THE ALIMENTARY TRACK OF GLASSY-WINGED SHARPSHOOTER AS A TARGET FOR CONTROL OF PIERCE'S DISEASE, AND DEVELOPMENT OF MIMETIC INSECTICIDAL PEPTIDES FOR GLASSY-WINGED SHARPSHOOTER CONTROL

Project Leader:
Brian A. Federici
Dept. of Entomology
University of California
Riverside, CA 92521

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ABSTRACT

Transgenic insecticidal crops expressing *Bacillus thuringiensis* (*Bt*) toxins have been successfully developed to control major chewing insect pests of agriculture, such as caterpillars and beetles. The same *Bt* toxin technology also has been used with *Bacillus sphaericus* for the control of mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus*, important vectors of human diseases. However, this transgenic technology has not yet been applied to economically important xylem-feeding sucking insect pests such as the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Our goal is to use a genomics approach to develop novel, highly specific mimetic insecticidal proteins derived from the variable binding domains of immunoglobulin molecules. "Mimetic" peptides mimic the normal substrates of key components of essential processes to block the activities of these proteins. Our research is targeting the exposed active domains of transport proteins on the surface of the GWSS midgut microvillar membrane and enzymes found in GWSS saliva. Degenerate PCR amplification of genes characterized in other insect species encoding proteins involved in gut transport and saliva activity and screening a cDNA microarray to identify novel gut and saliva protein encoding genes are the approaches being used to identify GWSS target proteins. Due to the target specificity, mimetic peptide technology can provide an environmentally sound approach to the control of vasculature feeding insect pests and could thereby provide a means of controlling Pierce's disease and crop losses due to GWSS feeding.

INTRODUCTION

Mimetic technology is new to agriculture, but has been used extensively and successfully in medicine (Clemens, 1996). Examples of medical uses include the inactivation of disease-related enzymes (Burke et al., 2001), blockage of metabolic receptors important to disease (Berezov et al., 2000), and the use of antibodies developed against disease constituents (Moe et al., 1999). Human cancers (Monzayi-Karbassi and Keiber-Emmons, 2001), diabetes (Deghenghi, 1998), and heart disease (Lincoff et al., 2000) all have been treated successfully through these applications of mimetic technology. In spite of lacking a history of application of mimetics to agriculture problems, its development should be straight forward. Antibody proteins have been synthesized successfully in plants for the production of antibodies to be used in medical applications (Larrick et al. 2001; Stoger et al., 2002), and the production of transformed lines of crop plants in which promoters that have been isolated by other researchers (Shi et al., 1994; Springer, 2000), which direct expression to the cell wall and vascular structures of plants, will assure that our antibody peptides are synthesized in a tissue-specific manner. Last year we succeeded in isolating portions of five GWSS genes by degenerate PCR: the A and c V-ATPase subunits, genes encoding trypsin-like and maltase-like saliva proteins, and a membrane transporter. This year we have added another membrane transporter gene clone, most closely related to the potassium coupled amino acid transporter isolated from Manduca sexta, KAAT1 (Castagna et al., 1998). These clones and others isolated from our normalized cDNA are being analyzed using bioinformatics tools to identify functional domains which will be effective and specific targets. The identified target peptides will be synthesized in a *Baculovirus* expression system. Peptides produced will be used as antigens for polyclonal antibody production, the products of which will be cloned into phage display libraries. Screening the phage display antibody libraries will identify the mimetic peptides that bind most efficiently to the targeted GWSS proteins. Ultimately these peptides will be used in feeding studies to identify those which are the best candidates for GWSS control.

OBJECTIVES

1. Determine the structure and cell types in the midgut epithelium and salivary glands of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*;
2. Prepare a normalized cDNA microarray of GWSS using pooled cDNAs isolated from each developmental stage.
3. Screen the microarray using cDNA probes derived from midgut and salivary gland tissue-specific probes to determine the tissue-specific expression of key midgut microvillar and saliva proteins;
4. Clone and sequence genes encoding one or more key midgut microvillar and saliva proteins and determine their suitability as targets for a molecular biological approach to GWSS and Pierce's disease control.
5. Predict functional domains of key GWSS midgut epithelium- and salivary gland-specific proteins based on sequences of genes using bioinformatics;
6. Express functional domain peptides for antibody production;
7. Clone single-chain fragment variable antibody genes into recombinant phage libraries and screen the libraries;
8. Conduct feeding studies to identify efficacious mimetic peptides effective in killing or deterring GWSS.

RESULTS

We have had a normalized cDNA library constructed by Evrogen JSC from total RNA isolated from whole GWSS of both sexes and all life stages, as well as from GWSS that have fed on grape infected with *X. fastidiosa*. We've had 10,752 clones isolated, glycerol stocks prepared, and PCR products of all inserts amplified and purified for microarray spotting. This August three members of our laboratory were trained at the Custom Microarray Facility at the University of Arizona and we are currently repeating the results obtained there at the Core Instrumentation Facility in the Institute for Integrative Genome Biology on the Riverside campus. A subset of 1,536 clones was spotted in duplicate (side by side spots) and the entire array duplicated on the same slide. These arrays were hybridized to Cy3 labeled control cDNA and Cy5 labeled cDNA reverse transcribed and amplified from total RNA isolated from GWSS treated with a sub-lethal dose or an LD50 dose of esfenvalerate. Dye swap experiments were performed. These experiments are part of a collaborative related project funded by CDFA with Frank Byrne as Project Leader. Our results are presented in his report for the project entitled "Evaluation of resistance potential in the glassy-winged sharpshooter (GWSS) using toxicological, biochemical and genomics approaches." The arrays detected obvious differences in gene expression levels between the two treatments. These experiments were chosen for our test study because it is known that several genes encoding cytochrome P450 proteins are up-regulated dramatically in response to pesticide treatment. We have succeeded in cloning the entire GWSS V-ATPase A gene (Figure 1) by RLM-RACE. Differences in both the 5'- and 3'- sequences between the clones obtained indicate more than one copy of the V-ATPase A gene exists in the GWSS genome

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   1 ctcgtgatatcagctggtgactgggtgaggtagctgcttcgtctgatcattacagtaagggatagctgaagatgcctttgcagaagattaaagatgag
                                                                            M  P  L  Q  K  I  K  D  E
  99 gacaaggagtccaagtttggatatgtgtacgctgtatccggaccggtcgttacggccgagaagatgtccgggtcagctatgtacgagttggtgcgtgtc
     D  K  E  S  K  F  G  Y  V  Y  A  V  S  G  P  V  V  T  A  E  K  M  S  G  S  A  M  Y  E  L  V  R  V
 198 ggctactttgagctggtaggagagatcattcgtctggaaggtgacatggcaactattcaggtatacgaagagacgtcaggtgtgacggttggtgaccct
     G  Y  F  E  L  V  G  E  I  I  R  L  E  G  D  M  A  T  I  Q  V  Y  E  E  T  S  G  V  T  V  G  D  P
 297 gtattgcggactggcaagcctctgtctgtggaactcggtcccgggatcctgggcgctattttgatggtatccagcgacccttgaaggacatcaatgaa
                                              t  t   a     aag  c     c
     V  L  R  T  G  K  P  L  S  V  E  L  G  P  G  I  L  G  A  I  F  D  G  I  Q  R  P  L  K  D  I  N  E
                                                     M     S
 396 ctgtctcacagcatctacatccctaagggagtcaacgttcccgcactctccagaacagccacttgggagtttaaccccttaacatcaagactggaagc
                                                 t                          c
     L  S  H  S  I  Y  I  P  K  G  V  N  V  P  A  L  S  R  T  A  T  W  E  F  N  P  L  N  I  K  T  G  S
 495 cacattaccggaggagatatttatggaatcgtccacgagaataccctggtgaaacacaagatgctgctgcctcctcgagctaagggaacagtgacgtac
                                                                                  g
     H  I  T  G  G  D  I  Y  G  I  V  H  E  N  T  L  V  K  H  K  M  L  L  P  P  R  A  K  G  T  V  T  Y
 594 atcgcatcccctggcaactacactgttgatgatgttgttctggaaacggagtttgacggtgagaagtctaagtatacaatgttgcaagtgtggcctgta
     I  A  S  P  G  N  Y  T  V  D  D  V  V  L  E  T  E  F  D  G  E  K  S  K  Y  T  M  L  Q  V  W  P  V
 693 cgtcagcccagacctgtgaccgagaagctgccagccaatcatccactgctcactggtcaacgtgtgctcgactctctattcccttgtgtgcaaggtggt
     R  Q  P  R  P  V  T  E  K  L  P  A  N  H  P  L  L  T  G  Q  R  V  L  D  S  L  F  P  C  V  Q  G  G
 792 accacggccatccctggagccttcggttgcggtaaaactgtcatctctcaggccctgtccaaatactccaactcggatgtcatcatttacgtaggatgt
     T  T  A  I  P  G  A  F  G  C  G  K  T  V  I  S  Q  A  L  S  K  Y  S  N  S  D  V  I  I  Y  V  G  C
 891 ggagagcgaggaaatgaaatgtctgaggtattgcaagacttccctgagctgtctgtagagattgatggagtcacggagagtataatgaagcgaactgct
     G  E  R  G  N  E  M  S  E  V  L  Q  D  F  P  E  L  S  V  E  I  D  G  V  T  E  S  I  M  K  R  T  A
 990 ctggtagccaacacctccaacatgcctgtcgccgcccgagaagcttccatttatacaggaatcacactgtctgaatatttccgagacatgggctacaat
     L  V  A  N  T  S  N  M  P  V  A  A  R  E  A  S  I  Y  T  G  I  T  L  S  E  Y  F  R  D  M  G  Y  N
1089 gtttctatgatggctgactctacatcccgttgggccgaagccctgagagaaatttctggacgattggcagaaatgcctgctgacagtggttaccccgca
     V  S  M  M  A  D  S  T  S  R  W  A  E  A  L  R  E  I  S  G  R  L  A  E  M  P  A  D  S  G  Y  P  A
1188 tacctgggtgcccgtctagcctccttctacgagagagccggtcgagtcaagtgcctgggaaatcccgatcgtgaaggctctgtcagtattgtcggtgct
     Y  L  G  C  P  S  S  L  L  L  R  X  S  R  S  S  Q  V  P  G  N  P  D  R  E  G  S  V  S  I  V  G  A
1287 gtgtcacctcctggtggtgacttctcagatcccgtcacctccgccacccttggtatcgtacaggtcttctggggtcttgacaagaaattggcacaaagg
     V  S  P  P  G  G  D  F  S  D  P  V  T  S  A  T  L  G  I  V  Q  V  F  W  G  L  D  K  K  L  A  Q  R
1386 aaacactttccctccattaactggctcatatcatacagtaaatacatgagagcactagatgacttctatgacaagagcttcccagagtttgtacccctg
     K  H  F  P  S  I  N  W  L  I  S  Y  S  K  Y  M  R  A  L  D  D  F  Y  D  K  S  F  P  E  F  V  P  L
1485 aggaccaaggtaaaggagattctgcaggaggaagaagatttgtcagaaattgtacaactggttggcaaagcatcacttgccgaaactgacaaaatcacc
                                                                                      gagtcc
     R  T  K  V  K  E  I  L  Q  E  E  E  D  L  S  E  I  V  Q  L  V  G  K  A  S  L  A  E  T  D  K  I  T
                                                                                         S
1584 ctcgaggtcaataggctactgaaagaagattttctgcaacagaacagctactctccgtatgatcgtttctgtcccttctacaagacagtgggcatgctc
     L  E  V  A  R  L  L  K  E  D  F  L  Q  Q  N  S  Y  S  P  Y  D  R  F  C  P  F  Y  K  T  V  G  M  L
1683 cgcaacatgattgcattctttgacatgtcaaggcatgccgtcgagtcgacagcacaaagtgaaaacaagatcacctggagtgtcatcaaggacggcatg
     R  N  M  I  A  F  F  D  M  S  R  H  A  V  E  S  T  A  Q  S  E  N  K  I  T  W  S  V  I  K  D  G  M
1782 ggcaacattctgtaccaactgtcgtcaatgaaattcaaggatcccgtgaaagatgaaaaaaaaaaaa
     G  N  I  L  Y  Q  L  S  S  M  K  F  K  D  P  V  K  D  E
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Figure 1. The complete cDNA and translated protein of GWSS V-ATPase A. The atg indicates the translational start site. Nucleic acid and protein sequence variations are indicated in bold. Sequence variations were determined from both sense and antisense sequences.

We have dissected and identified all of the components of the GWSS alimentary canal, performed ultrastructural studies of these tissues, and developed *in situ* hybridization techniques for the localization of gene expression (Figure 2). As expected the genes encoding the V-ATPase A and c subunits and that expressing HcMT1 are all expressed throughout the GWSS gut. HcMT1 clearly also is expressed in the salivary glands. The studies localizing the expression of the trypsin-like and maltase-like genes are in progress.

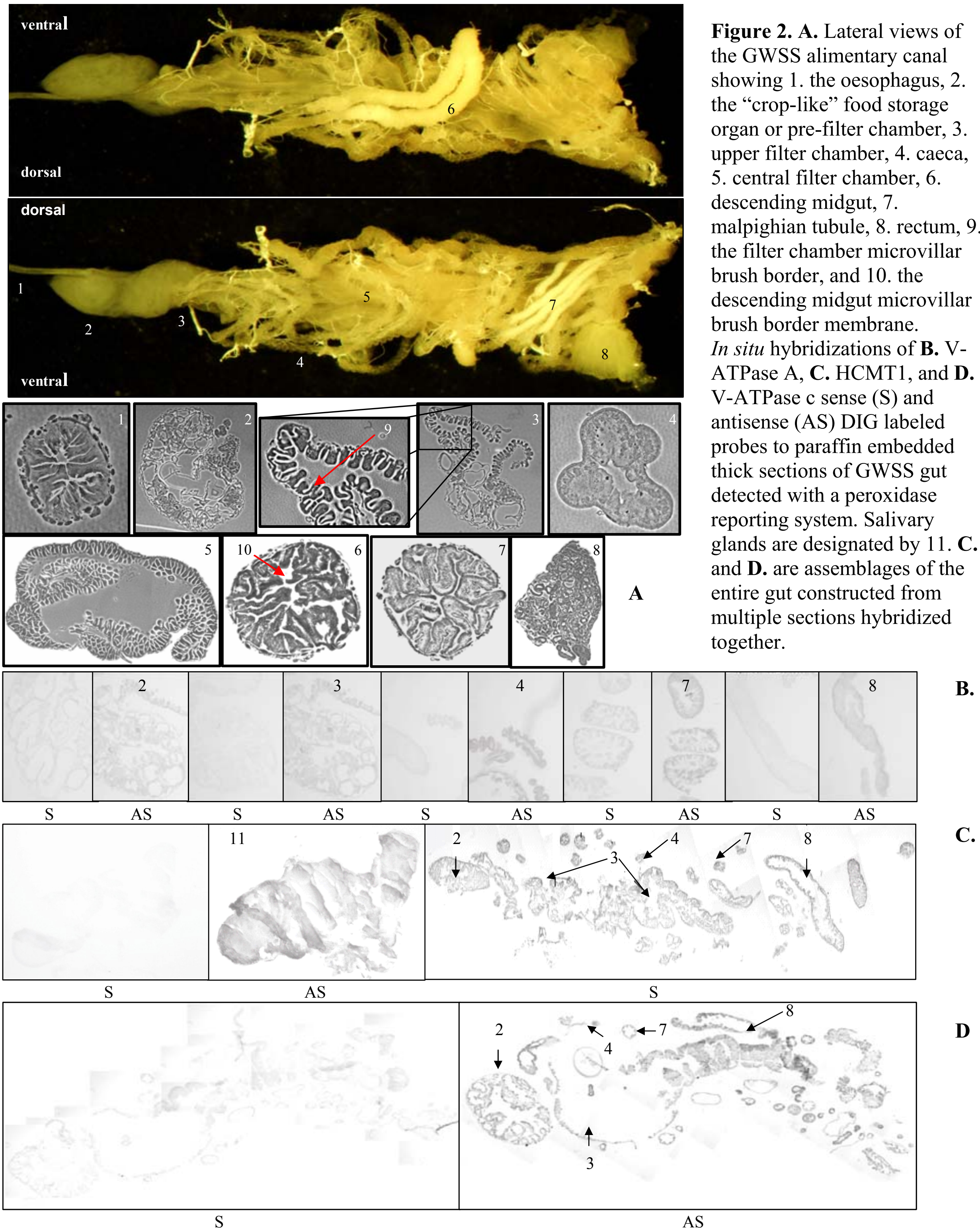

Figure 2. A. Lateral views of the GWSS alimentary canal showing 1. the oesophagus, 2. the "crop-like" food storage organ or pre-filter chamber, 3. upper filter chamber, 4. caeca, 5. central filter chamber, 6. descending midgut, 7. malpighian tubule, 8. rectum, 9. the filter chamber microvillar brush border, and 10. the descending midgut microvillar brush border membrane. *In situ* hybridizations of **B.** V-ATPase A, **C.** HCMT1, and **D.** V-ATPase c sense (S) and antisense (AS) DIG labeled probes to paraffin embedded thick sections of GWSS gut detected with a peroxidase reporting system. Salivary glands are designated by 11. **C.** and **D.** are assemblages of the entire gut constructed from multiple sections hybridized together.

Transcript sizes for each of the genes partially cloned have been determined by RNA blot hybridization (Figure 3). The transcript sizes were determined as: ~1,900 bp for V-ATPase A, which corresponds well with that determined from the cDNA sequence of 1,849 bp, ~1,200bp for V-ATPase c, and ~875 bp for HCMT1 and the trypsin-like gene. These values

correspond to those in the literature for each of these genes (van Hille *et al.* 1993; Pietrantonio and Gill, 1993; Zeng *et al.*, 2002; Liu *et al.,* unpublished data).

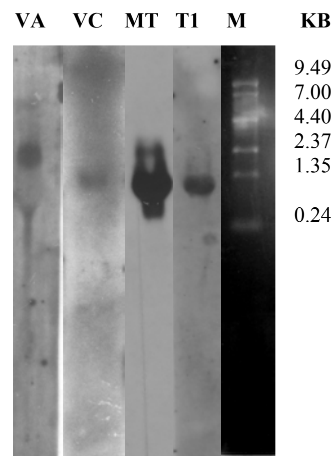

Figure 3. RNA blot hybridizations of 10μg GWSS total RNA hybridized to V-ATPase A (VA), V-ATPase c (VC) and the trypsin-like gene (T1) clones labeled with DIG and detected with chemiluminecsence and the HCMT1 (MT) gene clone labeled with 32P.

CONCLUSIONS

The presence of more than one GWSS V-ATPase A subunit gene will be confirmed by DNA blot hybridization. We have developed a clone capture technique which will allow us to isolate all gene clones with sequence similarity from our cDNA library in a single experiment. This procedure involves the formation of a RecA-mediated triple-stranded molecule between our biotinylated partial clone and full length cDNA clones with sequence similarity. Triple-stranded molecules are then removed from the reaction using streptavidin magnetic beads. This approach will allow us to much more quickly analyze all the members of specific gene families already partially cloned. Thus far we have succeeded in isolating clones similar to the KAAT-like gene clone recently obtained (data presented in the report of a related project: Development of Glassy-winged Sharpshooter Mimetic Insecticidal Peptides, and an Endophytic Bacterial System For Their Delivery to Mature Grape.). The clones isolated are being analyzed to identify the regions best suited for antibody targeting using bioinformatics tools. We anticipate that this approach also will allow us to isolate gene families of genes identified by microarray screening as being tissue-specifically expressed. This will be important in determining that a potential target does not have similarity to genes expressed other than in the organs we want to target.

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