ABSTRACT

Title of dissertation: SYMBIONTS ASSOCIATED WITH THE SALIVARY GLANDS OF THE POTATO LEAFHOPPER, *EMPOASCA FABAE*, AND THEIR FUNCTION WHEN FEEDING ON LEGUMINOUS HOSTS

Bridget Diane DeLay, Doctor of Philosophy, 2013

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Many species of phloem feeding insects are host to symbiotic bacteria, which provide their hosts with nutrients lacking from phloem. The potato leafhopper, *Empoasca fabae*, feeds on the phloem and cell contents of a wide variety of plants. In this study, I identified two taxa of symbiotic bacteria present in the salivary glands, midgut, bacteriomes and saliva of the potato leafhopper: *Sulcia muelleri* and *Wolbachia*. Treatment of the leafhoppers with 0.01% oxytetracycline-HCl resulted in the creation of aposymbiotic leafhoppers. Longevity and fecundity of aposymbiotic leafhoppers on alfalfa, *Medicago sativa*, and fava bean, *Vicia faba*, were significantly lower than that of symbiotic leafhoppers. In addition, aposymbiotic leafhoppers caused less of a decrease in photosynthesis rates on both alfalfa and fava bean in comparison to symbiotic leafhoppers. The salivary gland transcriptome of the potato leafhopper revealed the presence of potential salivary components, including lipase, pectin lyase and alkaline phosphatase, all of which were expressed at higher levels in salivary glands than in midgut or hind femur tissue. In addition, transcripts attributed to *Wolbachia* were

discovered in the sialotranscriptome, providing more evidence that this bacterium is present in the salivary glands of the potato leafhopper. Finally, expression of alfalfa wound response genes after exposure to potato leafhopper saliva was measured. Endo 1-3 ß-D-glucanase, isoflavone reductase, chalcone synthase and phenylalanine ammonia-lyase gene expression were higher in plants exposed to leafhopper saliva than in unexposed controls. Treatment of saliva with heat, filter sterilization, DTT, EDTA and K_2HPO_4 led to different plant wound response gene expression patterns. I conclude that the symbionts present in the potato leafhopper are necessary for the normal development and reproduction of this species, in addition to playing a potential role in plant wound response to feeding.

SYMBIONTS ASSOCIATED WITH THE SALIVARY GLANDS OF THE POTATO LEAFHOPPER, *EMPOASCA FABAE*, AND THEIR FUNCTION WHEN FEEDING ON LEGUMINOUS HOSTS

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Foreword

With the recommendation of the dissertation director (William O. Lamp), and with the endorsement of the Entomology program Graduate Director (Paula M. Shrewsbury), chapter 4 of this dissertation is included as a previously published work. The citation for this publication is as follows:

DeLay, B., Mamidala, P., Wijeratne, A., Wijeratne, S., Mittapalli, O., Wang, J., Lamp, W., 2012. Transcriptome analysis of the salivary glands of the potato leafhopper, *Empoasca fabae*. Journal of Insect Physiology 58(12), 1626-1634.

As directed in the graduate catalog for chapters previously published as coauthored works, I state that I was responsible for the inception of the manuscript and the majority of the manuscript preparation. This work was reformatted to meet the requirements for dissertations, but all other aspects of the published manuscript, including the use of first person plural, were used in this document. A letter was sent to the Dean of the Graduate School certifying that inclusion of this previously published work in this dissertation has the approval of the dissertation committee, the dissertation advisor, and the Graduate Director. A copy of this letter is included in Appendix A.

Dedication

I dedicate this work to the two who call me ‘Gitte.

To Nick: I can not imagine life without you, and am grateful for all of the support that you have shown to me thoughout the completion of this work. I could not have asked for a better husband, and count myself blessed to have met you.

To Gpa: although you are no longer around, I owe so much of who I am to you. You showed me how to work with tools, instilled in me a sense of craftsmanship, and gave me unwavering love. I only wish that you were here to share this work with me – I know that it would have made you smile.

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In addition to working with the potato leafhopper, I have also worked on the symbionts present in the brown marmorated stink bug, *Halyomorpha halys*. I would like to specifically thank Sadia Naseem for her help with stink bug dissections and symbiont screening. In addition, I would like to thank Chris Taylor, Galen Dively and Karen Rane for their input with this project and for providing stink bugs for me. Finally, I would like to thank Leslie Pick, Jian Wang, Cerruti Hicks, Galen Dively, Chris Taylor, Ellie Stevens, Alan Leslie and Ryan Gott for reviewing the stink bug manuscript and providing recommendations on how to improve it.

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In addition to those mentioned above, I would like to specifically thank Amy Beaven for her guidance in using the Zeiss LSM710 confocal microscope to produce the FISH images in Chapter 1. She also trained me in the use of the LightCycler 480 used for qRT-PCR in Chapters 4 and 5 of this dissertation, and in the use of the 3730xl DNA Analyzer used in Chapter 1.

In addition to those mentioned above, I would like to specifically thank my coauthors for Chapter 4: Jian Wang, Omprakash Mittapalli, Praveen Mamidala, Asela Wijeratne and Saranga Wijeratne. Jianhua Huang helped with the salivary gland dissections, and funding for the transcriptome work was provided by the Maryland Agricultural Experiment Station Grant Program, 2010–11, and MAES Hatch Project MD-ENTM-1016.

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Chapter I: Characterization of potato leafhopper salivary symbionts

Abstract

Symbiotic bacteria are present in many species of plant-feeding insects, and supplement the diet of their host with amino acids and vitamins lacking from the plant tissue consumed by the insect. The potato leafhopper, *Empoasca fabae*, is a pest of leguminous crops, on which it causes characteristic feeding damage known as hopperburn. Although this insect is an economically important pest in the United States, its biology is not entirely known. Here, I describe two taxa of symbiotic bacteria present in the salivary glands, midgut and bacteriomes of the potato leafhopper: *Sulcia muelleri* and *Wolbachia*. These symbionts were detected with primer sets designed to amplify the 16S rDNA gene of known leafhopper symbionts, the FtsZ gene of *Wolbachia* and a universal primer set designed to amplify the eubacterial groEL gene in leafhopper salivary gland, midgut, bacteriome and femur tissue. The resulting amplified DNA was sequenced, and BLAST was used to identify the species of bacteria detected. In addition, florescent *in situ* hybridization was used to visualize the symbionts in potato leafhopper tissue. An understanding of the symbionts present in this hopperburning pest may lead to novel pest management strategies in the future.

Introduction

Insect associations with symbiotic bacteria have only recently been studied in great detail. One well-studied relationship between an insect and its symbiotic bacterium is that of aphids and their symbiont *Buchnera aphidicola* (Douglas 1998). *Buchnera* is a vertically-transmitted obligate symbiont that resides in enlarged cells lining the midgut,

whose greatly reduced genome contains operons dedicated to the production of amino acids (Russell et al. 2003). These amino acids are produced by the bacterium to supplement the insect's diet of phloem, which is deficient in many essential amino acids (Douglas 2005). In return, the aphid houses *Buchnera* in specialized cells called bacteriocytes and provides the proteins necessary for the synthesis of the bacterium's cell walls (Gil et al. 2002).

Insect symbionts can be classified as either primary symbionts, which are necessary for the survival of the host, or secondary (facultative) symbionts, which provide additional benefits to the host but are not necessary for survival. Secondary symbionts may provide additional vitamins or amino acids to complement those produced by the primary symbiont, provide protection from disease or parasitization, or influence insect host range (Hurst and Hutchence 2010, Hansen et al. 2007, Li et al. 2011). Primary symbionts are transmitted vertically, are usually found within the vacuole of specialized cells (bacteriocytes) associated with the midgut of the host, and are unable to be cultured in vitro (Moran 2001). In contrast, secondary symbionts are not necessary for the survival of their host, and are often transmitted horizontally in addition to vertically (Gonella et al. 2012). Secondary symbionts may be located in bacteriocytes, or found in association with the salivary glands or fat body of the insect (Chiel et al. 2009).

Vertical transmission of obligate primary symbionts has led to concurrent evolution of the symbiont and the insect host. Insect bacterial symbionts are believed to have evolved from free-living bacteria that were taken into the vacuoles of insect cells 50 to 250 million years ago (Moran 1996). Over the course of their evolution, their genomes have become greatly reduced in size (as low as 300kbp), and they have lost many genes

that are necessary for independent survival, such as pathways for the creation of cellular membrane components (McCutcheon et al. 2009). Although the genomes of insect symbionts are greatly reduced, they often contain multiple operons for amino acids and vitamins. The genome of *Buchnera* contains most of the genes necessary for the biosynthesis of the essential amino acids that are lacking in plant phloem, with the genome of the pea aphid complementing the bacterial genome by containing the missing genes (Hansen and Moran 2011). Likewise, the symbiont *Wigglesworthia glossinidia* supplements the diet of blood consumed by the tsetse fly, *Glossina palpalis*, with the biosynthesis of B vitamins that are lacking in human blood (Snyder et al. 2010). The complementarity of symbiont and host genomes has allowed insects to specialize on hosts that would otherwise be unsuitable for development.

Interest in the aphid-*Buchnera* symbiosis has led to the discovery of symbiotic bacteria in other insect species that feed on diets that are lacking essential amino acids. Symbiotic bacteria have been found in association with the midgut of whiteflies, psyllids, mealybugs, tsetse flies and mosquitoes (Skaljac et al. 2010, Fukatsu and Niko 1998, von Dohlen et al. 2001, Snyder et al. 2010, Klyachko et al. 2007). In addition, symbionts have been reported in the salivary glands of leafhoppers, mosquitoes and ticks (Sacchi et al. 2008, Damiani et al. 2008, Klyachko et al. 2007). The role of these salivary symbionts is not well known, although they are presumed to play a role in the production of salivary components, as they are sometimes injected into the insect's food along with the saliva.

Leafhoppers are sap-feeding insects that form obligate associations with symbiotic bacteria (Dietrich et al. 2001). Some leafhoppers are strict xylem or phloem feeders, while others will opportunistically feed on the contents of plant cells that are

ruptured by their probing stylets while feeding (Wu et al. 2006, Welker et al. 1996, Hunter and Backus 1989). Phloem contains high levels of sugar and low levels of essential amino acids, while xylem is used mainly to transport minerals and water from the roots to the leaves, and is therefore lower in sugar and essential amino acids (Moran et al. 2002, Wu et al. 2006). To overcome the lack of essential amino acids in their diet, leafhoppers harbor vertically-transmitted symbiotic bacteria in bacteriomes located near the midgut or in association with the fat body or salivary glands (Moran et al. 2002, Wu et al. 2006, Marzorati et al. 2006). These symbionts use energy from sugar obtained from their insect host to produce components that are lacking in the insect's diet. For example, *Baumannia cicadellinicola* and *Sulcia muelleri*, the two symbionts found in the glassy-winged sharpshooter, *Homolodisca coagulata*, produce vitamins and essential amino acids respectively (Wu et al. 2006).

The potato leafhopper, *Empoasca fabae*, is a highly polyphagous species that feeds on a number of agricultural crops such as alfalfa, *Medicago sativa* (Lamp et al. 1994). It feeds on both phloem and cell contents, causing a cascade of plant wound responses in susceptible host plants which leads to damage known as hopperburn (Ecale Zhou and Backus 1999). Hopperburn is characterized by chlorosis of leaf tissue, stunting of plant stems, a decrease in photosynthesis and transpiration, accumulation of starch in the leaf tissue and in severe cases, death of the plant. Although the exact mechanism behind the production of symptoms is unknown, hopperburn appears to be caused by a combination of mechanical damage and plant reaction to the leafhopper's saliva (Backus et al. 2005). Unlike other economically important leafhopper pests, there have been no studies characterizing the symbionts present in the potato leafhopper.

This study used universal eubacterial primers and specific diagnostic primers designed to detect the 16S rDNA of known leafhopper symbionts to clone symbiont rDNA from the salivary glands, midgut and bacteriomes of the potato leafhopper to isolate symbiont rDNA for later identification. This approach led to the discovery of two taxa of symbionts in the potato leafhopper: *Sulcia muelleri* and *Wolbachia*. The genetic sequences obtained from the cloning reactions were used to create phylogenetic trees comparing the two symbionts of the potato leafhopper to the symbionts of other leafhopper species. In addition, the symbionts were visualized in the salivary glands, midgut and bacteriomes of the potato leafhopper using florescent *in situ* hybridization with primers designed to bind to the 16S sequences of the symbionts. The presence of symbionts in the potato leafhopper's salivary glands, midgut and bacteriomes may contribute to the leafhopper's ability to feed on a wide range of host plants and may also play a role in the hopperburn symptoms displayed by susceptible host plants.

Materials and methods

Insect collection and rearing

Potato leafhopper colonies were established by placing a single mated female into a collapsible screen cage (Bioquip, Rancho Dominguez, CA) containing fava bean, *Vicia faba*, plants. The female was allowed to lay eggs, and the resulting offspring allowed to interbreed in order to establish a colony with minimal genetic variation. The screen cages were kept in a walk-in growth chamber kept at 25°C with 14 hours of daylight and 10 hours of dark, and the fava bean plants were watered and replaced as needed. The females used to establish colonies were collected from alfalfa fields at the Western

Maryland Research and Education Center in Keedysville, Maryland, and at the Central Maryland Research and Education Centers in Beltsville and the Clarksville, Maryland. All leafhoppers screened for symbionts were obtained from these colonies.

Insect dissection and DNA extraction

Male, female and fourth instar nymphs were killed by placing them in a -20°C freezer for 30 minutes. Insects were dissected under a dissecting microscope at 60X in 1X PBS using sterilized forceps. Tissue from five individual leafhoppers was pooled into one sample in order to obtain enough tissue for subsequent DNA extraction. The salivary glands, midguts, bacteriomes and hind femurs were dissected and placed into 1.5mL microcentrifuge tubes containing 200µL 1X PBS. Salivary glands were removed from the leafhoppers by grasping the abdomen with one pair of fine-tipped forceps and the head with a second pair of fine-tipped forceps and gently pulling the head apart from the rest of the body. This generally resulted in the head and prothorax separating from the mesothorax and remainder of the body. The paired salivary glands were visible emerging from the prothorax, where they could be carefully removed with forceps. The paired yellow bacteriomes were located beneath the third abdominal tergite, and were removed by carefully prying the tergite up to access the bacteriome. The midgut was removed by opening the abdomen with forceps, and lifting out the entire gut before separating out the midgut.

Total DNA was extracted from the tissue samples using a DNeasy kit (Qiagen, Gaithersburg, MD) following the manufacturer's directions. DNA samples were stored at -80°C for future processing.

PCR of symbiont genes

DNA samples were screened with primers specifically designed to detect the 16S rDNA of known leafhopper symbionts. The primer pair 10F and 1507R was used to detect the 16S rDNA sequence of *Baumannia cicadellinicola*, yielding a fragment of approximately 1500 base pairs. The primers 10FF and 1307R were used to screen for *Sulcia muelleri*, amplifying a 1350 base pair region of the 16S rDNA. In addition, DNA samples were screened with the primers FtsZF1 and FtsZR1, which were designed to detect the FtsZ gene of *Wohlbachia,* and yield a fragment approximately 1050 base pairs long (Table 1.1). Primer sequences were taken from Takiya et al. 2006. In addition, the degenerate universal eubacterial primers GroF and GroR were used to amplify a 1.65 kb region of the *groEL* gene for subsequent cloning.

Primer Name	**Bacterium Detected**	**Gene Name**	**Primer Sequence (5' to 3')**
10F	*Baumannia*	16S	AGTTTGATCATGGCTCAGATTG
1507R	*Baumannia*	16S	TACCTTGTTACGACTTCACCCCAG
10FF	*Sulcia*	16S	AGTTTGATCATGGCTCAGGATAA
1307R	*Sulcia*	16S	CGTATTCACCGGATCATGGC
FtsZF1	*Wohlbachia*	FtsZ	GTTGTCGCAAATACCGATGC
FtsZR1	*Wohlbachia*	FtsZ	CTTAAGTAAGCTGGTATATC
GroF	Universal	GroEL	ATGGCAGCTAAAGAMGTAAAATTYGG
GroR	Universal	GroEL	TTACATCATRCCRCCCAT

Table 1.1: Primers used for the identification of symbionts in the potato leafhopper, *Empoasca fabae*

Each 50µL PCR reaction consisted of 2µL total DNA from leafhopper tissue in a final concentration of 1X Taq buffer, 0.2mM of each dNTP, 1µM of each primer, 1.25

units TaKaRa Ex Taq proofreading DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA), 2mM $MgCl_2$ and 2μL template DNA in PCR grade water. The PCR reaction consisted of a denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light to verify the presence of bands before sequencing.

Cloning and sequencing of symbiont genes

Forward and reverse primers (Table 1.1), along with 50ng of leafhopper template DNA were added to a PCR reaction as described above. The cycling conditions designed to add a poly-A tail to the fragments to be cloned into vectors were as follows: 5 minutes at 95°C, followed by 40 cycles of 1 minute at 95°C, 1 minute at 55°C and 3 minutes at 72°C, with a final extension step of 5 minutes at 72°C. The PCR products were cloned using a TOPO TA chemically competent vector kit (Invitrogen, Grand Island, NY), following the manufacturer's directions. Individual colonies were subjected to colony PCR, where one individual colony was added to a PCR reaction instead of total DNA, and amplified as described above. The resulting PCR products from 20 individual colonies were digested with the restriction enzymes EcoRI and HinDIII and visualized on a 1% agarose gel stained with ethidium bromide to check for different banding patterns.

PCR products from all of the primer sets and selected clones were sequenced using an ABI Prism Big Dye v3.1 sequencing kit (Applied Biosystems, Grand Island, NY), following the manufacturer's instructions. Sequencing reactions were performed on a 3730xl DNA Analyzer (Applied Biosystems, Grand Island, NY) at the University of Maryland Genomics Core Facility. Sequencing reactions were purified prior to

sequencing by adding 1μL 125mM EDTA, 1μL 3M NaOac and 25μL 100% EtOH to each well of a skirted plate (Applied Biosystems, Grand Island, NY) containing the sequencing reaction mix and template. The plate was then incubated at room temperature for 15 minutes, spun at 1650g for 45 minutes at room temperature and inverted onto paper towels and spun at 185g for 2 minutes. The reactions were then washed with 70μL 70% EtOH, spun at 1650g for 15 minutes at room temperature, then inverted onto paper towels and spun at 185g for 2 minutes. 20μL of Hi-Di formamide (Invitrogen, Grand Island, NY) was added to each well, and the plate was sealed with a septa seal cover and loaded onto the sequencer.

Phylogenetic analysis

The sequences obtained by both the forward and reverse primer sets were assembled and checked by hand. The assembled potato leafhopper symbiont sequences were then compared to known insect bacterial symbiont sequences in GenBank using blastn (http://blast.ncbi.nlm.nih.gov). The top blastn hits with 95% or greater identity to the potato leafhopper symbiont sequences were downloaded, and ClustalW (Thompson et al. 1994) was used to create multiple alignments. The multiple alignments were then used to create maximum likelihood (ML) trees using Seaview v.4.4.2 (Guo et al. 2010) using the GTR model with 1500 bootstrap resamplings. Bootstrap values greater than 50 were reported on the phylogenetic tree. For the phylogeny of *Sulcia muelleri*, the Blattobacterium symbiont of *Mastotermes darwiniensis* (GenBank accession number Z35665.1) was used as an outgroup, as described by Moran et al. (2005b). The 16S rDNA (GenBank accession AF397408.1), and FtsZ (GenBank accession JN616286.1)

gene sequences of the *Wolbachia* symbiont of *Aedes albopictus* were used as outgroups for the *Wolbachia* 16S rDNA and FtsZ phylogenies, respectively.

Microscopy

The florescent *in situ* hybridization procedure described here is a modified version of that reported by Tsuchida et al. (2004). To detect *Wolbachia* in the bacteriome, salivary gland and midgut tissue of the potato leafhopper, the modified primer FtsZFm was high-performance liquid chromatography purified and 5' end-labeled with TAMRA (FtsZFm; 6-carboxytetramethylrhodamine [TAMRA]-5'-GTTGTCGCAAATACCGATGC -3'). This primer was designed to specifically bind to the FtsZ gene. Likewise, the modified primer 10Fm (10Fm; Fluorescein isothiocyanate-5'-TTGATCATGGCTCAGA-3') was 5' labeled with fluorescein isothiocyanate (FITC) in order to detect *Sulcia* in potato leafhopper tissue. The nuclei of potato leafhopper cells were counterstained with DAPI (4',6'-diamino-2-phenylindole).

The bacteriome, salivary gland and midgut tissue of adult potato leafhoppers was dissected as described above, then placed onto a silane-coated slide and allowed to air-dry. Hybridization buffer (150µL; 20mM Tris-HCl pH 8, 0.9M NaCl, 0.01% SDS, 30% formamide, 50pmol/mLeach of the probes 10Fm and FtsZFm, and 0.5µg/mL DAPI) was added to each slide. Slides were incubated at room temperature in a dark humidified chamber overnight, then washed with 150µL washing buffer (20mM Tris-HCl pH 8, 0.9M NaCl, 0.01% SDS, 30% formamide) for 10 minutes. Slides were then washed three times with 150µL 1X SSC (0.015M sodium citrate, 0.15M NaCl), and a drop of ProLong Gold Antifade Reagent (Molecular Probes, Invitrogen, Grand Island, NY, USA) was added to each tissue sample. The tissue samples were covered with a glass cover slip,

which was sealed by applying clear nail polish to the cover slip edges. To confirm the specificity of the results, a no-probe control and a competitive suppression control containing excess unlabelled probe (at a concentration of 30nmol/mL) were run in parallel with the labelled probe slides.

Slides were viewed with a Zeiss LSM 710 Confocal Microscope at the University of Maryland's Department of Cell Biology and Molecular Genetics Imaging Core Facility using a 63x oil immersion lens (1.4 Oil DIC Plan-Apochromat). The images were processed (cropped, annotated and adjusted for brightness and contrast) using Zeiss Zen 2009 LE software.

Results

Symbiont presence/absence

I used primer sets designed specifically to amplify DNA from *Baumannia* (10F and 1507R), *Sulcia* (10FF and 1370R) and *Wolbachia* (FtsZF1 and FtsZR1). All three primer sets amplified DNA from the salivary glands, midguts and bacteriomes of the potato leafhopper (Figures 1.1, 1.2). The two negative controls (water and hind femur) did not yield bands with these primer sets. The universal eubacterial primer set groF and groR did not yield bands in any of the tissue samples tested (Figure 1.2).

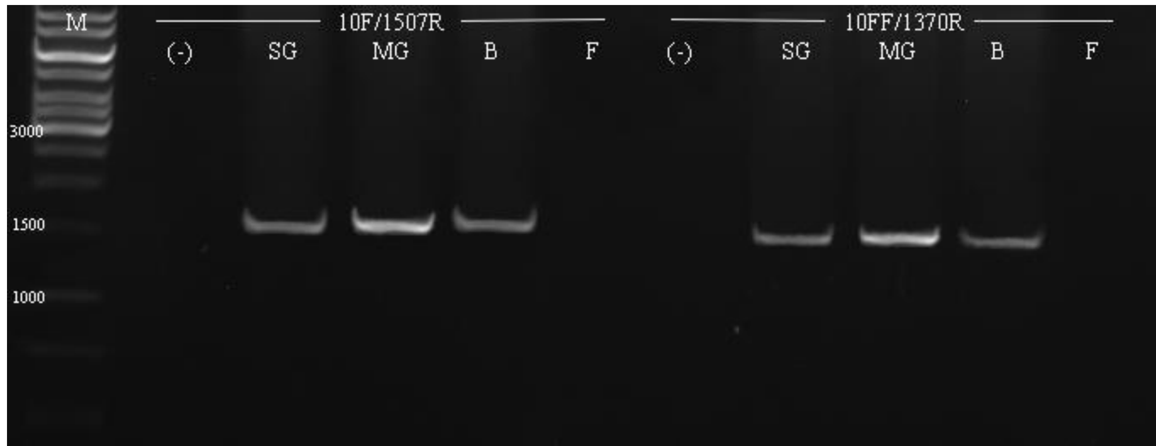

Figure 1.1: Specific amplification of bacterial symbionts from potato leafhopper, *Empoasca fabae* tissues using primers designed to detect *Baumannnia cicadellinicola* (10F/1507R) and *Sulcia muelleri* (10FF/1370R). M: Marker, (-): negative water control, SG: salivary glands, MG: midgut, B: bacteriome, F: hind femur.

Figure 1.2: Amplification of bacterial symbiont genes from potato leafhopper, *Empoasca fabae,* tissues using primers designed to detect *Wolbachia* FtsZ (FtsZF/FtsZR) and eubacteria groEL (GroF/GroR). M: Marker, (-): negative water control, SG: salivary glands, MG: midgut, B: bacteriome, F: hind femur.

Restriction enzyme digestion of bacterial clones created using rDNA obtained from the primer set 10F and 1507R yielded a single distinct pattern, and blastn analysis of the sequenced rDNA showed that the sequence was most closely related to the *Wolbachia*

symbiont of the spittlebug, *Philaenus maghresignus* (GenBank accession number AB772263.1, 99% identity, E value 0) (Tables 1.2, 1.3). The top BLASR hits for the *Wolbachia* 16S rDNA gene sequence obtained from the potato leafhopper were from the *Wolbachia* superfamily B, so the 16S rDNA sequence from the *Wolbachia* symbiont of *Aedes albopictus* (GenBank accession AF397408.1), which is in *Wolbachia* superfamily A, was used to root the tree in future phylogenetic analysis.

TGGCGGCAGGCCTAACACATGCAAGTCGAACGGAGTTATATTATAGCTTGCTATG GTATAACTTAGTGGCAGACGGGTGAGTAATGTATAGGAATCTACCTAGTAGTACG GAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCCTACGGGGGAAAAATT TATTGCTATTAGATGAGCCTATATTAGATTAGCTAGTTGGTGGAGTAATAGCCTAC CAAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGCCACACTGGAACTGAG ATACGGTCCAGACTTCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGA AAGCCTGATCCAGCCATGTCGCATGAGTGAAGAAGGCCTTTGGGTTGTAAAGCTCT TTTAGTGAGGAAGATAATGACGGTACTCACAGAAGAAGTCCTGGCTAACTCCGTG CCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAATTATTGGGCGTA AAGGGCGCGTAGGCTGATTAATAAGTTAAAAGTGAAATCCCGAGGCTTAACCTTG GAATTGCTTTTAAAACTATTAATCTAGAGATTGAAAGAGGATAGAGGAATTCCTG ATGTAGAGGTAAAATTCGTAAATATTAGGAGGAACACCAGTGGCGAAGGCGTCTA TCTGGTTCAAATCTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGA TACCCTGGTAGTCCACGCTGTAAACGATGAATGTTAAATATGGGAAGTTTACTTTC TGTATTACAGCTAACGCGTTAAACATTCCGCCTGGGGACTACGGTCGCAAGATTAA AACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTC GATGCAACGCGAAAAACCTTACCACTTCTTGACATGGAAATCATACCTATTCGAAG GGATAGGGTCGGTTCGGCCGGATTTTACACAGGTGTTGCATGGCTGTCGTCAGCTC GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCCTTAGTTG CCATCAGGTAATGCTGAGCACTTTAAGGAAACTGCCAGTGATAAGCTGGAGAAGA TGGGGATGATGTCAAGTCATCATGGCCTTTATGAAGTGGGCTACACACGTGCTACA ATGGTGTCTACAATGGGCTGCAAGGTGCGCAAGCCTAAGCTATCCCTAAAGACAC TCTCAGTTCGATTGAACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAAT CGTGGATCAGCATGCCACGGTGAATA

Table 1.2: 1300bp fragment of *Wolbachia* 16S rDNA gene sequence obtained from potato leafhopper.

Accession	**Insect host**	**Identity**	**Query cover**	**E value**	**Max score**
AB772263.1	*Philaenus maghresignus*	99.46	1304	0	2364
AB772260.1	*Aphrophora quadrinotata*	99.39	1304	0	2359
GU124506.1	*Nilaparvata lugens*	99.31	1304	0	2353
JQ726767.1	*Nysius expressus*	99.23	1304	0	2348
JQ726770.1	*Kleidocerys resedae*	99.31	1301	0	2348
M84686.1	*Nasonia vitripennis*	98.7	1304	0	2309
EF433793.1	*Diaphorina citri*	98.62	1304	0	2305
JX281793.1	*Aleurocanthus woglumi*	98.62	1304	0	2303
GQ206310.1	*Sogatella furcifera*	98.62	1304	0	2303
EU499317.1	*Bryobia praetiosa*	98.54	1304	0	2298
JN204506.1	*Bemisia tabaci*	98.54	1304	0	2298
DQ412081.1	*Drosophila pseudoananassae*	98.47	1304	0	2292
EF433794.1	*Diaphorencyrtus aligarhensis*	98.47	1304	0	2292
DQ115538.1	*Pseudolynchia canariensis*	98.47	1304	0	2292
NR_074127.1	*Culex quinquefasciatus*	98.39	1304	0	2292

Table 1.3: Top BLAST hits for potato leafhopper *Wolbachia* 16S rDNA gene.

Likewise, restriction enzyme digestion of PCR products amplified with the primer set 10FF and 1370R gave a single restriction pattern. DNA obtained from PCR amplification using 10FF/1370R was sequenced, and was most similar to the *Sulcia muelleri* symbiont of the native United States leafhopper, *Jikradia olitoria* (GenBank accession number AY676913.1 (Tables 1.4, 1.5).

Restriction enzyme digestion of clones obtained using the primer set FtsZF1 and FtsZR1 resulted in a single restriction pattern. The sequenced DNA was most closely related to the *Wolbachia* symbiont of the mulberry leafhopper, *Hishimonoides sellatiformis* (GenBank accession number AB073734.1, 99% identity, E value 0) (Tables 1.6, 1.7). The top BLAST hits for the *Wolbachia* ftsZ gene sequence obtained from the potato leafhopper were from the *Wolbachia* superfamily B, so the ftsZ sequence from the

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TGATCATGGCTCAGGAAAAACGCTAGCGGAGGGCTTAACACATGCAAGTCGAGGG
GCAGCAAATATAATATACGTTAACTTATGTATTTTGGCGACCGGCAAACGGGTGA
GTAATACATACGTAACTTTCCTTATGCTGAGGGATAGCCTGAGGAAACTTGGATTA
ATACCTCATAATACAATATTTTTATAAATATTGATAAAGTTTATTATGGCATAAGA
TAGGCGTATGCCCAATTAGTTAGTTGGTAAGGTAATGGCTTACCAAGACTATGATT
GGTAGGGGGCCTGAGAGGGGCGTTCCCCCACATTGGTACTGAGACACGGACCAAA
CTTCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGAGGAAACTCTGAACCA
GCCAGTCCGCGTGCAGGATGAAAGCCTTATTGGTTGTAAACTGCTTTTGTGTATGA
ATAAAAAATTCTAATTAAAGAAATAATTGAAGGTATTATACCAATAAGTATCGGC
AAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATACAAGCGTTATCCGGATTT
ATTGGGTTTAAAGGGTGCGTAGGCGGTTTTTAAGTCAGTAGTGAAATCTTAAAGCT
TAACTTTAAAATTGCTATTGATACTGAGAAACTAGAGTTAGTTTGGGGTAGCTGGA
ATGTGTGGTGTAGCGGTGAAATGCGTAGATATCACACAGAACACCGATAGCGAAA
GCAGGTTACTAAGCCTATACTGACGCTGAGGCACGAAAGCTTGGGGAGGAAACAG
GATTAGATACCCTGGTAGTCCATGCTGTAAACGATGATCACTGACTATTGGATTTT
GTATGTTGTAATTCAGTGGTTAAGCGAAAGTGTTAAGTGATCCACCTGAGGAGTAC
GACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAATCGGTGGAGC
ATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCATGACTTAAATGTACTACG
AATAAATTGGAAACAATTTAGTCTTACCACGGAGTACAAGGTGCTGCATGGTTGTC
GTCAGCTCGTGCCGTGAGGTGTAAGGTTAAATCCTTAAACGAGCGCAACCCTTATT
ATTAGTTGCCATCGAGTAATGTTGGGGACTCTAATAAGACTGCCGGCGCAAGCCG
AGAGGAAGGTGGGGACGACGTCAAATCATCACGGCCCTTACGTCTTGGGCCACAC
ACGTGCTACAATGGTCGCTACAAAGGGAGCGACTGGGTGACCAG
```

Table 1.4: 1265bp fragment of *Sulcia muelleri* 16S rDNA gene sequence obtained from potato leafhopper.

Wolbachia symbiont of *Aedes albopictus* (GenBank accession JN616286.1), which is in *Wolbachia* superfamily A, was used to root the tree in future phylogenetic analysis.

These results obtained from sequencing the 16S rDNA and ftsZ gene fragments that were amplified with the primer sets 10F/1507R, 10FF/1370R and FtsZF/FtsZR suggest that the potato leafhopper harbors two species of symbiotic bacteria: *Sulcia muelleri* and *Wolbachia*. As the primers amplified sequences from the salivary gland, midgut and bacteriome tissue of the potato leafhopper, it is likely that both of these symbionts are present in all three tissue types. Symbionts are not known to be present in insect femur tissue, so the hind femurs of the potato leafhopper were used as a negative control when screening for symbionts. The primers sets used in this study did not amplify DNA from the hind femur tissue of the potato leafhopper, suggesting that the symbionts

Accession	Insect host	Identity	Query cover	E value	Max score
AY676913.1	*Jikradia olitoria*	98.58	1269	0	2241
AY676897.1	*Helochara communis*	95.63	1282	0	2039
AY676911.1	*Pagaronia tredecimpunctata*	96.52	1235	0	2032
NR_102796.1	*Homalodisca vitripennis*	95.48	1282	0	2026
AY676915.1	*Cicadella viridis*	95.33	1286	0	2023
AY676903.1	*Acrogonia virescens*	95.32	1281	0	2017
AY676908.1	*Pamplona spatulata*	95.63	1258	0	2001
AY676900.1	*Paraulacizes irrorata*	96.11	1234	0	1999
AY676910.1	*Diestostemma* sp.	96.03	1234	0	1993
AY676907.1	*Cyrtodisca major*	95.08	1281	0	1993
JQ898318.1	*Matsumuratettix hiroglyphicus*	94.97	1272	0	1984
AY676902.1	*Homoscarta irregularis*	95.79	1236	0	1978
AY676906.1	*Proconosama alalia*	95.57	1241	0	1967
AY676904.1	*Diestostemma stesilea*	95.92	1224	0	1967
DQ066642.1	*Philya ferruginosa*	95.62	1234	0	1965
DQ066641.1	*Publilia modesta*	95.62	1233	0	1965

Table 1.5: Top BLAST hits for potato leafhopper *Sulcia muelleri* 16S rDNA gene.

TTATGTGATAAAAAAATTCACTTGGTATCAACTTAACTAAGGGTCTTGGTGCTGGT GCTTTGCCTGATGTTGGCAAAGGTGCAGCAGAAGAATCAATCGATGAAATTATGG AGCATATAAAAGATAGCCATATGCTCTTTATCACAGCAGGGATGGGTGGTGGTAC TGGAACAGGTGCTGCACCGGTAATTGCAAAAGCAGCCAGAGAAGCAAGAGCGGT AGTTAAAGATAAAGGAGCAAAAGAAAAAAAGATACTGACTGTTGGAGTTGTAACT AAGCCGTTCGGTTTTGAAGGTGTGCGACGTATGCGCATTGCAGAACTTGGACTTGA AGAGTTGCAAAAATACGTAGATACACTTATTGTCATTCCCAATCAAAATTTATTTA GAATTGCTAATGAGAAAACTACATTTGCTGACGCATTTCAACTCGCCGATAATGTT CTGCATATTGGCATAAGAGGAGTAACTGATCTGATGGTTATGCCAGGGCTTATTAA CCTTGATTTTGCTGATATAGAAACAGTAATGAGTGAGATGGGTAAAGCAATGATT GGTACTGGAGAGGCAGAAGGAGAAGATAGGGCAATTAGTGCTGCAGAGGCTGCG ATATCTAATCCATTACTTGATAATGTATCAATGAAAGGTGCACAAGGAATATTAAT TAATATTACTGGTGGTGGAGATATGACTCTATTTGAGGTTGATTCTGCAGCCAATA GAGTGCGTGAAGAAGTAGATGAAAACGCAAATATAATATTTGGTGCCACTTTTGA TCAGGCGATGGAGGGAAGAGTTAGAGTTTCTGTTCTTGCAACTGGCATTGATAGCT GTAACAACAATTCATCTGTTAATCAAAACAAGATCCCAGCAGAGGAAAAAAATTT TAAATGGCCTTATAATCAAATTCCAATATCAGAAACAAAAGAATATGCTTCAACTG AGCAACAAACGAAAGGGTTAAGTGGGGCAGCAATGTTATGATATACCAGCTTATC TAAGAAG

Table 1.6: 1004bp fragment of *Wolbachia* FtsZ gene sequence obtained from potato leafhopper.

Accession	Insect host	Identity	Query cover	E Value	Max Score
AB073734.1	*Hishimonoides sellatiformis*	99.69	983	0	1797
AB045315.1	*Franklinothrips vespiformis*	99.19	993	0	1788
AJ271201.1	*Acraea equitorialis*	99.18	971	0	1751
U28197.1	*Sitophilus oryzae*	99.68	945	0	1729
U28196.1	*Encarsia formosa*	99.58	945	0	1724
DQ256473.1	*Lissorhoptrus oryzophilus*	97.76	983	0	1692
HQ404789.1	*Bemisia tabaci*	97.56	983	0	1691
HQ404765.1	*Macrosteles fascifrons*	97.56	983	0	1691
HQ404753.1	*Nilaparvata muiri*	97.56	983	0	1691
AB073733.1	*Sogatella furcifera*	97.56	983	0	1691
AB039038.1	*Laodelphax striatellus*	97.38	993	0	1688
AB073733.1	*Hishimonoides sellatiformis*	97.38	993	0	1688
AB039280.1	*Elenchus japonicus*	97.38	993	0	1688
HQ404789.1	*Tetranychus urticae*	97.46	983	0	1676
AB078034.1	*Hishimonus sellatus*	97.56	983	0	1691

Table 1.7: Top BLAST hits for potato leafhopper *Wolbachia* FtsZ gene.

present in the leafhopper are not found in the hind femurs. In addition, the primer set 10F/1507R, which was reported to specifically amplify *Baumannia cicadellinicola* 16S rDNA by Takiya et al. (2006), amplified *Wolbachia* DNA. Therefore, the primer set 10F/1507R should not be considered to be a diagnostic primer set for *Baumannia cicadellinicola* 16S rDNA in all leafhopper species. *Baumannia cicadellinicola* has only been reported in the leafhopper subfamily Cicadellinae, so it was not expected to be present in the potato leafhopper. Leafhoppers from all three colonies had both *Sulcia* and *Wolbachia* present in their salivary gland, midgut and bacteriome tissue, and tested negative for symbionts in their hind femur tissue.

Phylogenetics of potato leafhopper symbionts

Maximum likelihood phylogenetic analysis of the *Sulcia* symbiont 16S rDNA showed that the potato leafhopper symbiont was closely related to the *Sulcia* symbionts of other leafhopper species (Figure 1.3). The potato leafhopper *Sulcia* symbiont was located in a strongly supported clade (93% of 1500 bootstrap replicates) with the *Sulcia muelleri* symbiont of the cicadellid leafhopper *Jikradia olatoria*, also native to the United States. The *Sulcia* symbiont detected in the potato leafhopper was also closely related to other leafhopper *Sulcia* symbionts, including the *Sulcia muelleri* symbiont of the glassy-winged sharpshooter, *Homolodisca vitripennis*.

Phylogenetic analysis of the *Wolbachia* 16s rDNA gene (Figure 1.4) grouped the potato leafhopper symbiont with the *Wolbachia* symbionts of the brown planthopper (*Nilaparvata lugens*), the four-spotted spittlebug (*Aphrophora quadrinotata*) and the spittlebug *Philaenus maghresignus*. The 16s rDNA phylogeny of the *Wolbachia* symbiont of the potato leafhopper grouped it most closely with the *Wolbachia* symbionts of other sap-feeding auchenorrhynchans.

Phylogenetic analysis of the FtsZ gene (Figure 1.5) of the *Wolbachia* symbiont present in the potato leafhopper showed that it is most closely related to the *Wolbachia* symbionts of the mulberry leafhopper (*Hishimonoides sellatiformis*), the vespiform thrips (*Franklinothrips vespiformis*), and the rice weevil (*Sitophilus oryzae*). *Hishimonoides sellatiformis*, is a species of leafhopper that is native to Japan, and which vectors mulberry dwarf phytoplasmas (Kawakita et al. 2000). Similar to the results obtained from the potato leafhopper, *Wolbachia* (superfamily B) has been detected in the salivary glands, intestines and bacteriomes of the mulberry leafhopper (Mitsuhashi et al. 2002).

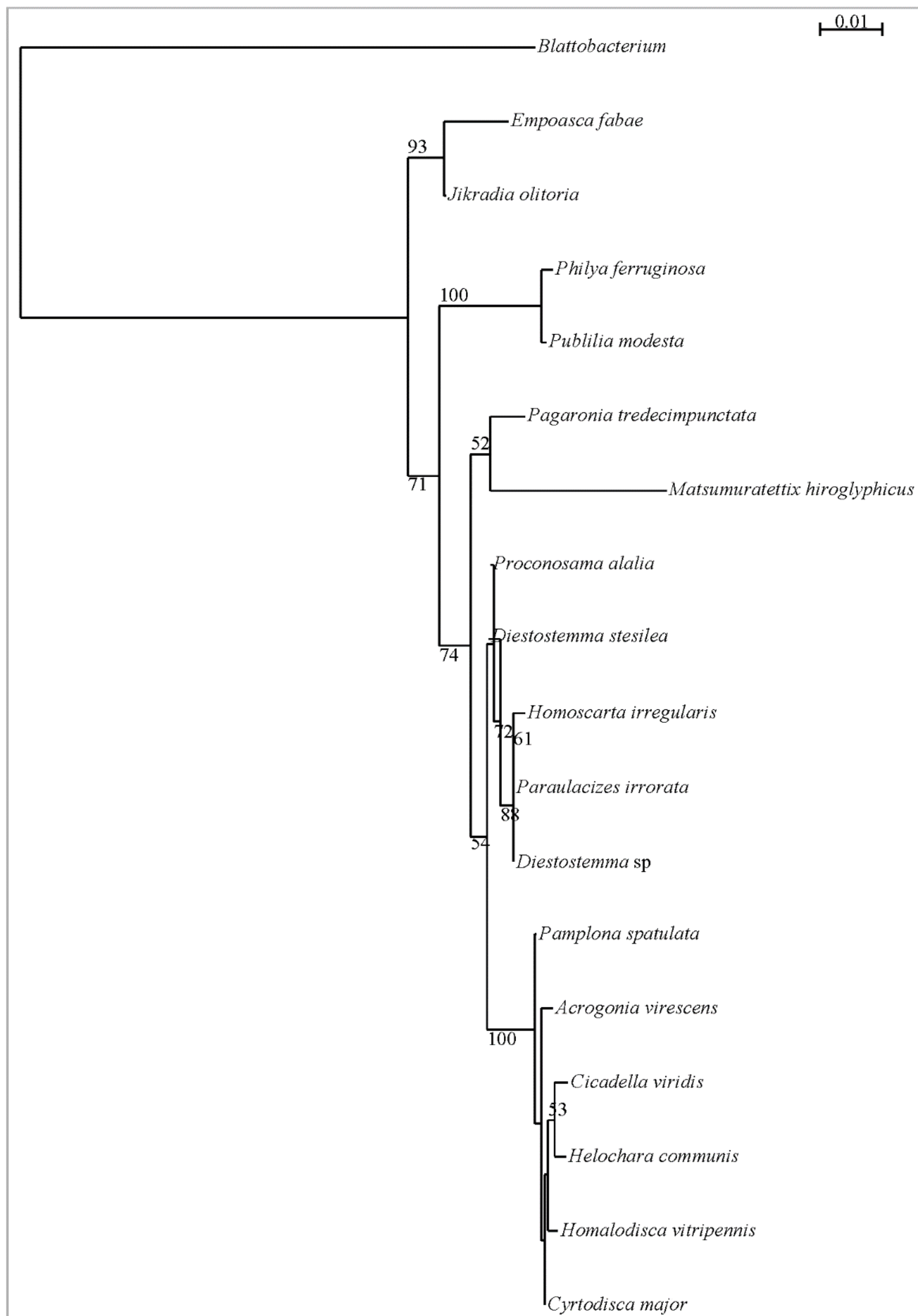

Figure 1.3: Maximum likelihood phylogenetic analysis of partial 16S rRNA sequences of *Sulcia* obtained from *Empoasca fabae* and sequences deposited in GenBank. Host species are included for each tree entry. Numbers indicate support greater than 50% for each clade.

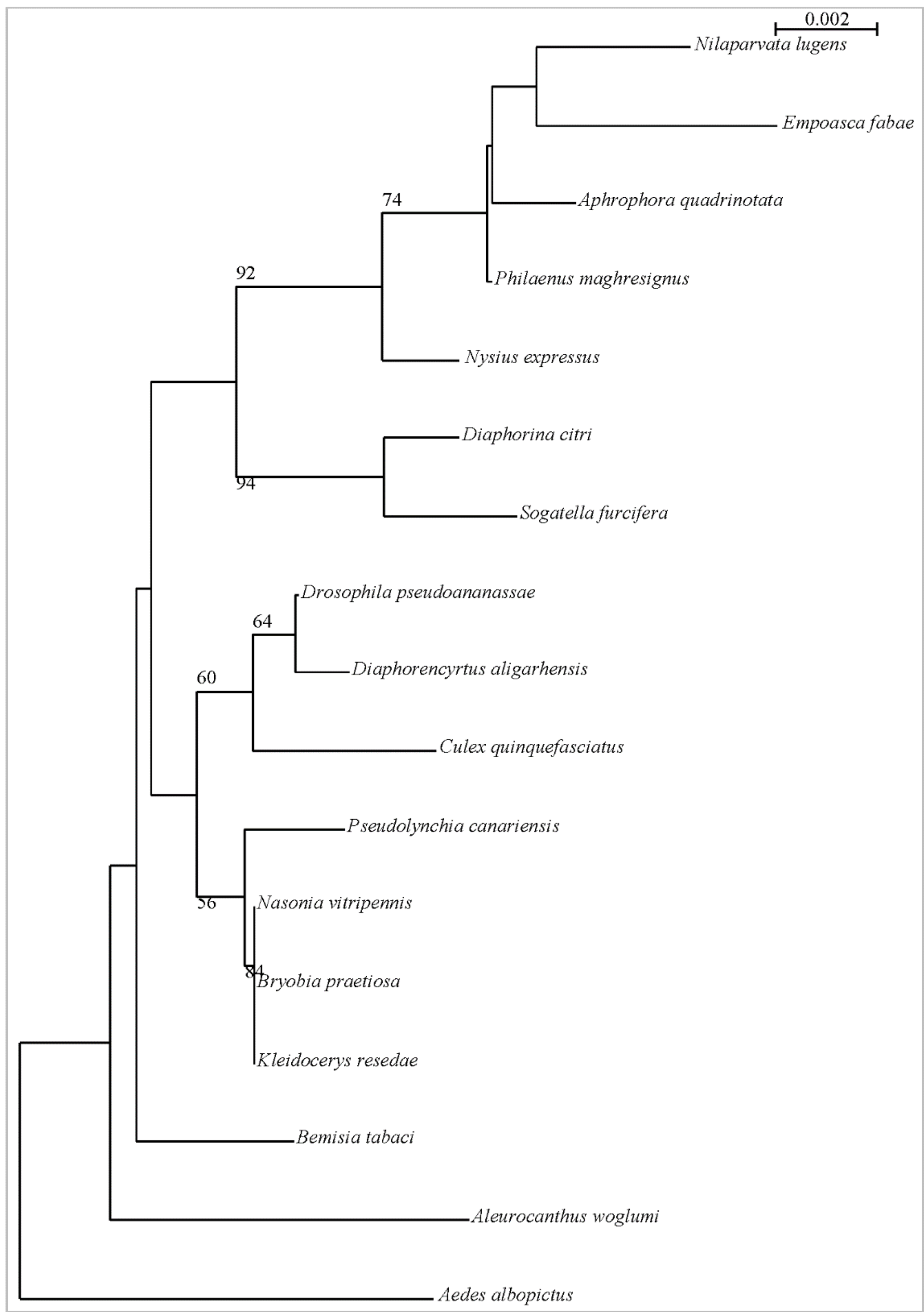

Figure 1.4: Maximum likelihood phylogenetic analysis of partial 16S rRNA sequences of *Wolbachia* obtained from *Empoasca fabae* and sequences deposited in GenBank. Host species are included for each tree entry. Numbers indicate support greater than 50% for each clade.

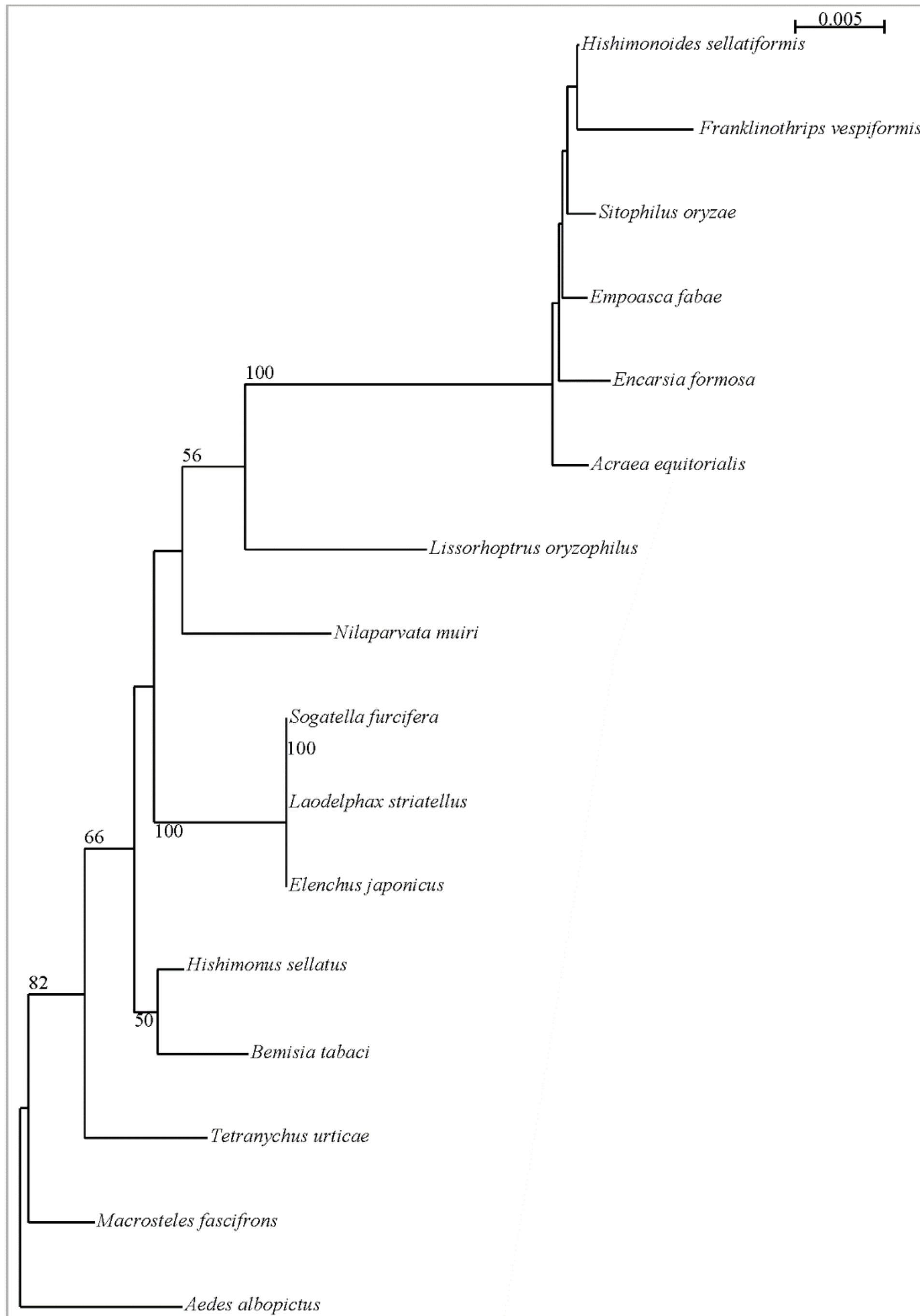

Figure 1.5: Maximum likelihood phylogenetic analysis of partial FtsZ sequences of *Wolbachia* obtained from *Empoasca fabae* and sequences deposited in GenBank. Host species are included for each tree entry. Numbers indicate support greater than 50% for each clade.

Phylogenetic analysis of the *Wolbachia* symbiont of the mulberry leafhopper yielded a similar branching pattern grouping the leafhopper symbiont with the *Wolbachia* present in the nymphalid butterfly, *Acraea equitorialis* and the parasitoid *Encarsia formosa*.

Microscopy

Florescent *in situ* hybridization of potato leafhopper tissues showed that *Sulcia* and *Wolbachia* are present in the salivary glands, midgut and bacteriomes of the potato leafhopper (Figure 1.6). The specificity of the probes was verified through no probe and

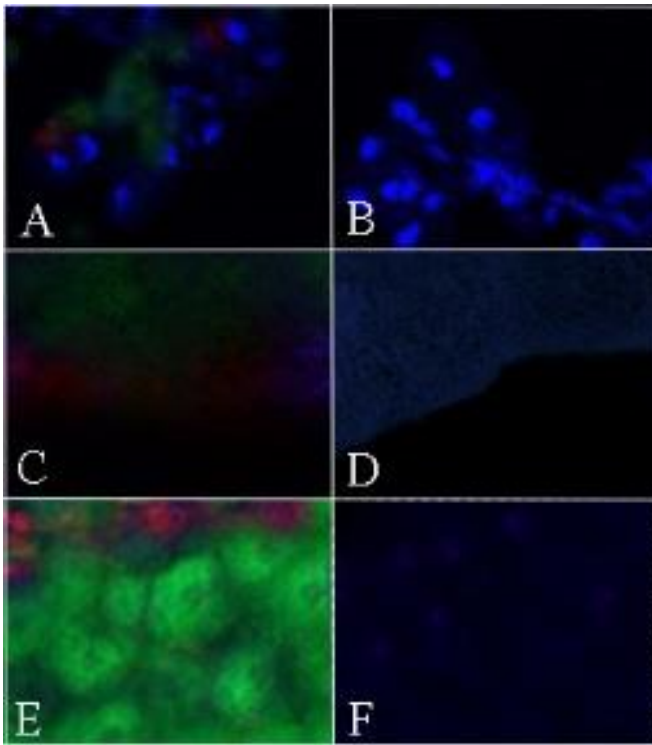

Figure 1.6: Florescent *in situ* microscopy of potato leafhopper, *Empoasca fabae*, tissues. A, B: Salivary glands; C, D: Midgut; E, F: Bacteriome. For A, C, E: red color corresponds to Wolbachia, green color corresponds to *Sulica* muelleri, and blue color corresponds to DAPI stained host nuclei. For B, D, F: Competitive suppression negative control, blue color corresponds to DAPI stained host nuclei.

competitive suppression controls. In the salivary gland tissue, individual lobes were characterized by enlarged cells. *Wolbachia* and *Sulcia* appeared to be located in separate

lobes, with only one taxa of bacteria present per cell. The midgut tissue showed larger areas of florescence corresponding to *Sulcia* than to *Wolbachia. Wolbachia* appeared to be present in small clumps near the exterior of the tissue, while *Sulcia* was pervasive throughout the midgut. The bacteriome tissue appeared to be comprised of enlarged cells, each of which housed a single type of symbiont. The majority of the bacteriome tissue housed *Sulcia*, with smaller areas devoted to *Wolbachia*. The no probe and competitive suppression controls did not exhibit red or green florescence.

Discussion

Many species of phloem feeding insects are hosts to symbiotic bacteria (Douglas 2005). The potato leafhopper feeds on phloem, so it is also likely host to symbiotic bacteria, but there have been no previous studies investigating the presence of symbionts in this species. Therefore, I screened the salivary glands, midguts and bacteriomes of the potato leafhopper for the presence of symbiotic bacteria. Two taxa of symbionts, *Sulcia muelleri* and *Wolbachia*, were found in the salivary glands, midguts and bacteriomes of the potato leafhopper. Both of these species have been reported in other cicadellid leafhoppers (Wu et al. 2006), so their presence in the potato leafhopper was not surprising. *Baumannia insecticola*, a symbiont which has only been reported in the subfamily Cicadellinae (Moran et al. 2005b), was not found in the potato leafhopper. The diagnostic primers 10F/1507R designed to amplify *Baumannia* 16S rDNA instead amplified the 16S rDNA of *Wolbachia* in potato leafhopper tissue, suggesting that these primers are not suitable as diagnostic primers for all species of cicadellids. Below, I discuss the rationale behind the experiments used in the chapter, importance of symbiosis

in leafhoppers, how the symbionts present in the potato leafhopper may contribute to its ability to feed on phloem, and how knowledge of the potato leafhopper symbionts may be used for future pest management strategies.

Experimental rationale

This chapter screened leafhoppers for symbionts using primers designed to detect the 16S rDNA gene of eubacteria. Many previous studies have used the 16S rDNA gene to identify insect symbionts, resulting in a large number of insect symbiont 16S rDNA sequences being deposited into GenBank (Moran et al. 1993, Takiya et al. 2006, Wangkeeree et al. 2012, Woyke et al. 2010). This gene is highly conserved, with a divergence rate between paired lineages of 2-4% per 50 million years in *Buchnera* (Moran et al. 1993). The leafhopper symbionts *Baumannia* and *Sulcia* have higher divergence rates than *Buchnera*, ranging from 7-19% per 50 million years (Takiya et al. 2006). Due to the relatively slow evolutionary rate exhibited by the 16S rDNA gene of insect bacterial symbionts, 98% or greater identity to known sequences in GenBank is necessary to identify a symbiont to species level (Bourtzis and Miller 2012). The 16SrDNA sequences of the two symbionts identified in the potato leafhopper in this chapter, *Sulcia* and *Wolbachia*, both displayed at least 98% identity to the 16S rDNA sequences of *Sulcia* and *Wolbachia* found in other insect species.

Although the 16S rDNA gene is commonly used to identify insect symbionts, the slow rate of evolution of this gene can make it difficult to resolve the phylogenetic relationships of closely-related strains of symbionts (Bourtzis and Miller 2012). One way to resolve this problem is to also sequence genes which are not as highly conserved, such as genes involved in cell division. *ftsZ* is a bacterial gene which forms the Z ring that

constricts to divide bacterial cells during binary fission (Erickson et al. 2010). This gene is not as highly conserved as the 16S rDNA gene, and has therefore been used to resolve the identity of *Wolbachia* strains in insects (Hinrich et al. 2000). To further confirm the identity of the two symbionts detected in the potato leafhopper, primers designed to detect *ftsZ* were used to amplify symbiont DNA from leafhopper tissue. Sequencing of the amplified DNA confirmed the identity of *Wolbachia* in the potato leafhopper, but did not result in the amplification of the *ftsZ* gene of *Sulcia*. Due to higher variation in the gene sequences of symbiont *ftsZ*, it was not surprising that the primers used in this study were unable to amplify the *ftsZ* gene of both symbionts.

In addition to screening leafhoppers for symbionts using primers designed to amplify 16S rDNA and *ftsZ* genes, a universal eubacterial primer designed to amplify a region of *groEL* was used. This primer set did not yield PCR products in any of the tissue samples tested. The *groEL* PCR reactions were performed at the same time as the other (successful) 16S rDNA and *ftsZ* reactions, using the same insect DNA samples, suggesting that the DNA and PCR reagents were not degraded. However, it is possible that the *groEL* primers used in the reaction were degraded. A positive control containing bacterial DNA known to be amplified by the *groEL* primer set would have ruled out primer degradation as a cause for the negative results obtained for this primer set in this study. To further improve the PCR screens used in this study, a potato leafhopper gene known to be expressed in all leafhopper tissue, such as a ribosomal gene, could have been used as an internal positive control for all of the reactions. PCR amplification using this internal control gene would have provided evidence that the femur tissue DNA used in this study was of good quality, and would have strengthened the negative results seen in

the hind femur tissue for the insect symbiont primer sets. The PCR reactions performed in this chapter all used TaKaRa ex Taq DNA polymerase, which is a proofreading DNA polymerase, to minimize sequencing errors.

Restriction enzyme digests were performed on plasmids that had leafhopper symbiont DNA inserts. There were two reasons for performing the restriction digests: confirmation that the plasmid contained leafhopper symbiont DNA, and identification of clones containing different leafhopper symbiont sequences. The TOPO TA plasmid has 3956 bps, and is cut twice by both EcoRI and HinDIII, the two restriction enzymes used in this experiment. If the TOPO TA plasmid has a properly inserted leafhopper symbiont gene fragment, it would be cut at least twice. For example, if the plasmid contained symbiont DNA amplified with the primer set 10F/1507R, a restriction digest performed with EcoRI would yield a band of approximately 3940 bp (corresponding to the TOPO TA plasmid) and a band of approximately 1500 bp (corresponding to the 1500 bp fragment amplified by 10F/1507R), assuming that EcoRI does not cut the symbiont gene insert. If the digest only yields fragments of 3940 and 20 bps, then the plasmid did not contain the symbiont gene insert.

The restriction digests were also used to screen subclones for differences in symbiont gene inserts. If a primer set amplified the DNA of two insect symbionts, a restriction digest may show which bacterial colonies contained plasmids with different symbiont gene inserts. Different restriction digest results, such as variations in the length of DNA fragments or extra DNA fragments, would indicate that the symbiont gene inserts contain different restriction enzyme cut sites. Therefore, sequencing clones containing inserts with variant restriction enzyme cut sites would allow for the

identification of multiple symbionts whose genes are amplified with the same primer set. The restriction digests used in this study did not yield differences in restriction enzyme digests. One explanation for this result is that each primer set only amplified the gene from one species of bacteria. As 20 clones were screened with restriction enzymes for each primer set used, this is a likely explanation. If, however, the tissue contained high levels of one species of symbiont and low levels of a second species, the second species may not have been detected in the 20 clones tested. One way to make this study more robust would have been to subject more than 20 clones to restriction enzyme digestion. This study used two restriction enzymes to screen for differences in restriction patterns, but the use of more restriction enymzes may have resulted in the detection of variations in restriction patterns not present in the two digests used. The restriction enzyme digest protocol used in this experiment was similar to other published insect symbiont detection protocols, which use two restriction enzymes to test 10 clones (Kikuchi et al. 2005).

In situ hybridization was used to visualize *Sulcia* and *Wolbachia* in potato leafhopper tissue. A negative, no probe, control was used to determine if the florescence pattern detected was due to autoflorescence of the leafhopper tissue. Likewise, a competitive suppression negative control was also used to detect autoflorescence. These negative controls did not result in florescence, indicating that the results seen with the florescent probes were due to binding of the probes to the leafhopper tissue. Various insect symbiont studies use these two controls when performing *in situ* hybridization (Kikuchi et al. 20005, Marzorati et al. 2006, Takiya et al. 2006). However, it is possible that the florescent primers used in this study were able to bind non-specifically to the leafhopper tissue, yielded florescence that does not correspond to the presence of

symbionts. A second control containing fluorescently-labeled probe designed with the same base pairs as the specific probe, but with the base pairs randomly assembled, would control for non-specific binding of the specific probe. If the random control probe bound to the leafhopper tissue in the same pattern as the specific probe, then the florescence pattern could not be attributed to symbiont presence in the tissue.

Importance of symbiosis in leafhoppers

Leafhoppers have a wide variety of feeding habits, ranging from xylem and phloem feeding, to lacerating cells and ingesting the contents (Redak et al. 2004, Backus and Hunter 1989). Symbiosis is common among insects that feed on phloem and xylem due to the low levels of essential amino acids and vitamins in this plant tissue (Douglas 2005). Buchner (1965) referred to the Auchenorrhyncha, the suborder of Hemiptera containing cicadas, planthoppers, treehoppers and froghoppers, as “a fairyland of symbiosis”. Müller later hypothesized that the common ancestor of all Auchenorrhyncha became infected with a bacterium that he called the “a symbiont”, and that this symbiont co-evolved and was vertically transmitted within the Auchenorrhyncha, with some lineages losing the “a symbiont” or gaining additional symbionts (Müller 1962, Chang and Musgrave 1972, Houk and Griffiths 1980). This “a symbiont” was later identified as *Sulcia muelleri* by Moran et al (2005b), and was found to be present in various treehoppers, froghoppers, planthoppers, cicadas and leafhoppers.

Most of the published studies available on the symbionts of cicadellid leafhoppers focus on leafhoppers in the subfamily Cicadellinae (Cottret et al. 2010, McCutcheon et al. 2009, Moran et al. 2002, Moran et al. 2005b, Wu et al. 2006). These leafhoppers, commonly known as sharpshooters, feed primarily on plant xylem (Son et al. 2012).

Xylem has lower levels of sugars, vitamins, and amino acids than the phloem that the potato leafhopper feeds on, therefore making the presence of symbionts in xylem-feeding insects crucial for survival (McCutcheon and Moran 2007).

In the case of sharpshooters, two species of symbiotic bacteria, *Baumannia cicadellinicola* and *Sulcia muelleri*, inhabit the midgut and provide the amino acids and vitamins missing from the insect's diet of xylem (Wu et al. 2006). Of these two species of bacteria, *Baumannia* appears to be present only in sharpshooters, while *Sulcia* is found throughout Auchenorrhyncha (Moran et al. 2005b). The genomes of both of these species of bacteria have been sequenced, revealing that together these symbionts provide all 10 amino acids necessary for insect development, as well as vitamins and cofactors. *Baumannia* synthesizes B vitamins, biotin, folic acid and histidine, as well as cofactors necessary for the production of amino acids (Wu et al. 2006). *Sulcia* complements the genome of *Baumannia* by utilizing the cofactors produced by *Baumannia* to synthesize amino acids, as well as providing ubiquinone and menaquinone to *Baumannia* (Wu et al. 2006, McCutcheon and Moran 2007).

In addition to *Baumannia* and *Sulcia*, other species of symbiotic bacteria have been reported in leafhoppers. The American grapevine leafhopper, *Scaphoideus titanus*, harbors an unnamed symbiont in the genus *Asaia* in its midgut, salivary glands and oviducts (Gonella et al. 2012). This symbiont is transmitted vertically by the female smearing her eggs with the symbiont after oviposition, but can also be transmitted horizontally through feeding on contaminated plant tissue or through mating (Gonella et al. 2012). In addition to *Asaia*, the symbiont ST1-C (most closely related to "Candidatus Cardinium hertigii") has been reported in the midgut, salivary glands and fat body of *S.*

titanus (Marzorati et al. 2006, Bigliardi et al. 2006, Sacchi et al. 2008). Like *Asaia*, ST1-C can be transmitted horizontally due to its presence in the salivary glands. It also coexists in the salivary glands with the bacterium "Candidatus Phytoplasma vitis", which causes the grapevine disease known as Flaescence doree (Marzorati et al. 2006, Sacchi et al. 2008). This coexistence of a symbiont and a plant pathogen within the same insect tissue opens up the potential of genetically modifying the symbiont in order to disrupt transmission of a plant pathogen, a process known as paratransgenic control (Marzorati et al. 2006, Bextine et al. 2004).

Sulcia as a symbiont

This study found *Sulcia muelleri* present in the salivary glands, midgut and bacteriomes of the potato leafhopper. This corresponds to the "a symbiont" originally described by Müller, and later characterized by Moran et al (2005b). *Sulcia* has been reported in the gut, oviduct, ovarioles and bacteriomes of various leafhoppers (Wangkeeree et al. 2012, Woyke et al. 2010), so it was expected to be present in the gut and bacteriomes of the potato leafhopper. Previous studies have not looked for *Sulcia* in the salivary glands of its insect hosts, so the discovery of *Sulcia* in the salivary glands of the potato leafhopper is novel. Other leafhopper bacterial symbionts, such as the ST1-C symbiont of *Scaphoideus titanus*, have been reported in the salivary gland tissue (Marzorati et al. 2006) which allows them to be horizontally transmitted during insect feeding on plant tissue. Therefore, it is possible that the *Sulcia* present in the salivary glands of *E. fabae* may be injected into the insect's plant host during feeding, allowing for potential horizontal transmission of this symbiont and the potential for inducing a plant wound response.

The genome of *Sulcia* has been sequenced, which allows speculation about what role it plays in the biology of its host (Woyke et al. 2010). Pathways for leucine, valine, isoleucine, threonine, tryptophan, lysine and arginine biosynthesis are present in the *Sulcia* genome, which means that it supplies seven out of the ten amino acids essential for insect development (Wu et al. 2006). Although *Wolbachia* was also found in the potato leafhopper, it is unlikely that *Wolbachia* supplies the remaining three essential amino acids because the *Wolbachia* genomes that have been sequenced lack amino acid biosynthesis pathways (Wu et al. 2004, Foster et al. 2005). Therefore, it is possible that the potato leafhopper harbors an additional symbiont that was not detected by the universal bacterial primers used in this study. It is also possible that the ability of the potato leafhopper to feed on ruptured cell contents allows it to obtain all of the amino acids that it needs through its diet, and it does not need an additional symbiont to complement *Sulcia.* The wide host range of the potato leafhopper may also explain a lack of complementary symbionts, because the leafhopper is capable of moving from one host plant species to another in order to exploit the nutritional profiles of different plant species. In spite of its wide host range and varied feeding habits, the presence of *Sulcia* in all potato leafhopper individuals screened suggests that *Sulcia* is necessary for the survival of the leafhopper, a hypothesis that will be tested in chapter 2 of this dissertation.

Wolbachia as a symbiont

Wolbachia is estimated to be present in over 60% of insect species (Hilgenboecker et al. 2008). It has been reported in various insect tissues, including ovaries, testes, fat body, gut, salivary glands and bacteriomes (Zouache et al. 2009, Bian et al. 2010, Hosokawa et al. 2010). This study found *Wolbachia* present in the salivary

glands, midgut and bacteriomes of the potato leafhopper, although the role that this bacterium plays in the biology of the leafhopper is unknown. The literature on *Wolbachia* is divided over the classification of this bacterium as a symbiont – in some cases it seems to provide no benefit or to be detrimental to its host, whereas in others it provides resistance to diseases or manufactures nutrients for its host. One example of *Wolbachia's* deleterious effects on an insect population is through cytoplasmic incompatibility, where males infected with *Wolbachia* can only successfully reproduce with females that are infected with the same bacterial strain, but infected females can mate successfully with any male. Because *Wolbachia* is vertically transmitted, it is in the best interest of the bacterium to give a selective reproductive advantage to infected females, even if it is not providing a tangible benefit to its host (Landman et al. 2009). *Wolbachia* is also known to skew sex ratios through male killing. In *Drosophila bifasciata*, low temperatures result in higher *Wolbachia* populations in the insect host, which in turn are responsible for selectively killing male embryos. As the females transmit *Wolbachia* to the next generation, it appears that the bacterium alters the population's sex ratio to favor the gender that is responsible for its vertical transmission (Riparbelli et al. 2012). It is unknown if *Wolbachia* causes cytoplasmic incompatibility or male killing in leafhoppers.

Although *Wolbachia* infections can negatively affect an insect population, they can also be beneficial. For example, *Wolbachia* confers resistance to *Drosophila* C Virus, Nora Virus and Flock House Virus in *Drosophila melanogaster* without disrupting the reproductive biology of the host (Teixeira et al. 2008). It is also present in the bacteriomes of bed bugs, *Cimex lectularius*, where it provides its host with B vitamins that are lacking in human blood (Hosokawa et al. 2010). It is possible that the *Wolbachia*

strain present in the potato leafhopper could confer viral resistance to its host or be responsible for producing vitamins to supplement the leafhopper's diet.

Implications for insect pest management

The presence of symbionts in the potato leafhopper opens up the potential for the development of symbiotic control strategies. Insect pests can be managed through their symbionts in two possible ways: the destruction of symbionts necessary for the insects's survival, or the manipulation of symbionts which are involved in the insect's ability to damage plants (Douglas 2007). The glassy-winged sharpshooter, *Homalodisca vitripennis*, is a leafhopper that vectors Pierce's disease in grapes. Pierce's disease is caused by the bacterium *Xylella fastidiosa*, which resides in the sharpshooter's gut and is egested into the xylem during feeding (Backus et al. 2012). The bacterium *Alcaligenes xylosoxidans* var. *denitrificans* is present in the gut of the glassy-winged sharpshooter, and genetic manipulation of this bacterium allows it to produce toxins which kill *X. fastidiosa.* The modified symbiont is fed to the insect, and then spread to the plant through feeding, where it encounters the plant pathogen in the xylem (Bextine et al. 2004). Although the potato leafhopper is not known to vector plant pathogens, the ability of researchers to manipulate a gut symbiont of the glass-winged sharpshooter suggests that it would be possible to alter the symbionts of the potato leafhopper in a way that would negatively affect their survival.

Chapter II: Role of symbionts in the population ecology of the potato leafhopper

Abstract

The potato leafhopper, *Empoasca fabae*, feeds on a wide variety of plants, including agriculturally important legumes such as alfalfa, *Medicago sativa*. Using stylets, it probes into plant tissue to feed on phloem and cell contents. Like many insects that rely on phloem as a food source, the potato leafhopper hosts symbiotic bacteria. This study examines the role that symbionts play in the population ecology of the potato leafhopper. To study the effects of symbionts on the leafhopper's lifecycle, leafhoppers were first cured of their symbionts with 0.01% oxytetracycline-HCl in a 3% sucrose feeding solution. The resulting aposymbiotic leafhoppers were allowed to feed on two leguminous host plants: alfalfa and fava bean, *Vicia faba*. Aposymbiotic adults had a 38% and 39% shorter lifespan than normal adults on alfalfa and fava, respectively. Likewise, egg production was reduced 23% and 17% in aposymbiotic leafhoppers on alfalfa and fava bean, respectively. Fewer nymphs hatched from the eggs produced by aposymbiotic leafhoppers than by normal leafhoppers and the number of nymphs surviving into adulthood was also lower. These results suggest that symbiotic bacteria play an important role in the population ecology of the potato leafhopper, and therefore targeting the symbionts may be a novel way to decrease the damage done by this polyphagous pest.

Introduction

Many hemipteran insects, including the potato leafhopper, feed on the phloem of plants. Phloem contains low levels of vitamins and lipids, and has a high carbon to

nitrogen ratio. It also rarely contains the proper concentrations of amino acids for insect nutrition (Sasaki et al. 1991). For example, the fava bean plant has phloem that is dominated by the amino acid asparagine, which accounts for 72% of the amino acids in the phloem. The nine other essential amino acids combined account for only 8.2% of the amino acids present in the phloem, leading to an imbalance of essential amino acids. In fact, histidine is the only essential amino acid that is present in a higher concentration in phloem than it is in aphid tissue (Douglas 2005). The concentration of essential amino acids present in the phloem is not high enough to support the observed growth rate of many insects, and insects in the suborders Sternorrhyncha and Auchenorrhyncha possess intracellular symbiotic bacteria that produce the essential amino acids that are lacking in the insect's diet (Douglas 2005). Insect bacterial symbionts are obligate, vertically transmitted, and often have a greatly reduced genome (Russell et al. 2003). In many insects, the relationship between host and symbiont arose at the same time that differentiation into major taxonomic groups occurred (Moran et al. 2005a).

The most widely-studied insect symbiont is *Buchnera aphidicola*, the primary symbiont of aphids in the family Aphidoidea. The genome of *B. aphidicola* is highly reduced, as small as 450kb in some strains, and yet approximately 10% of the genome consists of amino acid biosynthesis pathways (Gil et al. 2002, Moran et al. 2005a). Operons for the production of methionine, tryptophan, threonine, isoleucine, leucine, valine and phenylalanine, amino acids which are not present in high enough concentrations in phloem for an aphid to survive, have been reported in *Buchnera* (Douglas 1998, Douglas and Prosser 1992, Febvay et al. 1995). In addition, *Buchnera* is also capable of producing riboflavin (Nakabachi and Ishikawa 1999). Although *Buchnera*

is able to synthesize amino acids, it has lost the regulatory genes responsible for the production of all amino acids except for methionine, suggesting that it is not able to tailor amino acid biosynthesis levels to changes in the aphid host's diet (Moran et al. 2005a).

In addition to the primary symbiont, many insect species harbor secondary symbionts. These symbionts are found in lower concentrations than the primary symbiont, and often complement the functions of the primary symbiont (Wille and Hartman 2009, Sandstrom et al. 2001). These secondary symbionts may supplement the amino acids produced by the primary symbiont, confer disease or parasitoid resistance or influence plant-insect interactions (Hosokawa et al. 2007, Scarborough et al. 2005, Oliver et al. 2003, Tsuchida et al. 2004). Although secondary symbionts are beneficial to their host, they are not always necessary for the host's survival (Oliver et al. 2003). Unlike primary symbionts, many secondary symbionts are horizontally as well as vertically transmitted, allowing them to move into different populations or species (Oliver et al. 2010).

Insects that are cured of their symbionts (aposymbiotic insects) through feeding on artificial diets containing antibiotics or injection of antibiotics into the hemolymph often have higher mortality and lower fecundity rates than untreated insects (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006). For example, experimental elimination of *Buchnera* from aphids results in a slower growth and increased mortality, but the addition of amino acids, particularly phenylalanine, to the aphids' diets allows them to develop normally (Douglas and Prosser 1992). Aposymbiotic pea aphids, *Acyrthosiphon pisum*, exhibit a decrease in embryo production in comparison to symbiotic aphids, due to a lack of phenylalanine and tryptophan in their diet (Douglas 1996). In addition to aphids,

aposymbiotic stink bugs, cockroaches, tsetse flies and parasitic wasps have been created to study the role that symbionts play in the population ecology of these insects (Prado and Almeida 2009, Brooks and Richards 1955, Nogge 1978, Dedeine et al. 2001).

Previous studies have shown that the potato leafhopper, *Empoasca fabae*, is host to the symbionts *Sulcia muelleri* and *Wolbachia* (Chapter 1 of this dissertation), although the role that they play in the population ecology of this species is unknown. The potato leafhopper is found throughout the United States, and is an important pest of many leguminous crops (Poos and Wheeler 1943). The potato leafhopper is a highly polyphagous species, and adults been reported to feed and reproduce on over 220 species of plants in 26 different families (Lamp et al. 1994). Although adults have an even wider feeding host range, females lay eggs on fewer species of plants, and nymphs are unable to develop on all of the known adult host plants (Lamp et al. 1984). Potato leafhoppers prefer to feed on the leaves and tender stems of their host plants, but will also occasionally attack fruit such as oranges (Poos and Wheeler 1943). Although adults will feed on both leaves and stems of plants, nymphs preferentially feed on the underside of leaves (Lamp et al. 2004). Like aphids, the potato leafhopper has piercing sucking mouthparts, which it uses to feed on plant phloem and occasionally on ruptured plant cells (Backus and Hunter 1989).

To date, the effect of symbionts on the biology of the potato leafhopper has not been studied. This study attempts to establish the role that bacterial symbionts, including *Sulcia muelleri* and *Wolbachia*, play in the longevity, egg production and nymph survival of the potato leafhopper. Establishing an obligate relationship between the potato

leafhopper and its bacterial symbionts would open up the possibility of developing symbiont-based control strategies for managing this economically important pest species.

Materials and methods

Plant culture

All plants used in this study were grown in the University of Maryland Greenhouse Research Complex. Broad Windsor fava bean, *Vicia faba*, seeds were planted 10cm pots containing Metro-Mix potting medium (Sun Gro Horticulture). Three seeds were planted in each pot, and flats containing 15 pots were placed into screen cages (Bioquip) sitting on greenhouse benches and watered three times per week. Three week old fava bean plants were used for maintaining leafhopper cultures, symbiont transmission, and leafhopper longevity and fecundity experiments.

'Ranger' alfalfa, *Medicago sativa*, seeds were planted in perlite and allowed to germinate in a mist room. Two week old seedlings were transplanted into 15cm pots containing Metro-Mix potting medium (Sun Gro Horticulture), and placed into screen cages sitting on greenhouse benches. Each pot was placed in a plastic tray to collect water during watering, and were thoroughly watered three times per week. Alfalfa plants were ready to use five weeks after transplanting.

Insect culture

Potato leafhoppers were originally collected with sweep nets from an alfalfa field at the Western Maryland Research and Education Center in Keedysville, Maryland. A continuous leafhopper culture was established by placing leafhoppers in collapsible screen cages (Bioquip) containing fava bean, *Vicia faba*, plants in an MB-60 plant growth chamber (Percival Scientific Inc., Boone, Iowa). The growth chamber was kept at a

constant 25°C, with 14 hours of light and 10 hours of dark for all experiments. Relative humidity in the chamber was kept at 80%, and the light intensity at plant height was 120 μmol/m^2/sec during the day. Each week, three-week-old fava bean plants were placed into the screen cages to replenish plants that were killed due to leafhopper feeding.

Week-old potato leafhopper adults were obtained by placing adult leafhoppers in a screen cage containing fava bean plants, and allowing the adults to lay eggs for three days. After this oviposition period, the plants were removed from the cage, shaken to displace any adult leafhoppers, and placed into a new screen cage. The eggs were allowed to hatch, and the resulting nymphs were allowed to mature into adults, a process which took approximately three weeks from oviposition to adult emergence. Four weeks after the original oviposition period, the new adults were collected for use in longevity and fecundity experiments.

Feeding cage construction

Feeding cages (Figure 2.1) were contructed using two Nunc Lab-Tek II eight chamber media slides (Thermo Scientific). To do this, first the glass slides were removed from each chamber apparatus, and then the plastic gaskets holding the glass slides to the chambers were removed. One eight chamber apparatus had small holes drilled into each chamber from the side, to allow leafhoppers to be aspirated into the assembled chamber. The same chamber apparatus also had fine-mesh Organza cloth glued to the bottom, both so that leafhoppers could not escape from the assembled chamber and to allow air flow. To assemble the feeding cage, a Parafilm membrane was stretched across the bottom of the unmodified chamber apparatus, and the eight chambers were filled with the feeding

solution (3% sucrose, for example). The filled chamber was placed on top of the modified chamber, which had the Organza mesh facing the bottom. The assembled

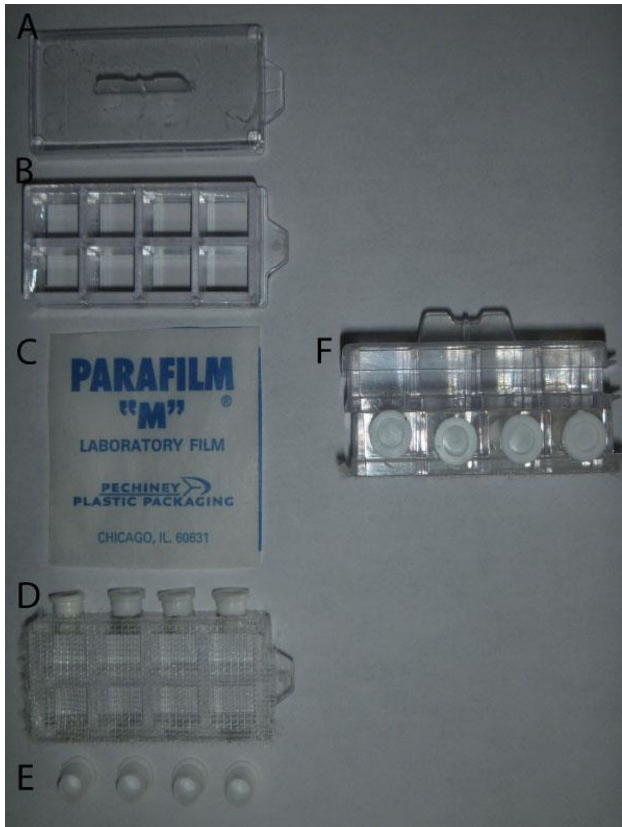

Figure 2.1: Expanded view of feeding cage. A: lid; B: divided chamber holding feeding solution; C: Parafilm membrane; D: divided chamber holding leafhoppers; E: Plugs for leafhopper chamber; F: fully assembled feeding cage, side view.

feeding cage was then topped with the chamber slide lid, and leafhoppers were aspirated into the individual chambers, which were sealed with plastic plugs.

Creation of aposymbiotic leafhoppers

Potato leafhoppers were allowed to feed on a 3% sucrose solution containing three different concentrations (0.1%, 0.05% and 0.01%) of the antibiotic oxytetracycline-HCl. The experiment was set up in a randomized complete block design with four treatments consisting of 0.01%, 0.05% and 0.1% oxytetracycline-HCl in 3% sucrose and

a 3% sucrose control. Four replications were carried out, with each replication consisting of one eight chamber feeding cage containing 8 leafhoppers for each treatment. Therefore, 32 leafhoppers were tested for each antibiotic treatment level. Week-old adult leafhoppers were aspirated into the feeding cages, which were kept in a reach-in growth chamber, and allowed to feed for 48 hours. After feeding, the surviving leafhoppers were removed from the feeding cages and individually screened for symbionts. Leafhopper mortality was also recorded during this period, and ANOVA testing was conducted with SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA). Fisher's LSD test was used to test for significant differences between treatment means.

PCR screening of leafhoppers for symbionts

To screen leafhoppers for symbionts, the paired salivary glands, midgut and hind femur from individual adult leafhoppers were dissected under a dissecting microscope in 1X PBS buffer and placed into 1.5 mL microcentrifuge tubes containing 200µL 1X PBS. The tissues were sorted into tubes by type, and DNA was extracted using a DNeasy kit (Qiagen).

DNA samples were screened with primers specifically designed to detect potato leafhopper symbionts. The primer pair 10F and 1507R was used to detect the 16S rDNA sequence of the primary γ-proteobacterial symbiont, yielding a fragment of approximately 1500 base pairs. In addition, DNA samples were screened with the primers FtsZF1 and FtsZR1, which were designed to detect the FtsZ gene of *Wolbachia,* and yield a fragment approximately 1000 base pairs long (Table 2.1). Each 50µL PCR reaction consisted of a final concentration of 1X Taq buffer, 0.2mM of each dNTP, 1µM of each primer, 1.25 units Taq DNA polymerase, 2mM $MgCl_2$ and 2µL template DNA in PCR

grade water. The PCR reaction conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light. To verify that bands present in the gel were in fact the symbiont in question, positive reactions were subjected to DNA sequencing.

Primer Name	Bacterium Detected	Gene Name	Primer Sequence (5' to 3')
10F	*Sulcia muelleri*	16S	AGTTTGATCA TGGCTCAGATTG
1507R	*Sulcia muelleri*	16S	TACCTTGTTACGACTTCACCCCAG
FtsZF1	*Wohlbachia*	FtsZ	GTTGT CGCAA ATACC GATGC
FtsZR1	*Wohlbachia*	FtsZ	CTTAA GTAAG CTGGT ATATC

Table 2.1: Primers used for the detection of symbionts in the potato leafhopper, *Empoasca fabae*

Sequencing reactions were prepared by following the manufacturer's protocol in a Big Dye v3.1 sequencing kit (ABI). PCR amplification of the samples was carried out in skirted ABI plates using the appropriate diagnostic primers (10F/1507R or FtsZF1/FtsZR1). The PCR cycle protocol was 25 cycles of 94°C for 15 sec, 50°C for 4 sec and 60°C for 2 min. PCR reactions were cleaned by adding 2µL 125mM EDTA, 2µL 3M NaOac and 50µL 100% EtOH to each PCR reaction in the plate and incubated at room temperature for 15 min. The samples were then spun at 1650xg for 45 min, inverted onto a paper towel, and spun at 185xg for 2 min. Each sample was then washed with 70µL 70% EtOH and spun at 1650xg for 15 min. The plate was then inverted onto a paper towel and spun for 2 min at 185xg to dry the samples. 20µL of Hi-Di formamide was then added to each well, and the plate was sealed with a septa seal before loading

onto on an Applied Biosystems 3730xl DNA Analyzer in the University of Maryland's Genomics Core Facility. Sequencing results were compared to known potato leafhopper symbiont sequences to verify positive bands from the diagnostic PCR reactions.

Aposymbiotic leafhopper longevity and egg production

Week-old, mated adult female potato leafhoppers were allowed to feed on either 3% sucrose solution or 3% sucrose solution containing 0.01% oxytetracycline-HCl for 48 hours. Five females from each treatment were dissected and subjected to PCR screening for symbionts. The remaining leafhoppers were then caged singly in dialysis tube cages on individual stems of fava bean and alfalfa plants in a growth chamber, and allowed to feed and oviposit. Plants were watered three times per week throughout the experiment, and cages were checked daily to record female mortality. After each female died, the plant stem was removed and stained for egg detection. The experiment consisted of 10 females for each treatment type (3% sucrose or 3% sucrose + 0.01% oxytetracycline-HCl) per replication, with three replications for each plant type. Fava bean and alfalfa plants were tested at different times. ANOVA testing was conducted with SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA). Fisher's LSD test was used to test for differences between treatment means.

Aposymbiotic potato leafhopper fecundity and nymph survival

Fecundity was measured by allowing week-old mated adult females to feed on either 3% sucrose or 3% sucrose with 0.01% oxytetracycline-HCl for 48 hours. Five females from each treatment were set aside and screened for the presence of symbionts to verify that antibiotic treatment was effective in establishing aposymbiotic leafhoppers. The remaining females were then aspirated singly into a dialysis tube cage on individual

stems of alfalfa or fava bean plants kept in a growth chamber. Females were allowed to oviposit for 48 hours, and were then removed from the plants. The dialysis tube cages were then replaced on the plant stems, and the eggs were allowed to hatch. Plants were watered three times per week, and checked daily for the presence of nymphs. Nymph longevity and the time to reach adulthood were also recorded. This was accomplished by transferring second instar nymphs onto new plants with a paintbrush, then caging the nymphs singly on the plant with dialysis tube cages. Cages were checked daily for nymph mortality and adult emergence. The experiment consisted of 3 replications with 10 females per treatment (3% sucrose or 3% sucrose + 0.01% oxytetracycline-HCl) for each plant type (alfalfa or fava bean). Alfalfa and fava bean plants were tested at different times. SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA) was used to conduct ANOVA tests on the fecundity of and nymph survival into adulthood of aposymbiotic leafhoppers, and Fisher's LSD test was used to test for differences between treatment means.

Potato leafhopper egg detection in plant stems

Potato leafhopper eggs were stained *in situ* in plant stems following a modified protocol of Backus et al. 1988. Briefly, plant stems were stained at room temperature for one week in McBride's stain (0.2% acid fuschin in one part each of ethanol and glacial acetic acid), then placed into a clearing solution (one part each of distilled water, 99% glycerine and 85% lactic acid). After the stained plant stems were submerged in the clearing solution, the solution was heated to boiling for 20 minutes. The plant stems were then removed from the clearing solution, rinsed with deionized water, and examined

under a dissecting microscope for the presence of dark oval leafhopper eggs in the cleared plant tissue.

Results

Aposymbiotic leafhoppers

Potato leafhoppers were exposed to three levels of oxytetracycline-HCl (0.1%, 0.5% and 0.01%) in an effort to cure them of their bacterial symbionts. A total of 32 leafhoppers were tested for each oxytetracycline-HCl level, with a control consisting of leafhoppers fed 3% sucrose without antibiotics. The salivary glands and midguts of all the leafhoppers from each antibiotic treatment level tested negative for the two known potato leafhopper symbionts (primary γ-Proteobacteria symbiont and *Wolbachia*) after being fed a 3% sucrose solution containing antibiotics for 48 hours, while the all of the leafhoppers fed the control 3% sucrose solution tested positive for the symbionts (Figure 2.2). In each case, the negative tissue control (hind femur) was negative for the presence of symbionts.

Leafhopper survival on 3% sucrose was significantly different for the four levels of oxytetracylcine-HCl tested ($F = 4.23$, $P = 0.04$) (Figure 2.3, Table 2.2). Using a least significant difference (LSD) test for planned comparisons, leafhopper survival on 0.05%, 0.01% and 0% oxytetracyline-HCl was not significantly different ($P > 0.05$), but survival on 0.10% oxytetracycline-HCl was significantly lower than on the 0% control ($P < 0.05$). The mean survival of potato leafhoppers on 0.10% oxytetracycline-HCl was 71.9%, compared to 85.4% in leafhoppers fed 0.05%, 0.01% and 0% oxytetracycline-HCl, a 15.8% decrease in survival. These results suggest that potato leafhoppers can be cured of

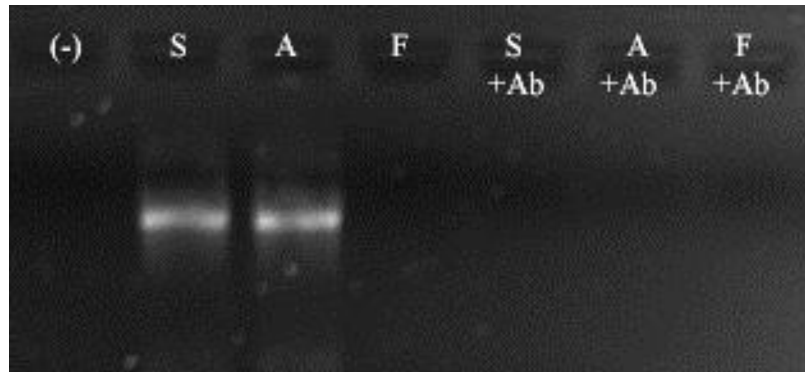

Figure 2.2: Antibiotic treatment of potato leafhoppers, *Empoasca fabae*, to kill symbionts. The first lane (-) is the negative control. All other lanes used the primers 10F and 1507R to detect the presence of the primary γ-Proteobacterial symbiont in the salivary glands (S), midgut (A) and hind femurs (F). Leafhoppers in the first three treatment lanes were fed a 3% sucrose solution, and leafhoppers in the last three lanes were fed a 3% sucrose solution containing 0.01% oxytetracycline-HCl.

their known bacterial symbionts with 0.05% and 0.01% oxytetracycline-HCl with minimal mortality. Therefore, the 0.01% oxytetracycline-HCl treatment was used for subsequent studies requiring aposymbiotic leafhoppers.

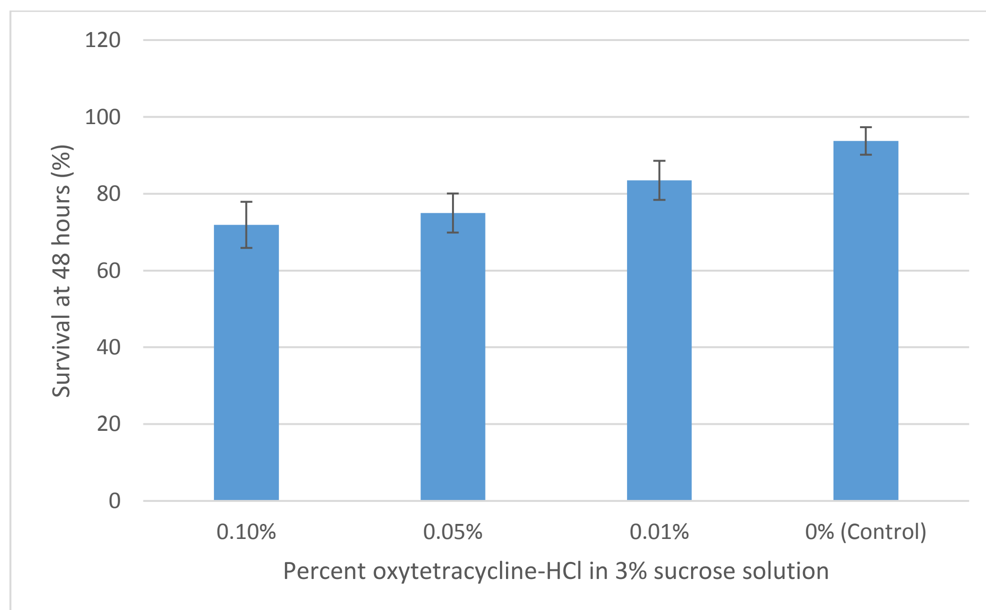

Figure 2.3: Mean ± SE of potato leafhopper survival at 48 hours after feeding on different concentrations of oxytetracycline-HCl in 3% sucrose.

Source of variation	**Degrees of freedom**	**F**	***P* (degrees of freedom)**
Block	3	3.52	0.06 (3,9)
Treatment	3	4.23	0.04 (3,9)
Experimental error	9	1.79	0.07 (9,112)
Sampling error	112		
Total	127		

Table 2.2: ANOVA table for potato leafhopper feeding on different concentrations of oxytetracycline-HCl in 3% sucrose.

Longevity, egg production, nymph survival and fecundity of aposymbiotic leafhoppers

Week-old adult female potato leafhoppers fed a 3% sucrose solution with 0.01% oxytetracycline-HCl had a 61.8% and a 61.0% decrease in longevity compared to leafhoppers fed the 3% sucrose control solution when reared on both alfalfa and fava bean, respectively (Table 2.3). Egg production of aposymbiotic leafhoppers was the same as for untreated leafhoppers on both host plants. Aposymbiotic potato leafhoppers produced 50.1% and 54.7% fewer nymphs after 48 hours of egg laying than untreated leafhopperson alfalfa and fava bean, respectively (Table 2.3). The percent of nymphs

Host plant	**Treatment**	**Longevity (days)**	**Egg production (eggs laid/day)**	**Nymphs produced (after 48 hours)**	**% Nymph survival into adulthood**
Alfalfa	Antibiotic	5.33 ± 2.14 a	1.19 ± 0.29 a	1.47 ± 0.31 a	29.6 ± 5.7 a
	Control	8.63 ± 1.09 b	1.42 ± 0.28 a	2.93 ± 0.16 b	67.6 ± 4.2 b
Fava bean	Antibiotic	5.72 ± 1.97 a	1.23 ± 0.31 a	1.52 ± 0.44 a	34.2 ± 5.7 a
	Control	9.37 ± 0.83 b	1.48 ± 0.25 a	2.78 ± 0.23 b	73.9 ± 5.4 b

Table 2.3: Longevity, egg and nymph production and nymph survival to adulthood of aposymbiotic week-old adult female potato leafhoppers. Treatments given for 24 hours: Antibiotic, 3% sucrose + 0.01% oxytetracycline-HCl; Control, 3% sucrose. Values followed by the same letter are not significantly different ($P = 0.05$).

surviving into adulthood was 43.8% lower in alfalfa and 46.3% lower in fava bean for aposymbiotic leafhoppers than for leafhoppers with symbionts present. The nymphs produced by aposymbiotic leafhoppers appeared to be lighter in color and smaller in size than symbiotic nymphs reared on the same host plant. They also appeared to be less active, suggesting that they were not as healthy as the symbiotic nymphs.

Discussion

Symbiotic bacteria are known to play a role in the ability of phytophagous insects to exploit amino-acid deficient food sources such as phloem and xylem (Douglas 2005). The potato leafhopper is known to have two species of symbiotic bacteria, *Sulcia muelleri* and *Wolbachia* (Chapter 1), both of which are known to have effects on the fitness of other insect hosts (Takiya et al. 2006, Fry et al. 2004). The goals of this study were to first create aposymbiotic leafhoppers and to then study the effect of asymbiosis on the adult longevity, egg production, nymph mortality and ability of nymphs to progress to adulthood. Asymbiosis caused a significant decrease in longevity and egg production, while also affecting the second generation by decreasing the number of nymphs that survived to become adults.

Aposymbiotic leafhoppers

Aposymbiotic potato leafhoppers can be produced with minimal mortality by feeding adults 0.01% oxytetracycline-HCl in a 3% sucrose solution, while higher levels of antibiotic in the diet greatly increased mortality. *Wolbachia* is not strictly associated with bacteriocytes like many of the primary insect symbionts (Dobson 1999). As *Wolbachia* is known to be present in the potato leafhopper, it is important that the antibiotics used to cure the potato leafhopper of symbionts are able to kill symbionts

living in the cytoplasm. Oxytetracycline is known to kill free-living symbiotic bacteria as well as symbionts that are harbored in bacteriocytes, and is therefore commonly used in the creation of aposymbiotic insects (Fouda 2009). Due to its ability to kill free-living symbionts, tetracycline has been shown to be highly effective in curing insects of *Wolbachia* infections (Teixeira et al. 2008). The creation of aposymbiotic insects is the first step in studying the role that symbionts play in an insect's biology, and also allows for future experiments involving selective manipulation of symbiont communities.

Potato leafhoppers treated with oxytetracycline in this study tested negative for the presence of *Sulcia* and *Wolbachia*, but it is possible that small quantities of symbionts survived the antibiotic treatment and were present at undetectable levels. To prevent false negative results, the PCR reactions conducted in this study had 30 cycles, which would amplify even small quantities of symbiont DNA. In addition, positive controls consisting of symbiotic leafhopper tissue were run at the same time to verify that the PCR reagents were not degraded. To improve the study, a positive control consisting of a potato leafhopper gene, such as ribosomal DNA, could have been run on all samples at the same time. This positive control would have ensured that the DNA used for the detection of symbionts in leafhopper tissue was not degraded.

Longevity, egg production, nymph survival and fecundity of aposymbiotic leafhoppers

Aposymbiotic potato leafhoppers had decreased longevity on both alfalfa and fava bean plants in comparison to leafhoppers that had their complement of symbionts intact. Similar results have been reported in other insect species. For example, the Japanese stink bug, *Megacopta punctatissima*, exhibits decreased longevity, longer times between instars, sterility and increased adult mortality when cured of its symbionts by antibiotics

(Fukatsu and Hosokawa 2002). Unlike the Japanese stinkbug, aposymbiotic adult female potato leafhoppers were able to lay viable eggs that hatched into nymphs. The number of eggs laid by aposymbiotic leafhoppers was lower than the number laid by untreated leafhoppers, and the hatch rate of the eggs was also decreased. Adult aphids fed antibiotics can reproduce successfully without their full complement of symbionts because the adults were able to obtain the proper nutrition during their nymphal development before they were cured of symbionts (Douglas 2005). Therefore, the females are able to lay viable eggs because they already had oocytes that were not lacking in amino acids due to symbiont loss. This is a likely explanation for why aposymbiotic adult potato leafhoppers are able to produce viable eggs even if their lifespan is shortened due to nutritional deficiencies caused by a lack of symbionts.

Aposymbiosis often causes more severe effects in the second generation after the loss of symbionts due to severe amino acid deficiencies (McLean et al. 2010). Therefore, the second generation after induction of symbiosis often exhibits greater generation time, higher mortality and decreased fecundity, or complete sterility. Second generation aposymbiotic potato nymphs showed increased mortality in comparison to untreated potato leafhopper nymphs, with fewer nymphs reaching adulthood. The behavioral, size and color differences noted in the aposymbiotic nymphs also support the idea that aposymbiosis greatly impairs the growth and reproduction of the potato leafhopper, because the aposymbiotic nymphs appear to be less robust. The nymphs produced by aposymbiotic leafhoppers were not screened for the presence of *Sulcia* and *Wolbachia*, so it is possible that some of the nymphs harbored symbionts. If that was the case, these nymphs may have caused the results to become skewed towards that of symbiotic

nymphs. Therefore, future testing of the effect of aposymbiosis on the second generation of potato leafhoppers should include screening of the second generation for symbionts to verify the results.

The potato leafhopper adults used in this study were already about one week old, and the lifespan of adult leafhoppers is approximately one month. Therefore, the leafhoppers used in the adult longevity experiment were already nearing the end of their natural lifespan. To improve the understanding of how symbionts influence potato leafhopper longevity, future studies should start with treated nymphs, which have a longer natural lifespan ahead of them. This may provide more insight into how *Sulcia* and *Wolbachia* effect the longevity of the potato leafhopper.

Symbionts can play a role in an insect's ability to survive on different host plants. For example, pea aphids, *Acyrthosiphon pisum*, have unique complements of facultative symbionts that allow them to specialize in feeding on a particular host plant. Moving a pea aphid to a new host plant can cause nutritional deficiencies if the aphid is not injected with the facultative symbionts necessary for growth on the new host (McLean et al. 2010). In the case of the potato leafhopper, aposymbiotic leafhoppers did not display differences in longevity or fecundity when reared on two different legumes, alfalfa and fava bean. The potato leafhopper is highly polyphagous, with each adult feeding on multiple host species throughout its lifespan (Lamp et al. 1994), so it is possible that the potato leafhopper has evolved to have a complement of symbionts that allow it to feed on multiple hosts instead of having distinct symbiont assemblies that allow host specialization. The host plants used in this study were all non-native to the United States, whereas the potato leafhopper is native. Using native host plants, such as a species of

Trifolium, may have yielded different results because the potato leafhopper may have coevolved with the native host and is therefore better adapted to surviving on it. It is possible that the symbionts present in the potato leafhopper allow it to feed on plants that it has not coevolved with, which could explain the decrease in fitness seen in aposymbiotic leafhoppers feeding on alfalfa and fava bean plants.

Summary

The ability to cure potato leafhoppers of their symbiotic bacteria makes it possible to study the effect that symbionts have on the leafhopper's life cycle. The decrease in longevity and fecundity seen in aposymbiotic potato leafhoppers suggests that the relationship between the symbionts and the potato leafhopper is mutualistic. The symbionts presumably provide nutrients that are necessary for the survival of the leafhopper, while the leafhopper provides the symbionts with nutrients and a stable growing environment. The leafhopper-symbiont mutualism may be exploited in the future to manage pest populations on agricultural crops. This study found no evidence that aposymbiotic leafhoppers have different longevity or fecundity rates on two leguminous hosts, alfalfa and fava bean, suggesting that at least on these two host plants, symbionts do not play a role on host plant specialization.

Chapter III: Physiological response of leguminous plants to feeding by aposymbiotic potato leafhoppers

Abstract

The potato leafhopper, *Empoasca fabae*, has two species of symbiotic bacteria, *Sulcia muelleri* and *Wolbachia*, both of which are present in its salivary glands. *Wolbachia* and other insect symbionts are known to alter plant wound response to insect feeding, so here I investigate the role that symbionts play in the physiological response of three legumes to feeding by the potato leafhopper. The saliva of potato leafhoppers contains both species of symbionts, and plant stems screened for symbionts after leafhopper feeding tested positively for *Sulcia* and *Wolbachia*. Aposymbiotic leafhoppers were allowed to feed on alfalfa, fava bean (*Vicia faba*) and soybean (*Glycine max*) plants, and the rates of photosynthesis and transpiration, as well as relative leaf chlorophyll levels, were measured. Alfalfa and fava bean plants fed upon by symbiotic leafhoppers had significantly lower photosynthesis rates than plants fed upon by aposymbiotic leafhoppers. Aposymbiotic leafhoppers caused less of a decrease in transpiration rate in alfalfa than symbiotic leafhoppers, but they did not have a significantly different effect on the transpiration rates of fava bean and soybean in comparison to symbiotic leafhoppers. The photosynthesis rates of soybean plants fed upon by aposymbiotic and symbiotic were not significantly different, but were lower than for plants that were not exposed to leafhoppers. Relative leaf chlorophyll levels did not significantly vary between plants fed upon by aposymbiotic and symbiotic leafhoppers. These results suggest that the

symbionts present in the potato leafhopper may play a role in plant response to leafhopper feeding, but their effect on the plant varies among legume species.

Introduction

Plant physiological responses to insect herbivory can range from compensatory growth, to a decrease in photosynthetic and transpiration rates, to a reallocation of resources (McNaughton 1983, Delaney et al. 2008, Trumble et al. 1993). In addition to compensating for tissue lost due to herbivory, these physiological changes may make the plant less suitable as a food source by sequestering resources such as photoassimilates in forms or places that are not readily available to sap-feeding insects (Singh and Shah 2012). Recent evidence suggests that insect symbiotic bacteria are able to interfere with the plant wound responses induced by insect feeding (Barr et al. 2010).

The endosymbiotic bacterium *Wolbachia* is the most abundant insect symbiont, infecting an estimated 44% of all terrestrial insect species (Zug and Hammerstein 2012). In addition to providing its insect host with vitamins and resistance to viruses, *Wolbachia* has been shown to mediate plant responses to insect feeding (Teixeira et al. 2008, Hosokawa et al. 2010). The apple leaf miner, *Phyllonorycter blancardella*, causes green islands, or areas that remain green and actively photosynthesizing, to occur where it is feeding on leaves that would otherwise senesce in the fall. Cytokinins secreted in the leaf miner's saliva delay the senescence of the leaf and prevent mobilization of photoassimilates from the leaves to the roots (Giron et al. 2007). *Wolbachia* has been shown to produce cytokinins, and when apple leaf miners were treated with antibiotics to

cure them of their symbionts, the levels on cytokinins detected in the mines decreased, the green islands did not develop, and leaf miner mortality increased (Kaiser et al. 2010). This evidence suggests that *Wolbachia* is able to influence plant response to insect feeding in a way that benefits the insect host.

In addition to altering the physiology of plants, insect symbionts are able to manipulate gene activity in a plant wound response. Western corn rootworms, *Diabrotica virgifera*, cured of *Wolbachia* showed increased mortality and caused a relative increase in corn wound response gene expression in comparison to rootworms with their symbionts intact (Barr et al. 2010). Similarly, high concentrations of the endosymbiont '*Candidatus* Liberibacter psyllaurous' in the tomato psyllid, *Bactericerca cockerelli*, are associated with a reduction of plant wound response gene expression in tomatoes (Casteel et al. 2012). Therefore, in addition to providing nutrients to their hosts, insect symbionts may influence the ability of an insect to utilize a plant as a food source.

The potato leafhopper is a highly polyphagous species that is an economically important pest of legumes (Lamp et al. 1994). On alfalfa, it causes injury known as hopperburn, which is characterized by leaf yellowing, stunting, and a subsequent reduction in yield (Kindler et al. 1973, Hower and Flinn 2004, Hutchins and Pedigo 1989). Hopperburn is caused by a combination of mechanical wounding and leafhopper saliva, and the resulting plant wound results in cell hypertrophy at the feeding site and eventual collapse of the phloem sieve elements (Ecale and Backus 1995). Phloem collapse causes a buildup of photoassimilates to occur in the tissue above the injured area, which in turn results in a decrease in photosynthesis rate in the leaves above the injured site (Lamp et al. 2004).

Previous studies have shown that the potato leafhopper harbors two taxa of symbionts in its salivary glands: *Sulcia muelleri* and *Wolbachia* (Chapter 1 of this dissertation). Aposymbiotic leafhoppers that have been experimentally cured of their symbionts with antibiotics exhibit decreased longevity and nymph survival, indicating that the symbionts are necessary for proper development and reproduction (Chapter 2 of this dissertation). Although they are important in the population ecology of the potato leafhopper, the reason that these symbionts are present in the salivary glands is unknown. Therefore, this study examined the role that the symbionts of the potato leafhopper play in the physiological response of leguminous plants to leafhopper feeding. Leafhopper saliva was screened for the presence of both species of symbionts, and alfalfa, *Medicago sativa*, fava bean, *Vicia faba*, and soybean, *Glycine max*, stems were screened for symbionts after leafhopper feeding. In addition, leafhoppers were cured of their symbionts using antibiotics, then allowed to feed on alfalfa, fava bean and soybean plants. If salivary gland symbionts play a role in the plant wound response to potato leafhopper feeding, plant physiological response, in the form of net photosynthesis and transpiration rates, would be expected to be lower in plants exposed to aposymbiotic leafhoppers than in plants exposed to symbiotic leafhoppers.

Materials and methods

Plant culture

All soybean, *G. max*, plants used in this study were grown in an MB-80 plant growth chamber (Percival Scientific, Inc., Boone, Iowa) kept at 25°C, 80% relative humidity, with 14 hours of daylight and 10 hours of dark. The light intensity at plant height was 120 µmol/m^2/sec during the day. One ‘Williams 82’ soybean seed was planted

in a 10cm pot containing Metro-Mix potting medium (Sun Gro Horticulture). Flats containing 15 pots each were then placed into the growth chamber and watered three times per week. Plants were used three weeks after planting, after the first trifoliate leaves emerged.

'Ranger' alfalfa, *M. sativa*, seeds were planted in perlite in flats containing 48 individual planting cells. The flats were placed in a mist room at the University of Maryland's greenhouse, and allowed to germinate. Two week old seedlings were removed from the perlite medium, and transplanted into 15cm pots containing Metro-Mix potting medium (Sun Gro Horticulture). The pots were put into screen cages (BioQuip) to exclude pests, and then placed on a greenhouse bench. Each individual pot was placed in a plastic tray to collect water during watering, and were thoroughly watered three times per week. Four weeks after transplanting, the alfalfa plants were moved into an MB-60 plant growth chamber as described above, and allowed to equilibrate for one week prior to being used for the experiment.

'Broad Windsor' fava bean, *V. faba*, plants used for photosynthesis measurements were grown in a Percival environmental growth chamber set at the conditions described above. One seed was placed into a 10cm pot filled with Metro-Mix potting medium (Sun Gro Horticulture), and flats of 15 pots were placed into the growth chamber and watered three times per week. Plants were ready for use three weeks after planting.

Three week old 'Broad Windsor' fava bean plants were also used for maintaining leafhopper cultures. These plants were grown in the University of Maryland greenhouse, where 3 fava bean seeds were planted in a 10cm pot filled with Metro-Mix potting medium (Sun Gro Horticulture). Flats filled with 15 individual pots were placed into

screen cages (BioQuip) on greenhouse benches to exclude insect pests and watered three times per week.

Insect culture

Three week old ‘Broad Windsor’ fava bean plants were also used for maintaining leafhopper cultures. These plants were grown in the University of Maryland greenhouse, where 3 fava bean seeds were planted in a 10cm pot filled with Metro-Mix potting medium (Sun Gro Horticulture). Flats filled with 15 individual pots were placed into screen cages (BioQuip) on greenhouse benches to exclude insect pests and watered three times per week. Potato leafhoppers were reared in collapsible screen cages (BioQuip) kept in an MB-60 plant growth chamber (Percival Scientific, Inc., Boone, Iowa) set at 25°C, 80% humidity, 14 hours of daylight and 10 hours of dark, and light intensity at plant height of 120 $\mu mol/m^2/sec$. The leafhopper colony was established with adults collected from alfalfa at the University of Maryland’s Western Maryland Research and Education Center in Keedysville, Maryland. Leafhoppers were reared on fava bean plants, which were watered three times per week. Each week, the plants were replaced with new plants to provide the leafhoppers with new, uninjured host plants.

To obtain week-old potato leafhopper adults, adult leafhoppers were placed into a collapsible screen cage containing fava bean plants. The adults were allowed to lay eggs for three days, after which the plants were removed from the cage containing the leafhoppers. The plants were then shaken to remove any adult leafhoppers present on the stems, then placed into a new screen cage without leafhoppers present. The eggs that were laid in the stems of the plants were allowed to hatch, and the nymphs were allowed

to mature into adults. Four weeks after the end of the oviposition period, the new week-old adults were collected for plant response studies.

Collection of leafhopper saliva for symbiont screening

Potato leafhopper saliva was collected for subsequent screening for *Sulcia* and *Wolbachia*. To do this, 16 adult potato leafhoppers were aspirated into each eight chamber feeding cage (Figure 2.1), with two leafhoppers placed into each chamber. A total of four feeding cages were used. Chambers containing caged leafhoppers were placed into an MB-60 plant growth chamber under the same conditions described above for plant culture. Each chamber held 50µL of filter sterilized 3% sucrose solution, which the leafhoppers fed on through a Parafilm membrane stretched across the feeding cage. Leafhoppers were allowed to feed for 48 hours, and the 3% sucrose solution containing leafhopper saliva from each individual chamber was collected and placed into a 1.5mL microcentrifuge tube. As a negative control, four feeding cages were assembled as described above, but did not have leafhoppers placed into the chambers. The 3% sucrose was collected from each chamber, and the saliva and negative control samples were stored at -80°C for later PCR screening for symbionts.

Screening of plant stems for symbionts

Potato leafhoppers were caged singly on alfalfa, fava bean and soybean stems to test for transfer of symbionts from the leafhopper saliva into plant stem tissue during feeding. The soybean plant stems were shaved with a scalpel to remove the trichomes prior to putting the clip cages on the plants. For all plants, a clip cage (Figure 3.1) was

Figure 3.1: Clip cage fastened on an alfalfa stem.

attached to the stem below the uppermost fully expanded leaf, and a single adult potato leafhopper aspirated into the cage and on the plant stems. The clip cages were assembled with clear hinged plastic boxes (1 by 2.5 by 2.5cm). A hole was cut on the top face of the box, and mesh was glued over the hole to ventilate the cage. On one side face of the box, an 8mm hole was drilled to allow for the introduction of leafhoppers into the closed cage. This hole was sealed by inserting a foam plug into the hole after aspirating the leafhopper into the cage. To allow the cage to clip over the stem of a plant, small holes were drilled into opposing sides of the box.

To expose plant stems to leafhoppers, a metal stake was inserted into the soil next to the stem, and a clip cage was clipped onto the stem and taped to the stake for stabilization. One leafhopper was then aspirated into each clip cage. A total of twenty plants of each plant species were exposed to leafhoppers in this manner, with only one

species of plant tested at a time in a randomized complete block design. As a negative control, 20 plants of each species had clip cages fastened to the stem below the uppermost fully expanded leaf, with no leafhopper present in the cage. Plants with and without leafhoppers were placed into an MB-60 plant growth chamber kept at the conditions described above for 48 hours. After 48 hours, the section of the plant stem enclosed by the cage was removed from the plant with a sterile scalpel, and stored at -80°C for later DNA extraction.

Creation of aposymbiotic leafhoppers

Week-old adult potato leafhoppers were aspirated into eight chamber feeding cages. The feeding cages were kept in an MB-60 plant growth chamber with the same settings as described above, and allowed to feed on either a 3% sucrose control solution or a 3% sucrose solution containing 0.01% oxytetracycline-HCl for 48 hours. After feeding, the leafhoppers were removed from the feeding cages using an aspirator and placed into individual clip cages on alfalfa, fava bean or soybean plants.

Screening of leafhoppers, saliva and plant stems for symbionts

To screen both untreated and antibiotic treated leafhoppers for the presence of symbionts, the total DNA from whole leafhoppers stored in 100% ethanol after feeding on alfalfa, fava bean or soybean plants was extracted using a DNeasy kit (Qiagen). Saliva collected from eight chamber feeding cages was subjected to direct PCR by adding 2µL of saliva to the PCR reaction instead of DNA. Alfalfa, fava bean and soybean plant stem sections fed upon by potato leafhoppers were ground with a mortar and pestle in liquid nitrogen, and total DNA extracted using a DNeasy Plant Mini Kit (Qiagen).

The primer pair 10F and 1507R (10F: AGTTTGATCA TGGCTCAGATTG; 1507R: TACCTTGTTACGACTTCACCCCAG) was used to detect the 16S rDNA sequence of *Sulcia muelleri*, yielding a fragment of approximately 1500 base pairs. The primer set FtsZF and FtsZR (FtsZF: GTTGTCGCAAATACCGATGC; FtsZR: CTTAAGTAAGCTGGTATATC), was used to detect the FtsZ gene of *Wolbachia,* producing a fragment approximately 1000 base pairs long. 50µL PCR reactions were assembled, consisting of a final concentration of 1X Taq buffer, 0.2mM of each dNTP, 1µM of each primer, 1.25 units Taq DNA polymerase, 2mM $MgCl_2$ and 2µL template DNA in PCR grade water. The PCR reactions were: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by a final extension step of 72°C for 7 min. The resulting PCR products were run on a 1% agarose gel, stained with ethidium bromide, and then visualized under UV light.

Results were scored as positive for a particular symbiont if they yielded a band of expected length, and negative for a symbiont if they did not produce a PCR product. For plant response to leafhopper feeding experiments, if an antibiotic-treated leafhopper scored as positive for either symbiont, the data for that leafhopper's block was considered invalid, and was discarded. Likewise, if an untreated leafhopper was scored as negative for either symbiont, the data for that leafhopper's block was also discarded.

Photosynthesis, transpiration and chlorophyll measurements

For each of the experiments described below, photosynthesis and transpiration rates were measured using an Licor 6400 XT photosynthesis system (Li-Cor, Inc., Lincoln, Nebraska) (Figure 3.2). The Licor conditions were as follows: light at 1500 µmol photons m^{-2} s^{-1}, CO_2 level at 400 µmol CO_2 mol^{-1}, air flow rate at 500 µmol s^{-1} and

block temperature at 25°C. The leaf area was set to 1 cm^2 for alfalfa plants and 6 cm^2 for fava bean and soybean plants. For alfalfa and soybean plants, the leaf chamber was clamped on the apical leaflet of the uppermost fully-expanded leaf. For fava bean plants, the leaf chamber was clamped on the uppermost fully expanded leaf. In all cases, the leaf chamber was clamped above the clip cage used to cage leafhoppers on the plant stem. Leaf area measurements for alfalfa were adjusted because the leaflet did not fill the entire leaf chamber of the Licor. This was done by first setting the leaf area on the Licor 6400XT to 1 cm^2, then after taking measurements removing the leaflet from the plant and photographing the leaflet next to a strip of graph paper. The leaf area of the alfalfa leaflet was then calculated with ImageJ using the graph paper to calibrate the length of the leaf in the photograph. Licor values were then transformed to reflect the individual leaf areas

Figure 3.2: Licor 6400XT Photosynthesis meter clamped on an alfalfa, *Medicago sativa*, plant leaflet.

of different alfalfa leaflets by dividing the values obtained by the Licor by the leaf area calculated by ImageJ in cm^2.

Relative leaf chlorophyll levels were measured using a Konica-Minolta SPAD 502 chlorophyll meter. After gas exchange rates were measured using the Licor 6400XT, the SPAD meter was used to take five readings from the same leaf used for the Licor gas exchange measurements. The five SPAD meter readings were then averaged to obtain the relative leaf chlorophyll level for each leaf tested.

Alfalfa response to leafhopper feeding

The experiment was conducted as a randomized complete block design with four treatments and twenty blocks, repeated three times with the same plants used for each repetition. Each block consisted of a single alfalfa plant, with each experimental unit consisting of a single stem on the plant. Each alfalfa stem used in the experiments had a clip cage (Figure 3.1) fastened onto the internode between the topmost fully-expanded trifoliate leaf and the leaf directly below it. To prevent the cage from bending the stem due to its weight, it was taped to a metal stake that was inserted into the soil of the pot. The four treatments consisted of a control with no leafhopper in the clip cage, a leafhopper control where the adult leafhopper had been fed alfalfa for 24 hours prior to the experiment, a second leafhopper control with a single adult leafhopper fed 3% sucrose for 24 hours, and an experimental treatment with a single antibiotic-treated leafhopper placed into the clip cage.

Plants were placed in an MB-60 plant growth chamber set at the previously described conditions, with the cages in place for 24 hours. After 24 hours, each clip cage was removed, and the leafhoppers were aspirated into individual 1.5mL tubes and placed

in a -20°C freezer for future PCR screening for symbionts. Photosynthesis and transpiration rates were measured using a Licor 6400XT photosynthesis measurement system, and relative chlorophyll levels were taken with a SPAD 502 chlorophyll meter (Konica Minolta) as described above. Analysis of Variance was used to determine if aposymbiotic leafhoppers have a lesser effect on plant physiological response to feeding than leafhoppers containing their symbionts. This analysis was completed using the PROC MIXED procedure of SAS (SAS Institute Inc., 2011).

Fava bean response to leafhopper feeding

Fava bean response to leafhopper feeding was conducted as a randomized complete block design with four treatments and ten blocks, repeated three times. The experimental unit was a single fava bean plant. A clip cage (Figure 3.1) was fastened onto the internode beneath the topmost fully-expanded leaf, and the cage secured to a stake in the soil with tape. The four treatments consisted of a control with no leafhopper, a leafhopper control of leafhoppers fed fava beans for 24 hours before the experiments, a leafhopper control with a 3% sucrose-fed adult leafhopper, and an experimental treatment with a single antibiotic-treated leafhopper.

The fava bean plants were placed into an MB-60 plant growth chamber set at the previously described conditions, with the clip cages kept in place for 24 hours. After 24 hours, the clip cages were removed, and the leafhoppers were placed into individual 1.5mL tubes. The tubes were stored at -20°C for future PCR screening for symbionts. Photosynthesis, transpiration and relative chlorophyll levels were measured and the data analyzed as described above.

Leafhopper ability to feed on soybean

'Williams 82' soybean is a pubescent variety with antibiotic resistance to potato leafhopper (Broersma et al. 1972). To determine if shaving of trichomes allows survival and feeding on soybean, an experiment was set up as a randomized complete block design with three treatments and ten blocks, repeated three times. A clip cage (Figure 3.1) containing a single potato leafhopper served as the experimental unit. For the control treatment, a single potato leafhopper was placed into a clip cage with no food source. A second treatment consisted of placing the clip cage containing the leafhopper onto a soybean stem on the internode below the uppermost fully expanded leaf. For the third treatment, the clip cage containing a leafhopper was placed onto the internode below the uppermost fully expanded leaf of a soybean plant where the trichomes had been removed from the internode by shaving them off with a scalpel. Leafhopper mortality in each treatment was checked daily until all leafhoppers had died. Analysis of Variance (ANOVA) was used to analyze the mortality of potato leafhoppers on the three different food sources to determine if leafhoppers are able to feed on soybean plants with or without trichomes. This was done using the PROC MIXED procedure in SAS (SAS Institute Inc., 2011).

Soybean response to leafhopper feeding

The response of soybeans to potato leafhopper feeding was also conducted as a randomized complete block design with four treatments and ten blocks, repeated three times. The experimental unit was a single soybean plant. One clip cage (Figure 3.1) was fastened onto the internode below the uppermost fully-expanded leaf. The clip cage was then secured to a stake with tape. The four treatments were unshaved stem control with

no leafhopper, shaved stem control with no leafhopper, shaved stem 3% sucrose-fed leafhopper control, and a shaved stem experimental treatment with an antibiotic-treated leafhopper.

Soybeans were placed into an MB-60 plant growth chamber as described above for 24 hours after treatment. After 24 hours, the clip cages were removed, and the leafhoppers stored at -20°C in 1.5mL tubes for future PCR screening for symbionts. Photosynthesis, transpiration and relative chlorophyll levels were measured as described above. Data was analyzed using SAS (SAS Institute Inc., 2011) as described above.

Results

Presence of symbionts in the saliva of the potato leafhopper

Saliva from 32 feeding chambers containing two adult potato leafhoppers per chamber was collected and screened for the presence of *Sulcia* and *Wolbachia*. The saliva of all but 2 of the feeding chambers (94%) tested positive for the presence of both symbionts (Figures 3.3 and 3.4). The two feeding chambers that tested negative for symbionts had leaked, and the leafhoppers inside the chambers had died before the 48 hour feeding period had elapsed. The small amount of fluid left in these two chambers was collected and screened for symbionts, but it is not known if the leafhoppers fed on the solution before the chamber leaked and the leafhoppers starved. All 32 of the negative control chambers that did not contain leafhoppers tested negative for the presence of both symbionts, and none of the negative control chambers leaked.

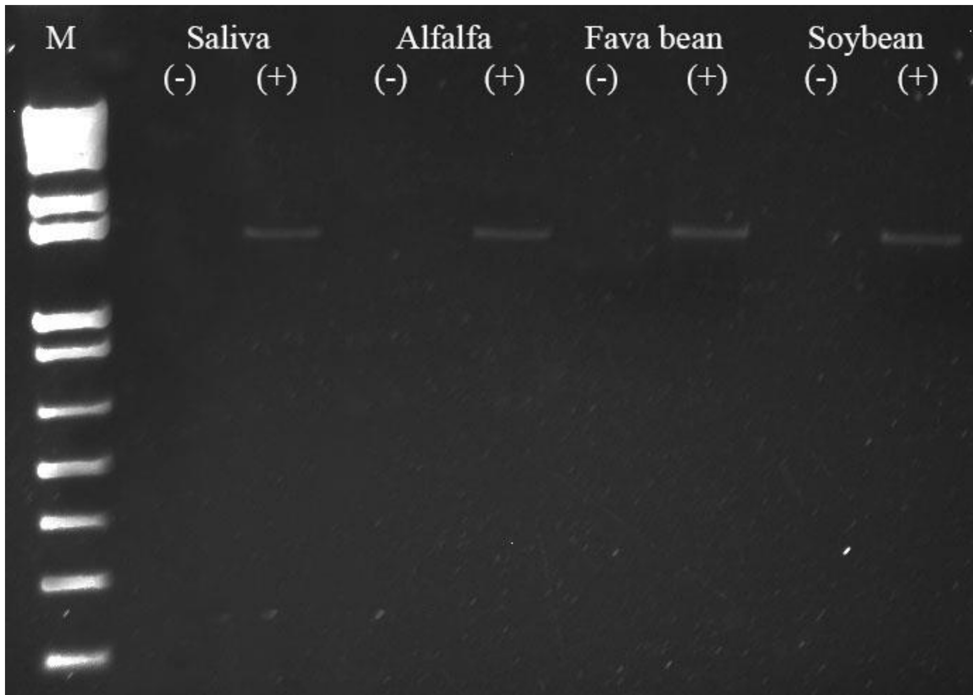

Figure 3.3: Screening of potato leafhopper saliva and alfalfa, fava bean and soybean plant stems for the presence of *Sulcia muelleri*. M: 1kb ladder, (-): Negative control, (+): Sample exposed to potato leafhopper.

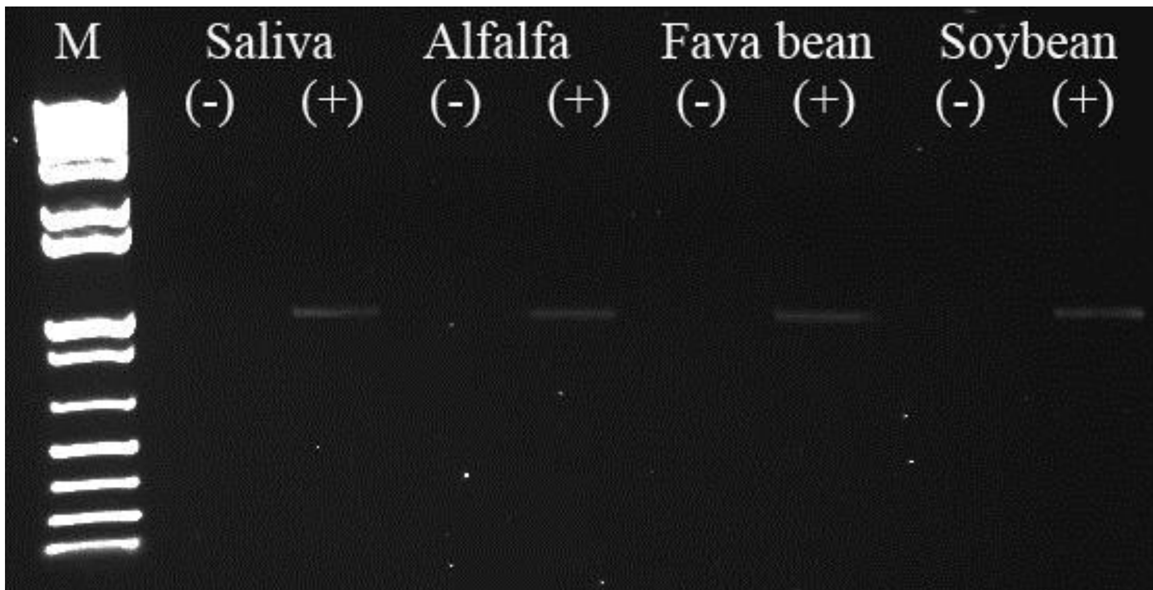

Figure 3.4: Screening of potato leafhopper saliva and alfalfa, fava bean and soybean plant stems for the presence of *Wolbachia*. M: 1kb ladder, (-): Negative control, (+): Sample exposed to potato leafhopper.

Presence of symbionts in plant stems after leafhopper feeding

Alfalfa, fava bean and soybean plants stems were screened for the presence of *Sulcia muelleri* and *Wolbachia* after feeding by potato leafhoppers. The negative controls for all three plant species (no leafhopper on the plant stem) did not test positively for either of the symbiont species (Figures 3.3, 3.4). In alfalfa, 19 out of the 20 plant stems exposed to leafhoppers tested positive for *Sulcia muelleri*, and all of the plant stems tested positive for *Wolbachia*. All fava bean stems exposed to the potato leafhopper tested positively for both *Sulcia muelleri* and *Wolbachia*. Two of the potato leafhoppers caged on soybean died during the experiment, and the plant stems for both of those samples were negative for the presence of *Sulcia muelleri* and *Wolbachia*. The remaining 18 leafhoppers survived the duration of the experiment, and the plant stems that they were caged on tested positively for both symbiont species.

Effect of aposymbiotic leafhoppers on alfalfa

Alfalfa plants were exposed to four different treatments to determine if aposymbiotic leafhopper feeding causes less of a reduction in photosynthesis and transpiration rates than feeding by leafhoppers with symbionts. Alfalfa plant stems with no leafhoppers had the highest photosynthesis (8.25 ± 1.45 μmol CO_2 s^{-1}) and transpiration (7.36 ± 0.71 μmol H_2O m^{-2} s^{-1}) rates of all four treatments (Table 3.1). Transpiration rates for plant stems fed upon by alfalfa-fed leafhoppers (3.85 ± 0.69 μmol H_2O m^{-2} s^{-1}) and plant stems fed upon by leafhoppers fed a 3% sucrose control solution (3.92 ± 0.57 μmol H_2O m^{-2} s^{-1}) were not significantly different, but were significantly lower than the rates for plant stems fed upon by aposymbiotic leafhoppers (5.63 ± 0.64 μmol H_2O m^{-2} s^{-1}). Photosynthesis rates were lowest for leafhoppers with symbionts

Parameter	Photosynthesis (μmol CO_2 s^{-1})	Transpiration (μmol H_2O m^{-2} s^{-1})	Relative chlorophyll level (SPAD)
ANOVA (source = treatment)			
Df	3, 87	3, 87	3, 87
F value	3.73	6.46	0.07
Probability	0.03	0.002	0.93
Treatment (mean ± standard error)			
1. No leafhoppers	8.25 ± 1.45 a	7.36 ± 0.71 a	31.1 ± 8.5 a
2. Alfalfa-fed leafhoppers	2.38 ± 1.43 c	3.85 ± 0.69 c	27.2 ± 6.9 a
3. 3% sucrose-fed leafhoppers	2.75 ± 1.32 c	3.92 ± 0.57 c	26.7 ± 8.1 a
4. Aposymbiotic leafhoppers	5.19 ± 1.40 b	5.63 ± 0.64 b	27.3 ± 7.4 a

Table 3.1: Alfalfa plant response to feeding by aposymbiotic and symbiotic potato leafhoppers. Values followed by the same letter are not significantly different (P = 0.05).

intact: 2.38 ± 1.43 μmol CO_2 s^{-1} and 2.75 ± 1.32 μmol CO_2 s^{-1} for alfalfa-fed and 3% sucrose solution-fed leafhoppers, respectively. The photosynthesis rate for aposymbiotic leafhoppers (5.19 ± 1.40 μmol CO_2 s^{-1}) was intermediate between the no leafhopper control and the symbiotic leafhopper controls. Relative chlorophyll levels of the leaves were not significantly different between the four treatments.

Effect of aposymbiotic leafhoppers on fava bean

Fava bean plants were exposed to four different treatments to determine if aposymbiotic potato leafhoppers have less of an impact on photosynthesis, transpiration and relative chlorophyll levels than symbiotic potato leafhoppers. Like alfalfa, fava bean plants that were not fed upon by leafhoppers had the highest photosynthesis (10.7 ± 1.30 μmol CO_2 s^{-1}) rates of all four treatments (Table 3.2), followed by aposymbiotic leafhoppers (6.67 ± 1.09 μmol CO_2 s^{-1}). Leafhoppers with their symbionts intact had the lowest photosynthesis rates, 4.48 ± 0.91 μmol CO_2 s^{-1} and 4.13 ± 0.98 μmol CO_2 s^{-1} for

Parameter	Photosynthesis (μmol CO_2 s^{-1})	Transpiration (mmol H_2O m^{-2} s^{-1})	Relative chlorophyll level (SPAD)
ANOVA (source = treatment)			
Df	3, 87	3, 87	3, 87
F value	7.84	0.06	0.02
Probability	< 0.001	0.94	0.98
Treatment (mean ± standard error)			
1. No leafhoppers	10.71 ± 1.30 a	4.21 ± 1.48 a	37.2 ± 7.7 a
2. Fava bean-fed leafhoppers	4.48 ± 0.91 c	3.62 ± 1.39 a	35.5 ± 8.1 a
3. 3% sucrose-fed leafhoppers	4.13 ± 0.98 c	3.55 ± 1.46 a	34.7 ± 8.4 a
4. Aposymbiotic leafhoppers	6.67 ± 1.09 b	4.19 ± 1.32 a	35.8 ± 7.1 a

Table 3.2: Fava bean plant response to feeding by aposymbiotic and symbiotic potato leafhoppers. Values followed by the same letter are not significantly different (P = 0.05).

fava bean-fed and 3% sucrose solution-fed leafhoppers, respectively. Transpiration and relative chlorophyll levels in the leaves were not significantly different between the four treatments.

Longevity of leafhoppers on soybean

To determine if potato leafhoppers can feed on soybeans, leafhoppers were subjected to three different treatments: no food source, soybeans with trichomes intact, and soybeans with trichomes removed from the stem with a scalpel. The effect of treatment on leafhopper longevity was significant ($F_{(2, 58)} = 22.19$, $P < 0.001$) (Table 3.3). Potato leafhoppers that were not provided a food source showed no significant difference in longevity (1.10 days) than leafhoppers fed on soybeans with trichomes intact (1.20 days). In contrast, leafhoppers allowed to feed on soybean stems that had their trichomes removed with a scalpel lived for a significantly longer time (6.50 days) than leafhoppers that were starved.

Treatment	**Mean longevity (in days) ± Standard error of the mean**
No food source	1.10 ± 0.30 a
Williams 82 with trichomes	1.20 ± 0.40 a
Williams 82 without trichomes	6.50 ± 1.02 b

Table 3.3: Longevity of potato leafhoppers on soybean. Values followed by the same letter are not significantly different ($P = 0.05$).

Effect of aposymbiotic leafhoppers on soybean

Soybeans were exposed to aposymbiotic and symbiotic leafhoppers in an effort to determine if aposymbiotic leafhoppers cause a lower decrease in photosynthesis, transpiration and relative chlorophyll levels in leaves than symbiotic leafhoppers. Photosynthesis (15.34 ± 1.82 µmol CO_2 s^{-1}) and transpiration (3.48 ± 0.31 mmol H_2O m^{-2} s^{-1}) rates were highest in soybean plants that were not exposed to leafhoppers and that did not have their stems shaved to remove trichomes (Table 3.4). The photosynthesis rate (7.29 ± 1.33 µmol CO_2 s^{-1}) for soybean plants with shaved stems but no leafhoppers was significantly lower than for the unshaved stem no leafhopper control. The lowest photosynthesis rates were found in the soybean plants with shaved stems and symbiotic (4.35 ± 1.36 µmol CO_2 s^{-1}) and aposymbiotic (4.18 ± 1.39 µmol CO_2 s^{-1}) leafhoppers, which were not significantly different from one another. Although transpiration rate was highest in the unshaved stem no leafhopper control, the transpiration rates for the remaining three treatments were not significantly different. Like alfalfa and fava bean plants, relative chlorophyll levels in the leaves of the four different treatments were not significantly different.

Parameter	Photosynthesis ($\mu mol\ CO_2\ s^{-1}$)	Transpiration (mmol $H_2O\ m^{-2}\ s^{-1}$)	Relative chlorophyll level (SPAD)
ANOVA (source = treatment)			
Df	3, 87	3, 87	3, 87
F value	12.34	14.82	0.03
Probability	< 0.001	< 0.001	0.99
Treatment (mean ± standard error)			
No leafhoppers, unshaved stem	15.34 ± 1.82 a	3.48 ± 0.31 a	34.8 ± 6.2 a
No leafhoppers, shaved stem	7.29 ± 1.33 b	1.21 ± 0.29 b	36.4 ± 6.3 a
3% sucrose-fed leafhoppers, shaved stem	4.35 ± 1.36 c	1.28 ± 0.34 b	34.2 ± 6.5 a
Aposymbiotic leafhoppers, shaved stem	4.18 ± 1.39 c	1.02 ± 0.26 b	36.1 ± 6.7 a

Table 3.4: Soybean plant response to feeding by aposymbiotic and symbiotic potato leafhoppers. Values followed by the same letter are not significantly different ($P = 0.05$).

Discussion

Symbiont presence in leafhopper saliva

The saliva of potato leafhopper adults tested positively for both *Sulcia muelleri* and *Wolbachia*, suggesting that it is possible for the symbionts to be transmitted to plants during feeding. Testing of plant stem tissue dissected from areas fed upon by potato leafhopper adults gave positive results for both symbionts. These results suggest that the potato leafhopper's symbionts are injected into the host plant during feeding, although the reason for this is unclear. *Wolbachia* has been shown to be horizontally transmitted through pumpkin plants from the silverleaf whitefly, *Bemisia tabaci*, to the white-winged planthopper, *Nisia nervosa*, and flea beetles, *Phyllotreta* sp. (Sintupachee et al. 2006).

Therefore, it is possible that the symbionts of the potato leafhopper are capable of horizontal transmission from one potato leafhopper to another, or from the potato leafhopper to another insect species, through feeding on a common host plant.

Alfalfa response to leafhoppers

Potato leafhopper adults are highly mobile, and will feed on both the leaves and stems of alfalfa. Previous studies have shown that adults prefer to feed on the stems of alfalfa plants, and that when a potato leafhopper is confined to a stem, the leaves above the feeding site show a decrease in photosynthesis and transpiration rates after one day of leafhopper feeding (Lamp et al. 2004). In this study, alfalfa plants fed upon by leafhoppers had significantly lower photosynthesis and transpiration rates than plants that were not exposed to leafhoppers. Aposymbiotic leafhoppers caused less of a decrease in photosynthesis and transpiration rates than symbiotic leafhoppers did, suggesting that a lack of symbionts is associated with less of a plant response to insect feeding.

Potato leafhoppers cause distinctive injuy on alfalfa known as hopperburn, which is characterized by yellowing of the leaves and stunting of the stems (Hower and Flinn 2004). In addition to these phenotypical changes, movement of the leafhopper's stylets during feeding combined with saliva causes changes in the structure of the phloem due to hypertrophy of phloem cells (Ecale and Backus 1995). These vascular changes result in an accumulation of photosynthesis products in the tissue due to blockage of the phloem, resulting in a decrease of photosynthesis due to leafhopper feeding (Flinn et al. 1990). The resulting hopperburn is believed to be due to a combination of mechanical damage sustained during feeding and plant wound response to the potato leafhopper saliva (Ecale and Backus 1995, Backus et al. 2005).

This study found that both symbiotic and aposymbiotic potato leafhoppers cause a reduction in photosynthesis and transpiration rates in alfalfa. Chapter 1 of this dissertation found that the symbionts *Sulcia muelleri* and *Wolbachia* are both present in the salivary glands of the potato leafhopper, suggesting that they may play a role in the production of saliva, or that the bacteria may be present in the saliva itself. As hopperburn is caused in part by the saliva of the potato leafhopper, and aposymbiotic leafhoppers have less of an effect on photosynthesis and transpiration than symbiotic leafhoppers, symbionts may be partially responsible for the plant wound response that leads to hopperburn either by producing salivary components that incite injury or by inciting a plant wound response on their own. It is also possible that aposymbiotic leafhoppers do not feed as readily as symbiotic leafhoppers, causing less mechanical damage to the plants during feeding.

In addition to measuring the photosynthesis and transpiration rates of alfalfa plants exposed to symbiotic and aposymbiotic leafhoppers, relative leaf chlorophyll measurements were also taken. The relative leaf chlorophyll levels were not significantly different for any of the treatments in this study (no leafhopper, alfalfa-fed symbiotic leafhopper, 3% sucrose-fed symbiotic leafhopper and aposymbiotic leafhopper), so leaf chlorophyll levels in the plants should not have impacted the photosynthesis and transpiration rates of the plants in the different treatments. I did not expect to see a reduction in leaf chlorophyll levels in plants fed upon by potato leafhoppers, even though leafhopper feeding on alfalfa can cause leaf yellowing, because yellowing of alfalfa leaves due to potato leafhopper feeding occurs approximately five days after initial feeding (Granovsky 1928).

Fava bean response to leafhoppers

Although the physiological response of alfalfa to potato leafhopper has been studied in detail, little is known of the effect that potato leafhoppers have on fava bean, which are another suitable host plant. Adult potato leafhoppers have similar survival rates on both Broad Windsor fava beans and Ranger alfalfa, and have higher oviposition rates on fava beans than on alfalfa (Lamp et al. 2011). Due to the ability of the potato leafhopper to feed and reproduce on fava beans, I decided to test the response of fava beans to feeding by symbiotic and aposymbiotic leafhoppers.

Fava beans that were not exposed to leafhoppers had the highest photosynthesis levels, suggesting that leafhopper feeding causes injury to fava bean. Similar to the results for alfalfa, aposymbiotic leafhoppers caused less of a decrease in photosynthesis rates than symbiotic leafhoppers did. The greater reduction in photosynthesis levels caused by symbiotic leafhopper feeding suggests that the presence of symbionts is correlated with greater feeding injury in fava beans, although the exact way that the symbionts contribute to plant injury is unknown.

Unlike in alfalfa, transpiration rates were the same for plants that were not exposed to leafhoppers as they were for both symbiotic and asymbiotic leafhoppers. Although the response of fava bean to potato leafhopper feeding has not been studied, the gas exchange rate changes due to potato leafhopper feeding are similar in grapes, *Vitis* spp., and fava bean. In both Chambourcin and Chardonnay grapes, potato leafhopper feeding causes a significant decrease in the rate of photosynthesis after one day of feeding, but does not cause a decrease in transpiration rate (Lamp et al. 2011). Therefore, it appears that although potato leafhopper feeding tends to decrease photosynthesis rates

in multiple host plants, the effect that it has on transpiration rates varies according to host plant species. As transpiration rates did not differ between fava bean plants fed upon by symbiotic and aposymbiotic leafhoppers, the symbionts present in the potato leafhopper do not appear to effect the transpiration rate of fava bean.

Soybean response to leafhoppers

The ability of potato leafhoppers to feed on soybean plants is affected by the type of pubescence that the plant possesses. Modern commercial varieties of soybean are generally covered in trichomes, small hairs that cover the stems and undersides of the leaves, which deter or prevent potato leafhoppers from feeding (Bernard and Singh 1969). There are a few older experimental varieties of soybeans that are glabrous (lacking trichomes), and are susceptible to feeding by the potato leafhopper (Broersma et al. 1972). I chose to use the pubescent soybean variety 'Williams 82' for this study because it is the model soybean as its genome has been sequenced (Schmutz et al. 2010).

I tested the ability of adult potato leafhoppers to feed on soybean by comparing the longevity of adults that were not provided with a food source to adults provided unshaved (pubescent) plant stems and experimentally shaved plant stems. Leafhoppers that were starved did not significantly differ in longevity from leafhoppers that were provided with unshaved soybean stems, suggesting that the trichomes present on the stem of soybeans prevent potato leafhoppers from feeding. In contrast, leafhoppers provided with shaved stems survived for a significantly longer length of time. This evidence suggests that potato leafhoppers are able to feed on 'Williams 82' soybean plants as long as the trichomes have been removed.

After determining that potato leafhoppers are able to feed on shaved soybean stems, I tested the response of this variety of soybean to feeding by the potato leafhopper. There were two controls for this experiment: unshaved soybean without leafhoppers, and shaved soybean without leafhoppers. Plants with shaved stems had lower photosynthesis and transpiration rates than plants with unshaved stems, suggesting that the mechanical damage caused by shaving the plant stems caused a plant wound response. I also put symbiotic and aposymbiotic leafhoppers onto soybean plants with shaved stems. Photosynthesis rates for shaved soybean exposed to leafhoppers were lower than for both the unshaved and shaved controls, suggesting that potato leafhoppers cause a plant wound response in soybean. There was no difference in the rate of photosynthesis of plants fed upon by aposymbiotic and symbiotic leafhoppers, indicating that symbionts do not play a significant role in the depression of photosynthesis caused by potato leafhopper feeding on soybean. Transpiration rates for shaved soybeans, shaved soybeans with symbiotic leafhoppers and shaved soybeans with aposymbiotic leafhoppers were not significantly different from each other, but were significantly lower than for unshaved soybeans. This suggests that transpiration rates in soybean are affected most by mechanical damage, because the addition of leafhopper saliva (injected by the living adult leafhopper) did not cause an increased reduction in transpiration.

Conclusions

The symbionts present in the potato leafhopper, *Sulcia muelleri* and *Wolbachia*, appear to negatively influence the photosynthesis and transpiration rates of alfalfa and fava bean, but do not have the same effect on soybean. As both of these symbionts are present in the salivary glands of the potato leafhopper, there are two possible ways that

the symbionts could influence plant wound response to leafhopper feeding: the symbionts may be involved in the production of salivary components which cause a plant wound response cascade to occur when injected into the plant in the insect's saliva, or the symbionts themselves may be injected into the plant along with the saliva.

There is evidence in other insect species that *Wolbachia* produces compounds which are injected into a plant in the saliva of the insect host. For example, *Wolbachia* produceds cytokinins, which when injected into leaves in the saliva of the apple leaf miner, cause green island production and an increase in photosynthesis within the leaf (Kaiser et al. 2010). Although symbiotic potato leafhoppers cause a reduction in photosynthesis rates in both alfalfa and fava bean, the symbionts present in the salivary glands of the potato leafhopper may be producing other components which influence the plant's biology.

Some species of insect symbionts are closely related to plant pathogens. For example, *Pantoea agglomerans* is a species of symbiotic bacteria that is present in multiple species of stink bugs (Prado and Almeida 2009). This species of bacteria is also able to cause rot in plants after being injected into the plants during feeding by the southern green stink bug, *Nezara viridula* (Medrano et al. 2007). This example of an insect symbiont behaving as a plant pathogen suggests that symbionts are able to be transmitted in the saliva of an insect and cause disease in plants. Since insect symbionts such as *Pantoea* are closely related to plant pathogens (De Maayer et al. 2012), it is possible that a plant may mistakenly recognize an insect symbiont as a pathogen and initiate a wound response.

In chapter 2 of this dissertation, aposymbiosis was associated with lower longevity, fecundity and nymph survival into adulthood. Lower leafhopper performance may not simply be due to a lack of symbionts, but may also be due to changes in the leafhopper's feeding behavior. The antibiotics used to create aposymbiotic leafhoppers in this study would kill *Sulcia* and *Wolbachia* along with any other gut bacteria present in the potato leafhopper. If a lack of normal gut microbes causes the leafhopper to change its feeding behavior, either through a reduction in feeding or an increase in feeding to compensate for a lack of nutrients which are normally produced by the symbionts, plant response may be changed. The aposymbiotic leafhoppers used for this study did eat, because leafhoppers that are starved die within 24 hours. The aposymbiotic leafhoppers survived for the 24 hours that they were caged on the host plants, suggesting that they consumed plant tissue during the exposure time. Although the aposymbiotic leafhoppers did feed on the host plants, it is not known how much the leafhoppers consumed over the course of the study. If the aposymbiotic leafhoppers consumed less than the symbiotic leafhoppers, the decrease in plant response to aposymbiotic leafhopper feeding may not have been due to a lack of symbiont-produced salivary components, but instead due to less mechanical injury and saliva exposure. The exact reason why aposymbiotic potato leafhoppers causes less of a decrease in photosynthesis and transpiration rate in alfalfa and fava beans is unknown, and in Chapter 5 of this dissertation, I look at how plant wound response gene expression is affected by introducing potato leafhopper saliva directly into the stem of alfalfa and soybean plants.

Chapter IV: Transcriptome analysis of the salivary glands of the potato leafhopper, *Empoasca fabae*

Abstract

The potato leafhopper, *Empoasca fabae*, is a pest of economic crops in the United States and Canada, where it causes damage known as hopperburn. Saliva, along with mechanical injury, leads to decreases in gas exchange rates, stunting and chlorosis. Although *E. fabae* saliva is known to induce plant responses, little knowledge exists of saliva composition at the molecular level. We subjected the salivary glands of *E. fabae* to Roche 454-pyrosequencing which resulted significant number (30,893) of expressed sequence tags including 2805 contigs and 28,088 singletons. A high number of sequences (78%) showed similarity to other insect species in GenBank, including *Tribolium castaneum*, *Drosophila melanogaster* and *Acrythosiphon pisum*. KEGG analysis predicted the presence of pathways for purine and thiamine metabolic, biosynthesis of secondary metabolites, drug metabolism, and lysine degradation. Pfam analysis showed a high number of cellulase and carboxylesterase protein domains. Expression analysis of candidate genes (alpha amylase, lipase, pectin lyase, etc.) among different tissues revealed tissue-specific expression of digestive enzymes in *E. fabae*. This is the first study to characterize the sialotranscriptome of *E. fabae* and the first for any species in the family of Cicadellidae. Due to the status of these insects as economic pests, knowledge of which genes are active in the salivary glands is important for understanding their impact on host plants.

Introduction

Insect saliva plays an important role in the ingestion of food and in the interaction between an insect and its host. Labial salivary glands are the most common type of insect salivary gland, followed by hypopharyngeal and mandibular salivary glands (Poiani and Da Cruz-Landim, 2010). The paired secretory lobes of the labial salivary glands of hemipterans are located in the head, and are connected to the labium by excretory ducts (Tsai and Perrier, 1996). Hemipterans produce two different types of saliva: sheath saliva and watery saliva (Miles, 1999). Sheath saliva hardens upon contact with air, and helps to stabilize the mouthparts of the insect as it probes into a host plant and prevents plant wound response to components in the watery saliva (Miles, 1964; Will and van Bel, 2006). Watery saliva contains a mixture of amino acids, proteins and digestive enzymes, and is thought to lubricate the stylets inside of the salivary sheath, aid in the digestion of plant material and prevent plant wound response (Carolan et al., 2009; Harmel et al., 2008; De Vos and Jander, 2009).

Leafhoppers are sap-feeding insects in the hemipteran family Cicadellidae and have long been recognized as significant pests of agricultural crops (Nault and Rodriguez, 1985; Poos and Wheeler, 1943). Species are usually classified as either sheath feeders or cell rupture feeders (Miles, 1972). Sheath feeders secrete saliva that hardens into a sheath surrounding their stylets as they feed from a single phloem cell (Hollebone et al., 1966). Cell rupture feeders lacerate multiple cells with their stylets and ingest the phloem that leaks out of the wounded cells while secreting watery saliva to prevent plant wound response (Backus and Hunter, 1989). Leafhopper feeding can cause a generalized plant response known as hopperburn, which is characterized by leaf chlorosis, stunted

growth and reduced yield (Backus et al., 2005). In spite of their agricultural importance, sialotranscriptomes are unknown for any species in the family Cicadellidae.

The potato leafhopper, *Empoasca fabae* (Harris), feeds and reproduces on over 220 species of plants in 26 families and is an especially important agricultural pest of legumes (Lamp et al., 1994). On alfalfa, *Medicago sativa*, *E. fabae* adults feed on the vascular tissues of leaves and stems of plants, while nymphs preferentially feed on leaves (Lamp et al., 2004). *E. fabae* are dynamic feeders, using both cell rupture and modified sheath feeding methods (Backus et al., 2005). As a cell rupture feeder, *E. fabae* mechanically injures phloem and parenchyma cells while injecting saliva to feed on the leaking cell contents. In addition, *E. fabae* can feed directly from vascular tissue, making a short-lived partial salivary sheath to stabilize its stylets during feeding (Zhou and Backus, 1999). Within 24 h of feeding on alfalfa stems, plants display reduced rates of photosynthesis and transpiration, disruption in the transport of photoassimilates, and accumulation of starch in the leaves (Pirone et al., 2005; Lamp et al., 2004; Nielsen et al., 1990). Subsequent to feeding, the generalized wound response caused by the feeding injury leads to the production of hopperburn associated characteristics in alfalfa (Pirone et al., 2005). While past studies have focused on mechanical injury by the mouthparts, saliva plays a role in the response (Ecale and Backus, 1995; DeLay and Lamp, unpublished data), yet the constituents of saliva involved in the plant response are unknown.

To date much of the sialotranscriptomes (salivary gland transcriptomes) have been deciphered in blood feeding insects viz., *Ixodes scapularis* (Nielsen et al., 1990; Valenzuela et al., 2002; Francischetti et al., 2005), *Anopheles gambiae* (Ribeiro et al.,

2006; Arca et al., 2006; Calvo et al., 2006; Neira et al., 2009), *Dermacentor andersoni* (Das et al., 2010), Triatoma brasiliensis (Alarcon-Chaidez et al., 2007), *Ixodes ricinus* (Santos et al., 2007), *Triatoma infestans* (Chmelar et al., 2008), *Glossina morsitans* (Assumpcao et al., 2008) and *Amblyomma variegatum* (Alves-silva et al., 2010), with little studies on phytophagous insects (Ribeiro et al., 2011). Roche® 454 pyrosequencing has in the recent past revolutionized functional genomic studies in non-model organisms, particularly in insects wherein little to no genetic information is available (Francischetti et al., 2007; Morozova and Marra, 2008; Margulies et al., 2005; Vera et al., 2008; Pauchet et al., 2009; Mittapalli et al., 2010; Bai et al., 2011). The developed transcriptomic database can subsequently be used as a reference for future functional studies like RNA seq and to mine for candidate targets for RNAi experiments. The primary goal of this study is to develop a sialotranscriptomic database for *E. fabae* (Expressed Sequence Tags, ESTs of saliva) using 454 pyrosequencing. Results obtained from this study provide insight into potential salivary components that play significant role(s) in the host response subsequent to *E. fabae* feeding injury.

Materials and methods

Insect samples

Potato leafhoppers were collected on alfalfa from the Western Maryland Research and Education Center in Keedysville, Maryland on the morning of June 30, 2009. Leafhoppers were caught with sweep nets, and individual adult *E. fabae* were aspirated into cages containing excised alfalfa sprouts. The cages were then brought back to the laboratory for salivary gland dissection in the afternoon.

Dissection of leafhopper salivary glands

Leafhopper adults were anesthetized by carbon dioxide and placed in a Petri dish that was kept cold on ice. Salivary glands (Figure 4.1) were dissected in a microplate-well with a drop of the sterilized 1X Phosphate Buffered Saline (1X PBS) solution using finetipped forceps. This was accomplished by first pulling the head from the thorax with forceps, then carefully removing the salivary glands that emerged from the distal region of the severed head. A total of 200 salivary glands were dissected and directly dipped into 200 μL Trizol solution (Invitrogen, CA) for RNA preparation.

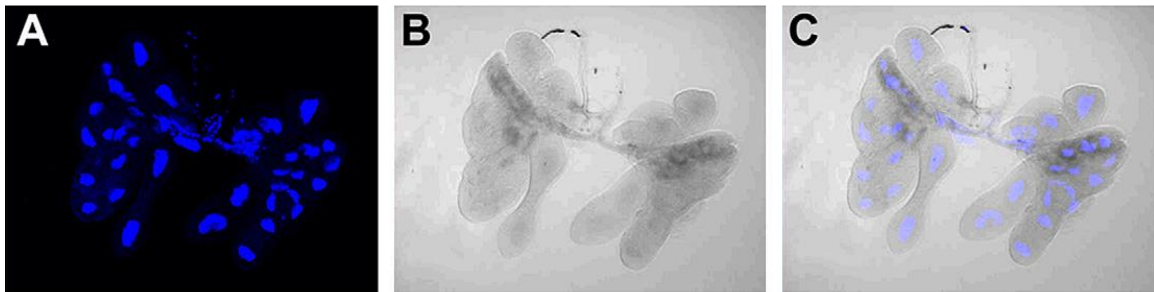

Figure 4.1: Excised salivary glands of the potato leafhopper, *Empoasca fabae*. (A) DAPI staining, showing nuclei. (B) Paired salivary glands of an adult leafhopper, showing the complex acinar structure. (C) Merged image. Image by Jian Wang.

RNA isolation and 454 pyrosequencing

Two hundred pairs of salivary glands of *E. fabae* were used for total RNA isolation using TRIzol® (Invitrogen). QC of the total RNA was assessed with RNA 6000 Nanochip. The library preparation and pyrosequencing was done at Purdue Genomics Core Facility, West Lafayette, IN as per Mittapalli et al., 2010. In brief, a SMART cDNA library construction kit (Clonetech, Mountain View, CA) was used following manufacturer's instructions followed by shearing and nebulization of cDNA with subsequent extraction. The isolated DNA was blunt ended, ligated to adapters and

immobilized on beads. Single stranded DNA was later isolated from these beads and subjected to QC using RNA 6000 (Agilent Technologies). The emPCR reactions were performed to amplify a single template onto a single sequencing bead. One-quarter of a pico-titer plate was sequenced at the Purdue Genomics Core Facility (West Lafayette, IN) using the GS FLX Titanium chemistry (Roche Diagnostics, Indianapolis, IN).

Bioinformatic data analysis

The 454 transcriptomic reads were assembled (after removal of adapters and low quality regions) using Newbler program (Roche) by the Purdue University Genomics Core facility. Initial annotation of assembled sequences, namely isotigs and contigs and the sequences that were not assembled into contigs or isotigs was done using Blast2Go software suite (Conesa et al., 2005; Gotz et al., 2008). Briefly, sequences were searched against GenBank nonredundant database with using BLASTx algorithm (Altschul et al., 1990) with E value cutoff of 10^{-6}. The blast results were mapped to gene ontology terms and annotation was carried out using default annotation parameters in the Blast2Go software suit (Conesa et al., 2005; Gotz et al., 2008). For further functional annotation, the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping was carried out in Blast2Go. To obtain, species distribution, top blast hits were exported from the Blast2Go project and a modified version of a python script available in NCBI taxonomy tree made easy repository (https://github.com/jhcepas/ncbi_taxonomy) was used to retrieve lineage from NCBI taxonomy database. For comparative genomics the sequences were compared to the protein sequences of *Drosophila melanogaster*, *Tribolium castaneum* and *Acrythosiphon pisum* using BLASTx program with expect value of 10^{-5}. Protein domains

were identified using the HMMER v3 program (Eddy, 1998) by importing the Blast2Go project generated by the Purdue Genomics Facility to a local server.

Quantitative real-time PCR

Quantitative real-time PCR was performed on total RNA extracted from the salivary glands, midguts and hind femurs of *E. fabae* collected on *M. sativa* at the University of Maryland's Western Maryland Education and Research Center. Tissues were dissected from individual leafhoppers and pooled by tissue type before total RNA extraction using a Qiagen RNeasy mini RNA extraction kit. cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche), following the provided manufacturer's protocol. This cDNA was used as the template for the qRT-PCR reactions, which were performed using a LightCycler 480 SYBR Green I Master Kit (Roche) on a LightCycler 480 qRT-PCR system (Roche), with the cycling parameters of 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. Primers used in the study (Table 4.1) were designed using IDT SciTools RealTime PCR software (Integrated DNA Technologies).

Relative expression analysis was performed with *E. fabae* specific ribosomal protein 15 as the internal control which was demonstrated to be a suitable internal control in other insects (Mamidala et al., 2011b). Gene expression levels for each of the three tissue types were determined with the Relative Standard Curve method (Mittapalli et al., 2010), using threshold cycle (Ct) values, as detailed in the LightCycler 480 Instrument Operations Manual Version 1.0 (Roche). Relative expression values (REV) were calculated by dividing the quantity of mRNA detected in the target sample by the quantity of mRNA detected in the ribosomal protein sample. Analysis of Variance

Primer target	**Primer sequence**
Alkaline phosphatase (forward)	AGCCACTTGACTGTTCACAC
Alkaline phosphatase (reverse)	CAGCCTCCAGGATATACAAAGG
Alpha-amylase (forward)	CTGGGTAAAGAACTCGGAAGG
Alpha-amylase (reverse)	CTCTGTGTCGTGGTTCTCTATG
Lipase (forward)	CGTTCATGTCCCCTATCTTCAG
Lipase (reverse)	GTTAAAGGCAGGTTTCGGTG
Pectin lyase (forward)	GTGGGAGGCTACTGATAACTAAG
Pectin lyase (reverse)	GCCCCTCTTGTGTAGTTCTG
Laccase (forward)	CATATACTGTCCTCTGCCCTG
Laccase (reverse)	GAGAGTACGACTTTGACCTGC
Wolbachia membrane protein (forward)	AGATTATAATTCTGACTTTTTACTCCTGG
Wolbachia membrane protein (reverse)	AGGAAGCATTAAACTGACAGAGAC
Ribosomal protein 15 (forward)	GGACTAGACACCTTGTATGCAG
Ribosomal protein 15 (reverse)	TCCAAATATTCTCGCTCCAGTG

Table 4.1: Primers used for quantitative real-time PCR of *E. fabae* tissues.

(ANOVA) was used to analyze the REVs of each target gene using the PROC MIXED procedure in SAS (SAS Institute Inc., 2011). For each sample, two biological replicates and two technical replicates were used for the statistical analysis, with the biological replicates used as a random effect in the model. Relative fold changes in tissue gene expression were calculated by setting the tissue type with the lowest REV for the gene (calibrator) at 1X. The calculated standard error shows the variance in the two biological replicates, each of which contained two technical replicates.

Data deposition

The Roche 454 reads of *E. fabae* were deposited to the NCBI Sequence Read Archive under the accession number SRA037848.1 and assembled isotigs which are

above 200 nucleotides were deposited in Transcriptome Shotgun Assembly (TSA) under accession number, 175548 TSA.

Results and discussion

454 pyrosequencing

The 454 pyrosequencing of *E. fabae* sialotranscriptome resulted in 255,491 transcriptomic reads (102,069,574 bp) from which 86.51% and 85.45% were aligned respectively with an inferred read error of 1.77%. These reads were further assembled (after removal of adapters and low quality regions) using Newbler program version 2.5 (Roche). The post assembly of the sequences resulted in 30,893 high quality ESTs including 2805 contigs and 28,088 singletons. The contigs ranged between 60 and 6199 bp with an average length of 1093 bp and totaled to 3031,962 bp. Singletons ranged from 50 to 919 bp with an average length of 396 bp totaled to 11,133,319 bp (Table 4.2). To date, no ESTs are available for *E. fabae* in GenBank and to our knowledge this is the first comprehensive study on sialotranscriptome for this species. A sequence similarity search was done using BLASTx algorithm, which revealed high similarity of *E. fabae* (for 11,322 sequences with taxonomy information) with other insect species (83%) (Figure 4.2). However, a portion of the sequences showed similarity to other eukaryotes (12%), bacteria (4%), fungi (0.4%) and virus (0.3%) as observed in other insect transcriptomic studies (Fig. 4.2) (Pauchet et al., 2009; Mittapalli et al., 2010).

	Contigs	Singleton
<199	13	2182
200:399	77	8906
400:599	743	16975
600:799	491	24
800:999	376	1
1000:1199	249	
1200:1399	226	
1400:1599	136	
1600:1799	109	
1800:1999	99	
2000:2199	79	
2200:2399	24	
2400:2599	42	
2600:2799	28	
2800:2999	13	
3000:3199	23	
3200:3399	37	
3400:3599	5	
3600:3799	3	
3800:3999	1	
4000:4199	18	
4200:4399	1	
4400:4599	0	
4600:4799	0	
4800:4999	4	
5000:5199	0	
5200:5399	1	
5400:5599	4	
5600:5799	0	
5800:5999	1	
6000:6199	2	

Table 4.2: Summary of *Empoasca fabae* sialotranscriptomic sequences.

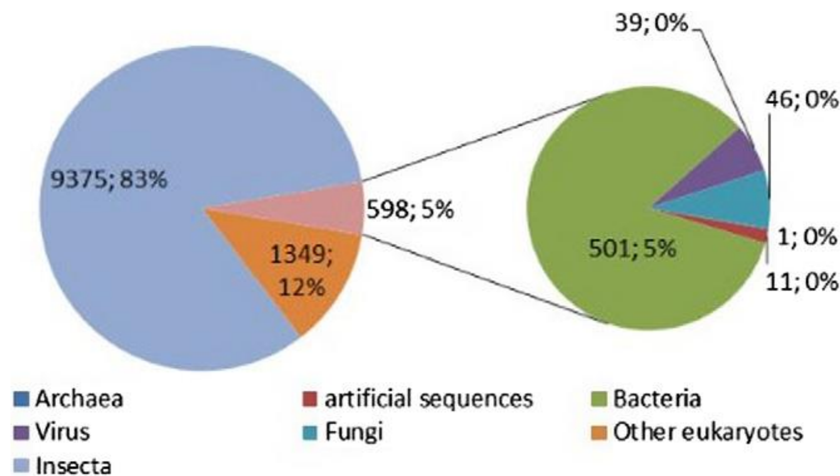

Figure 4.2: A pie-chart showing species distribution of the top blast hits of the *Empoasca fabae* sequences to various taxa.

Comparative analysis

The derived sequences of E. fabae were compared to the proteins of model insect species including the fruit fly (*D. melanogaster* Meigen) of Diptera, red flour beetle (*T. castaneum* Hebst) of Coleoptera and pea aphid (*A. pisum* Harris) of Hemiptera whosegenomes are available (Karatolos et al., 2011; Adams et al., 2000; Richards et al., 2008). The majority of the *E. fabae* sequences showed similarity with those of *A. pisum* (33.24%) followed by *T. castaneum* (33.12%) and *D. melanogaster* (30.75%) (Figure 4.3). Similar observations were reported in a recent study on the brown planthopper (*Nilaparvata lugens* Stal) wherein *N. lugens* sequences shared a higher similarity with *T. castaneum* than with *A. pisum* (Xue et al., 2010). At the current time, it is difficult to explain the similarity of *E. fabae* with *T. castaneum*, but future genomic studies may reveal the evolutionary relationship among these two species. A high percentage (62.58%) of sequences was unique to *E. fabae*, i.e., no significant similarity with the sequences of Gen-Bank non-redundant database. This might be due to novel genes of *E.*

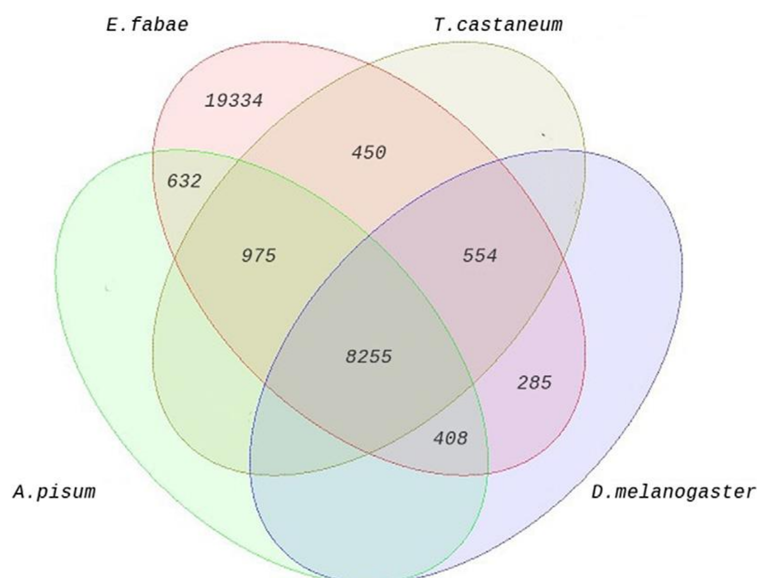

Figure 4.3: Venn diagram showing the comparisons of the sequences from *Empoasca fabae* with the protein sequences of *Drosophila melanogaster*, *Tribolium castaneum* and *Acrythosiphon pisum*. Diagram by Praveen Mamidala.

fabae or untranslated regions of 50 and 30 transcripts and/or assemblage errors as observed in other transcriptomic studies (Pauchet et al., 2009; Mittapalli et al., 2010). We have also compared the *E. fabae* salivary transcripts with recent transcriptomic data of *N. lugens*, which resulted in 11.76% similarity (Xue et al., 2010). The lesser percentage of similarity with *N. lugens* might be due to the comparison with transcriptome data, instead of a fully sequenced genome (Table 4.3). Comparative genomics using the ESTs obtained from these studies will potentially reveal putative function of novel genes (Mittapalli et al., 2010). Moreover, ESTs retrieved from such studies could serve as a useful resource for rapid identification of transcripts involved in a particular biological process (Xue et al., 2010).

Gene ontology

Gene Ontology (GO) terms were assigned to a total of 18,027 transcriptomic sequences based upon their homology to GenBank protein sequences. The GO terms were grouped

Databases	Number of EST hits	%
T. castaneum (Protein_9928)	10,234	33
D. melanogaster (Protein_ 22316)	9,502	31
A. pisum (Protein_17689)	10,270	33
N.lugens (EST_23534)	3635	12

Table 4.3: Comparative summary of *Empoasca fabae* sialotranscripts with *Nilaparvata lugens* and other model insects.

into three main divisions: biological processes, molecular function and cellular components. The majority of the biological processes (Figure 4.4) in the sialotranscriptome represented metabolic processes (4487 sequences) and cellular process (3597 sequences). These results indicate that the cells in the salivary glands are metabolically active, which correlates well with the biological function of the tissue of interest. In addition, the sialotranscriptome contained many sequences involved in biological regulation (1836 sequences), localization (1343) stimulus response (736 sequences). Therefore, it is possible that the sequences coding for these physiological processes are involved in variation in the saliva composition (Guo et al., 2010). However, further functional studies need to be performed to validate these hypotheses.

Among the molecular function GO terms identified the majority were predicted to be involved in binding (4687 sequences) and catalytic (4572 sequences) functions (Figure 4.5). This number includes sequences annotated to be involved in protein, nucleic acid, ion, cofactor and enzyme binding. Hydrolase activities were also identified, wherein 656 *E. fabae* sequences showed homology to hydrolase sequences deposited in GenBank. Hydrolases are enzymes that catalyze chemical bond formation, and have been described in the saliva of the Russian wheat aphid (*Diuraphis noxia*), bird cherry-oat aphid

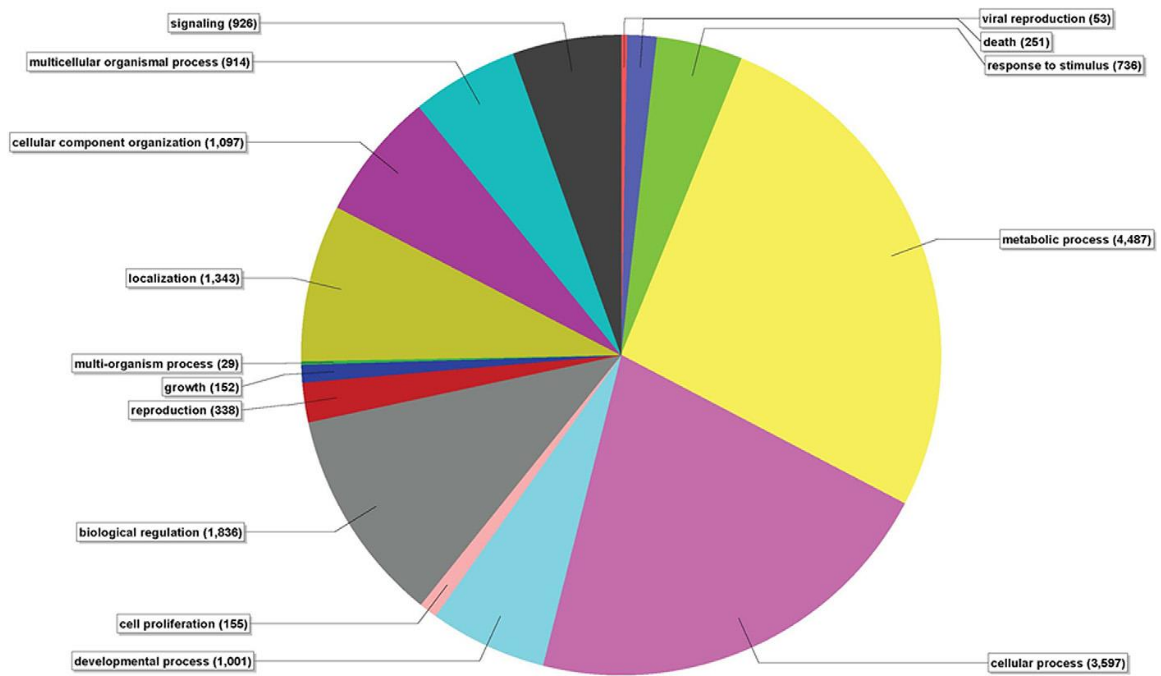

Figure 4.4: Biological process gene ontology terms for the *Empoasca fabae* salivary gland transcriptome.

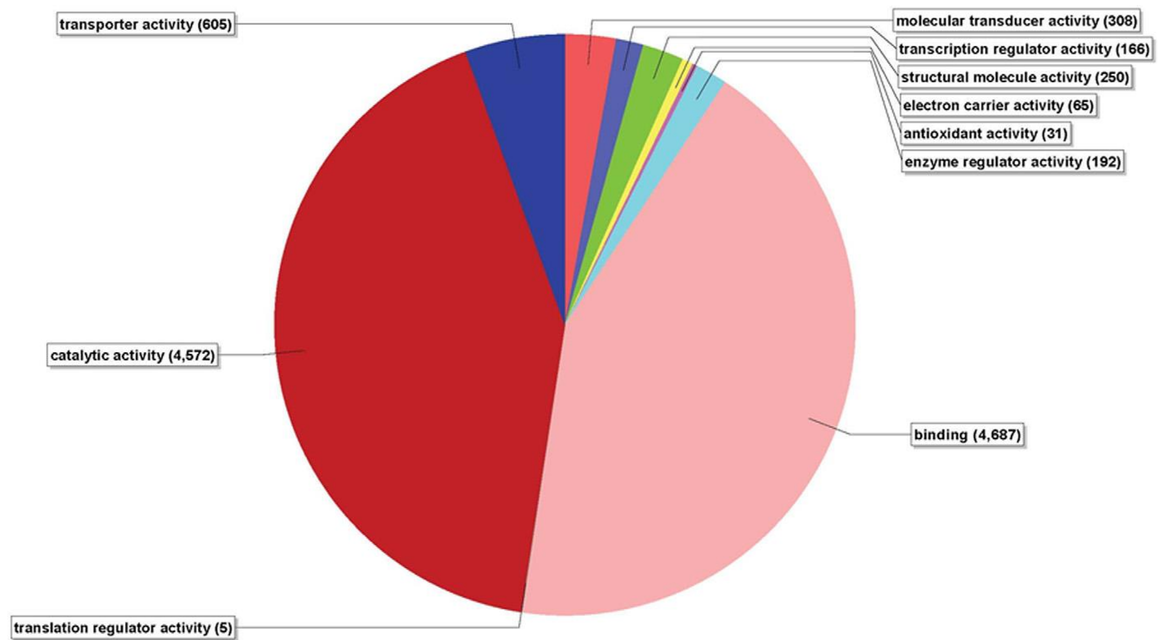

Figure 4.5: Molecular function gene ontology terms for the *Empoasca fabae* salivary gland transcriptome.

(*Rhopalosiphum padi*), and the mosquito *Culex quinquefasciatus* (Bede et al., 2006; Ni et al., 2000).

The majority of cellular component GO terms showed homology with cell (4447) and organelle (2754) sequences in Gen-Bank (Figure 4.6). In addition, 1711 sequences appeared to be involved in macromolecular complex, and 422 sequences had homology to sequences of membrane enclosed lumen.

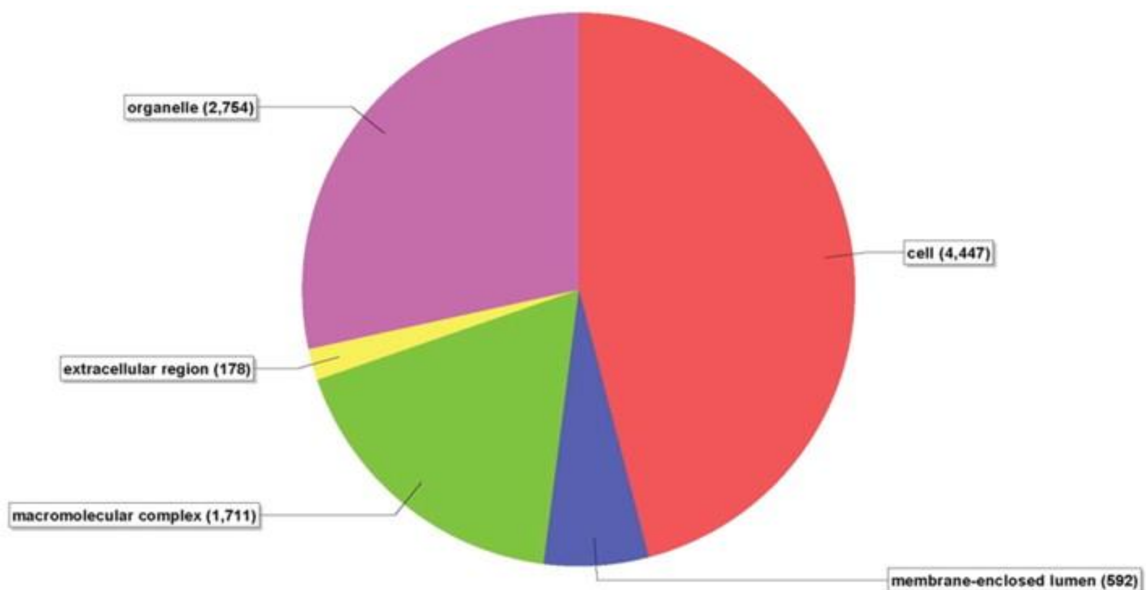

Figure 4.6: Cellular component Gene Ontology (GO) terms for the *Empoasca fabae* salivary gland transcriptome.

KEGG analysis

The KEGG pathways predicted in the sialotranscriptome of *E. fabae* were purine metabolism (215), thiamine metabolism (36), drug metabolism (48), and lysine degradation (43) (Table 4.4). Besides these, we also recovered transcripts involved in

Pathway	#ESTs
Oxidative phosphorylation	103
Tropane, piperidine and pyridine alkaloid biosynthesis	102
Thiamine metabolism	36
Beta-alanine metabolism	27
Drug metabolism–other enzymes	48
Lysine degradation	43
Drug metabolism–cytochrome P450	19
Starch and sucrose metabolism	50
Metabolism of xenobiotics by cytochrome P450	20
Fatty acid metabolism	63
Amino sugar and nucleotide sugar metabolism	80
Purine metabolism	215
Pyrimidine metabolism	86

Table 4.4: Predicted KEGG pathways in sialotranscriptome of *E. fabae*.

pathways of fatty acid and amino acid metabolism (63), starch and sucrose metabolism (50) and 20 sequences involved in metabolism of xenobiotics by cytochrome P450.

Protein domains

We identified 676 distinct domains in 3298 sialome transcripts of *E. fabae* using HMMER3 software. Among the top Pfam domains, a cellulase (273) domain was the highest in occurrence (Table 4.5). Phytophagous insects feeding on plant biomass degrade cellulose to glucose and utilize the latter as an energy source. However, the mechanism of carbohydrate metabolism (breakdown) in insects is poorly understood (Ribeiro and Francischetti, 2001). Initially, it was thought that the source of cellulases in insects was from their endosymbionts such as bacteria, fungi and protozoa (Watanabe and Tokuda, 2010). However, Watanabe et al., 1997 described the first insect-origin cellulase gene from *Retticulitermes speratus* RsEG, which encodes a endo-b-1,4-glucanase. There is further evidence that insects from Dictyoptera, Orthoptera and

Pfam accession	Pfam domain	Pfam domain description	#Occurrence
PF00135.21	COE	Carboxylesterases	268
PF00150.11	Cellulase	Cellulase	273
PF00096.19	Zinc finger	Zinc finger C_2H_2	77
PF00151.12	Lipase	Lipase	80
PF00128.17	Alpha amylase	Alpha amylase	56
PF00704.21	Glyco hydro_18	Glycosyl hydrolase	48
PF00069.18	Protein kinase	Protein kinase	68
PF00076.15	RRM_1	RNA recognition motif (a.k.a RPM, RBD, or RNP domain)	54
PF00089.19	Trypsin	Trypsin	44
PF01607.17	CBM_14	CBM_14	47
PF00067.15	p450	Cytochrome P450	39
PF03723.7	Hemocyanin	Hemocyanin	33
PF00071.15	Ras	Ras family	28
PF00227.19	Proteasome	Proteasome	27
PF02798.13	GST	Glutathione-*S*-transferase	22

Table 4.5: Top Pfam domains identified in *E. fabae* sialotranscriptome.

Coleoptera produce their own cellulases in the foregut, midgut or salivary glands (Martin, 1983; Scharf et al., 2003; Sugimura et al., 2003). Insect cellulases which possess high relative activity at alkaline pH are believed to have potential applications in the biofuel industry (Martin, 1983; Wei et al., 2006).

Next to cellulase domains, we found a high number of carboxylesterases (COE) domains (=268). COEs are multigene families and are widely distributed among prokaryotes and eukaryotes. Theseare primarily involved in detoxification, development and neurogenesis (Willis et al., 2011; Satoh and Hosokawa, 1998; Marshall et al., 2003; Ranson et al., 2002; Bornscheuer, 2002). The role of COEs in detoxification is well

studied in insects and is thought to play important roles in defense against plant allelochemicals and various synthetic chemicals within the context of metabolic resistance (Mittapalli et al., 2005; Small and Hemingway, 2000). However, the function of salivary COEs in phytophagous insects remains elusive.

A high number of lipase domains (84) were predicted in the current study. Lipases are multifunctional proteins involved in lipid acquisition, storage and mobilization besides their involvement in reproduction and development (Mamidala et al., 2011a; Horne et al., 2009). Lipases perform a diverse and unique array of functions in insects and are well documented in several blood feeding insects, however, these are poorly understood in phytophagous insects (Arrese and Soulages, 2010; Tunaz and Stanley, 2004; Anderson et al., 2006). In a recent study of *Mayetiola destructor*, a lipase salivary transcript (MdesL1) was shown to be involved in extra-oral digestion and host cell permeability (Anderson et al., 2006). The other Pfam domains of digestive enzymes in the current study include alpha amylase (56), glycosyl hydrolase (48) and trypsin (44) domains. Alpha amylase, glycosyl hydrolase and trypsin are known for their digestive role in insects and in recent studies have been reported to play an important role in salivary secretions of insects (Shukle et al., 2009; Hosseininaveh et al., 2009). Functional characterization of these digestive enzymes of E. fabae may shed light on their mode of extra-oral digestion.

Besides the above-mentioned digestive associated transcripts, we also found a high number of cytochrome P450 (39) and glutathione-S-transferase (GST) domains (22), which are often associated with detoxification of plant allelochemicals, insecticides and endogenous metabolites (Scharf et al., 2010; Small and Hemingway, 2000). The role of

cytochrome P450s and GSTs in particular are well documented in insect midgut and fat body tissues (Pauchet et al., 2009). However, little is known on the role of cytochrome P450s in insect saliva. Other protein domains that were predicted from the sialotranscriptome of *E. fabae* were Zinc finger C2H2 (77), Protein kinase (68), RNA recognition motif (54), Hemocyanin (33), Ras family (28) and Proteasome (27) domains (Table 4.5).

Genes of interest

We screened the *E. fabae* sialotranscriptomic database for known insect saliva gene products. The ESTs encoding for endobeta-glucanase (EBG), alpha-amylase, chitinase, lipase etc. were found to be predominant among top ten potential salivary gland specific proteins (Table 4.6). Among the candidate genes listed in Table 4.6, we found high occurrence of EBGs (58) putatively involved in the break-down of cellulose, a major component of plant cell walls. The salivary gland specific expression of these EBGs is well documented in termites (*R. speratus*), the blister beetle (*Mylabris pustulata*), and various species of aphids (Motoyama and Dauterman, 1980; Slaytor, 1992; Watanabe et al., 1997). EBGs are also reported in the foregut and midgut of termites and cockroaches, where they are involved in breaking down ingested plant matter (Motoyama and Dauterman, 1980). Further, the EBGs of *Lygus* are well demonstrated to degrade the cell walls of alfalfa (*M. sativa*), damage which was previously thought to be caused by the insect's mouthparts (Will and van Bel, 2006). Future studies on the function of *E. fabae* saliva may shed light on the role of EBGs and other cell wall degrading enzymes in the development of hopperburn associated symptoms (Harmel et al., 2008).

Expression analysis of an *E. fabae* alpha-amylase revealed the highest mRNA levels in the midgut followed by salivary glands and femur. Alpha-amylases break down polysaccharides by hydrolyzing alpha-D-(1,4)-glucan bonds. Starch is a common polysaccharide found in plants, and is known to be degraded into sucrose by the alpha-amylase found in honeybee (*Apis mellifera*) saliva (Ohashi et al., 1999). Alpha-amylases have also been found in the saliva of several other insects including the silkworm (*Bombyx mori*) (Ngernyuang et al., 2011), the mosquito (*Aedes aegypti*) (Grossman and James, 1993), the rice weevil (*Sitophilus oryzae*), red flour beetle (*Tribolium castaneum*), and yellow meal worm (*Tenebrio molitor*) (Feng et al., 1996). In all of these cases, alpha-amylase breaks ingested polysaccharides down to sucrose, and the role that alpha-amylase plays in the saliva of *E. fabae* is likely to be similar. The high EST occurrence (36) and higher mRNA levels of alpha-amylase better explains their putative role in digesting ingested plant compounds.

A high number of ESTs coding for chitinases (20) in *E. fabae* sialotranscriptome is intriguing (Table 4.6). Insects are known to produce chitinases for chitin degradation during molting (Feng et al., 1996). However, chitinases have also been reported in the saliva of ants that feed on fungi, where the enzyme is necessary to degrade the fungal cell wall (Merzendorfer and Zimoch, 2003). The occurrence of these chitinases in *E. fabae* suggests that their role is to degrade polysaccharides found in plant cell walls, allowing the insect to feed more easily.

Among the identified lipases (14) in the current EST database, we detected transcript levels for one candidate lipase (Isotig 00445) which was specifically expressed in the salivary glands of *E. fabae* (Fig. 4.7A). Lipases have been reported in the salivary

Candidate genes	Number of occurrences in salivary glands
Endo-beta-glucanase	58
Alpha-amylase	36
Chitinase, acidic mammalian	14
Chitinase 10	4
Chitinase 1	2
Lipase	7
Pancreatic lipase	7
Carbonic anhydrase	6
Pectin lyase	3
Aldehyde dehydrogenase	2
Beta-glucosidase	2
Protein phosphatase	2
Superoxide dismutase	2
Alpha-mannosidase	1
Catalase	1

Table 4.6: Genes of interest identified in the salivary gland transcriptome of *Empoasa fabae*.

glands of multiple species of insects, including the Hessian fly (*M. destructor*) (Shukle et al., 2009), the mosquito (*Anopheles stephensi*) (Valenzuela et al., 2003), the froghopper (*Aeneolamia varia saccharind*) (Hagley, 1966), and the milkweed bug (*Oncopeltus fasciatus*) (Francischetti et al., 2007). In these insects, lipases are reported to break down lipids of the host cells, facilitating ingestion and digestion. Therefore, it is likely that the lipases found in the sialotranscriptome of *E. fabae* are also used to break down cell membranes in order to facilitate feeding. In the case of the froghopper (*A. varia saccharind*), salivary lipases cause the red streaking of leaf tissue associated with blight symptoms in sugarcane (Hagley, 1966). Other studies have shown that phospholipases in saliva can induce plant response cascades that can cause symptoms of feeding damage

(Munnik et al., 1995; Wang, 1999). These studies suggest that lipases are capable of causing wound response cascades that are similar to the hopperburn caused by *E. fabae* feeding. Therefore, the role that salivary lipases play in the plant response to *E. fabae* should be investigated further.

In addition to the above putative salivary proteins, we also examined mRNA transcript levels for alkaline phosphatase, pectin lyase, laccase and *Wolbachia* surface membrane protein putatively involved in digestion. Among these, alkaline phosphatase expression was detected only in the salivary glands (Fig. 4.8B), whereas the transcript levels of pectin lyase were found to be higher in the salivary glands (20) followed by the midgut (16) (Fig. 4.7B). While alkaline phosphatases have important functions in the salivary glands, development, cuticle sclerotization, and neural and renal function of insects, the pectin lyases are major insect salivary proteins involved in pectin (the major polysaccharide in plant cell walls) degradation and modification, facilitating digestion of ingested plant material (Harper and Armstrong, 1972; Chang et al., 1993; Yang et al., 2000; Funk, 2001). The peak mRNA transcript levels of laccase in midgut (650) and salivary glands (450) (Fig. 4.7C) suggests their induced expression for rapid oxidation of phytotoxic compounds, which is evident with other insect species (Hattori et al., 2005).

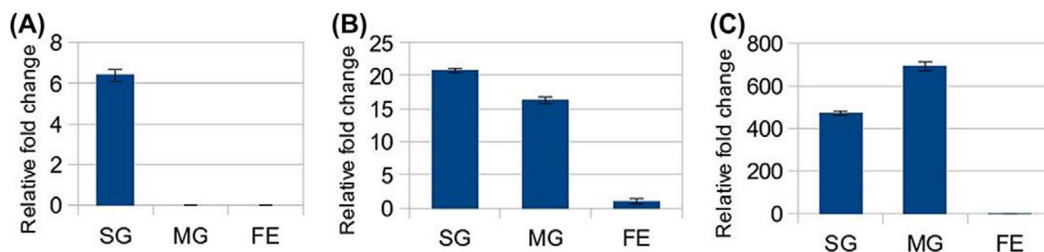

Figure 4.7: Transcript levels of *Empoasca fabae* lipase (A); pectin lyase (B); and laccase (C) in salivary gland (SG), midgut (MG) and hind femur (FE) tissues. A ribosomal protein of *E. fabae* (EfRPL15) was used as the internal control for calculating relative expression. Error bar represents standard error for two biological replicates (each with two technical replicates).

The expression levels of *Wolbachia* membrane protein were exclusively seen in the midgut (Fig. 4.8C). *Wolbachia* is an insect-associated bacterium found in the midgut of many other insect species, and can be transferred horizontally via saliva injection into an insect's host plant (Sintupachee et al., 2006).

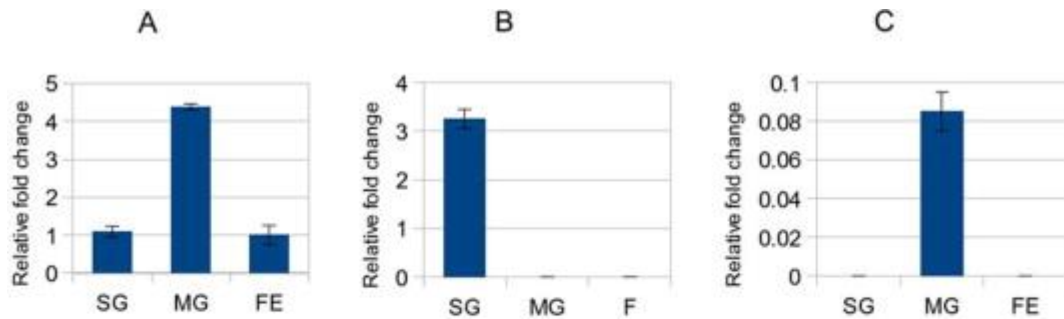

Figure 4.8: Transcript levels of *Empoasca fabae* alpha amylase (A); alkaline phosphatase (B); and *Wohlbachia* membrane protein (C) in salivary gland (SG), midgut (MG) and hind femur (FE) tissues. A ribosomal protein of *E. fabae* (EfRPL15) was used as the internal control for calculating relative expression. Error bar represents standard error for two biological replicates (each with two technical replicates).

This study focused on the sialotranscriptome of adult *E. fabae* that have fed on alfalfa. Analysis of gene expression in the salivary glands of *E. fabae* nymphs or individuals that have fed on different food sources may show different expression patterns. Plant-feeding insects are known to secrete different salivary components depending on the developmental stage of the insect (Gouinguene et al., 2003; Takabayashi et al., 1995), and we sought to minimize variance in gene expression by using only adult *E. fabae* in this study. In addition, phytophagous insects are known to vary their salivary protein composition in response to the nutritional content and defensive compounds of different host plants (Peiffer and Felton, 2005). Therefore, we collected leafhoppers from a single plot of alfalfa to minimize the potential effects of host plant composition on the sialotranscriptome of *E. fabae*. As adult *E. fabae* are

polyphagous and capable of flight, the leafhoppers used in the study may have fed on host plants other than alfalfa. Therefore, we pooled the salivary glands of 200 adult *E. fabae* collected from the interior of an alfalfa field into one sample in order to minimize the genetic contribution of the few individuals that may have fed on an alternative host plant to the assembled sialotranscriptome data.

Conclusions

This is the first comprehensive study of the *E. fabae* sialotranscriptome, and the first of any cicadellid leafhopper species. The goal of this study was to understand which genes are active in the salivary glands of adult leafhoppers in an attempt to understand the components of the saliva produced by this economic pest. A number of known insect salivary enzymes were detected in the sialtotranscriptome of *E. fabae*, as well as sequences involved in cellular metabolism and biological processes. Tissue-specific expression analysis suggests that some putative digestive enzymes identified in the sialotranscriptome are produced at higher levels in the salivary glands than in the midgut. These results, along with the sequences deposited in GenBank, provide insight into the functioning of the salivary glands of adult *E. fabae* that have fed on alfalfa.

Acknowledgments

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Chapter V: Change in gene expression levels in plants after exposure to saliva from potato leafhopper

Abstract

The potato leafhopper, *Empoasca fabae*, causes a generalized wound response known as hopperburn on alfalfa, *Medicago sativa*, plants. This study measured the relative gene expression rates of four plant wound response genes (Endo 1-3 ß-D-glucanase, chalcone synthase, isoflavone reductase, and phenylalanine ammonia-lyase) in response to potato leafhopper saliva. In addition to exposing plants to untreated saliva, potato leafhopper saliva was collected and manipulated with heat, filter sterilization, DTT, EDTA, and K_2HPO_4 treatments before being applied to wounded alfalfa stems. All five saliva manipulations led to a decrease in the relative gene expression of isoflavone reductase, chalcone synthase and phenylalanine ammonia-lyase in alfalfa plants stems in comparison to untreated potato leafhopper saliva. Endo 1-3 ß-D-glucanase relative expression rates were more variable, with heat treated saliva causing no difference in expression in comparison to untreated saliva, but filter sterilized saliva causing a significant increase in gene expression in comparison to untreated saliva. DTT, EDTA and K_2HPO_4 saliva manipulation resulted in a decrease in endo 1-3 ß-D-glucanase relative expression rates in relation to untreated saliva. The results obtained from this study suggest that compounds present in untreated potato leafhopper saliva act as elicitors of plant wound response genes in alfalfa.

Introduction

Mechanical wounding of plant tissue has been used to study the effect that chewing insect herbivory has on plant wound response (Green and Ryan 1972). Although

it is easier to control the amount of damage that a plant sustains through mechanical wounding alone, plant response to insect herbivory also relies upon elicitors in insect saliva to upregulate wound response genes (Kessler and Baldwin 2002). For example, proteinase inhibitor II transcription levels are higher in potato, *Solanum tuberosum*, plants that have been fed upon by tobacco hornworms, *Manduca sexta*, than in plants that have been mechanically wounded. When caterpillar regurgitant is added to cut potato leaf petioles, proteinase inhibitor II transcription levels increase to that of plants exposed to the caterpillars themselves, indicating that a combination of wounding and elicitors in the saliva contribute to the plant's wound response (Korth and Dixon 1997). Damage to leaf tissue initiates a series of events, starting with local processes such as sealing of the wounded tissue to prevent opportunistic pathogens from invading the wound and production of defensive compounds to limit insect feeding (de Bruxelles and Roberts 2001). In addition, volatile compounds may be produced, which can function to induce defensive compound production in nearby plants or to attract natural enemies of insect herbivores (Paré and Tumlinson 1999).

Plant damage due to phloem-feeding insects is not as well-understood as the damage caused by chewing insects. Aphids feed by inserting their stylets intercellularly through the plant's epidermis and mesophyll cells in order to reach the phloem (Pollard 1972). During feeding, aphids produce watery saliva containing potential wound response elicitors such as β-glucosidases and peroxidases (Miles 1999). Unlike chewing insects, which may easily move to an unwounded plant to escape plant defensive compounds produced in response to herbivory, phloem feeding insects generally stay in one place to feed, making them more vulnerable to defensive compounds (Giordanengo et al. 2010).

Therefore, phloem feeders must access the phloem cells with a minimum of damage to plant tissue, prevent the induction of plant wound responses, and keep the phloem sieve tube cells alive during feeding (Miles 1999). The resulting plant wound response to phloem feeders is more similar to that caused by plant pathogens than to the response due to damage caused by chewing insects (Inbar et al. 1999).

Plant wound response due to phloem feeding insects is often elicited by watery saliva injected at the feeding site. Oligogalacturonides are produced when pectinases in aphid watery saliva degrade plant cell walls during feeding, and are capable of inducing plant wound response (Will and van Bel 2008). For example, wheat, *Triticum* spp., produces the volatile defense compounds sulcatol and sulcatone when exposed to pectinases in the saliva of the wheat aphid, *Sitobion avenae*, attracting the parasitoid *Aphidius avenae* (Liu et al. 2009). In addition to pectinases, the watery saliva of aphids contains peroxidases and polyphenoloxidases (Cherqui and Tjallingii 2000). These enzymes convert phenolic compounds produced by plants in response to aphid feeding into less toxic forms (Urbanska et al. 1998). Polymerization of toxic phenolic compounds by the spotted alfalfa aphid, *Therioaphis trifolii*, causes brown precipitates to occur near the aphid's feeding site (Jiang and Miles 1993).

Phloem feeding insects activate salicylic acid and jasmonic acid regulated genes, as well as inducing the production of hormones involved in plant response to pathogens (Thompson and Goggin 2006). The aphids *Schizaphis graminum*, *Macrosiphum euphorbiae* and *Myzus persicae* induce salicylic acid-dependent genes, while inhibiting the jasmonic acid-dependent genes (Zhu-Salzman et al. 2004, de Ilardurya et al. 2003, Moran et al. 2002). Jasmonic acid-dependent defense has been shown to increase aphid

mortality and decrease fecundity, while the less effective salicylic acid-dependent defense causes a less severe impact on aphid growth and reproduction (Ellis et al. 2002, Walling 2008). Salicyclic acid-dependent defense is involved in the protection of plants from pathogen attacks, so upregulation of salicyclic acid-dependent defense pathways may increase the suitability of the plant for the prolonged feeding observed in phloem feeding insects (Giordanengo et al. 2010).

Potato leafhopper, *Empoasca fabae*, feeding on alfalfa, *Medicago sativa*, creates characteristic damage known as hopperburn. Approximately 24 hours after adult leafhoppers feed on the stems of alfalfa, a decrease in photosynthetic rate of leaves above the feeding site can be detected (Lamp et al. 2004). In addition, a decrease in transport of photoassimilates through the phloem near the injury site leads to an accumulation of starch in the leaves above the injured stem (Lamp et al. 2004, Nielson et al. 1990). Phloem cells injured due to leafhopper feeding are repaired through the creation of new phloem cells that bypass the injured site, a process aided by the accumulated starch (Ecale Zhou and Backus 1999, Pirone et al. 2005). Stomate closure following leafhopper feeding results in lower levels of CO_2 available for the chloroplasts in the leaves, causing a decrease in functionality of the xylem (Lamp et al. 2007). Seven days after feeding, alfalfa plants exhibit a generalized wound response characterized by chlorosis, stunted growth and reduced stand yield (Ecale and Backus 1995, Lamp et al. 2004). Phloem cells in the stems of alfalfa plants fed upon by the potato leafhopper often become blocked or necrotic (Smith and Poos 1931, Nielson et al. 1990). In addition, plants begin to accumulate carbohydrates in the leaves above the leafhopper feeding site (Ecale and Backus 1995). The symptoms of hopperburn are thought to be due to the blockage of the

phloem, often due to the hypertrophy of surrounding cells which crush the phloem tissue, and accumulation of carbohydrates in the upper leaves (Nielson et al. 1990). Symptoms of injury occur within 24 hours of leafhopper feeding, and damage to the vascular system starts to be repaired eight days after cessation of feeding (Zhou and Backus 1999).

When potato leafhoppers feed on a plant, they inject saliva into the tissue and physically damage phloem and parenchyma cells (Zhou and Backus 1999). Electrical penetration graph (EPG) studies have shown that the potato leafhopper is able to modify its feeding method in order to obtain food more easily. A leafhopper may continuously probe the plant tissue while feeding, alternate the injection of watery saliva with ingestion of plant tissue, or leave its mouthparts in one position while feeding (Backus et al. 2005).

Potato leafhoppers may use one or more of three different feeding strategies while feeding on a plant: lacerate-and-sip, lacerate-and-flush and lance-and-ingest. The lacerate-and-sip feeding strategy involves the leafhopper rapidly moving its stylets in and out of the plant tissue (laceration) while simultaneously producing watery saliva and "sipping" the contents of the column of lacerated cells (Backus and Hunter 1989). The lacerate-and-flush style of feeding consists of the leafhopper either slowly moving its stylets down through the mesophyll and parenchema cells or holding the stylets steady, while producing saliva and alternating the ingestion of cell contents and saliva (Hunter and Backus 1989, Kabrick and Backus 1990). Both lacerate-and-sip and lacerate-and-flush feeding strategies are cell rupture feeding techniques, where the leafhopper ruptures a cell and ingests the contents (Backus et al. 2005). The final feeding strategy, lance-and-ingest, results in ingestion of phloem from leaking sieve elements and excretion of honeydew while the stylets remain motionless (Backus et al. 2005).

Lacerate-and-sip feeding in alfalfa stems is thought to trigger hopperburn in afalfa (Ecale and Backus 1994, Ecale and Backus 1995, Ecale Zhou and Backus 1999). This type of probing results in disorganization of the vascular tissue, hyperplasia and hypertrophy of phloem cells, and the eventual collapse of phloem sieve tube elements (Kabrick and Backus 1990). Blockage of the phloem cells leads to accumulation of photoassimilates in leaves above the feeding site, which in part contributes to the symptoms of hopperburn that occur after leafhopper feeding injury (Kabrick and Backus 1990, Ecale and Backus 1995).

Previous studies have shown that mechanical damage alone is not sufficient to induce hopperburn, as symptoms of hopperburn are only induced when the vascular system of the plant is completely girdled, which does not occur through feeding by the potato leafhopper (Johnson 1934). The mechanical damage inflicted on plant stems during leafhopper feeding is not enough to cause the tissue damage associated with hopperburn, implying that the saliva of the potato leafhopper induces the plant wound response (Ecale and Backus 1995). Exposure of cut alfalfa stems to potato leafhopper saliva has been shown to decrease the photosynthetic rate of leaves above the cut, indicating that hopperburn can be induced through saliva alone (Lamp and DeLay, unpublished data).

Although the physical effects of potato leafhopper feeding on alfalfa tissue have been well documented, there have been no studies published on the plant wound response gene expression elicited by potato leafhopper feeding. The aim of this study was to determine if the saliva of the potato leafhopper induces plant wound response pathways in alfalfa. Potato leafhopper saliva was heat treated to denature heat sensitive proteins,

and treated with DTT and EDTA, both of which have been shown to inhibit insect salivary enzyme activity (Funk 2001). Alfalfa plant stem vascular tissue was then exposed to the treated saliva, along with adult potato leafhoppers, and wound response gene expression levels were measured using quantitative real-time PCR (qRT-PCR). In addition, potato leafhopper saliva was collected and filter sterilized to remove bacteria, then applied to cuts in the vascular tissue of alfalfa stems. Plant wound response gene expression was then measured using qRT-PCR to determine if symbionts present in the leafhopper's saliva play a role in plant wound response gene induction.

Materials and methods

Plant culture

'Ranger' alfalfa seeds were sown in perlite in flats containing 48 individual planting cells. After planting, the flats were placed on greenhouse benches in a mist room at the University of Maryland's greenhouse. Two week old seedlings were carefully removed from the perlite medium, then transplanted into 15cm pots filled with Metro-Mix potting medium (Sun Gro Horticulture). Eight 15cm pots were placed into plastic saucers inside a single screen cage (BioQuip) to exclude greenhouse pests. The screen cages were kept on a greenhouse bench, and were watered three times per week. Four weeks after transplanting the seedlings into 15cm pots, the alfalfa plants were moved into an MB-80 plant growth chamber (Percival Scientific, Inc., Boone, Iowa), and allowed to equilibrate for one week prior to being used for saliva exposure experiments. The environmental growth chamber was kept at 25°C, with 14 hours of light and 10 hours of dark, light intensity at plant height of 120 µmol/ m^2/sec, and 80% relative humidity.

‘Windsor’ fava bean, *Vicia faba*, plants were used for maintaining leafhopper cultures. Three fava bean seeds were planted in a 10cm pot filled with Metro-Mix potting medium (Sun Gro Horticulture), and flats filled with 15 individual pots were placed into screen cages (BioQuip), with one flat per screen cage. The screen cages were placed on greenhouse benches at the University of Maryland’s greenhouse, and watered three times per week.

Insect culture

Adult potato leafhoppers were collected from alfalfa at the University of Maryland’s Western Maryland Research and Education Center in Keedysville, Maryland to establish a laboratory colony. The leafhoppers were placed into collapsible screen cages (BioQuip) containing eight pots of Broad Windsor fava bean plants per cage. Cages were kept in an MB-60 plant growth chamber as described above. Fava bean plants were watered three times per week, and the old plants were replaced with new plants weekly to provide the leafhoppers with new, uninjured host plants.

Week-old potato leafhopper adults were used for all experiments. They were obtained by placing adult leafhoppers into a collapsible screen cage containing fava bean plants. After the leafhoppers were allowed to oviposit in the stems of the plants for three days, the plants were removed from the cage and shaken to remove any leafhoppers still present on the stems. The leafhopper-free plants were then placed into a new screen cage, and the eggs were allowed to hatch. The nymphs were allowed to mature into adults, a process that took approximately three weeks. Four weeks after the end of the oviposition period, the new week-old adults were collected for use in saliva collection and plant response experiments.

Collection of leafhopper saliva

Potato leafhopper saliva was collected by aspirating 100 adult potato leafhoppers into a tube cage sitting on top of a glass petri dish containing 50mL filter sterilized 3% sucrose solution. The petri dish had a layer of Parafilm stretched across the top of it, through which the leafhoppers would probe to feed on the sucrose solution. The cages were placed into a Percival environmental growth chamber under the same conditions described above for plant culture. Leafhoppers were allowed through the Parafilm membrane for 48 hours, and the 3% sucrose solution containing leafhopper saliva was collected and placed into 50 mL Falcon screw top tubes. As a negative control, cages were assembled as described above, but leafhoppers were not placed into the chambers. A total of 5 cages containing leafhoppers and 5 cages without leafhoppers were set up in this manner.

Treatment of saliva

Potato leafhopper saliva was treated in five ways prior to application to alfalfa plant vascular tissue. For each saliva treatment method, the saliva collected from one feeding cage containing leafhoppers and the sucrose solution from one feeding cage without leafhoppers were treated. The first treatment consisted of heating the saliva and the control to 65°C for one hour to denature heat sensitive proteins in the saliva. The second treatment consisted of filter sterilizing the saliva and sucrose solution controls using a 0.2µm Millex Durapore syringe filter unit (Millipore) to remove bacterial cells. For the remaining three treatments, the saliva was treated by adding DTT, EDTA or K_2HPO_4, known insect salivary enzyme inhibitors, just prior to application to alfalfa stems (Funk 2001). For the DTT treatment, 1mL of 0.05M DTT was added to 49mL

saliva or sucrose solution just prior to application to alfalfa stems for a final concentration of 1mM DTT. One mL of 5mM EDTA was added to 49mL saliva or sucrose solution, for a final concentration of 0.1mM EDTA for the EDTA treatment, and 1mL of 0.05M K_2HPO_4 was added to 49mL saliva or sucrose solution for a final concentration of 1mM potassium phosphate.

Application of saliva to alfalfa stems

Alfalfa stems were wounded with a sterile scalpel in the internode beneath the uppermost fully expanded leaf to expose the vascular tissue, and glass wool was applied to the stem and secured with tape (Figure 5.1). The scalpel was used to carefully cut through the stem into the vascular tissue, removing a small wedge of tissue without severing the stem completely. 500uL of treated saliva, untreated saliva or treated sucrose solution was then pipetted onto the wound in the stem, with the glass wool serving to hold the solution in place. The application of 500uL of saliva was the equivalent of the saliva from one leafhopper, because 100 leafhoppers originally fed on 50mL of sucrose solution.

Experimental design

Each of the five saliva manipulations (heat, filter sterilization, DTT, EDTA, K_2HPO_4) were tested in separate experiments. For each experiment, a total of 40 plants were exposed, with 20 plants at a time to a given saliva manipulation. After exposure, leaf samples were collected, and total RNA extracted from each leaf sample. RNA (5uL) from 10 plants was then pooled into a single sample for subsequent cDNA amplification. The experiment was then repeated with a second set of twenty plants, and the RNA was

extracted and pooled as for the first replication. This gave four biological replications for the qRT-PCR analysis for each of the five saliva manipulation experiments.

For each plant used in the experiment, five treatments were applied to separate stems. All treatments were applied to the internode beneath the uppermost fully expanded leaf. The treatments consisted of: 1) an intact control stem, 2) a wounded stem without saliva or sucrose solution added, 3) a wounded stem with treated sucrose solution added, 4) a wounded stem with treated saliva added, and 5) a wounded stem with untreated saliva added. For the intact control stem and wounded stem without saliva or sucrose added, glass wool was applied to the internode beneath the uppermost fully expanded leaf and taped in place, but no solution was applied to the stem. Treatments remained in place for 24 hours, then the terminal leaflet in the uppermost fully expanded leaf was removed and immediately frozen in liquid nitrogen. After the leaves were frozen, they were stored at -80°C for subsequent RNA extraction.

Quantitative real-time PCR

Total RNA was extracted from alfalfa leaves using a Qiagen RNeasy mini RNA extraction kit. For each replication consisting of twenty plants exposed to one of the five different saliva manipulations, 5uL of RNA from ten of the plants was pooled into a single 1.5mL microcentrifige to create a single biological replicate, with 5uL of RNA from each of the remaining ten plants pooled into a second biological replicate. cDNA was then synthesized from each biological replicate using a Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the provided manufacturer's protocol. The qRT-PCR reactions were performed using a LightCycler 480 SYBR Green I Master Kit (Roche) on a LightCycler 480 qRT-PCR system (Roche), following the manufacturer's

instructions. The cycling parameters for the qRT-PCR reaction were 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. Primers used in the study (Table 5.1) were designed using IDT SciTools RealTime PCR software (Integrated DNA Technologies). For each of the five saliva treatments there were four biological replicates (each composed of RNA pooled from 10 plants), with three technical replicates for each biological replicate. qRT-PCR plate design for one replication of 20 plants is shown in Table 5.2. Two plates were used for each saliva treatment/gene of interest combination.

Primer target	**Primer sequence (5' to 3')**
Tubulin (forward)	ACTCTGCTCATATCT
Tubulin (reverse)	GAAAGGAATGAGGTTCACTG
Endo 1-3 ß-D-glucanase (forward)	TCTACCGCGATAGACACAACACTAA
Endo 1-3 ß-D-glucanase (reverse)	ACCACTTGCAGCGTCACTAAAA
Isoflavone reductase (forward)	ACGAGGCAGTTGAGCCAGTTAG
Isoflavone reductase (reverse)	GCGTGGCAACAAAGGTAAGTGT
Chalcone synthase (forward)	ACTATTTGGAGATGGAGCGGCT
Chalcone synthase (reverse)	GGAGCAATTGTTTGTGCAGTCC
Phenylalanine ammonia-lyase (forward)	TCTTGGTGGCGAAACACTGAC
Phenylalanine ammonia-lyase (reverse)	TCCATCACCCAATCACTGCTG

Table 5.1: Primers used for quantitative real-time PCR of alfalfa tissues.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TB 50ng std curve	TB 5ng un wound ctrl	TB 5ng un wound ctrl	TB 5ng un wound ctrl	TB 5ng un wound ctrl	TB 5ng un wound ctrl	CH 50ng std curve	CH 5ng un wound ctrl	CH 5ng un wound ctrl	CH 5ng un wound ctrl	CH 5ng un wound ctrl	CH 5ng un wound ctrl
B	TB 10ng std curve	TB 5ng wound ctrl	TB 5ng wound ctrl	TB 5ng wound ctrl	TB 5ng wound ctrl	TB 5ng wound ctrl	CH 10ng std curve	CH 5ng wound ctrl	CH 5ng wound ctrl	CH 5ng wound ctrl	CH 5ng wound ctrl	CH 5ng wound ctrl
C	TB 2ng std curve	TB 5ng trt sucrose	TB 5ng trt sucrose	TB 5ng trt sucrose	TB 5ng trt sucrose	TB 5ng trt sucrose	CH 2ng std curve	CH 5ng trt sucrose	CH 5ng trt sucrose	CH 5ng trt sucrose	CH 5ng trt sucrose	CH 5ng trt sucrose
D	TB 400pg std curve	TB 5 ng trt saliva	TB 5 ng trt saliva	TB 5 ng trt saliva	TB 5 ng trt saliva	TB 5 ng trt saliva	CH 400pg std curve	CH 5 ng trt saliva	CH 5 ng trt saliva	CH 5 ng trt saliva	CH 5 ng trt saliva	CH 5 ng trt saliva
E	TB 80pg std curve	TB 5ng untrt saliva	TB 5ng untrt saliva	TB 5ng untrt saliva	TB 5ng untrt saliva	TB 5ng untrt saliva	CH 80pg std curve	CH 5ng untrt saliva	CH 5ng untrt saliva	CH 5ng untrt saliva	CH 5ng untrt saliva	CH 5ng untrt saliva
F	TB 16pg std curve	TB 5ng un wound ctrl	TB 5 ng trt saliva				CH 16pg std curve	CH 5ng un wound ctrl	CH 5 ng trt saliva			
G	TB – RT ctrl std curve	TB 5ng wound ctrl	TB 5ng untrt saliva				CH – RT control std curve	CH 5ng wound ctrl	CH 5ng untrt saliva			
H	TB no cDNA ctrl	TB 5ng trt sucrose					CH no cDNA control	CH 5ng trt sucrose				

Table 5.2: qRT-PCR plate setup, showing the setup to analyze chalcone synthase gene expression levels in alfalfa stems exposed to heat treated potato leafhopper saliva. TB: Tubulin, –RT: no reverse transcriptase control, CS: chalcone synthase, light gray background: first pool of 10 plant leaf samples (biological replicate 1), dark gray background: second pool of 10 plant leaf samples (biological replicate 2).

Relative expression analysis was performed using tubulin as the internal control gene. Gene expression levels were determined with the Relative Standard Curve method (Mittapalli et al., 2010), using threshold cycle (Ct) values, as detailed in the LightCycler 480 Instrument Operations Manual Version 1.0 (Roche). To calculate relative expression values (REV), the quantity of mRNA that was detected in the target sample was divided by the quantity of mRNA detected in the tubulin control sample. The REVs of each target gene were subjected to analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (SAS Institute Inc., 2011). Four biological replicates and three technical replicates were used for the statistical analysis of each saliva treatment, and the

biological replicates were used as a random effect in the model. Relative fold changes in tissue gene expression were calculated by setting the treatment (uncut control stem, a cut stem without saliva or sucrose solution added, a cut stem with treated sucrose solution added, a cut stem with treated saliva added, and a cut stem with untreated saliva added) with the lowest REV for the gene at 1X. The calculated standard error is based on the variance in the four biological replicates, each of which contained three technical replicates, while significant differences between means were determined using Fisher's least significant difference test.

Results

Heat manipulation

Saliva heat treatment caused significant differences in the treatment means of endo 1-3 ß-D-glucanase gene expression (F = 3.21, $P_{3,12}$ = 0.05) (Table 5.3, Figure 5.1)

Source	Degrees of Freedom	F	*P* (degrees of freedom)
Total (samples)	59		
Biological replicate	19		
Blocks	3	1.11	0.38 (3, 12)
Treatments	4	3.21	0.05 (4, 12)
Experimental error	12	0.56	0.86 (12, 40)
Sampling error	40		

Table 5.3: Sample ANOVA table for Endo 1-3 β-D-glucanase heat treatment.

Heat treatment of saliva did not cause a significant decrease (2.54 ± 0.39, mean ± SE) in endo 1-3 ß-D-glucanase gene expression in relation to wounded stems (2.34 ± 0.38, 2.59

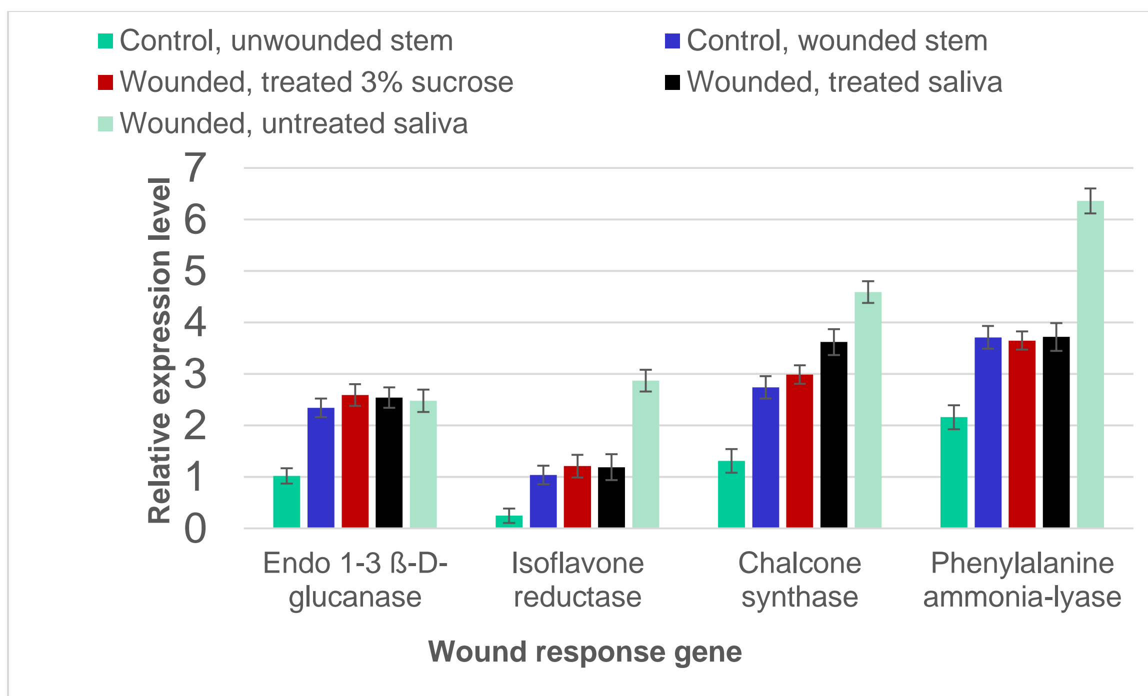

Figure 5.1: Effect of heat manipulation on wound response gene expression of alfalfa exposed to potato leafhopper saliva.

Stem treatment	Endo 1-3 ß-D-glucanase (mean ± SE)	Isoflavone reductase (mean ± SE)	Chalcone synthase (mean ± SE)	Phenylalanine ammonia-lyase (mean ± SE)
Control, unwounded stem	1.02 ± 0.35a	0.25 ± 0.24a	1.31 ± 0.43a	2.16 ± 0.39a
Control, wounded stem	2.34 ± 0.38b	1.04 ± 0.28b	2.74 ± 0.32b	3.71 ± 0.37b
Wounded, treated 3% sucrose	2.59 ± 0.37b	1.21 ± 0.32b	2.99 ± 0.22b	3.65 ± 0.32b
Wounded, treated saliva	2.54 ± 0.39b	1.19 ± 0.35b	3.62 ± 0.37c	3.72 ± 0.35b
Wounded, untreated saliva	2.48 ± 0.36b	2.87 ± 0.31c	4.59 ± 0.34d	6.36 ± 0.36c

Table 5.4: Transcript levels of alfalfa wound response genes when exposed to heat treated potato leafhopper saliva. Tubulin was used as the internal control for calculating relative expression. Standard error is for four biological replicates (each with three technical replicates), with letters indicating significant differences between treatments.

± 0.37, 2.54 ± 0.39b), but did result in higher endo 1-3 ß-D-glucanase expression than the unwounded control stems (1.02 ± 0.35) (Table 5.4). Similarly, the treatment effect for isoflavone reductase (F = 9.99, $P_{4,12}$ = <0.001) gene expression was also significant. Isoflavone reductase gene expression rates were lowest in the unwounded control stems (0.25 ± 0.24), with stems exposed to untreated saliva having the highest relative gene expression rates (2.87 ± 0.31). The relative gene expression rates of isoflavone reductase were not significantly different for wounded stems with no solution added (1.04 ± 0.28), wounded stems with heat treated 3% sucrose added (1.21 ± 0.32), and for wounded stems with heat treated saliva added (1.19 ± 0.35). The same pattern was seen for phenylalanine ammonia-lyase, which had a significant treatment effect (F = 17.89, $P_{4,12}$ = <0.001) with the unwounded control stems having the lowest relative gene expression rates (2.16 ± 0.39) and the stems exposed to unheated saliva have the highest relative expression rates (6.36 ± 0.36). The treatment effect for chalcone synthase was significant (F = 12.37, $P_{4,12}$ = <0.001), with relative expression rates lowest in the unwounded control stems (1.31 ± 0.43), followed by wounded stems with no solution added (2.74 ± 0.32) and wounded stems with heat treated 3% sucrose solution added (2.99 ± 0.22). Wounded stems with heat treated saliva had significantly higher relative expression levels of chalcone synthase (3.62 ± 0.37), with wounded stems exposed to untreated saliva having the highest relative expression levels (4.59 ± 0.34).

Filter sterilization

Potato leafhopper saliva was also filter sterilized to remove particles larger than 0.2µm in size, and relative gene expression levels of four wound response genes were measured after alfalfa stems were exposed to the saliva. The treatment effect of filter

sterilization on endo 1-3 ß-D-glucanase expression was significant (F = 5.47, $P_{4,12}$ = 0.01). Relative expression levels of Endo 1-3 ß-D-glucanase were lowest in unwounded control stems (1.23 ± 0.37, mean ± SE), and highest in wounded stems exposed to filter sterilized saliva (3.39 ± 0.32) (Table 5.5, Figure 5.2). The relative expression levels of

Stem treatment	Endo 1-3 ß-D-glucanase (mean ± SE)	Isoflavone reductase (mean ± SE)	Chalcone synthase (mean ± SE)	Phenylalanine ammonia-lyase (mean ± SE)
Control, uncut stem	1.23 ± 0.37a	0.32 ± 0.22a	1.39 ± 0.41a	2.18 ± 0.33a
Control, cut stem	2.44 ± 0.30b	0.98 ± 0.34b	2.68 ± 0.36b	3.72 ± 0.34b
Cut, treated 3% sucrose	2.52 ± 0.34b	1.13 ± 0.29b	2.82 ± 0.43b	3.51 ± 0.45b
Cut, treated saliva	3.39 ± 0.32c	1.11 ± 0.30b	3.05 ± 0.35b	3.76 ± 0.40b
Cut, untreated saliva	2.78 ± 0.35b	2.18 ± 0.35c	5.42 ± 0.48c	6.02 ± 0.43c

Table 5.5: Transcript levels of alfalfa wound response genes when exposed to filter sterilized potato leafhopper saliva. Tubulin was used as the internal control for calculating relative expression. Standard error is for four biological replicates (each with three technical replicates), with letters indicating significant differences between treatments.

wounded stems with no solution added (2.44 ± 0.30), wounded stems with filter sterilized 3% sucrose added (2.52 ± 0.34), and wounded stems exposed to saliva that had not been filter sterilized (3.21 ± 0.35) were intermediate in value but not significantly different from one another. The treatment effects for isoflavone reductase (F = 4.83, $P_{4,12}$ = 0.01), chalcone synthase (F = 12.80, $P_{4,12}$ = <0.001) and phenylalanine ammonia-lyase (F = 12.36, $P_{4,12}$ = <0.001) were all significant, and the relative gene expression levels all followed a similar pattern. The unwounded control stems having the lowest relative expression levels and the wounded stems with filter sterilized saliva having the

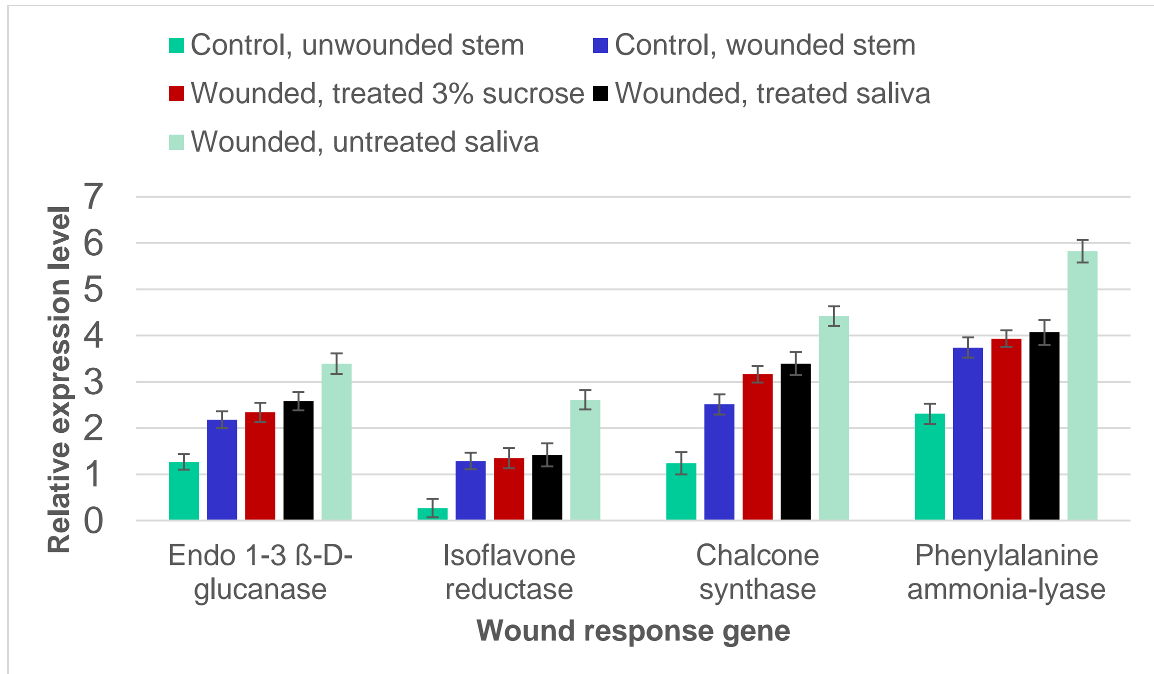

Figure 5.2: Effect of filter sterilization on wound response gene expression of alfalfa exposed to potato leafhopper saliva.

highest levels. Wounded stems with no solution added, wounded stems with filter sterilized sucrose solution added, and wounded stems with untreated saliva added did not have significantly different relative expression levels (Table 5.5).

DTT treatment

DTT (1mM) was also used to treat potato leafhopper saliva, and alfalfa plant wound response gene relative expression levels were measured after exposure to treated saliva. The treatment effects for both endo 1-3 ß-D-glucanase (F = 8.84, $P_{4,12} = 0.001$) and isoflavone reductase (F = 7.57, $P_{4,12} = 0.003$) were significant, and relative gene expression levels followed a similar pattern (Table 5.6, Figure 5.3). The unwounded control stems had the lowest relative gene expression levels, followed by the wounded stems with no solution added. The relative gene expression levels for wounded stems with 1mM DTT treated 3% sucrose added, wounded stems with 1mM DTT treated saliva

Stem treatment	Endo 1-3 ß-D-glucanase (mean ± SE)	Isoflavone reductase (mean ± SE)	Chalcone synthase (mean ± SE)	Phenylalanine ammonia-lyase (mean ± SE)
Control, unwounded stem	1.34 ± 0.31a	0.28 ± 0.24a	1.32 ± 0.43a	2.29 ± 0.40a
Control, wounded stem	2.21 ± 0.34b	1.06 ± 0.29b	2.51 ± 0.25b	3.42 ± 0.41b
Wounded, treated 3% sucrose	2.89 ± 0.28c	2.31 ± 0.41c	2.79 ± 0.35bc	3.66 ± 0.39b
Wounded, treated saliva	3.08 ± 0.26c	2.25 ± 0.35c	2.98 ± 0.23c	3.82 ± 0.43b
Wounded, untreated saliva	3.62 ± 0.25d	2.20 ± 0.34c	4.97 ± 0.35d	5.83 ± 0.38c

Table 5.6: Transcript levels of alfalfa wound response genes when exposed to potato leafhopper saliva treated with 1mM DTT. Tubulin was used as the internal control for calculating relative expression. Standard error is for four biological replicates (each with three technical replicates), with letters indicating significant differences between treatments (P = 0.05)

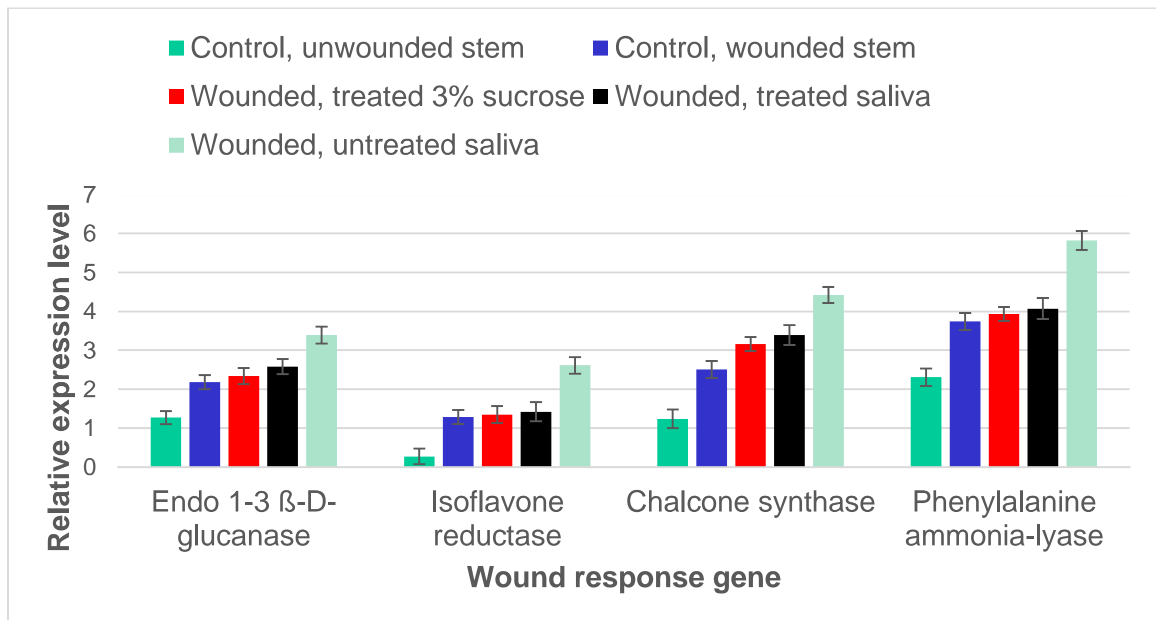

Figure 5.3: Effect of DTT manipulation on wound response gene expression of alfalfa exposed to potato leafhopper saliva.

added and wounded stems with untreated saliva added were higher than for the unwounded and wounded controls, but untreated saliva added were higher than for the unwounded and wounded controls, but were not significantly different from one another. The treatment effect for chalcone synthase was significant (F = 16.02, $P_{4,12} = <0.001$). Relative expression levels of chalcone synthase were lowest in the unwounded control (1.32 ± 0.43, mean ± SE), followed by wounded stems with no solution added (2.51 ± 0.25). Wounded stems with 1mM DTT treated 3% sucrose (2.79 ± 0.35) and wounded stems with 1mM DTT treated saliva (2.98 ± 0.23) had significantly higher expression rates than the unwounded control, but were not significantly different from one another. The highest chalcone synthase gene expression rates were found in stems exposed to untreated saliva (4.97 ± 0.35). Like chalcone synthase, the treatment effect for phenylalanine ammonia-lyase was significant (F = 10.14, $P_{4,12} = <0.001$), with the lowest expression levels in the unwounded control stems (2.29 ± 0.40), and the highest were found in the stems exposed to untreated saliva (5.83 ± 0.38). The relative chalcone synthase expression rates for the remaining three treatments were intermediate in value, but were not significantly different from one another.

EDTA treatment

Alfalfa plants were also exposed to potato leafhopper saliva treated with 0.1mM EDTA, and relative wound response gene expression was measured. The treatment effects for endo 1-3 ß-D-glucanase (F = 3.61, $P_{4,12} = 0.04$), isoflavone reductase (F = 4.76, $P_{4,12} = 0.02$), and phenylalanine ammonia-lyase (F = 13.34, $P_{4,12} = <0.001$) were all significant, and the relative expression levels all followed the same pattern (Table 5.7, Figure 5.4). For each of these genes, relative expression for stems exposed to untreated

Stem treatment	Endo 1-3 ß-D-glucanase (mean ± SE)	Isoflavone reductase (mean ± SE)	Chalcone synthase (mean ± SE)	Phenylalanine ammonia-lyase (mean ± SE)
Control, unwounded stem	1.36 ± 0.31a	0.38 ± 0.31a	1.19 ± 0.36a	2.25 ± 0.32a
Control, wounded stem	2.21 ± 0.32b	1.11 ± 0.40b	2.68 ± 0.26b	3.59 ± 0.35b
Wounded, treated 3% sucrose	2.57 ± 0.37b	1.32 ± 0.39b	3.02 ± 0.29bc	3.82 ± 0.39b
Wounded, treated saliva	2.62 ± 0.34b	1.39 ± 0.42b	3.15 ± 0.27c	3.95 ± 0.38b
Wounded, untreated saliva	3.25 ± 0.46c	2.73 ± 0.42c	4.27 ± 0.42d	6.03 ± 0.41c

Table 5.7: Transcript levels of alfalfa wound response genes when exposed to potato leafhopper saliva treated with 0.1mM EDTA. Tubulin was used as the internal control for calculating relative expression. Standard error is for four biological replicates (each with three technical replicates), with letters indicating significant differences between treatments (P = 0.05)

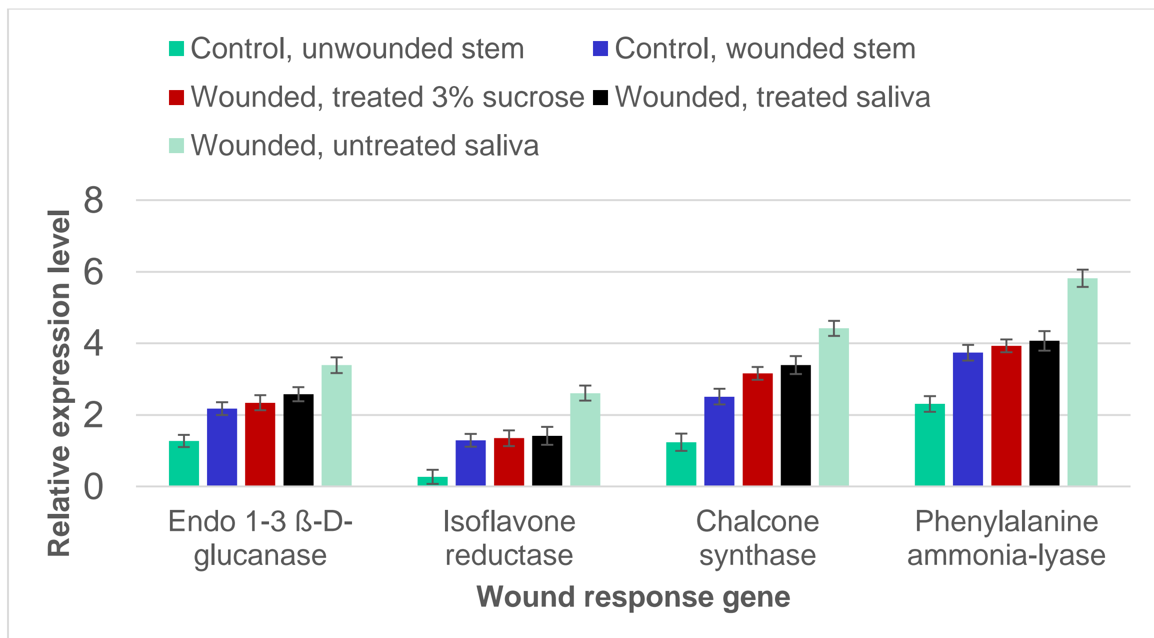

Figure 5.4: Effect of EDTA manipulation on wound response gene expression of alfalfa exposed to potato leafhopper saliva.

saliva, and lowest for unwounded stems. Expression levels for wounded stems, wounded stems with 0.01mM EDTA treated 3% sucrose added and wounded stems with 0.01mM EDTA treated saliva added were intermediate in value, but not significantly different from one another. Similarly, the treatment effect for chalcone synthase (F = 11.58, $P_{4,12}$ = <0.001) was also significant, and the relative expression levels were lowest in unwouned stems (1.19 ± 0.36, mean ± SE) and highest in stems exposed to untreated saliva (4.27 ± 0.42). The expression levels for the remaining three treatments were intermediate in value, with the wounded control stems having significantly lower levels of chalcone synthase expression (2.68 ± 0.26) than wounded stems with 0.1mM EDTA treated saliva applied (3.15 ± 0.27).

K_2HPO_4 treatment

Potato leafhopper saliva was treated with 0.05mM K_2HPO_4, then applied to alfalfa plant stems to measure relative gene expression of plant wound response genes to treated saliva. Like the relative gene expression patterns seen for potato leafhopper saliva treated with 0.1mM EDTA, the treatment effects for endo 1-3 ß-D-glucanase (F = 5.65, $P_{4,12}$ = 0.009), isoflavone reducatase (F = 10.32, $P_{4,12}$ = <0.001) and phenylalanine ammonia-lyase were all significant, and relative gene expression levels all followed the same pattern (Table 5.8, Figure 5.5). For these three genes, relative expression was highest for plants stems exposed to untreated potato leafhopper saliva, and lowest for unwounded plant stems. Relative gene expression was intermediate for wounded control stems, wounded stems with 0.05mM K_2HPO_4 applied and for wounded stems with 0.05mM K_2HPO_4 treated saliva applied. Similarly, the treatment effect for chalcone synthase (F = 10.50, $P_{4,12}$ = <0.001) was significant, and relative expression

Stem treatment	Endo 1-3 ß-D-glucanase (mean ± SE)	Isoflavone reductase (mean ± SE)	Chalcone synthase (mean ± SE)	Phenylalanine ammonia-lyase (mean ± SE)
Control, unwounded stem	1.27 ± 0.31a	0.27 ± 0.23a	1.24 ± 0.37a	2.31 ± 0.35a
Control, wounded stem	2.18 ± 0.28b	1.29 ± 0.27b	2.51 ± 0.33b	3.74 ± 0.39b
Wounded, treated 3% sucrose	2.34 ± 0.32b	1.35 ± 0.31b	3.16 ± 0.31c	3.93 ± 0.40b
Wounded, treated saliva	2.58 ± 0.36b	1.42 ± 0.32b	3.39 ± 0.36c	4.07 ± 0.42b
Wounded, untreated saliva	3.39 ± 0.33c	2.61 ± 0.36c	4.42 ± 0.43d	5.82 ± 0.38c

Figure 5.8: Transcript levels of alfalfa wound response genes when exposed to potato leafhopper saliva treated with 0.05mM K_2HPO_4. Tubulin was used as the internal control for calculating relative expression. Standard error is for four biological replicates (each with three technical replicates), with letters indicating significant differences between treatments.

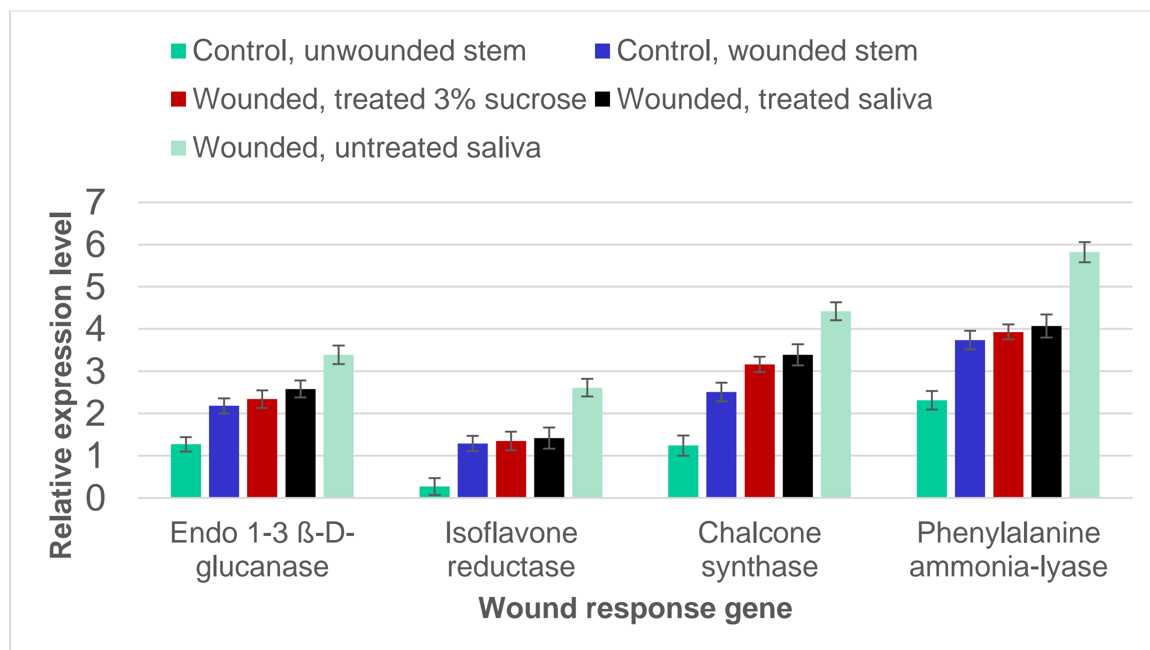

Figure 5.5: Effect of K_2HPO_4 manipulation on wound response gene expression of alfalfa exposed to potato leafhopper saliva.

levels were highest for plant stems exposed to untreated saliva (4.42 ± 0.43, mean ± SE). Relative expression of chalcone synthase was second highest in wounded stems with 3% sucrose treated with 0.05mM K_2HPO_4 (3.16 ± 0.31) and wounded stems with 0.05mM K_2HPO_4 treated saliva (3.39 ± 0.36), which were not significantly different from one another. The lowest expression values were found in the wounded control stems (2.51 ± 0.33) and unwounded control stems (1.24 ± 0.37).

Discussion

Plant resistance to insect feeding can be due to constitutive or induced defenses (Mauricio et al. 1997). Constitutive defenses against insect herbivory include toxins that are expressed at a constant level in a plant's tissue, whether the plant is wounded or not (Wittstock and Gershenzon 2002). Unlike constitutive defenses, induced defenses to herbivory are only expressed after the plant has perceived an attack, a process that is induced by an elicitor (Walling 2009). Herbivorous insects and plant pathogens can have unique sets of elicitors which allow plants to tailor their response to the particular attacker (Stout et al. 2006, Glazebrook 2005). Previous studies (chapters 1, 2, and 3 of this dissertation) found that the potato leafhopper has two species of symbionts present in salivary gland tissue, and that these symbionts contribute to the leafhopper's population ecology and plant physiological response to leafhopper feeding. In addition, *Wolbachia* genes were discovered in the sialotranscriptome of the potato leafhopper, along with genes for the production of enzymes known to initiate plant wound response gene expression (Chapter 4 of this dissertation). Therefore, this chapter looked at how plant wound response gene expression levels are effected by the presence of untreated potato leafhopper saliva, and saliva manipulated to inactivate salivary proteins or remove

symbionts through filter sterilization. Alfalfa plant stems were exposed to potato leafhopper saliva manipulated in five different ways (heat, filter sterilization, DTT, EDTA and K_2HPO_4) to determine if any of the treatments inactivated an elicitor in the leafhopper's saliva. The resulting gene expression levels suggested that there are plant wound response gene elicitors present in the saliva of the potato leafhopper, and that treatment of the saliva is able to at least partially inactivate the elicitors.

Experimental rationale

The heat, DTT, EDTA and K_2HPO_4 saliva manipulations used in this study were the same treatments used to inactivate alkaline phosphatase in the saliva of the whitefly, *B. tabici* (Funk 2001). DTT is often used in buffers where it stabilizes proteins with free sulfhydryl groups by reducing disulfide bonds. Although it stabilizes some proteins, DTT acts as a non-competitive inhibitor of the salivary enzyme alkaline phosphatase, which needs to be oxidized in order to be active (Zhang et al. 2000). Like DTT, EDTA is found in many laboratory buffer solutions. It is a chelating agent which forms bonds with metal ions, including ions such as magnesium, which are necessary for the function of many digestive and salivary enzymes (Conyers et al. 1967). K_2HPO_4 disassociates in solution to release potassium ions, which are known to inhibit the activity of enzymes such as alkaline phosphatase (Fernley and Walker 1967, Funk 2001). The filter sterilization manipulation was designed to remove bacterial cells from the saliva using a non-reactive nitrocellulose membrane designed to allow proteins to pass through unchanged. Therefore, the filter sterilization manipulation removed *Sulcia* and *Wolbachia* from the saliva of the potato leafhopper to test plant wound response gene expression to saliva in the absence of symbiont cells.

Alfalfa plants were exposed to saliva for 24 hours prior to extracting RNA for gene expression analysis. Acidic β-1,3-glucanase is expressed constitutively in alfalfa, but gene expression increases rapidly when the plant is exposed to pathogens, remaining elevated for over 48 hours (Baldridge et al. 1998). Isoflavone reductase gene expression levels in alfalfa increase to maximum levels within 12 hours of exposure to an elicitor, and remain at this heightened level for 48 hours after exposure (Paiva et al. 1991). Similarly, chalcone synthase expression levels increase within 6 hours of wounding of alfalfa, and stay elevated for up to five days (McKhann and Hirsch 1994). Alfalfa phenylalanine ammonia lyase gene expression levels are known to increase within 2 hours of exposure to an elicitor, and stay elevated for approximately 72 hours (Jorrin and Dixon, 1989). Therefore, testing the alfalfa plants after 24 hours of exposure to potato leafhopper saliva would fall within the time frame that endo-β-1,3-glucanase, isoflavone reductase, chalcone synthase and phenylalanine ammonia lyase gene expression is elevated.

Endo 1-3 ß-D-glucanase expression

Endo 1-3 ß-D-glucanase is known to be expressed constitutively in plants, although its expression increases due to pathogen presence (Sela-Buurlage et al. 1993). Mechanical wounding is also known to increase endo 1-3 ß-D-glucanase expression in tomato plants (Morohashi and Matsushima 2000). This study involved mechanically wounding alfalfa plants in order to apply leafhopper saliva to the stems, and all stems that were cut had higher relative expression levels of endo 1-3 ß-D-glucanase than uncut stems. Therefore, it is logical that at least some of the increase in endo 1-3 ß-D-glucanase expression in alfalfa stems is due to mechanical wounding.

Previous studies (chapter 2 of this dissertation) indicated that symbionts are present in the salivary glands and saliva of the potato leafhopper. The filter sterilization treatment used in this study was meant to remove bacterial cells from the saliva of the potato leafhopper. The relative expression levels of endo 1-3 ß-D-glucanase in filter sterilized potato leafhopper saliva were significantly higher than the expression levels of alfalfa stems exposed to untreated saliva or cut stems exposed to filter sterilized sucrose solution. This suggests that filter sterilization resulted in the removal of an inhibitor of endo 1-3 ß-D-glucanase expression in alfalfa stems. Bacterial symbionts are known to manipulate plant response to insects, and *Wolbachia* is known to produce compounds that inhibit senescence and cholorophyll loss (Kaiser et al. 2010). Therefore, it is possible that the symbionts present in the saliva of the potato leafhopper are able to manipulate plant wound response.

Potato leafhopper saliva treated with DTT, EDTA and H_2PO_4 resulted in significantly lower relative expression levels of endo 1-3 ß-D-glucanase than untreated potato leafhopper saliva, while heat treatment did not result in a significant decrease in endo 1-3 ß-D-glucanase expression levels. The heat, DTT, EDTA, H_2PO_4 treatments used in this study were all shown to inactivate alpha amylase in whitefly, *Bemisia tabaci*, saliva (Funk 2001). Alpha amylase was detected in the salivary gland transcriptome of the potato leafhopper, and was found to be expressed in higher levels in the salivary glands than in the midgut or femur, suggesting that it is a potential salivary component (DeLay et al. 2012). Plants are known to produce beta-1,3-glucanases, which degrade alpha amylase in response to insect feeding (Franco et al. 2002). Therefore, it is possible

that alpha amylases in potato leafhopper saliva are responsible for the induction of endo 1-3 ß-D-glucanase seen when alfalfa stems are exposed to untreated leafhopper saliva.

Isoflavone reductase, chalcone synthase and phenylalanine ammonia-lyase expression

Jasmonic acid is a growth regulator that is common in plants (Creelman and Mullet 1997). When experimentally applied to plant leaves, jasmonic acid has been shown to decrease photosynthesis, delay bud formation, and to cause an increase in ethylene production leading to fruit ripening (Koda 1997). Although jasmonic acid is able to influence plant physiology on its own, its main role in plant wound response is in the induction of plant wound response pathways. For example, tomato plants injured by the tobacco hornworm, *Manduca sexta*, produce jasmonic acid, which in turn initiates the production of secondary compounds involved in insect resistance (Thaler et al. 1996).

Jasmonic acid production induces the expression of secondary plant wound response compounds, including chalcone synthase and phenylalanine ammonia-lyase (Creelman et al. 1992, Gundlach et al. 1991). Chalcone synthase, produced by the gene *CHS*, catalyzes the first step of the biosynthesis of plant flavonoids (Hahlbrock and Scheel 1989). Flavonoids are a group of polyphenolic compounds which can polymerize to form tannins, and can influence insect feeding and oviposition (Simmonds 2001). Expression of *CHS* is increased due to plant exposure to insects, pathogens and mechanical wounding (Zhu et al. 1996). *CHS* gene expression is normally low in alfalfa leaf and stem tissue, but when the plant is wounded, gene expression greatly increases (McKhann and Hirsch 1994). Like *CHS* gene expression, expression of the gene *PAL*, which produces phenylalanine ammonia-lyase, increases when alfalfa plants are wounded by pathogens (Koike and Nanbu 1997). Increased gene expression of *PAL* in alfalfa also

corresponds to an increased deposition of lignin in stem tissue (Bidlack et al. 1995), which is thought to seal the wounded plant tissue from the environment. Isoflavone reductase is also involved in the production of flavonoids, and is necessary for the production of medicarpin in alfalfa (Oommen et al. 1994). Medicarpin is an antimicrobial phytoalexin that is produced due to wounding or pathogen exposure in alfalfa (Vaziri et al. 1981).

Importance of this research

Previous research has shown that the potato leafhopper has two species of symbiotic bacteria present in both its salivary glands and saliva (Chapters 1 and 2 of this dissertation). Although the exact role that these salivary symbionts play in the population ecology of the potato leafhopper is unknown, aposymbiosis decreases longevity and fecundity (Chapter 2 of this dissertation), suggesting that the symbionts are important for normal development and reproduction. In addition to altering development of the potato leafhopper, aposymbiosis also alters the physiological response of legumes to leafhopper feeding. Alfalfa and fava bean plants exposed to aposymbiotic leafhoppers had less of a decrease in their photosynthetic rates than plants exposed to symbiotic leafhoppers (Chapter 3). Likewise, transpiration rate was higher in alfalfa plants exposed to aposymbiotic leafhoppers than in plants exposed to symbiotic leafhoppers, indicating less of a plant response to leafhopper feeding. Therefore, symbionts may play a role in the physiological response of alfalfa and fava bean plants to potato leafhopper feeding. Soybean plants fed upon by aposymbiotic and symbiotic leafhoppers did not exhibit differences in photosynthesis and transpiration rates, suggesting that symbiosis does not play a role in soybean response to leafhopper feeding. It was for this reason that alfalfa

was used to test the relative expression rates of plant wound response genes in response to treated potato leafhopper saliva.

This study found that heat, filter sterilization, DTT, EDTA and H_2PO_4 treatment of potato leafhopper saliva led to a decrease of relative gene expression of isoflavone reductase, chalcone synthase and phenylalanine ammonia-lyase in alfalfa plants stems in comparison to untreated potato leafhopper saliva. This evidence suggests that one or more elicitors in the potato leafhopper's saliva were removed from the saliva by filter sterilization or inactivated by one the treatments used in this study. Filter sterilization should have removed any bacteria, including symbionts, from the saliva, leaving proteins intact. Therefore, symbionts in the saliva of the potato leafhopper appear to be able to act as elicitors of plant wound response genes in alfalfa. Heat, DTT, EDTA and H_2PO_4 are all able to denature proteins. These treatments would not discrimate between insect or bacterial proteins, making it impossible to conclude if the elicitors inactivated by these treatments were produced by the potato leafhopper or by symbionts.

Chapter 4 of this dissertation investigated the salivary gland transcriptome of the potato leafhopper, and identified genes (lipase, pectin lyase and alkaline phosphatase) whose expression was higher in the salivary glands than in the midgut or hind femurs. These enzymes would be denatured by the treatments used in this study, so it is possible that they are elictors of the plant wound response seen in alfalfa plants. The generalized wound response caused by potato leafhopper feeding on alfalfa, known as hopperburn, is caused by a combination of mechanical injury and leafhopper saliva. The research presented in this dissertation suggests that alfalfa may not be reacting solely to the leafhopper, but may also be reacting to the symbionts present in the saliva and salivary

glands. Therefore, future management strategies may be able to exploit the relationship between the leafhopper, *Sulcia muelleri* and *Wolbachia*.

Appendix A: Approval for previously published work.

COLLEGE OF COMPUTER, MATHEMATICAL AND NATURAL SCIENCES
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November 4, 2013

Dr. Charles Caramello
Associate Provost for Academic Affairs
& Dean of the Graduate School
The Graduate School
2123 Lee Building
University of Maryland

Dear Dean Caramello:

This letter is written to signify that Bridget DeLay (student, Department of Entomology, ID# 109627979) has the approval of her committee, dissertation director, and the graduate director of the Department of Entomology to include his own previously published work as part of her final dissertation. Bridget made significant contributions to the manuscript including being responsible for its inception and the majority of the manuscript preparation. The citation for the published work is:

DeLay, B., Mamidala, P., Wijeratne, A., Wijeratne, S., Mittapalli, O., Wang, J., Lamp, W., 2012. Transcriptome analysis of the salivary glands of the potato leafhopper, Empoasca fabae. Journal of Insect Physiology 58(12), 1626-1634.

In accordance with the Graduate School's policy, please accept this letter as notification of Bridget's inclusion of her own previously published material in her dissertation. All required documentation is included in the dissertation, including a copy of this letter and the required signatures.

Sincerely,

Paula Shrewsbury

Dr. Paula Shrewsbury
Dept. of Entomology Graduate Director

Continue on Page 2

Continued from Page 1

REQUIRED SIGNATURES

Graduate Student

By signing below, the graduate student acknowledge that he/she has satisfied all the requirements of the graduate school for inclusion of their own previously published work in his/her dissertation.

Bridget DeLay
Graduate Student, Name

Signature

Dissertation Committee:

By signing below, the dissertation committee members (including the advisor) acknowledge that the student in question has satisfied all the requirements as described above to include his/her previously published work.

William Lamp
Dissertation Director (Advisor), Name
Signature

Daniel Gruner
Committee Member 2, Name
Signature

David Hawthorne
Committee Member 3, Name
Signature

Leslie Pick
Committee Member 4, Name
Signature

Jian Wang
Committee Member 5, Name
Signature

David Straney
Committee Member 6- Dean's Rep, Name
Signature 11/5/13

Graduate Director

By signing below, the graduate director agrees with the dissertation committee that the student in question has satisfied all the requirements as described above to include his/her previously published work in his/her dissertation.

Paula Shrewsbury
Graduate Director, Name
Signature

Appendix B: Expanded discussion for Chapter 4.

Previous studies have shown that hopperburn is caused by a combination of mechanical damage and plant response to leafhopper saliva, but there was no data on the saliva compostion of the potato leafhopper (Ecale Zhou and Backus 1999). This chapter looked at the sialotranscriptome of the potato leafhopper, the first such study of any cicadellid leafhopper, with a focus on identifying genes responsible for the production of potential saliva components. In addition to identifying genes of interest, the sialotranscriptome verified the presence of *Wolbachia* in the salivary glands of the potato leafhopper, as first identified in chapter 1 of this dissertation. Below, I discuss the overall results of the 454 sequencing reaction, verification of *Wolbachia* in the sialotranscriptome and implications for future studies on plant wound response to leafhopper feeding.

Overall results of the 454 sequencing reaction

Pyrosequencing of the sialotranscriptome of the potato leafhopper resulted in 255,491 transcriptomics reads, which is similar in size to other insect transcriptomes sequenced using Roche 454 technology. For example, the whole body transcriptomes of the bed bug, *Cimex lectularius*, and six-spot burnet moth, *Zygaena filipendulae*, resulted in 216,419 reads and 319,956 reads respectively (Bai et al. 2011, Zagrobelney et al. 2009). The sialotranscriptome data presented in this chapter represents a snapshot of the genes which are active in the salivary glands at a particular point in time, and therefore does not contain data for all of the genes present in the potato leafhopper's genome. For example, the transcriptome of an individual human tissue sample is estimated to be less

that 5% of the total human genome size (Frith et al. 2005). Because the size of the transcriptome for a particular tissue varies by tissue and species, it is not possible to estimate the size of the potato leafhopper's genome from the sialotranscriptome data presented in this chapter. The sequencing primers used in this study were designed to complement the poly-A tail of mature mRNAs, so the sialotranscriptome data was not likely to include intron, ribosomal RNA or transfer RNA sequences.

The average length of the assembled contigs in the sialotranscriptome of the potato leafhopper (1093 bp) was slightly higher than that of other published insect transcriptomes, with average contig lengths of 329 to 965 bp (Karatolos et al. 2011, Zagrobelney et al. 2009, Zie et al. 2012, Bai et al. 2011). The average contig length is important because we scanned the assembled transcriptome data for contigs that potentially coded for full length genes. These contigs needed to include start and stop codons, and enough nucleotides to comprise a full length protein. Therefore, we only looked at contigs that were over 1000 bp in length for potential genes of interest.

At this time, there are no other gene sequenced for the potato leafhopper in GenBank. The majority of the contigs sequenced in this study showed similarity to other insect sequences in GenBank (83%), with the remainder of the sequences aligning with other eukaryotic, bacterial and viral genes. This result is similar to the findings of other insect transcriptome studies (Karatolos et al. 2011, Zagrobelney et al. 2009, Zie et al. 2012, Bai et al. 2011). We compared the sialotranscriptome of the potato leafhopper to the genomes of *A. pisum*, *T. castaneum*, and *D. melanogaster*. Although the sialotranscriptome of the potato leafhopper was most similar to that of *A. pisum*, the majority of sequences in the sialotranscriptome were unique. One explanation for this

result is that the unique sequences contained untranslated regions which would not alter the function of the completed protein. After publication of this data, the sialotranscriptomes of the beg bug was completed (Francischetti et al. 2010). Future comparison of the sialotranscriptome of the potato leafhopper to that of the bed bug may reveal that some of the sequences previously thought to be unique to the potato leafhopper are also present in other piercing/sucking insect species.

Symbiont presence in the sialotranscriptome

Approximately 5% of the contigs in the sialotranscriptome of the potato leafhopper corresponded to those of bacterial species in GenBank. Multiple genes corresponding to *Wolbachia* were detected in the salivary glands of the potato leafhopper, confirming the results of previous studies that *Wolbachia* is present not only in the midgut of the potato leafhopper, but in the salivary glands as well (Chapters 1 and 2 of this dissertation). Unlike *Wolbachia*, no sequences aligning to *Sulcia* were detected in the sialotranscriptome. One possible explanation for this result is that the primers used to create the sialotranscriptome were poly-T primers designed to bind to the poly-A tails of mature eukaryotic mRNA. If the *Sulcia* RNA present in the salivary gland tissue samples used for the sequencing reaction did not have large poly-A regions, they would not have been amplified by the primers used in this study. Another explanation for the lack of *Sulcia* sequences in the sialotranscriptome is that the *Sulcia* RNA that was sequenced was not assembled into contigs, or if it was, the assembly was incorrect. These singletons would not have been included in the megaBLAST search, leading to the negative result for *Sulcia* in the results. Likewise, if *Sulcia* RNA was incorrectly assembled into contigs, it would not have given positive results for *Sulcia* in the megaBLAST search.

The potato leafhopper is known to have two taxa of symbiotic bacteria present in its salivary glands, *Wolbachia* and *Sulcia* (Chapters 1 and 2 of this dissertation). The presence of other bacterial sequences in the sialotranscriptome of the potato leafhopper suggests that there may be more than two species of bacterial symbionts in the leafhopper's salivary glands. Future studies could use this sialotranscriptome data to screen the salivary glands for new species of symbionts. It would also be interesting to compare the sialotranscriptome to a transcriptome study of the midgut of the potato leafhopper. This type of comparison may reveal the presence of a different community of symbionts in the midgut compared to the salivary glands.

The presence of bacterial sequences in the sialotranscriptome of the potato leafhopper also opens up the possibility that horizontal gene transfer has occurred between the leafhopper and its symbionts. For example, horizontal gene transfer has occurred between the mealybug, *Planococcus citri*, and the symbiont *Moranella endobia*. In this case, 22 genes have been lost from the symbiont and transferred into the mealybug genome, which has allowed the symbiont's genome size to decrease without losing essential genes (Husnik et al. 2013). Similarly, horizontal transfer of two genes from *Wolbachia* to the mosquito *Aedes aegypti* has been demonstrated to occur with the help of a bacteriophage associated with *Wolbachia* (Klasson et al. 2009). Future analysis of the sialotranscriptome data could focus on searching for horizontal gene transfer events between *Wolbachia* and the potato leafhopper.

Implications for future studies of plant wound response to leafhopper feeding

Two genes of interest were highly expressed in the sialotranscriptome of *E. fabae*: endo-beta-glucanase, and alpha-amylase. Endo-beta-glucanase has been identified in the

saliva of multiple insect species, including the blister beetle, *Mylabris pustulata*, and the glassy-winged sharpshooter, *Xylella fastidiosa* (Sami et al. 2011, Backus et al. 2012). Endo-glucanases degrade cellulose, resulting in the release of glucose (Wilson and Irwin 1999). The sheath saliva of the glassy-winged sharpshooter contains endo-beta-glucanase, and when the sharpshooter feeds on xylem tissue, the endo-beta-glucanase is transported through the xylem away from the feeding site (Backus et al. 2012). Given the high level of expression of endo-beta-glucanase in the sialotranscriptome of the potato leafhopper, it is possible that the sheath saliva of the potato leafhopper also contains endo-beta-glucanase. In addition to being secreted in insect saliva, endo-beta-glucanase is also produced by plants in response to wounding or pathogen infection (Morohashi and Matsushima 2000, Sela-Buurlage et al. 1993). It is possible that the endo-beta-glucanase produced by the potato leafhopper may modulate the plant wound response in addition to aiding in glucose ingestion by manipulating the levels of endo-beta-glucanase that are present in the plant tissue during feeding.

Alpha amylase has been reported in the saliva of the honeybee, *Apis mellifera*, the mosquito *Anopheles merus* and the pea aphid, *A. pisum*, where it breaks down starch into glucose (Ohashi et al. 1999, Effio et al. 2003, Harmel et al. 2008). Many species of plants produce alpha-amylase inhibitors in response to insect feeding, including legumes (Marshall and Lauda 1975, Ishimoto et al. 1996). Transgenic plants expressing alpha-amylase inhibitors have been developed to reduce the damage caused by insect salivary alpha-amylases (Franco et al. 2002). The high expression levels of alpha-amylase in the sialotranscriptome of the potato leafhopper indicate that alpha-amylase is an important salivary component. Therefore, future management strategies could manipulate the plant

response to the alpha-amylases present in the potato leafhopper's saliva either through the creation of transgenic crops or through selection of plants that are tolerant to the leafhopper's alpha-amylase activity.

Studies after publication of Chapter 4 (e.g., Chapter 5 of this dissertation) focused on plant wound response gene expression after exposure to potato leafhopper saliva. In addition to exposing alfalfa plants to untreated leafhopper saliva, the saliva was filter sterilized to remove bacterial cells. Bacteria are able to produce endo-beta-glucanases, and since multiple species of bacteria were present in the sialotranscriptome of the potato leafhopper, it is possible that these bacteria are responsible for the production of the endo-beta-glucanase produced by the leafhopper's salivary glands. In fact, filter sterilization of potato leafhopper salia caused a significant reduction in the expression of plant wound response genes (endo 1-3 ß-D-glucanase, chalcone synthase, isoflavone reductase and phenylalanine ammonia-lyase) in relation to untreated leafhopper saliva. Filter sterilization of the saliva may prevent plant wound response by removing bacteria from the saliva, therefore preventing the release of endo-beta-glucanase into the plant tissue. Similiarly, alfalfa exposed to saliva treated with heat or denaturing compounds (DTT, EDTA, and K_2HPO_4) had significantly lower expression levels of chalcone synthase, isoflavone reductase and phenylalanine ammonia-lyase in comparison to plants exposed to untreated saliva. Therefore, heat and denaturing compounds may inactivate endo-beta-glucanase and alpha-amylase activity in the saliva of the potato leafhopper.

Appendix C: Expanded abstract for this dissertation.

The potato leafhopper, *Empoasca fabae*, is an economically important pest of agricultural crops in the United States, including alfalfa, *Medicago sativa*. A combination of mechanical damage caused by the insect's piercing/sucking mouthparts and injection of saliva into the plant tissue causes characteristic injury on alfalfa known as hopperburn. Although saliva is known to play a key role in the development of hopperburn symptoms, including a decrease in photosynthesis and transpiration rates, accumulation of starch in the leaves above the feeding site, and yellowing of the leaves, little is known about the saliva composition of the potato leafhopper. To clarify the role that the saliva of *E. fabae* plays in the plant wound response to feeding, this dissertation had five major goals: 1) Identify the symbiotic bacteria present in the salivary glands and midgut, 2) Examine the role that symbionts play in the population ecology of the potato leafhopper, 3) Determine the role that symbionts play in legume physiological response to potato leafhopper feeding, 4) Sequence the transcriptome of the salivary glands of the potato leafhopper, 5) Determine how plant wound gene expression is affected by potato leafhopper saliva.

Many species of phloem and xylem feeding insects harbor bacterial symbionts. These symbionts provide amino acids and vitamins that are lacking in the diet of their insect host, allowing the insect to survive on an otherwise nutritionally deficient diet. Other species of leafhoppers are known to harbor the symbionts *Sulcia muelleri*, *Baumannia cicadellinicola* and *Wolbachia*, but there have been no prior studies done on the symbionts present in the potato leafhopper. In chapter 1 of this dissertation, I describe two taxa of symbiotic bacteria present in the potato leafhopper: *Sulcia muelleri* and

Wolbachia. These symbionts are present in the salivary gland, bacteriome and midgut tissue of the leafhopper, but not in femur tissue. Although insect symbionts are often found in association with bacteriome and midgut tissue, symbiont presence in salivary gland tissue has not been reported in a wide variety of insects. The presence of symbionts in the salivary glands of the potato leafhopper suggests that the symbionts are in part responsible for the production of salivary components.

Although Chapter 1 of this dissertation established that the potato leafhopper harbors two taxa of symbionts, *Sulcia* and *Wolbachia*, the role that the symbionts play in the population ecology of the leafhopper was unknown. Therefore, adult leafhoppers were treated with the antibiotic oxytetracycline HCl to cure them of symbionts. These aposymbiotic leafhoppers were then allowed to lay eggs on alfalfa and fava bean, *Vicia faba*, plants. Aposymbiotic leafhoppers had a shorter lifespan, and produced fewer eggs on both alfalfa and fava bean. In addition, fewer eggs hatched from the eggs produced by aposymbiotic leafhoppers, and fewer of these nymphs survived into adulthood than nymphs produced by symbiotic females. Therefore, the symbionts present in the potato leafhopper form a mutualistic relationship with the leafhopper, with the leafhopper providing the symbionts with a stable environment and nutrients, and the symbionts positively affecting the population ecology of the leafhopper.

The two taxa of symbionts present in the potato leafhopper increase the longevity and fecundity of the leafhopper on alfalfa and fava bean, but the role that these symbionts play in plant response to leafhopper feeding is unknown. In chapter 3 of this dissertation, I investigate the role that *Sulcia* and *Wolbachia* play in the physiological response of alfalfa, fava bean and soybean, *Glycine max*, to potato leafhopper feeding. In addition to

being present in salivary gland tissue, *Wolbachia* and *Sulcia* were detected in the saliva of potato leafhoppers, and in alfalfa, fava bean and soybean stems after leafhopper feeding. This suggests that plants are exposed to symbionts during leafhopper feeding, and that the plant response to feeding may be in part due to the symbionts. To test this hypothesis, aposymbiotic leafhoppers were caged on alfalfa, fava bean and soybean plants, and the photosynthesis and transpiration rates of the plants were measured after 24 hours of leafhopper feeding. Symbiotic leafhoppers normally cause a decrease in the photosynthesis and transpiration rates of alfalfa plants, and this study found that aposymbiotic leafhoppers caused less of a decrease in photosynthesis in alfalfa and fava bean plants. In addition, aposymbiotic leafhoppers caused less of a decrease in transpiration rates in alfalfa, but caused the same reduction in transpiration rate as symbiotic leafhoppers on fava bean and soybean. Therefore, symbionts appear to play a role in alfalfa plant response to potato leafhopper feeding, but the effect of the symbionts varies depending on the species of legume exposed to leafhoppers.

Potato leafhopper saliva causes a reduction in photosynthesis and transpiration levels in alfalfa plants, but the protein compostition of the saliva has not been described. In chapter 4 of this dissertation, I sequenced the sialotranscriptome of the potato leafhopper, and identified genes involved in the production of potential salivary proteins. Two highly expressed genes, endo-beta-glucanase and alpha-amylase, are insect salivary enzymes known to initiate plant wound response. In addition, the presence of *Wolbachia* in the salivary glands of the potato leafhopper was verified through the sequencing of multiple *Wolbachia* genes from the salivary gland tissue.

The sialotranscriptome of the potato leafhopper identified proteins known to initiate plant wound response gene expression. Therefore, in chapter 5 of this dissertation, I explored the role that potato leafhopper saliva plays in the initiation of four plant wound response genes: endo 1-3 ß-D-glucanase, chalcone synthase, isoflavone reductase, and phenylalanine ammonia-lyase. Alfalfa plants were exposed to untreated potato leafhopper saliva, in addition to saliva manipulated in four different ways (heat, filter sterilization, DTT, EDTA, and K_2HPO_4), all designed to either denature salivary proteins or in the case of filter sterilization, to remove symbionts from the saliva. Saliva was introduced to the vascular tissue through cuts in the stems, and leaf tissue was subjected to rt-PCR to measure wound response gene expression levels after treatment. All five saliva manipulations led to a decrease in the relative gene expression of isoflavone reductase, chalcone synthase and phenylalanine ammonia-lyase in alfalfa plant stems in comparison to untreated potato leafhopper saliva. Heat treated saliva caused no difference in expression of endo 1-3 ß-D-glucanase in comparison to untreated saliva, but filter sterilized saliva caused a significant increase in gene expression in comparison to untreated saliva. DTT, EDTA and K_2HPO_4 saliva manipulation resulted in a decrease in endo 1-3 ß-D-glucanase relative expression rates in relation to untreated saliva. These results suggest that proteins present in the saliva of the potato leafhopper act as elicitors of plant wound response genes in alfalfa, and that symbionts present in the saliva may be involved in the upregulation of plant wound response pathways.

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