side. Here, we identified a 20-aa region of the protein PPC from *Vitis vinef*era, a key enzyme in the C4-photosynthetic carbon cycle from grapevine that we call PPC20 (Fig 8A). We also searched for the smallest peptide with a large hydrophobic moment and a high proportion of negatively charged residues on the hydrophilic side and identified a 15-aa region of isoprene synthase from grapevine that we call ISS15 (Fig 8B). Both of these proteins have a very large hydrophobic moment. Fig 9A is a PYMOL rendering that shows the highly hydrophobic surface of PPC20 and Fig 9B clearly shows the positively charged surface, with the exception of a single Asp that is the only negative residue in a positive surface. Fig 9C shows a PYMOL rendering of the highly hydrophobic protein surface of ISS15 and in Fig 9D one can clearly see the negatively charged surface.

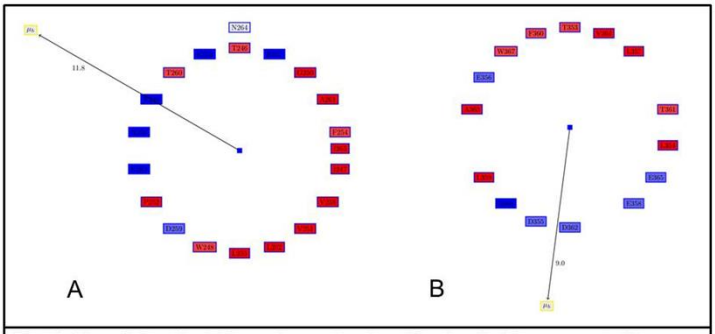

Fig. 8: Visualizing the Edmundson wheel and hydrophobic moment of two Vitis alpha helices: The color coding is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored blue - dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides. (A) The 20 aa residue PPC20 with positively charged residues. (B) 15 aa residue ISS15 with negatively charged residues.

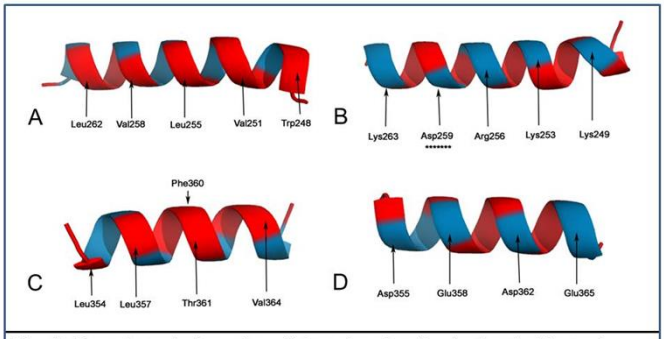

Fig. 9: Pymol rendering of peptides showing the hydrophobic and charged surfaces for the two potential lytic peptides extracted from grapevine genes. All hydrophobic residues are colored red, while hydrophilic residues are colored blue. (A) hydrophobic surface for PPC20 (B) charged surface for PPC20 (C) hydrophobic surface for ISS15 (D) charged surface for ISS15.

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

We used our recently developed bioinformatics tools PAGAL and SCALPEL to conduct a phase 1 search and identified three potential grapevine candidate proteins to replace the CecB lytic peptide domain of our previously described chimeric antimicrobial protein (CAP; Dandekar et al. 2012). Using the same tools, we further refined our search within these particular proteins to identify a smaller segment that was then chemically synthesized and tested for antimicrobial activity. The following grapevine proteins were chemically synthesized: a 22-aa version of HAT (VvHAT22; a 52-aa segment of this protein was previously identified), a 15-aa segment of ISS (VvISS15), and 20-aa segment of PPC (VvPPC20). These proteins were successfully tested for antimicrobial activity using to the Temecula strain of *Xf* and a strain of *Xanthomonas arbicola* (*Xa*) that was more sensitive than *Xf*. Using the same search criteria, the search identified a 22-aa N-terminal segment of the 34-aa Cecropin B (CBNT22) protein and a 12-aa segment of cathaylecitin (CATH15); these proteins with known antimicrobial activity served as a positive control for our bioassays. VvHAT22 and VvPPC22 inhibited *Xf* growth at levels comparable to CBNT22 and CATH15; however, VvISS15 displayed no detectable antimicrobial activity.

Table 1: Minimum inhibitory concentration of peptides tested

Indicator Bacteria	CecB	CBNT21	PPC20	CHITI25	ISS15
Xylella fastidiosa	100 μM	200 μM	150 μM	100μM	>300 μM
Xanthomonas arbicola	25 μM	25 μM	50 μM	150 μM	>300 μM

We have successfully validated our Vv peptide candidates described above using plating spot assays (Table 1, Fig 10). CecB lytic peptide was used as a control and was the most potent peptide tested, displaying a minimum inhibitory concentration (MIC) of 25μM for *Xa* and 100μM for *Xf*. Interestingly, CBNT21 has a slightly lower

MIC value, indicating and validating the need for the C-terminal alpha helix of CecB, which is comprised mostly of hydrophobic residues. This result corroborates a plausible mechanism suggested by others in which the anionic membrane of bacteria is targeted by the cationic N-terminal, followed by the insertion of the C-terminal AH into the hydrophobic membrane, creating a pore. PPC20 and CHITI25 have comparable potencies to CECB and CBNT21, although *Xa* appears to be slightly more resistant to CHITI25. Finally, the anionic peptide used as a negative control shows no effect on any of these pathogens.

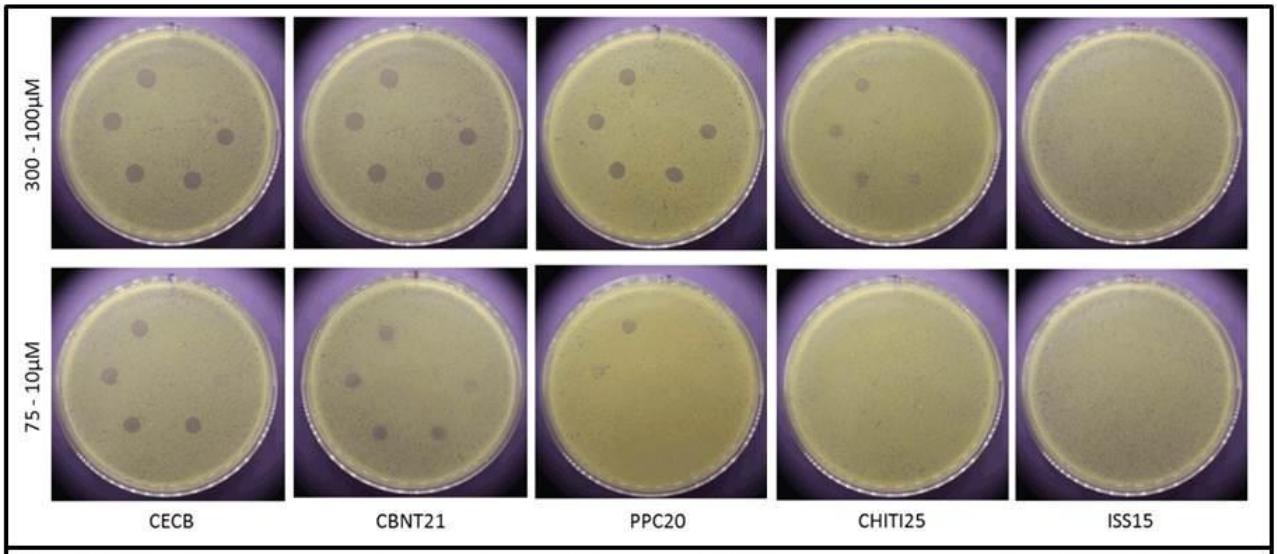

Fig 10: Plating assay to determine minimum inhibitory concentration (MIC) of SCALPEL identified peptides for *Xanthomonas arboricola*. Counter-clockwise: 300μM, 250μM, 200μM, 150μM, 100μM 75μM, 50μM, 30μM, 25μM, 10μM, PBS. CECB: MIC 25, CBNT2: MIC 10, PPC20: MIC 50, CHITI25: MIC 150, ISS15: MIC >300

Activity 6: Conduct *in planta* efficacy testing of the grape-derived CAP components using transient expression. We have successfully completed construction of all binary vectors for expression of VsP14a, VsP14a-CecB, VsP14a-VvHAT52 and VsP14a-VvPPC20, to test the efficacy of these proteins. We are currently infiltrating tobacco plants to isolate and purify proteins so that their efficacy can be tested as described above (Activity 5).

Objective 3: Construct and test a fully plant-derived CAP (PE-PLD) for the ability to confer resistance to PD in grapevine rootstocks.

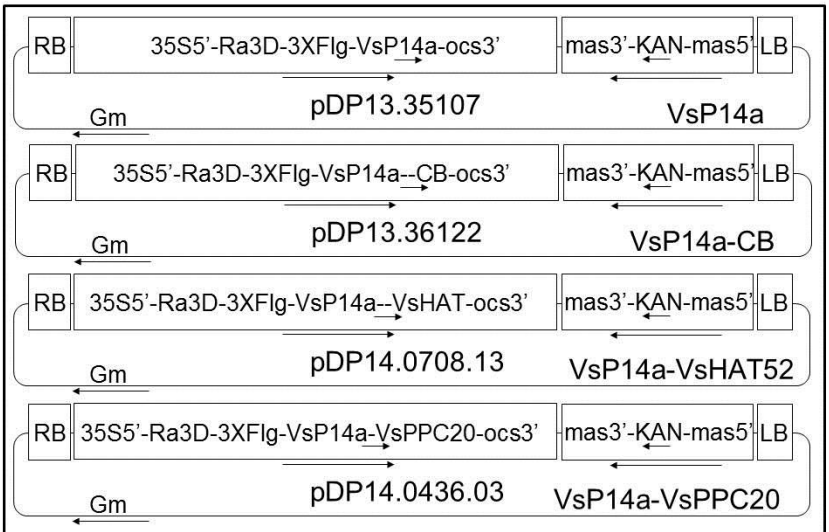

Fig 11: Binary vectors used for Agrobacterium-mediated transformation of grapevine 110-14 rootstocks for the expression of grapevine derived CAP for protection against PD

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

We have developed four binary vectors (Fig 11) that are currently undergoing plant transformation to create transgenic tobacco and 110-14 grapevine rootstocks. The first two are controls. pDP13.35107.02 is designed to express the P14a protein domain by itself and pDP13.36122.02 will express the P14a domain as a CAP, linking it to the previous lytic domain CB to test whether it enhances lysis of *Xf* more than P14a by itself. The next two vectors, pDM14.0708.13 and pDM14.0436.03, will express the *Vitis*-derived CAPs VsP14a-VvHAT52 and VsP14a-VvPPC20. These will be tested for their efficacy against the original CAP NE-CB and against the controls described above.

CONCLUSIONS

We have engineered transgenic grapevines that can protect themselves from Pierce's disease by making NE-CB, a chimeric antimicrobial protein (CAP) that kills the causative agent, *Xylella fastidiosa* (Xf). The NE and CB components of CAP are derived from two different proteins from two different non-plant sources, each with a distinct mode of action, but their action is cooperative when combined for killing Xf. The question that we addressed in our last proposal was to obtain NE and CB components from grapevines. To accomplish this, we used and developed some unique computational tools that can examine the relevant parts of a protein's 3-D structure. Using these tools, we found that the P14a protein from grapevine would be a good substitute for the NE component. The isolated protein P14a killed Xf at levels comparable to that observed for NE. Using an additional set of tools, we identified two grapevine candidates, HAT52 and PPC20, as replacements for the CB component. Both purified proteins killed Xf, but that PPC20 did so at levels comparable to that observed for CB. Binary vectors for the expression of these novel CAP are being used to create transgenic grapevine rootstocks for validating the efficacy against PD.

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