DEVELOPING A METHOD TO SCREEN POTENTIAL TARGETS FOR ARTIFICIAL RNAi IN APHIDS

Attorneys

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DEVELOPING A METHOD TO SCREEN POTENTIAL TARGETS FOR ARTIFICIAL RNAi IN APHIDS

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CHAPTER I

ECONOMIC DAMAGE DUE TO PESTS

1.1 PEST MANAGEMENT

Agricultural losses in the United States due to insect pest damage have steadily increased during the last century. Quantifying the absolute amount lost due to insect pests is difficult as there are many factors that lead to decreased yield or profit such as the cost of preventative measures, physical damage to plants, mechanical spread of disease, cosmetic damage to produce and loss after cultivation. The following sources illustrate the problem of insect pest herbivory throughout US history. In the late 1930s, it was estimated that the average loss in crop production due to infestation of a group of insect pests was approximately $527,000 a year (Hyslop 1938). In 1977, it was estimated that 25-39% of crops grown in the United States were lost due to insect pests (Pimentel et al. 1977). More recently, the Environmental Protection Agency (EPA) estimated approximately 382 million acres were used for agricultural crops, an industry worth an estimated $100 billion in revenue. If Pimentel's estimates are combined with the EPA's modern agricultural crop estimates, 95.5-149 million acres of crops and $25-39 billion in revenue are lost due to damage caused by insect pests every year in the US alone.

In an effort to combat the loss of produce to insect pests, US farmers apply over 600 different pesticides at an estimated annual cost of approximately $10 billion (Pimentel 2005). Adding to that figure, other factors such as public health effects, pesticide resistance, water contamination, as well as the cost to governmental agencies to monitor pesticide use brings losses due to pests closer to $12 billion (Pimentel 2005). Furthermore, in 2001 the United States Department of Agriculture (USDA) reported that over 90% of the most popular crops grown were treated with insecticides to protect crop yield and that at least 10% of pesticides used were applied just to combat increased resistance (Pimentel 2005).

Undoubtedly, the financial losses due to insect pests will continue to increase as insects become more resistant, the human population grows, and the amount of arable land diminishes. The practice of heavy and repeated use of pesticides has led to documented insecticide resistant pest populations in over 500 different species of insect pests (Gut et al. 2011; Toor et al. 2008). Included in the list of insecticide resistant pests are aphids, which have developed resistance to multiple classes of insecticides (Georghiou 1972; Moores et al. 1994; Needham et al. 1971).

Aphids are economically important pests that damage plants in four main ways. Firstly, aphids consume vital plant metabolites including large volumes of translocated sugars from the phloem sap diverting energy from the plant and reducing yield. Secondly, aphids secrete saliva into the plant tissues while probing the layers of leaf to find the phloem, in some instances the secreted saliva is thought to be phytotoxic (Dedryver et al. 2010). The physical damage done to the leaf along with the toxic attributes of the saliva leads to localized necrosis in some aphid plant interactions

(Klingler et al. 2009; Sauge et al. 2001; Villada et al. 2009). Thirdly, aphids act as virus vectors either by "circulant transmission" or "non-circulant transmission". The "circulant transmission" occurs when the aphid acquired plant virus passes through the lumen of the aphid into the hemolymph and to the salivary glands to be redistributed as long as the aphid lives. The "non-circulant transmission" occurs when virus particles non-specifically bind to the mouthparts of the aphid, are transmitted via mechanical injury, and do not persist within the aphid (Martiniere et al. 2009). Finally, large aphid infestations can coat the leaves of host plants with honeydew, a sugary excretion, which serves as a substrate for fungal growth that can limit photosynthesis.

The overall goal of this project was to examine the possibility of using genetically-based control methods such as RNA interference (RNAi) to target the expression of key genes in the aphid. In particular, developing methods for screening potential targets in the aphid and testing the efficacy of RNAi targeting in artificial diets and transgenic model organisms before applying knowledge of targets to more costly and time consuming crops.

1.2 APHID BIOLOGY

Aphids are small, soft bodied insects belonging to the order *Hemiptera* that feed on plant phloem sap using highly modified mouth parts. A protective beak known as the rostrum is made from the labrum (upper lip) and the labium (lower lip). This structure helps protect the inner mouth parts called stylets that are composed of modified maxilla (upper jaw) and mandible (lower jaw). Generally, the mandibles surround the maxilla. The maxilla have grooves that when pushed together form channels (Pollard 1973). Once

an aphid settles on a host plant it will penetrate the cuticle of the leaf with its stylet supported by the rostrum. The stylet then moves primarily through the interstitial space of the cells in the different layers of the leaf to find the phloem. While probing, the aphid periodically samples the contents of cells along the stylet pathway by puncturing them (Douglas 2003; Tabara et al. 1998) .

Previous studies have shown that aphids secrete one type of saliva while probing and another type while feeding. Electrical penetration graph (EPG) experiments have provided some of the strongest evidence in support of this idea (Tjallingii 2006). The "gelling" saliva is expelled from the aphid before and during probing. The gelling saliva hardens forming a sheath around the stylet to protect it from damage while penetrating the mesophyll to the phloem. A different type of saliva described as "watery" saliva is expelled by the aphid after it punctures a cell. This watery saliva is believed to assist the aphid in avoiding or minimizing detection by the plant and might prevent occlusion of the phloem sieve element (Cooper et al. 2010; Tjallingii 2006). When the sieve elements of the phloem are penetrated the aphid will ingest the phloem sap and excrete honeydew.

The aphid diet predominantly consists of phloem sap. Since they do not break down solid materials, aphids have a relatively simple digestive tract resembling a coiled tube. Their digestive system consists of an esophageal valve opening into the mid-gut, which flows seamlessly into the hind-gut (Smith 1938). Phloem sap consists mainly of water, carbohydrates and amino acids forming a medium to transport proteins, RNAs and various metabolites (Chen et al. 2006). Sucrose is the most abundant solute in the phloem sap comprising between 11 and 75 percent of samples, depending on the plant species (Byrne et al. 1990). Due to the high sugar content of this diet, aphids have

evolved mechanisms to regulate the osmotic effects of the phloem sap (Shakesby et al. 2009). To avoid dehydration, a sucrase-transglucosidase active in the aphid gut transforms excess sugar into long-chain oligosaccharides that are excreted from the body as honeydew (Douglas 2006). Another characteristic of phloem sap is that it does not contain all of the essential amino acids necessary for aphid survival. To overcome the deficiency, aphids have developed a symbiotic relationship with bacteria from the genus *Buchnera* to provide essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Douglas 1998, 2006). The bacteria are able to synthesize these amino acids from sucrose and aspartate which, are readily found in the phloem sap diet of the aphid (Douglas 1998). These bacteria live in the cytoplasm of specialized cells referred to as bacteriocytes in the hemocoel of the aphid and are transferred vertically from mother to nymph (Douglas 2006).

Aphids have a complex reproductive cycle that leads to high reproductive plasticity (Figure 1.1). Most aphid species reproduce asexually for several generations as long as temperature, day length, and food supplies are conducive and supportive of population growth (Douglas 2003; Simon et al. 2010). Nymphs undergo a series of four molts; each molt produces a different instar, or stage of development. It takes 8 to 10 days for a nymph to become a fully mature, reproductive adult. In most aphid species, nymphs develop without wings; however, if the colony is too large or the availability of food is scarce they can also develop wings (Douglas 2003). The formation of wings allows easier and faster dispersion of aphids in less than ideal environments to search for new food sources.

Aphids will reproduce sexually once environmental thresholds are crossed such as diminishing food quality/quantity, shortening days, or cooler temperatures (Douglas 2003; Simon et al. 2010). Once conditions are met, viviparous females produce sexual aphid forms. Sexually reproducing aphids will lay cold-hardy eggs allowing survival in areas too cold for overwintering live aphids. Emergent aphids are females that give live birth (vivipary) that develop from asexually formed embryos (parthenogenesis). For the majority of aphid species, sexual reproduction occurs on the same species of plant as asexual reproduction; however, there is also a small percentage of species that will reproduce asexually on herbaceous plants and sexually on woody plants (Simon et al. 2010; Trionnaire et al. 2008).

One of the reasons aphids are such a persistent agricultural pest is their very high reproductive rates. Telescoping generations, in which embryonic nymphs in an adult aphid contain developing embryos of their own, enables rapid increase in numbers when environmental conditions are conducive. The parthenogenetic nature of these insects is particularly useful experimentally as it is possible to create cohorts of same-age, genetically identical individuals.

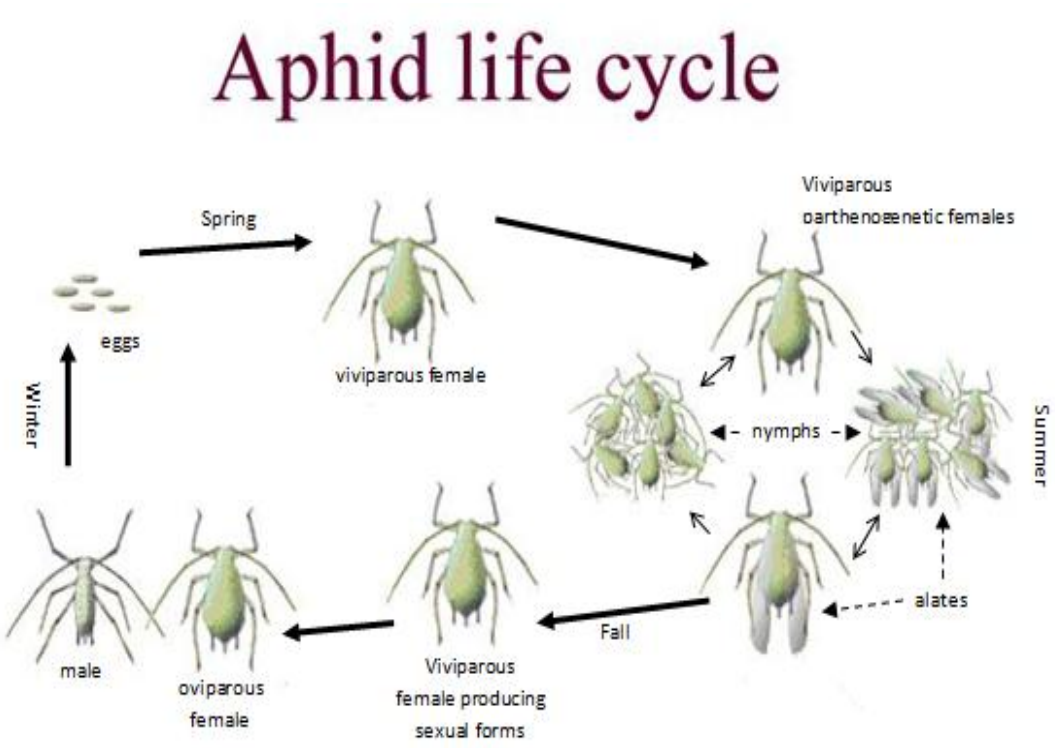


Figure 1.1 The aphid life cycle. Summer reproduction is asexual for multiple generations or throughout the year if conditions permit. Viviparous parthenogenetic females give live birth to nymphs with or without wings depending on environmental conditions. Towards the fall viviparous female will start producing sexual forms which will mate and lay eggs in diapause for overwintering. In the spring viviparous females emerge and start the cycle over again. Adapted from www.metapathogen.com/aphid.

1.3 APHID CONTROL

The excessive use of chemical pesticides to control agriculturally important insects has caused insecticide resistance to rapidly emerge within insect populations. Synthetically produced chemical insecticides first appeared in the 1930s with the well-recognized compound dichlorodiphenyltrichloroethane (DDT). The rapid and widespread popularity of DDT led to the development of insecticide resistance in many species. *Myzus persicae* (green peach aphid) provides a good example of the development of insecticide resistance within a species. This aphid species was first observed to have resistance to DDT in the United States during the 1950s and resistance was confirmed in 1963 (Georghiou 1972). In 1975, the first mechanism of insecticide resistance in *M. persicae* was identified while studying the translocation of holocentric chromosomes in several ecotypes. The resistant ecotype was reared in a glasshouse where it was subjected to regular sprayings with organophosphate insecticides. These insects had high levels of carboxyl esterase activity (Needham et al. 1971). Carboxyl esterase can sequester and detoxify insecticides with ester groups such as organophosphates, carbamates, and pyrethroids (Criniti et al. 2008).

The amplification of two esterase genes E4 and FE4 were responsible for the increase in carboxylesterases in the aphid (Field et al. 2002). As new classes of insecticides such as dimethylcarbamates and pyrethroids were developed and extensively used alternative insecticide resistance mechanisms were also discovered. Target site mutations in the genes encoding acetylocholinesterase (a target for dimethylcarbamates) and the insect sodium channel (a target for pyrethroids) in *M. persicae* were identified as conferring resistance to those classes of insecticides (Anstead et al. 2007; Moores et al.

1994; Toor et al. 2008). According to the Insecticide Resistance Action Committee (IRAC) international Mode of Action (MoA), there are currently over 28 classes of insecticides being used world-wide, and the IRAC estimate resistance is developed to each new class within 2 to 20 years of use (http://www.irac-online.org/wp-content/uploads/2009/09/MoA_Classification.pdf). These estimates pose a concern because the number of synthetic insecticides is limited and resistance has developed against all of the currently employed agents.

In light of the continual emergence of insecticide resistant insects, the use of host plant with genetic insect resistance is a viable control alternative to chemical insecticides. Genetic resistance derived from wild host plants or closely related taxa has enabled the introduction of resistant traits into domesticated cultivars through conventional plant breeding. Sources of aphid resistance have been identified in numerous insect-plant interactions; *Aphis gossypii* (cotton-melon aphid) and *Cucumis melo* (melon) (Kishaba et al. 1971); *Macrosiphum esculentum* (potato aphid) and *Solanum esculentum* (tomato) (Milligan et al. 1998); *Nasonovia ribisnigri* (lettuce aphid) and *Lactuca sativa* (lettuce) (Eenink et al. 1982); *Schizaphis graminum* (greenbug) and various cereals (Porter et al. 1997) and *Acyrthosiphon kondoi* (bluegreen aphid) and *Acyrthosiphon pisum* (pea aphid) and *Medicago trucatula* (barrel medic) (Gao et al. 2008; Klingler et al. 2009). Single gene resistance (R genes) is typical of many of the well characterized interactions (Porter et al. 1997); however, insect biotypes can have differential responses to a given R gene within a specific plant-insect interaction and only some cloned R genes can be effectively transferred to another host plant of the target insect (Goggin et al. 2001).

Transgenic approaches to generate aphid resistance in plants have primarily focused on targeting the expression of proteins in the phloem that are toxic to insects. *Dioscorea batatas* tuber lectin 1 (DB1) is a storage protein related to a lectin family known to inhibit growth and development of insects by binding to mid-gut mannose-containing glycoproteins (Kato et al. 2010). Expression of the DB1 gene directed by the phloem-specific rice sucrose synthase 1 (RSs1) promoter, (Rao et al. 1998) in transgenic tobacco exhibited resistance to *M. persicae* by reducing population size by 60% compared to non-transgenic plants (Kato et al. 2010). In addition to the RSs1 promoter, there are now many other phloem-specific promoters available that direct gene expression to immature sieve elements or companion cells.

1.4 SMALL REGULATORY RNAS

The central dogma of molecular biology is based on the fundamental concept that DNA is transcribed into RNA which in turn is translated into protein. Though the process of protein synthesis from a DNA template via an RNA intermediate appears straightforward, there are many regulatory processes that occur during the complex activities involved in transcription and translation. A relatively recent discovery involves regulatory processes that are carried out by small RNAs (smRNAs). The use of smRNAs in different model systems for gene regulation, epigenetics, or chromatin remodeling is described as RNA interference (RNAi) in mammalian systems, post-transcriptional gene silencing (PTGS) in plants, and quelling in fungi (Finnegan et al. 2003).

Small RNAs are a class of non-coding RNAs that are generally 17-31 nucleotides in length that act through homologous sequence interactions with their targets. Their

effects can vary from stabilizing mRNA, targeting epigenetic modifications, or silencing the expression of their target genes (Finnegan et al. 2003). There are two large and well documented classes of small RNAs known as miRNAs and siRNAs as well as a number of less ubiquitous classes such as transacting siRNA (tasiRNA), tiny non-coding RNAs (tncRNAs), small modulatory RNA (smRNA), piwi interacting RNA (piRNA), and small temporal RNAs (stRNAs). This literature review will primarily focus on the established genesis of miRNAs and siRNAs.

Mature miRNAs are usually derived from single stranded RNA molecules transcribed from intergenic regions by RNA polymerase II. Stretches of RNA fold-back on themselves to form a characteristic stem loop or hairpin structure including a 5'cap and polyadenylated tail. The stem of the structure commonly contains bulges where there are no complementary bases in the sequence. In order to become mature, this capped and polyadenylated hairpin precursor known as the primary miRNA (pri-miRNA) has to be processed.

In plants the primary -miRNA (pri-miRNA) transcript is processed in the nucleus by a series of proteins. It is believed that a RNA binding protein called DAWDLE (DDL) stabilizes the pri-miRNA transcripts in nuclear processing centers called D-bodies. These D-bodies facilitate the conversion of pri-miRNAs to pre-miRNAs which involves the participation of a C2H2 zinc finger protein, SERRATE (SE), the dsRNA binding protein HYPONASTIC LEAVES1 (HYL1), Dicer-like1 (DCL1), and the nuclear cap binding complex (CBC) (Voinnet 2009). DCL1 consists of a PAZ domain, two RNase III domains, a DEXD/H domain, and DUF domain. PAZ recognizes and binds the special ends of dsRNA destined to be smRNAs. The tandem RNase III domains cleave

the dsRNA precursor to create a short RNA duplex, while DEXD/H and DUF are domains of unknown function. Another characteristic of DCL1 is it's "ruler" helix which is thought to measure the appropriate number of nucleotides for the smRNA being produced. The pri-miRNA transcript can adopt a hairpin-like structure is cleaved by DCL1, assisted by HYL1, to release the miRNA/miRNA* duplex. A terminal methyl group is added to 3'-2nt overhangs by methyltransferase HEN1. This methylation stabilizes the duplex. The duplex is then exported via the nuclear transporter protein, HASTY into the cytoplasm (Du et al. 2005). The miRNA, but not the miRNA*, is loaded into the RNA induced silencing complex (RISC). A major component of the RISC complex is the Argonaute protein composed of PAZ, MID, and PIWI domains. The PAZ domain contains a pocket to hold the 3' end of the smRNA, the MID domain contains a pocket to hold the 5' end of the smRNA, and the PIWI domain acts as the cleavage site. The mature miRNA RISC complex is called the microRNA ribonucleoprotein complex (miRNP). The mature miRNA guides the complex via sequence complementarity with target transcripts that are either cleaved or transcriptionally blocked (Carthew et al. 2009).

The genesis of siRNA is very different from miRNA. Endogenous siRNA are transcribed either directly from transcription that is completed by RNA polymerase IV or derived from inverted repeats of transgenes/transposons. siRNA are composed of three classes: antisense-siRNAs (natsiRNAs), trans-acting-siRNAs (tasiRNAs), and heterochromatic small RNAs (hcRNAs) (Farazi et al. 2008). NatsiRNAs are generated from long dsRNA exported into the cytoplasm where RNase III enzymes such as Dicer cleave it into ~22 nt fragments leaving 3' 2nt overhangs and phosphorylated 5'ends.

TasiRNA are generated from genes that are transcribed in the nucleus by RNA polymerase II and transcripts cut by a microRNA ribonucleoprotein complex. DsRNA is synthesized from the fragment by RNA dependent RNA polymerase VI. DCL4, another dsRNA binding protein with endonucleolytic activity, will bind to the dsRNA and cleave it into ~22nt duplex fragments with phosphorylated 5' ends. HEN 1 will methylate the 3' overhangs then the siRNA duplex is incorporated into the RISC complex and one strand guides the complex to cleave target mRNA and the other strand is destroyed (Farazi et al. 2008).

While the general concepts are similar between plants and insects, the targeting principles are different between the two. Plant miRNAs have high sequence complementarity with the target mRNA, while animal miRNAs target genes if they have 7 nucleotide (seed region) from the 5' end of the miRNA. The length of smRNA products can vary depending on their origin; plant miRNAs are slightly shorter in length (~21nt) than most insect miRNAs that are slightly longer (~22nt).

1.4.1 SMALL RNAS AS AN APPROACH TO CONTROL APHIDS

The involvement of smRNAs in regulating plant growth and development as well as stress responses and defense is well documented (Baulcombe 2004; Eamens et al. 2008). The regulatory roles of smRNAs affecting plant physiological responses, especially stress and defense response is becoming increasingly understood enabling the development of smRNA technology as a way to improve plant health and to serve as an alternative to chemicals for crop protection. This technology has been used to produce crops that are resistant to various virus diseases and insect pest infestation (Franz et al.

2006; Mao et al. 2007). The growth and development of insects is also regulated in part by smRNAs (reviewed in Ambros 2004; Belles 2010). Unraveling the various roles of smRNAs has been accomplished through the development of different methods of delivery of dsRNA into insects. Methods include direct approaches such as microinjection, soaking, or feeding dsRNA to induce RNAi. Micro-injection of dsRNA, instead of siRNA successfully knocked down expression of target calreticulin and cathepsin L genes in aphids with equal sensitivity in different tissues; however, the efficiency of RNAi varied depending upon the gene target and aphid species (Jaubert-Possamai et al. 2007; Tabara, et al. 1998). Soaking nematodes in solutions containing dsRNA induced interference of gene expression that was displayed through the F_1 progeny (Tabara, et al. 1998). Others supplemented artificial diets with dsRNA and knocked down targets via RNAi (Jaubert-Possamai, et al. 2007). The diet of aphids consists predominantly of phloem sap that is sucked from sieve elements, providing an opportunity to exploit the phloem as an avenue to control this group of insects. Shakesby and coworkers (2009) administered dsRNA to aphids feeding on a liquid artificial diet as a proxy for phloem sap. A concentration of 1 µg/µL dsRNA in a liquid diet led to a two-fold decrease in the expression of a putative aquaporin, ApAQP1, gene that was manifested after 24 hours of feeding. In an effort to provide the "proof of concept" that RNAi could serve as a species-specific pesticide, Whyard and coworkers (2009) demonstrated RNAi to different species by using dsRNA in artificial diets that targeted unique regions of the genes of interest. The expression of targeted gene was down-regulated in pea aphid (*Acyrthosiphon pisum*) nymphs, four species of the genus *Drosophila (D. melanogaster, D. sechellia, D. yakuba and D. pseudoobscura*), flour

beetle (*Tribolium castaneum*) larvae and tobacco hornworm (*Manduca sexta*) larvae. Treating *A. pisum* with *vATPase* dsRNA in artificial diet led to decreased target transcript accumulation and mortality.

1.5 OVERVIEW OF THIS STUDY

Aphids have developed varying degrees of resistance against all classes of chemical insecticides that are used for their control in crops. Scientists are currently investigating alternative methods of aphid control including transgenic crops that produce proteins that are toxic to aphids (Hilder et al. 1994; Rahbe et al. 2003; Xu et al. 1996). The discovery of smRNAs and technologies to manipulate smRNA sequences offers an alternative approach to confer aphid resistance in plants by targeting the expression of aphid genes to decrease fertility, reproduction and lifespan of the insect. The studies described in this thesis focused on developing methods for the use of dsRNA in artificial diets to rapidly screen candidate smRNAs for their effects on aphid development, longevity, and reproduction. This rapid screening technique will inform which smRNAs serve as optimal candidates for controlling aphids when expressed in the phloem of transgenic plants. Studies also included the initial work to develop transgenic *Arabidopsis thaliana* plants expressing artificial miRNAs in the phloem that could potentially down-regulate a gut-specific gene target in green peach aphids.

CHAPTER II

TESTING ARTIFICIAL DIETS ON TWO SPECIES OF APHIDS

2.1 INTRODUCTION

Aphid artificial diets have been documented in the literature for nearly a century. Dadd and Mittler were among the first to create a chemically defined diet for aphids, specifically *Myzus. persicae* (Dadd et al. 1966). The development of artificial diets for aphids was initially derived from diets already established for chewing insects and adapted by the titration of various components of the diet to determine optimal concentrations required for aphid growth and development. A variety of sucrose concentrations were tested to determine that optimal level were between 10% and 20% of the diet composition for nymphs and adult aphids.

The total amino acid concentration of 0.5% was found to be required for both the growth of nymphs and high rates of asexual reproduction. Optimal levels for each individual amino acid in the diet were determined by measuring aphid growth. Potassium, magnesium and phosphorus were also found to be essential for the growth of nymphs, adult survival, and asexual reproduction. The last core diet component considered was water-soluble vitamins. Feeding adults on vitamin-deficient diet led to decreased birth rates and abnormal development of nymphs (Dadd et al. 1965).

Through years of research and optimization, a diet capable of sustaining aphids for long periods of time was established.

Research also showed that the feeding methods used had a substantial effect on aphid survival, weight, and fecundity. Feeding sachets containing diet were developed to increase ease of handling and decrease microbial contamination. Initially, diets were prepared using cholesterol containing water and adjusted to a neutral pH. Later, the cholesterol was eliminated as it did not prove to enhance uptake and the pH was adjusted to be slightly acidic to eliminate loss of insoluble magnesium phosphates (reviewed in Dadd et al. 1966). The frequency with which the sachets were changed was another parameter used to decrease the opportunity for contamination. Some authors changed sachets every one or two days (Fragoyiannis et al. 1998; Kato et al. 2010), while others would wait longer between changes (Douglas et al. 2006; Le-feuvre et al. 2007) or the same sachet would be used for the duration of the experiment (Carrillo et al. 2010; Shakesby et al. 2009).

As an alternative to full aphid diets, many studies have utilized a sucrose solution or amino acids in sucrose solution. These diets are much less complex and less expensive than complete diets. Simpler diets proved effective for delivery of treatments with low mortality to control groups when tested under shorter time spans. These diets were used for single generation studies and were not intended for raising nymphs for purposes other than seeing direct developmental effects of molecules being tested (Cooper et al. 2010; Douglas et al. 2006; Kim et al. 2007).

Determining the appropriate diet for the aphids in this study was important because it would be used as the solution to dilute dsRNAs for later feeding experiments. A diet the aphids would readily feed upon would ensure ingestion of the dsRNA by the aphids and promote maximal effects of the dsRNA on target genes in the aphid. Artificial diets were used as the choice mode of delivery of the artificial smRNAs because they provided a noninvasive way of distributing the dsRNAs into the insects that approximated the phloem sap. It also provided a way to directly control concentrations of smRNAs available to feeding aphids. Three diets and two aphid species were employed to determine the best suited combination for the purpose of this study. The diets considered were 0.5M sucrose, 0.5M sucrose + amino acids (Kim et al. 2007) and 0.5M sucrose + amino acids + minerals + vitamins also referred to as complete diet (Dadd et al. 1966) (Table 2.1). Two agriculturally important aphid species, the green peach aphid (*Myzus persicae*) and the cotton–melon aphid (*Aphis gossypii*) were used in these studies.

Complete Diet

Mixture of L-amino acids (see below) (g)	2.41
Sucrose (g)	15
Vitamin mixture (see below) (ml)	10
Ascorbic acid (mg)	100
KH_2PO_4	500
$MgCl_2\ 6H_2O$	200
U.S.P. salt mixture (see below) (mg)	10
Water, to 100 ml adjusted to pH 7 with KOH	
dissolve and filter through 0.2 µM filter	

L-Amino Acid Mixture (mg)

Alanine	100
Arginine	270
Asparagine	550
Aspartic Acid	140
Cysteine HCl	40
Glutamic Acid	140
Glutamine	150
Glycine	80
Histidine	80
Isoleucine	80
Leucine	80
Lysine HCl	120
Methionine	40
Phenylalanine	40
Proline	80
Serine	80
Threonine	140
Tryptophan	80
Tyrosine	40
Valine	80

10 ml of Vitamin Mixture (mg)

Thiamine	2.5
Riboflavin	0.5
Nicotinic Acid	10
Pyridoxine	2.5
Folic Acid	0.5
Calcium Patothenate	5
Meso-Inositol	50
Choline Chloride	50
Biotin	0.1

U.S.P. Salt Mixture (mg)

Calcium Biphosphate	13.58
Calcium Lactate	32.69
Ferric Citrate	2.96
Magnesium Sulfate	13.7
Potassium Phosphate	23.99
Sodium Biphosphate	8.73
Sodium Chloride	4.35

Amino Acid Diet

Sucrose (g)	2.14
Water to 15mL	
dissolve and filter through 0.2 µM filter	

L-Amino Acid Mixture (mg)

Alanine	2.14
Arginine	33.75
Asparagine	68.75
Aspartic Acid	17.5
Cysteine HCl	5
Glutamic Acid	17.5
Glutamine	18.75
Glycine	10
Histidine	10
Isoleucine	10
Leucine	10
Lysine HCl	15
Methionine	5
Phenylalanine	5
Proline	10
Serine	10
Threonine	17.5
Tryptophan	10
Tyrosine	5
Valine	10

Table 2.1 Components of artificial diets

2.2 MATERIALS AND METHODS

2.2.1 GENERATING COHORTS

To minimize the amount of variation in gene expression due to aphid life stage differences, cohorts were established by taking 10-15 adult aphids from mixed-age colonies of green peach aphids (*Myzus persicae*) or cotton melon aphids (*Aphis gossypii*) and placing them inside of a clip cage (Figure 2.1). Several clip cages with adult aphids were then attached to uninfested Pak Choi (*Brassica rapa*) plants and the aphids were allowed to produce nymphs for 24 hours at room temperature. After 24 hours, the adults were removed with a fine tipped paint brush and clip cages were replaced over nymphs. Nymphs were allowed to grow for 96 hours (4 days) then gently removed from the leaves with a paint brush ready for use in feeding experiments.

2.2.2 FEEDING SACHETS

Glass beakers (15 mL) were soaked in 10% bleach then cleaned with soap and water, rinsed with water, and allowed to air dry. Clean, dry beakers were treated with RNase Zap (Ambion catalog #9780) for 2 minutes at room temperature then rinsed twice with 3 mL each of DEPC treated water. Beakers were individually wrapped with aluminum foil, autoclaved for 30 minutes, and dried for 10 minutes. On the day the experiment was to be performed the beakers and 2 x 2 inch parafilm squares were exposed to UV light for 1 hour. A total of 10 nymphs were placed into a single 15 mL beaker and a square of parafilm was stretched over the mouth of the baker with UV exposed side facing up. Diet (75 µL) was dispensed on top of the parafilm and another piece of parafilm was stretched over the diet with UV exposed surface facing down. Diet

was manipulated to create maximum surface area possible for aphids to feed (Figure 2.2). Insects were allowed to feed on the artificial diets for 4-7 days in 16:8 light:dark cycles at 21^{o} C. Each treatment was replicated four times. The number of living adult aphids and nymphs was recorded every 24 hours. At the end of the assay, aphid performance on each diet was assessed to determine which diet formulation was optimal to use for future dsRNA feeding experiments.

2.2.3 ANALYSIS OF VARIANCE

Data collected on day four of feeding experiments were averaged then entered into an online ANOVA program N=4 (http://www.physics.csbsju.edu/stats/anova.html).

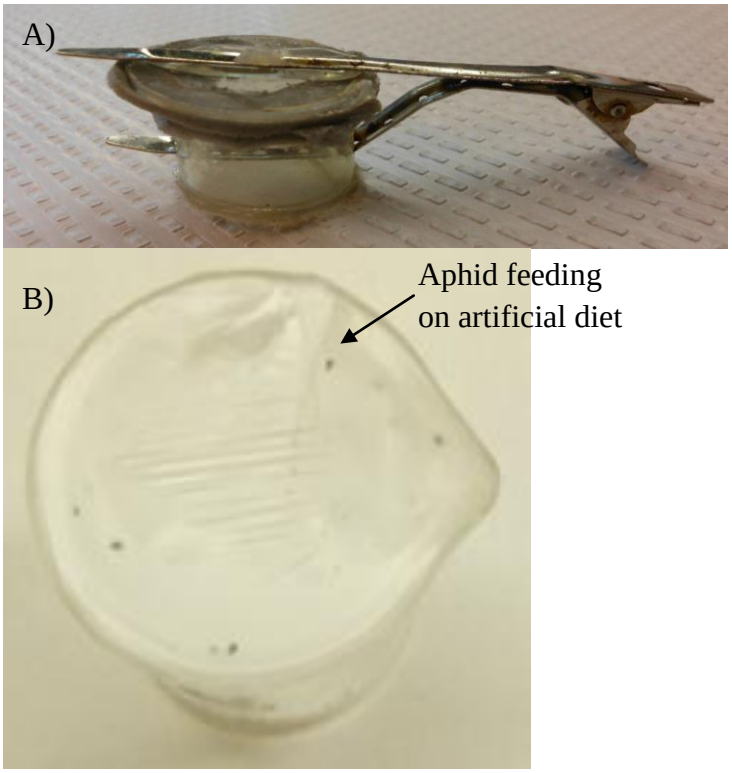


Figure 2.1 Experimental containment systems. A) Picture of a clip cage used to establish cohorts. B) Aphids feeding on an artificial diet sachet.

2.2.4 OPTIMUM COHORT AGE

The age of the cohort has the potential to skew results, so determining the appropriate cohort age for the experiment was critical. A range of ages have been used in previous artificial diet assays. Douglas et al. (2006) used two day old nymphs transferred from plants. Cooper et al. (2010) transferred 3rd and 4th instar aphids to feeding sachets. Fragoyiannis et al. (1998) allowed adult aphids to lay nymphs in beakers to be used for feeding experiments and started treating 12 hour old nymphs on artificial diet with low mortality. It is important to note that Fragoyiannis allowed nymphs to be laid in the arena in which they were to be tested and were reared on artificial diet, while the aphids in the thesis studies were transferred from plants to the feeding arena. Experiments performed by Shakesby et al. (2009) used six day old aphids for administering dsRNA in artificial diets, which produced low mortality in control groups. Whyard et al. (2009) fed one-day-old nymphs dsRNA in artificial diet with low mortality in control groups as well. As evident by these studies there is no single age that is best suited for using with artificial diets. Generally, literature employing artificial diets used younger cohorts, from 12 hours old to first instar (Douglas et al. 2006; Fragoyiannis et al. 1998; Le-feuvre et al. 2007; Sadeghi et al. 2009; Shakesby et al. 2009).

Bearing these previous studies in mind, we reasoned cohorts that are too young could show higher mortality due to their fragile nature. Moving individuals from leaf to artificial feeding arena when they are too young might damage or kill them leading to inaccurate results for the assay. Allowing mature aphids to lay nymphs in feeding arenas could lead to increased incidence of contamination as well as more difficulties in setting up assays because the number of nymphs laid could not be standardized. We also

reasoned using older cohorts might not be ideal as the effect of the dsRNA might not be detectable because target transcripts could have already established an adequate amount of protein or the proteins derived from the target transcripts have a longer half-life so effects would be delayed or not apparent at all. We therefore chose to transfer aphids to artificial diet feeding arenas after four days of growth on leaves.

2.3 RESULTS AND DISCUSSION

Determining the preferred aphid diet is crucial to the development of future assays. The purpose of these experiments was to establish the parameters necessary to give an optimum testing environment for future experiments. Providing a favorable diet to serve as the substrate for the dsRNA for aphid feeding ensures that aphids will feed and thus, consume more of the dsRNA optimizing the opportunity to attain results that are due to the treatment and not diet deficiencies or handling. This feeding assay served as the first step in determining a diet conducive to testing candidate genes from RNAi targeting through transgenic plant feeding the overall goal for optimizing this method was to facilitate faster validation of candidate gene targets.

Aphis gossypii survived equally well on the sucrose only diet and sucrose augmented with amino acids and minerals diet (complete diet) (Figure 2.2). After day one, the aphids feeding on complete diet seemed to survive better than the other diets. Survival of this group dropped quickly; however, between day one and day three survival of aphids feeding on sucrose augmented with amino acids (sucrose + aa) diet remained steady for two days before gradually decreasing. At the end of the experiment on day four, aphid survival appeared to be best on the sucrose with amino acids diet.

ANOVA analysis of *A. gossypii* survival at day four (Figure 2.3) did not show statistically significant differences between the diet treatments as the p value produced was greater than 0.05 (p=0.055, N=4). Results were generated by entering the mean of each of four replicates per treatment after the fourth day of feeding on artificial diets. Each treatment started with 10 aphids.

A. gossypii reproduction was best on the sucrose + aa diet (Figure 2.4). Aphids feeding on the sucrose diet never produced nymphs throughout the four day experiment. On the third day of the experiment aphids feeding on the sucrose + aa diet started producing nymphs at approximately 1 nymph per beaker (n=4). At day four of the experiment aphids feeding on complete diet started to reproduce with one nymph in each of two beakers (n=4). The reproduction rate of the aphids feeding on the sucrose + aa diet did not produce any additional nymphs after the third day.

M. persicae survival was optimal on either sucrose + aa or complete diet (Figure 2.5). The reduction of survival on sucrose only diets became evident at day one. At day two the gap between sucrose only and complete diet narrowed slightly with best survivability being with the sucrose + aa diet. Data collected at day three revealed 50% mortality of aphids feeding on sucrose only, while aphids feeding on the complete and sucrose + aa diets both performed well. Between days three and four the cohort feeding on sucrose only diet showed increased mortality, while the sucrose + aa diet dropped slightly below the survivability of the complete diet. ANOVA analysis of *M. persicae* survival at day four (Figure 2.6) showed statistically significant treatment differences as the p value produced was lower than 0.05 (p=0.002, N=4). Results were generated by

entering the mean of each of 4 replicates per treatment after the fourth day of feeding on artificial diets. Each treatment started with 10 aphids.

Figure 2.7 illustrates there is a clear advantage in reproductive rate for *M. persicae* feeding on complete diet. By the second day aphids feeding on complete diet started producing nymphs. By day three, aphids feeding on the sucrose + aa diet started to produce nymphs; more nymphs were produced on sucrose + aa (day three) than on the complete diet (day two) when the first nymphs were observed. Cumulative production of nymphs on day three was greater on the complete diet (3 $\pm$ 0.58) as compared to the sucrose + aa diet (1.25 $\pm$ 0.79). By the fourth day nymph production on both the complete diet (18 $\pm$ 1.89) and sucrose + aa diet (10.25 $\pm$ 1.38) increased dramatically. Throughout the duration of the experiment, nymphs were never produced on the sucrose diet.

Overall, none of the diets tested seemed to be optimal for *A. gossypii*, whereas the complete diet resulted in high levels of survivability and reproduction for *M. persicae* that would be acceptable for future experiments. These results are not completely unexpected since the complete diet used in these experiments was originally developed and optimized for *M. persicae* (Dadd et al. 1966; Dadd et al. 1965). Determining the preferred aphid diet is crucial to the development of future assays. Providing a favorable diet to dilute dsRNA for aphid feeding ensures that aphids will feed and thus, take up more of the dsRNA ensuing better results and that results are due to the treatment not diet deficiencies or handling. These results showed that *M. persicae* feeding on complete diet had highest survivability (Figure 2.5). Although the survival rate of *A. gossypii* was also

high while feeding on sucrose diet supplemented with amino acids its reproductive rate was much lower.

An important factor in determining the fitness of aphids on a diet is their fecundity rate. The fecundity rate will be an important observation during testing with dsRNA because it is a measure of fitness and direct reflection of how the cohort is reacting to the treatment. Clearly, *M. persicae* feeding on complete diet produced the most nymphs at nearly every time point (Figure 2.5). Fecundity combined with high survivability makes *M. persicae* feeding on complete diet the best system to use for dsRNA studies.

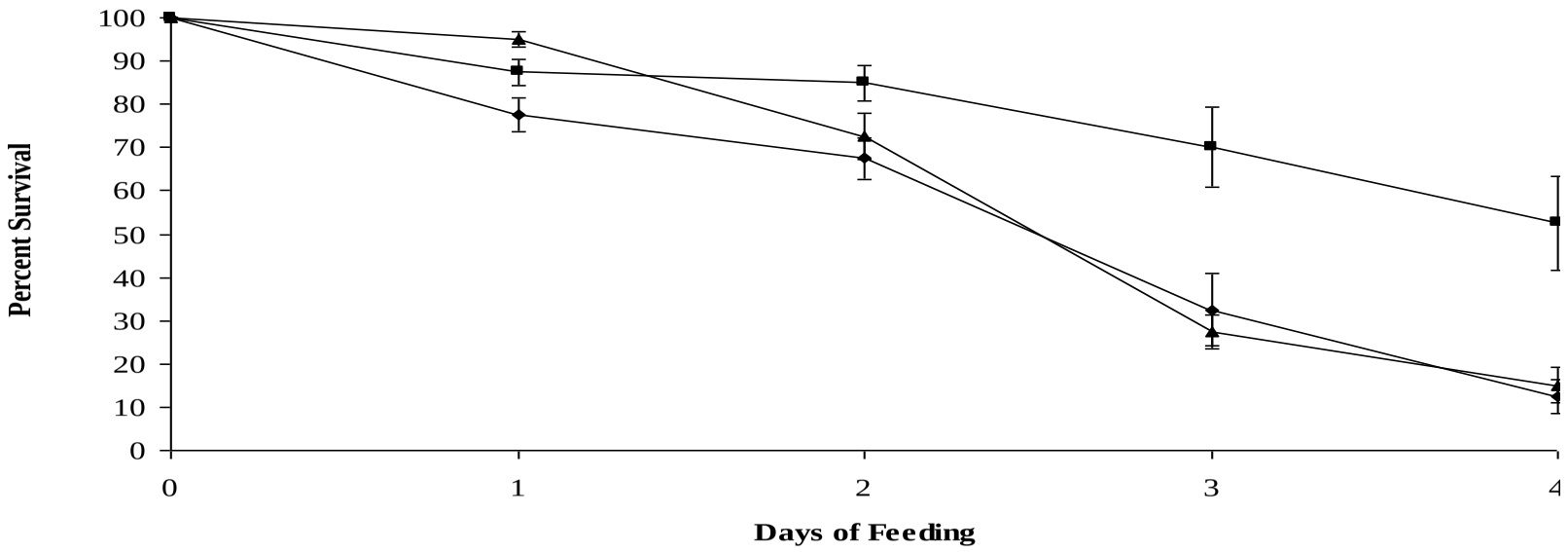


Figure 2.2 *Aphis gossypii* survival on artificial diets. Diets were composed of 0.5M sucrose (♦), sucrose augmented with amino acids (■), or a complete diet composed of sucrose, amino acids and minerals (▲). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean $\pm$ SE of four replicates.

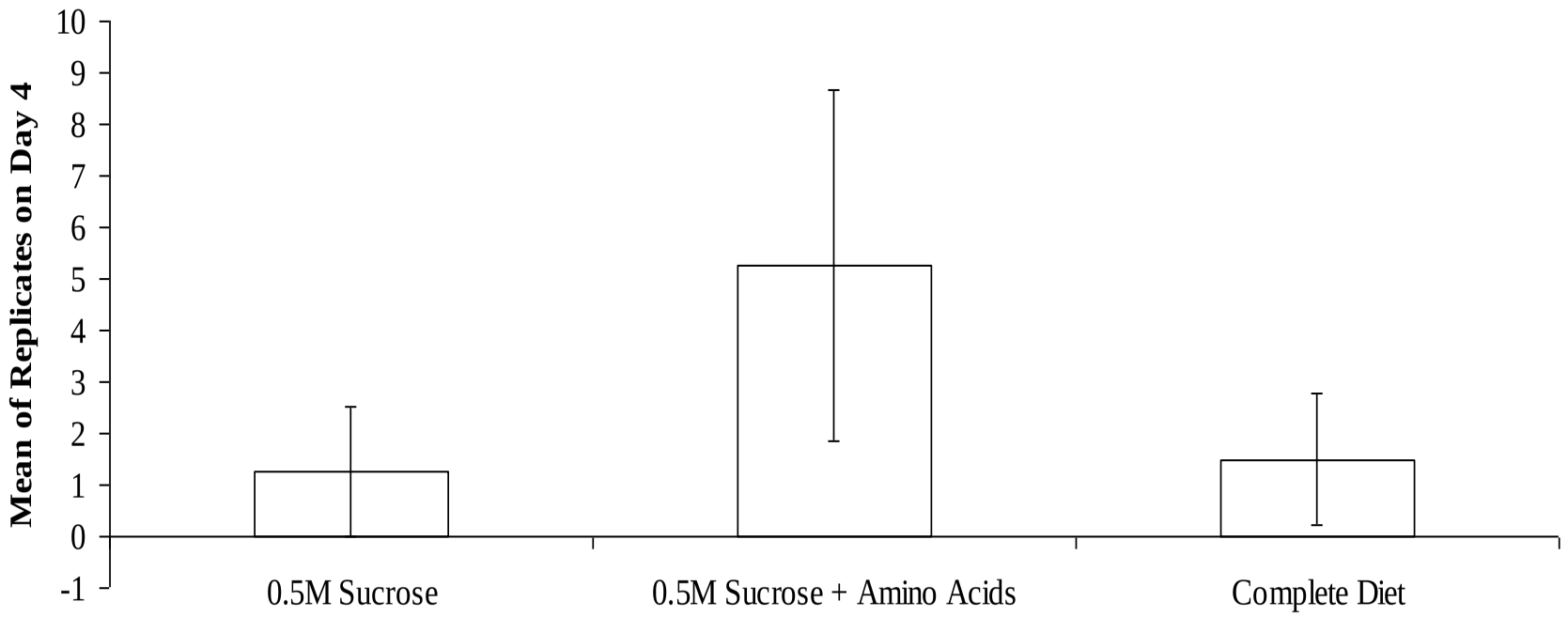


Figure 2.3 ANOVA of *Aphis gossypii* on artificial diets. Means are the mean average of 4 replicates containing 10 aphids per replicate for each treatment after 4 days of feeding on artificial diet. These results assume the null hypothesis that the result of treatment was not due to the treatment as p=0.055

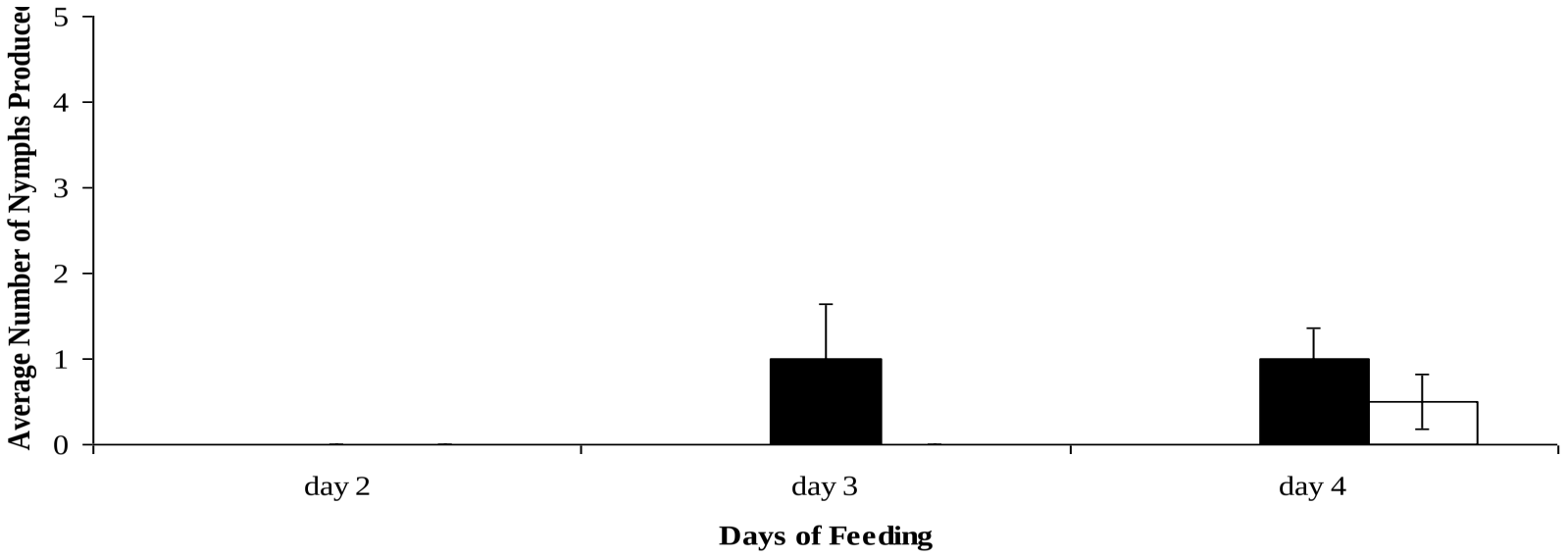


Figure 2.4 *Aphis gossypii* nymph production on artificial diets. Diets were composed of 0.5M sucrose (bars not visible because no nymphs produced), sucrose augmented with amino acids (black bars), or a complete diet composed of sucrose, amino acids and minerals (white bars). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean $\pm$ SE of four replicates.

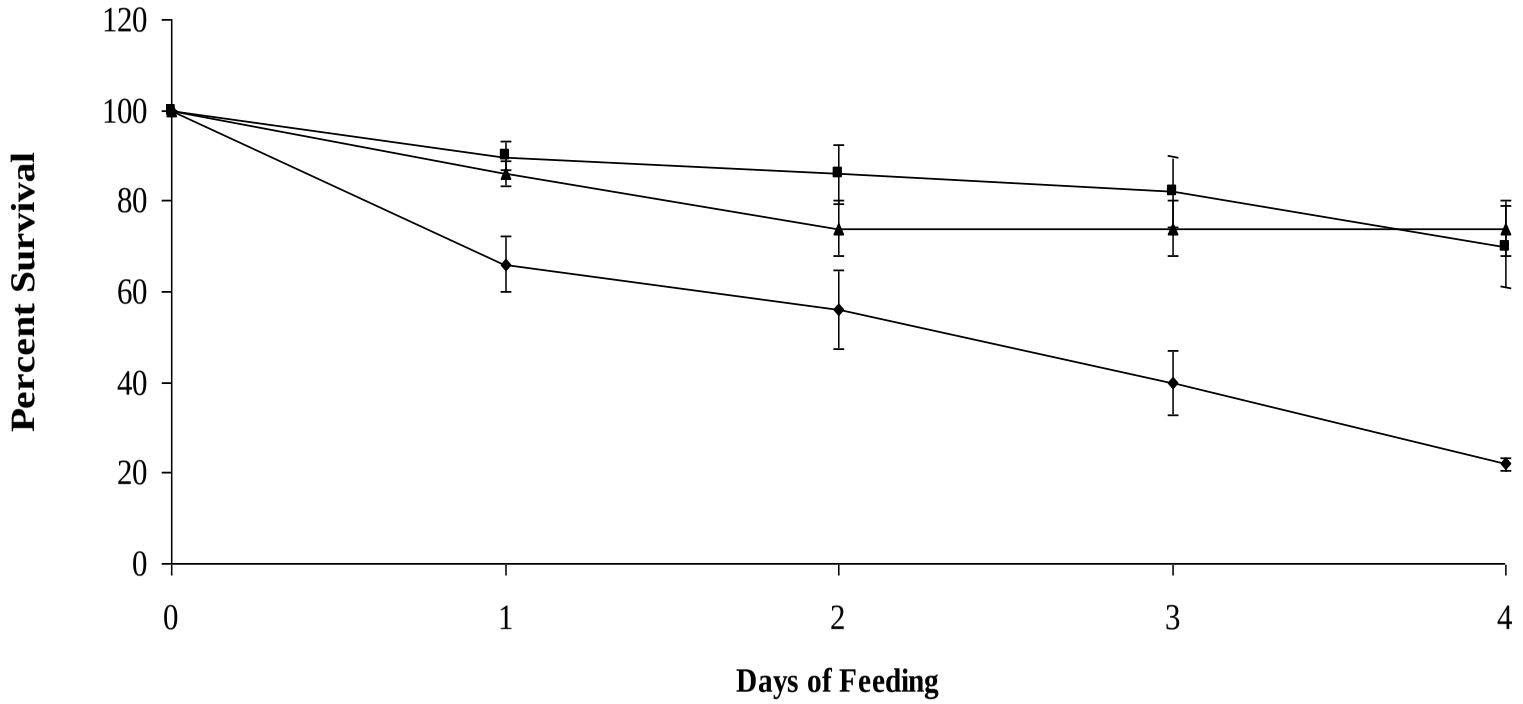


Figure 2.5 *Myzus persicae* survival on artificial diets. Diets were composed of 0.5M sucrose (♦), sucrose augmented with amino acids (■), or a complete diet composed of sucrose, amino acids and minerals (▲). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean $\pm$ SE of four replicates.

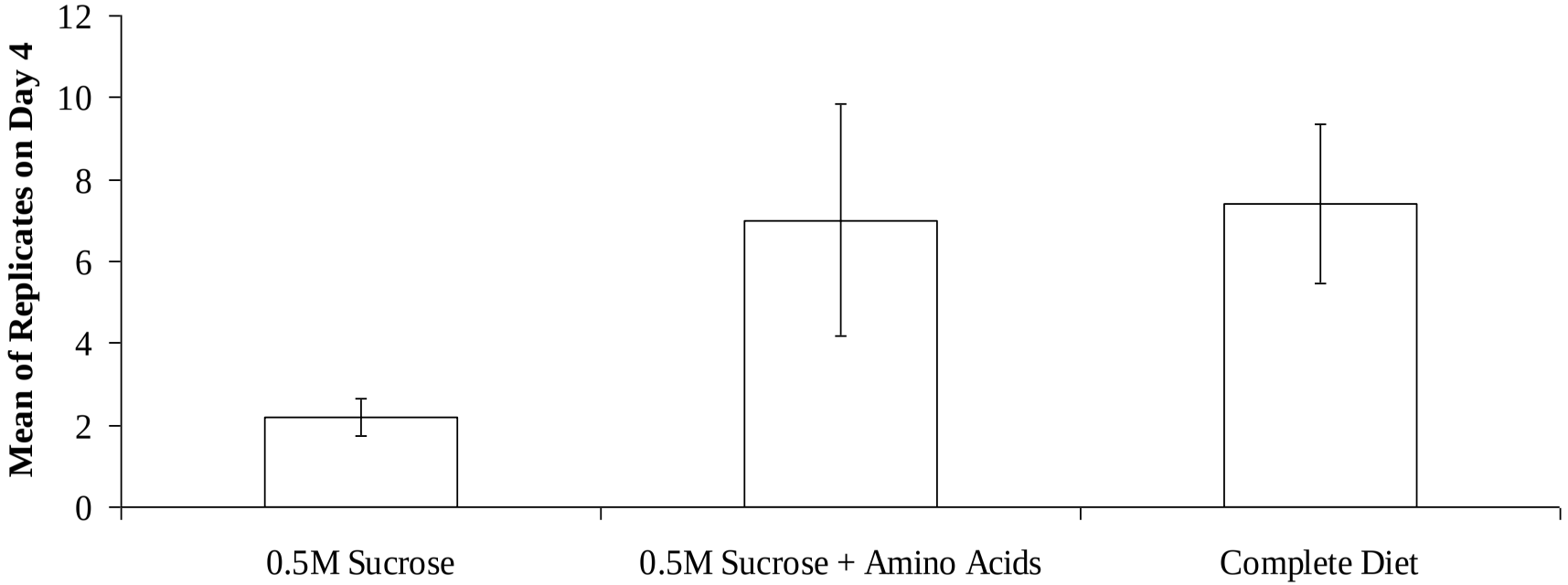


Figure 2.6 ANOVA of *Myzus persicae* on artificial diets. Means are the mean average of 4 replicates containing 10 aphids per replicate for each treatment after 4 days of feeding on artificial diet. These results assume the null hypothesis that the result of treatment was due to the treatment as p=0.002

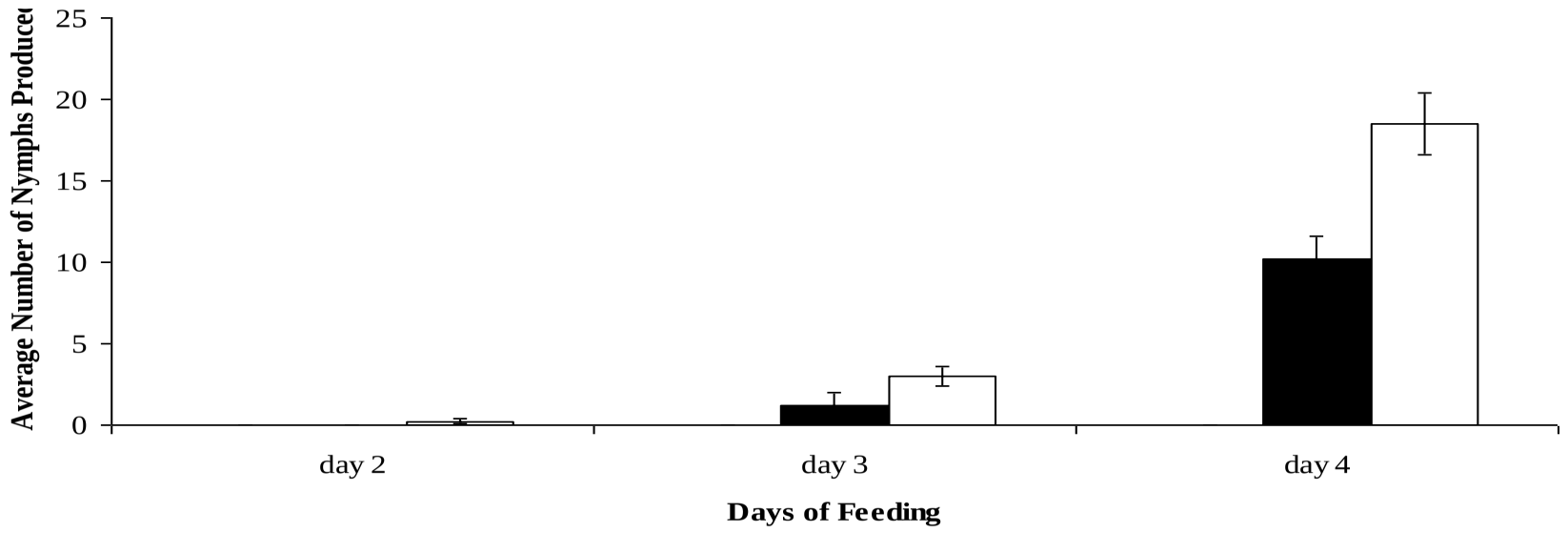


Figure 2.7 *Myzus persicae* nymph production on artificial diets. Diets were composed of 0.5M sucrose (bars not visible because no nymphs produced), sucrose augmented with amino acids (black bars), or a complete diet composed of sucrose, amino acids and minerals (white bars). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean $\pm$ SE of four replicates

CHAPTER III

USING DSRNA TO KNOCKDOWN TARGET GENE EXPRESSION IN INSECTS

3.1 INTRODUCTION

The increasing popularity of RNAi techniques has led researchers to start evaluating RNAi-based insect pest control (Huvenne et al. 2010; Mao et al. 2007; Price et al. 2008). These techniques are only useful if smRNA molecules can be easily and effectively introduced into an organism. Two potential RNAi uptake mechanisms are proposed in insects; the trans-membrane channel-mediated uptake mechanism and an endocytosis-mediated uptake mechanism (Huvenne et al. 2010). Briefly, the trans-membrane channel-mediated uptake mechanism utilizes proteins that recognize dsRNAs and transport the non-cell-autonomous dsRNA from the extracellular space to the cytoplasm through a trans-membrane channel. The endocytosis-mediated uptake mechanism has yet to be elucidated; however, dsRNA is introduced into the cell across the plasma membrane through endocytosis rather than through a membrane channel. These uptake models suggest that several key factors are important when designing RNAi based insect pest control studies, such as the concentration of the dsRNA, nucleotide sequence composition, length of the dsRNA fragment, persistence of the silencing effect, and life stage of the target organism. Other concerns include target specificity of the

dsRNA to impact only the intended insects and the potential development of insect resistance to plants producing dsRNA for RNAi (Huvenne et al. 2010). Spraying dsRNA onto the plants has been suggested as an alternative to genetically altering the plant genome to encode for the synthesis of the dsRNA (Huvenne et al. 2010). It has been suggested that the best target site would be the gut of the insect, which highlights the challenge of targeting phloem feeding insects because of the specialized food source.

An important first step in determining candidate genes for targeting by RNAi is testing the efficiency of the dsRNA to affect target gene expression by methods that are rapid and relatively easy to interpret. Mutti et al. (2006) used direct injection to deliver siRNAs into insects and found that injecting siRNA targeting a salivary gland-specific gene C002 resulted in lethality in a shorter time span than the green fluorescent protein (GFP) negative control. Direct injection of dsRNA has been popular as it administers dsRNA or siRNA into the hemolymph where most target tissues can be affected. While injection of siRNAs in insects has been shown to reduce gene expression, the effectiveness of the technique is largely dependent on the skill and experience of the experimenter as mechanical injection often leads to damaging tissues at the injection site.

Initial studies using artificial diets containing purified dsRNA as an alternative to micro-injection has resulted in transient reductions in the expression of the target gene (Shakesby et al. 2009). Administering dsRNA orally in an artificial diet is more practical than injection, especially when targeting genes that are expressed in the aphid gut. Important parameters such as whether the dsRNA is effective after feeding and the concentration needed for reducing gene expression must be empirically determined. The

experiments described in this chapter are designed to test if dsRNA can reduce the expression of an aphid gut target gene, *cathepsin L*.

3.2 MATERIALS AND METHODS

3.2.1 DSRNA ASSAY

Cathepsin L is a cysteine proteinase that is expressed in the epithelial cells that line the gut in aphids (Deraison et al. 2004). The *cathepsin L* (*CatL*) gene was selected as a target for these experiments based on the work of Jaubert-Possamai and coworkers (2007), who found that micro-injecting a 353 bp dsRNA targeting the *CatL* gene into the abdomen of fourth instar pea aphids (*Acyrthosiphon pisum*) resulted in a 40% decrease in *CatL* transcript levels. Furthermore, Carrillo and coworkers (2010) found that aphids feeding on a diet containing a cysteine-proteinase inhibitor had reduced performance (Carrillo et al. 2010; Jaubert-Possamai et al. 2007; Rahbe et al. 2003). A 396 bp region in the center of the *CatL* gene was selected for amplification in this study. A 466 bp region of the GFP gene was chosen as a negative control because it does not share significant sequence similarity with any aphid gene and previous studies showed its use had minimal impact on aphid performance (Mutti et al. 2006; Pitino et al. 2011).

Laboratory colonies of *M. persicae* were used to isolate aphid gut RNA and to clone the *CatL* cDNA. Small plastic pestles were cleaned by treating with RNase Zap for 1 minute, DEPC water for 30 seconds, 100% ethanol for 30 seconds, and DEPC treated water for 30 seconds. Aphid guts of 20-25 aphids of mixed ages were harvested under a dissection microscope using fine tipped tweezers. A drop of nanopure water was placed in and empty petri dish cover and a single aphid was placed on top of the droplet of water to immobilize the aphid. One set of tweezers was used to grab the rostrum of the aphid

while another set was used to grab the posterior; tweezers were gently pulled apart until the contents of the aphid spilled out into the droplet of water. Once the contents of the aphid were exposed the gut was recognized by its oval structure with a string like attachment (intestine). Isolated guts from individual aphids were immediately added to 100 µL of TRIzol (Invitrogen, catalog no. 15596018) in a 1.5mL Eppendorf tube on ice. The gut-TRIzol mixture was homogenized and allowed to incubate at room temperature for 5 minutes then centrifuged at 12,000 rpm for 10 min at 4^0 C. The supernatant was transferred to a 1.5 mL tube and 20 µL of chloroform was added then the tube was shaken vigorously for 15 sec, allowed to incubate at room temperature for 3 minutes, and centrifuged at 12,000 rpm for 10 minutes at 4^0 C. The clear liquid phase (~20 µL) was next added to 5 µL of isopropanol and 5 µL high salt buffer (0.8M Na citrate and 1.25M NaCl) and incubated over night at -20^0 C. The next day the RNA was centrifuged at 13,000 rpm for 10 minutes at 4^0 C then the liquid was decanted off and the pellet was washed with 200 µL 70% ethanol and centrifuged at 6,000 rpm for 5 minutes at 4^0 C. The washing step was repeated once more before the pellet was air dried for 10 minutes. RNA was resuspended in 10 µL DEPC treated water while incubating on ice for 2 hours. First-strand cDNA was synthesized from the gut enriched RNA using SuperscriptTM II Reverse Transcriptase (Invitrogen Catalog number 18064-022) and oligo dT following manufacturer's instructions.

Gene specific primers derived from NCBI database sequences were used to amplify the *CatL* cDNA (See Appendix 1 for primer sequences). The entire coding sequence (1026 bp) was amplified to serve as a template for 356 bp region of gene to be used for RNAi targeting by dsRNA. The template and primers were added to PCR

Master Mix (Promega, catalog no. M7502), and PCR amplification was performed following a standard program (94°C 5'> [94°C 15" > 50°C 30"> 68°C 40"] x 40 > 68°C 5') with Biorad DNA engine peltier thermo cycler. PCR amplicons were separated in a 1.5% agarose gel. The amplicon of 1026 bp was extracted and purified using the Omega gel extraction kit (Omega Bio-tech catalog #D2501-02). Purified PCR products were ligated into pGEM-T Easy (Promega catalog #A1360) and transformed into DH5alpha cells using standard laboratory procedures, plated on LB ampicillin medium, and incubated over night at 37^0 C. Several of the colonies on the plate were grown individually in 25mL LB ampicillin medium over night while shaking 100 rpm at 37^0 C. Harvested cells were lysed and plasmids purified using the Omega plasmid prep kit (Omega Bio-tek catalog #D6942-02). Purified plasmids were sequenced using either T7 or SP6 primers at the Oklahoma State University Recombinant DNA/Protein Core Facility.

Plasmids with the correct sequence were then used as templates to generate dsRNA using the MEGAscript Kit (Ambion catalog # AMB1334-5). A 396 bp region in the center of the *CatL* cDNA and a 466 bp region of the *GFP* cDNA were used to generate dsRNA from plasmids. The primers used in these experiments are shown in Appendix 1. Phenol chloroform extraction was used to purify dsRNA with an additional chloroform extraction and one pellet wash with 80% ethanol. The RNA pellet was resuspended in DEPC treated water on ice for 2 hours. RNA concentration was determined in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA was stored at -80^0C. The day of the experiment dsRNA was thawed on ice and diluted to desired concentrations (1 ng/µL, 0.5 ng/µL, 0.375

µg/µL, 0.25 µg/µL, or 0.125 µg/µL) with complete diet. Complete diet with no dsRNA was used as a negative control for the experiments.

Cohorts of aphids were established as described in Chapter II; newly emerged nymphs were allowed to grow for four days on Pak Choi (*Brassica rapa*). Artificial diet experiments were conducted using the material and methods described in Chapter II. Ten aphids were placed in each beaker and allowed to feed on complete diet with treatment (*CatL* or *GFP* dsRNA) or no treatment as a negative control. Each treatment was replicated 4 times. Experiments were terminated after seven days or when all the aphids had died.

3.2.2 PROBIT ANALYSIS

The lethal concentration at which 50% of the aphid population died (LC_{50}) was determined using probit analysis to assess the relative toxicity of the dsRNA. Probit analysis is commonly used to analyze dose response with binomial variables by turning sigmoidal data into linear (Bliss 1934). Data was entered into the Statistical Package for the Social Sciences (SPSS) software package (SPSS, Inc.) to transform percent mortality into probability units (probits). The log of the concentrations was plotted against the probits and a regression line was fitted to the data points.

3.2.3 REAL TIME-PCR ANALYSIS OF GUTS

Real time PCR was used to quantify the amount of target gene transcript in control and treated aphids. The concentration of *CatL* dsRNA, (0.375 µg/µL in complete diet) was chosen based on the probits analysis where 50% of the population died after four days. The assay consisted of ten aphids per beaker with six replicates of each

treatment. Aphids were allowed to feed for 48 hours on artificial diets with or without dsRNA. After 48 hours, guts extracted from living aphids in each beaker were pooled into one RNA isolation reaction by immediately transferring extracted guts to 100 uL of TRIzol in an Eppendorf tube on ice. RNA extraction was performed as previously described quantifying the RNA after resuspending in water as each replicate had a varying number of guts collected. First strand cDNA was synthesized according to manufacturer's instructions (Invitrogen SuperScriptTM II Reverse Transcriptase Catalog number 18064-022). RT-PCR was performed on cDNA using primers specific for two housekeeping genes; actin and elongation factor 1 alpha. Three primer sets were used for amplification of the *CatL* RNA (Appendix 1). RT-PCR was performed according to the manufacturer's instructions (Applied Biosystems RT-PCR) that was modified by omitting cDNA synthesis step and adding a disassociation curve. Pfaffl method was used to analyze the results (Pfaffl 2001) .

3.3 RESULTS AND DISCUSSION

The initial experiments were designed to test the efficacy of presenting a high concentration of dsRNA in the diet to induce RNAi of an essential gene that is expressed in aphid guts. Previous investigators reported that concentrations of 1 µg/µL dsRNA would decrease the expression of target genes and increase the mortality of aphids feeding on diets containing dsRNA (Shakesby, et al. 2009). Figure 3.1 illustrates initial comparison of comparison of the mortality of aphids feeding on a diet containing *CatL*-dsRNA (1 µg/µL), *GFP*-dsRNA (1 µg/µL), and no dsRNA. The average number of surviving aphids, recorded every 24 hours, was expressed as a percentage of the initial number of aphids and plotted on the graph. Aphids feeding on complete diet had no

mortality throughout the four days of treatment. Introducing *GFP*-dsRNA into the diet resulted in decreased survival (92.5% survival) after 24 hours and became more pronounced (72.5%) after 48 hours. The percent survival of the *CatL*-dsRNA treatment was the same as the *GFP*-dsRNA treatment after 24 hours; however, the survival rate decreased drastically after 48 (37.5%) and 72 (2.5%) hours by the end of the four days all of the *Cat L*-dsRNA treated aphids had died. In contrast, approximately 20% of the aphids in the *GFP*-dsRNA treatment aphids survived to the fourth day of treatment. The results of this experiment demonstrate that *Cat L*-dsRNA is more toxic than *GFP*-dsRNA; however the *GFP* dsRNA treatment did show a pronounced toxic effect. It is interesting to note that during the course of these experiments aphids feeding on no dsRNA diets generally produced nymphs as in Chapter II on complete diet but neither the *CatL*- or *GFP*-dsRNA diets produced many if any nymphs.

The toxic effects of both *CatL*- and *GFP*-dsRNA could be due to the high concentrations of dsRNA presented to the aphids in their diet. The concentrations of both *CatL*- and *GFP*-dsRNA were titrated to further test the efficiency of *CatL*-dsRNA. Four concentrations *CatL*-dsRNA (0.5, 0.375, 0.25, and 0.125 µg/µL), were compared with 0.5 µg/µL *GFP*-dsRNA and no dsRNA (Figure 3.2). Similar to the results of the previous experiment, aphids feeding on a complete diet devoid of dsRNA showed a consistently high level of survival throughout the experiment with 90% of the aphids surviving after four days. In contrast after 24 hours aphids feeding on the diet with 0.5 µg/µL *CatL*-dsRNA had a lower survival rate (77.5%) than any of the other concentrations of *CatL*-dsRNA (0.375 µg/µL = 100%, 0.25 µg/µL = 97.5%, and 0.125 µg/µL = 100%) and *GFP*-dsRNA (90%). At 48 hours the number of surviving aphids feeding on 0.5 µg/µL

CatL-dsRNA had dropped drastically (5%) and at a much faster rate than any other concentration of dsRNA (0.375 µg/µL = 65%, 0.25 µg/µL = 85%, and 0.125 µg/µL = 97.5%, *GFP* 0.5 µg/µL = 47.5 %). There was also a sharp decrease (0%) in the survival of aphids feeding on 0.5 µg/µL *GFP*-dsRNA after 72 hours. At the end of three days, all aphids feeding on a diet containing a concentration of dsRNA greater than 0.25 µg/µL had 0% survival (*CatL* 0.25 = 25%; *CatL* 0.125 = 65%).

Other studies have used *GFP*-dsRNA as a negative control for RNAi experiments and observed aphid mortality levels that were significantly lower than mortality levels in the treatments with target dsRNA (Mutti et al. 2006; Shakesby et al. 2009). Figure 3.3 illustrates the survival of aphids feeding on decreasing amounts of *GFP*-dsRNA (0.5, 0.375, 0.25, and 0.125 µg/µL), while the *CatL*-dsRNA (0.5 µg/µL) concentration treatment was held constant. All treatments were compared to a no dsRNA control diet on which the aphids had high levels of survival (90%) at the end of four days. Consistent with previous experiment (see Figure 3.2), aphids feeding on 0.5 µg/µL *Cat L*-dsRNA showed decreasing survival at one (65%) and two (20%) days with no aphids surviving after three days in any replicate. Unlike the previous experiment, aphids did survive (20%) at the end of the four day period while feeding on the 0.5 µg/µL concentration of *GFP* dsRNA. Nonetheless, decreasing concentrations of *GFP*-dsRNA resulted in increased aphid survival. Interestingly, the 0.5 and 0.375 µg/µL *GFP*-dsRNA treatments gave similar results at each time point and at the end of the four days (0.5 µg/µL=20%: 0.375 µg/µL=30%) of the experiment. Both the 0.25 µg/µL and 0.125 µg/µL concentrations showed similar high levels of survival with the no dsRNA control at three days; however by the fourth day the lowest concentration of *GFP*-dsRNA (0.125 µg/µL =

95%) had comparable results to complete diet while aphid survival decreased (90%) when feeding on the 0.25 µg/µL concentration (60%). For each of the titration experiments increasing concentrations of *CatL*- or *GFP*-dsRNA decreased the percent survival, while the dsRNA negative control remained consistent indicating that mortality was due to the treatments.

To assess the relative toxicity of the *CatL* and *GFP* dsRNAs the lethal concentration at which 50% of the aphid population died (LC_{50}) was determined using probit analysis (Bliss 1934). Probit analysis is an alternative data analysis method to analyze dose responses with binomial variables by converting sigmoidal data into linear. The dose to mortality ratio graph of the fourth day *CatL*- and *GFP*-dsRNA titration data allowed the calculation of lethal concentration (LC_{50}) values (Figure 3.4). The calculated LC_{50} values of the 0.279 for *CatL*-dsRNA and 0.294 for *GFP*-dsRNA are not significantly different indicating that the mortality after feeding on the diets containing the respective dsRNAs might be due to contaminants in the dsRNA preparations and not due to altering the levels of *CatL* target gene expression. Different techniques were used to eliminate the possibility of contaminants in the dsRNA preparations. The use of multiple chloroform extractions to remove residual phenol or spin columns to purify the dsRNA provided inconclusive results (data not shown). Despite potential technical difficulties among experiments, each set of experiments demonstrated that the *CatL*-dsRNA increased mortality above the *GFP*-dsRNA treatments.

Real time-PCR experiments were conducted to determine whether *CatL*-dsRNA presented to the aphids in the diet resulted in quantitative changes in the amount of *CatL*-mRNA that could be detected in aphid guts. Aphids were allowed to feed on complete

diet with no dsRNA or 0.375 µg/µL *CatL*-dsRNA for 48 hours. This concentration of *CatL*-dsRNA was selected based on the probits analysis where 50% of the population died after four days. A cDNA dilution series was initially used to determine the efficiency of the real time-PCR primers (Figure 3.5, Panel A) for *CatL* as well as elongation factor 1alpha (EF) and actin; two housekeeping genes that were used to compare the levels of *CatL* transcripts among treatments. The R^2 values (*CatL*=0.9906; EF= 0.9378; actin=0.9741) are all close to a value of 1.0000 indicating that all of the data from the dilutions were nearly linear. The efficiency values (E values) of the data calculated by $E=10^{[-1/slope]}$ all fell within the recommended 1.7-2.2 range (*CatL*=2.22; EF=2.11; and Actin= 1.88) which are acceptable efficiencies according to the Plaffl method (Pfaffl 2001).

Comparing the number of guts from which total RNA was extracted to the ratio in expression levels of *CatL* and actin did not reveal differences between aphids fed the complete diet with no dsRNA and those fed *CatL*-dsRNA (Figure 3.5, Panel B). The simplest explanation for the lack of differential expression between the *CatL*-dsRNA treatment and no dsRNA control is that the mortality observed in the experiments was not due to altering the levels of *CatL*-mRNA by RNAi. Several other alternative explanations could also be explored by further experimentation. The results could be due to the inability of this particular *CatL*-dsRNA sequence to effectively target transcripts from the *CatL*-gene. The lack of differential expression could also be due to the duration of time the aphids were allowed to feed. Forty-eight hours might not have been sufficient time for the RNAi machinery to alter the steady-state levels of *Cat* transcripts of other genes in the cathepsin gene family. Additional experimentation will be required to

optimize this technique so that it will be useful to assess the effects of dsRNA delivered to aphids through their diet.

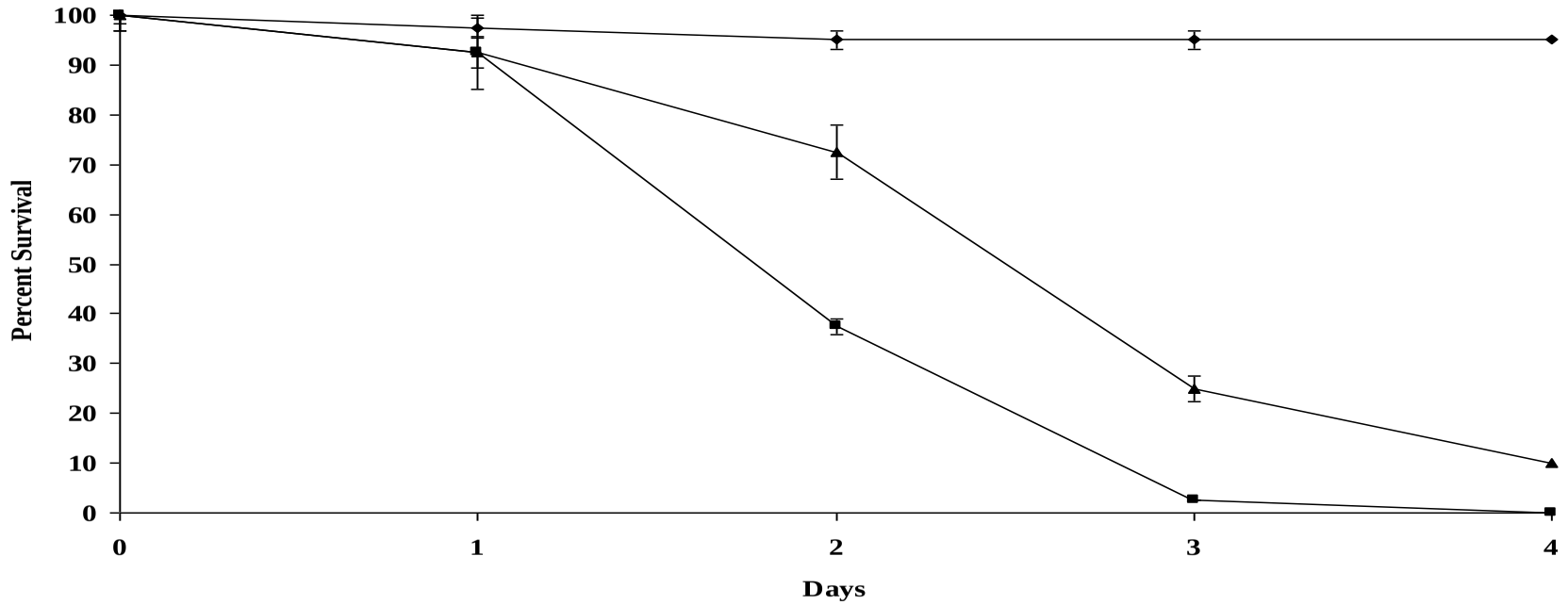


Figure 3.1 *Myzus persicae* feeding on high concentrations of dsRNA. Complete diet was supplemented with 1 µg/µL *GFP* dsRNA (▲), 1 µg/µL *CatL* (■), or DEPC treated water (♦). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean $\pm$ SE of four replicates.

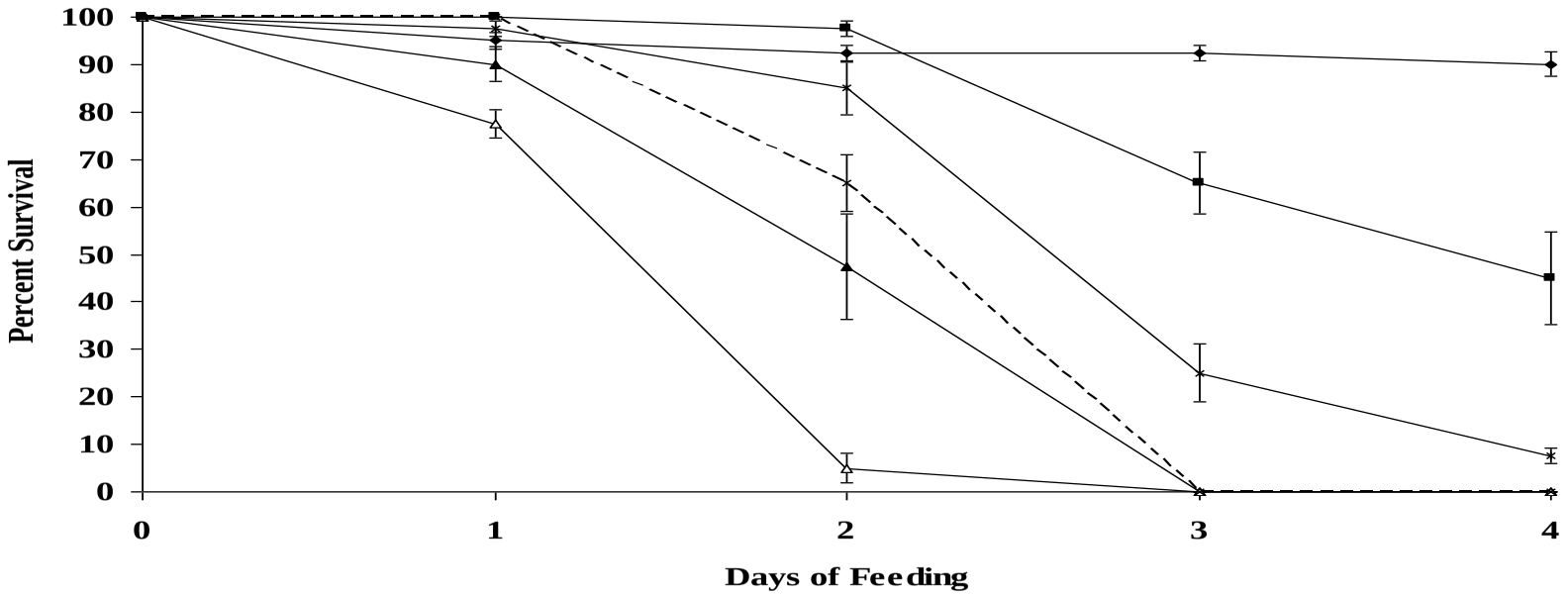


Figure 3.2 *Myzus persicae* feeding on different concentrations of *CatL* dsRNA. Complete diet was supplemented with titrated amounts of cathepsin L dsRNA. Complete diet with DEPC treated water (♦), 0.5 µg/µl *GFP* dsRNA (▲), 0.5 µg/µL *CatL* dsRNA (Δ), 0.375 µg/µL *CatL* dsRNA (X), 0.25 µg/µL *CatL* dsRNA (✶) and 0.125 µg/µL *CatL* dsRNA (■). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C. Bars represent the mean ± SE of four replicates.

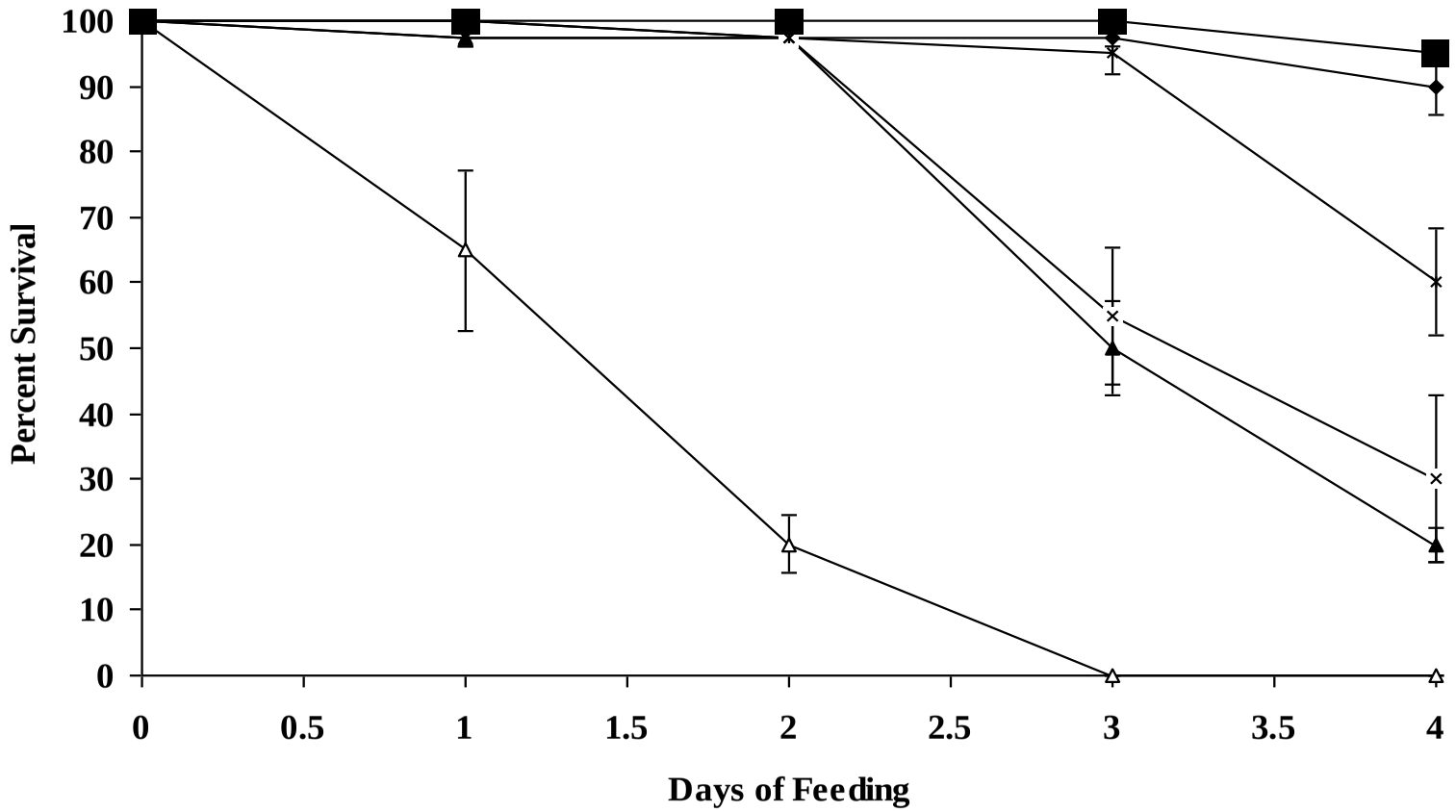
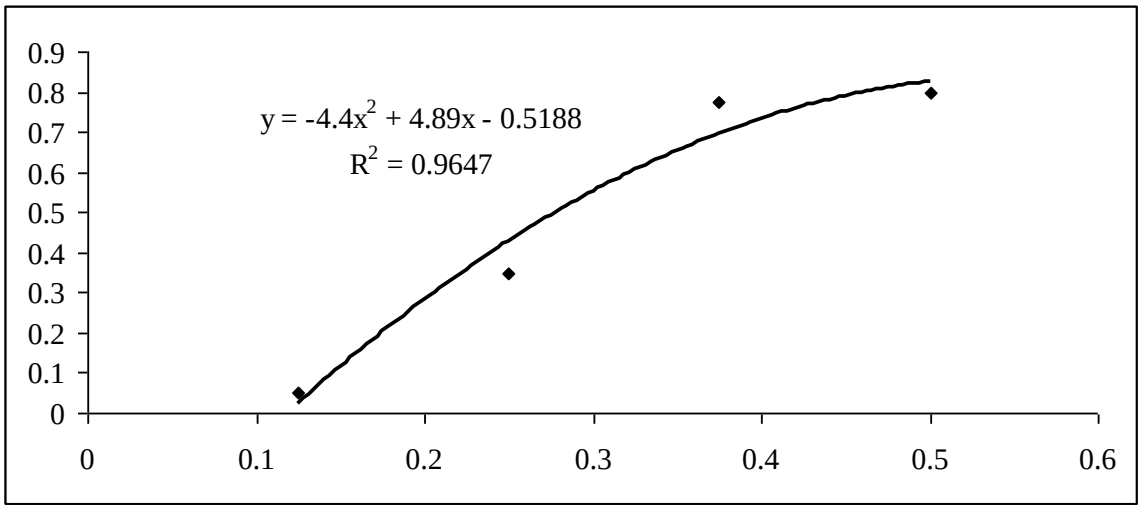


Figure 3.3 *Myzus persicae* feeding on different concentrations of *GFP* dsRNA. Complete diet was supplemented with titrated amounts of *GFP* dsRNA. Complete diet with DEPC treated water (♦), 0.5 µg/µL *CatL* dsRNA (Δ), 0.5 µg/µL *GFP* dsRNA (▲),0.375 µg/µL *GFP* dsRNA (X), 0.25 µg/µL l *GFP* dsRNA (∗) and 0.125 *GFP* dsRNA (■). Aphid cohorts were reared on Pak Choi then transferred to a sachet containing 75 µL of diet for four days with 16:8 h light:dark cycles at 24^{o} C.

A)



B)

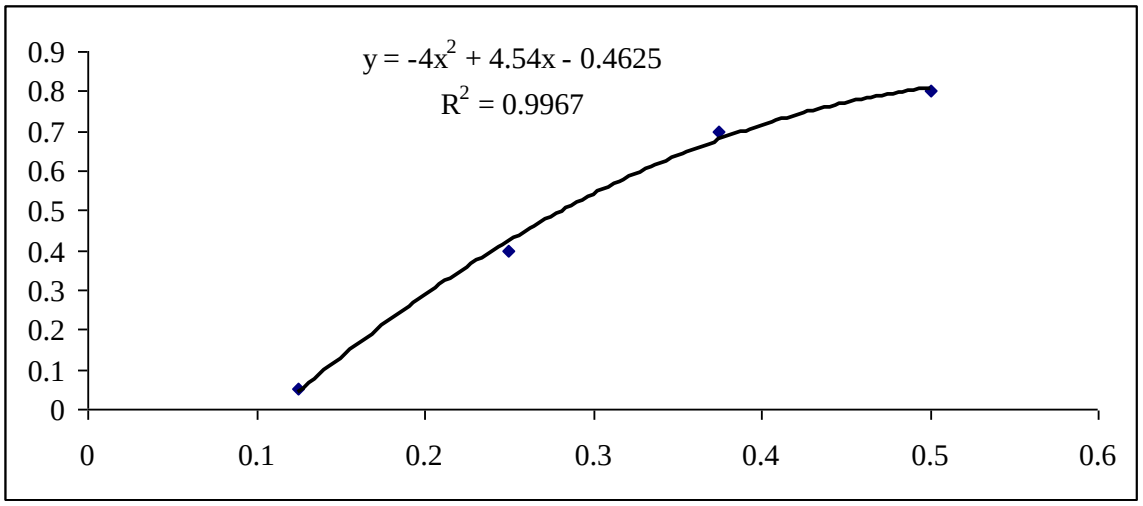
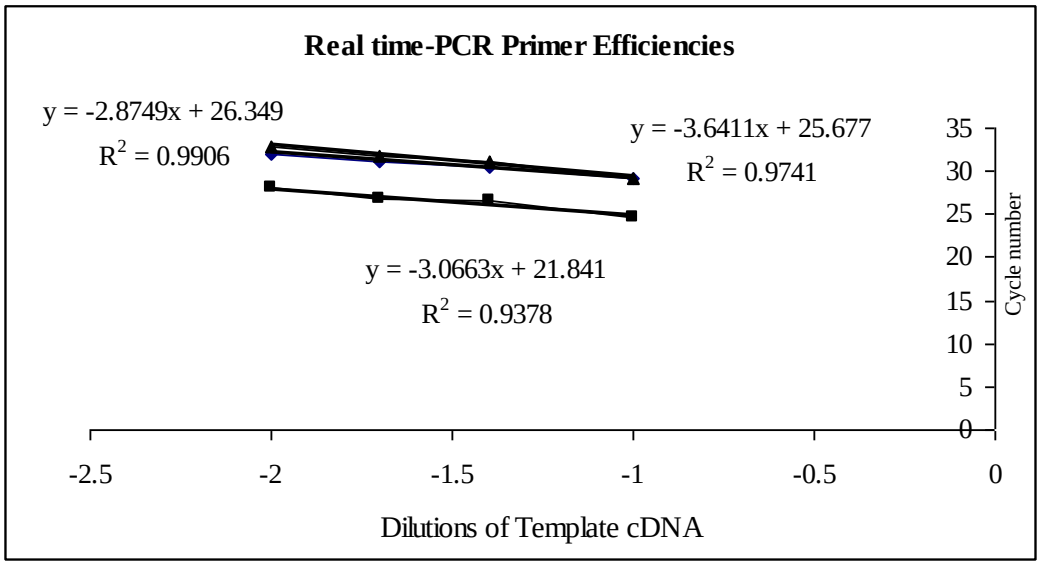


Figure 3.4 LC_{50} calculations for *CatL* and *GFP* dsRNA. Panel A. Dose to mortality ratio graph. Probits analysis of *CatL* titration calculated LC_{50} was 0.279 with 95% confidence intervals of 0.184-0.45 Panel B. Dose to mortality ratio graph. Probits analysis of *GFP* titration calculated LC_{50} was 0.294 with 95% confidence intervals of 0.228-0.371.

A)



B)

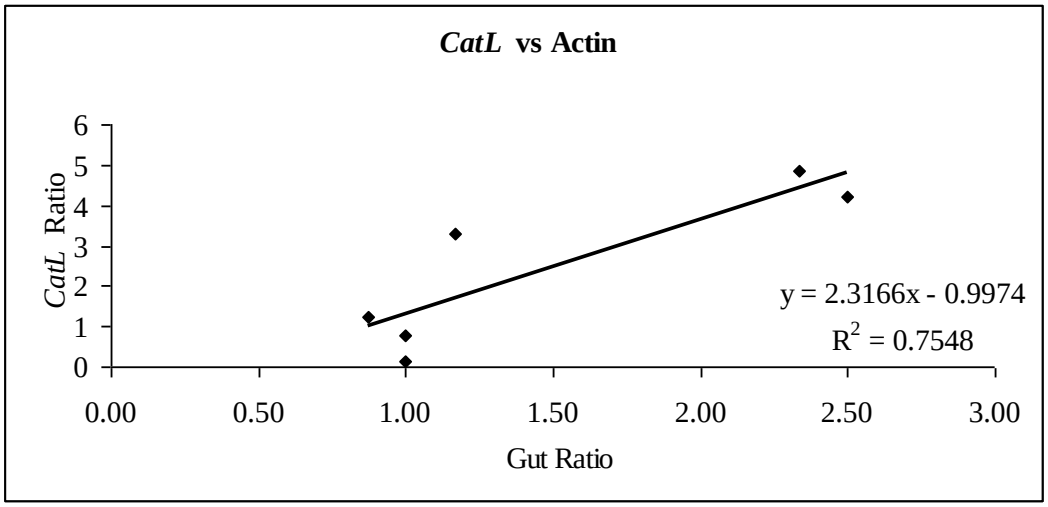


Figure 3.5 QRT-PCR analysis of *CatL*. Panel A. Graph of the dilution series used to determine the efficiency of the real time-PCR primers for *CatL*, elongation factor1-alpha (EF), and actin. All of the E values were between 1.7 and 2.2. Panel B. Correlation ratio of the number of guts extracted per replicate and the ratio of target gene *CatL* to housekeeping gene actin.

CHAPTER IV

TRANSGENIC ARABIDOPSIS PLANTS

4.1 INTRODUCTION

Arabidopsis plants have been utilized a model system to develop "proof of concept" for post-transcriptional gene silencing (PTGS) and to assess potential RNAi targets for pest control in crops. For example, Schwab et al. (2006) was able to create artificial miRNAs (amiRNAs) to target different endogenous mRNAs and determined that amiRNAs silencing was consistent with most natural plant miRNAs. Mutating the backbone sequences of endogenous Arabidopsis miRNA genes to target different sequences gave remarkable specificity in targeting transcripts. When amiRNAs were expressed under tissue-specific promoters the effect of the amiRNA was more pronounced than the same amiRNA under the control of a constitutive promoter (Schwab et al. 2006). Mao et al. (2007) made transgenic Arabidopsis and tobacco plants that expressed hairpin RNA directed against P450 monooxygenase CYPAE14 in cotton bollworms. When cotton bollworm larva fed on these plants they showed a dramatic decrease in tolerance to gossypol leading to larval growth retardation.

In other studies, Arabidopsis lines expressing dsRNA targeting the down regulation of two different aphid gene transcripts, Receptor of Activated Kinase C (Rack-1) (expressed in the gut) and MpC002 (expressed in the salivary glands), were examined (Pitino, et al. 2011). Inverted repeats of *MpC002*, *Rack-1*, and green florescent protein (GFP) were cloned into the pJawohl8-RNAi plasmid and expressed transiently via *Agrobacterium* infiltration in *Nicotiana benthamiana*. After 17 days of feeding on ds*MpC002* or ds*Rack-1* infiltrated *N. benthamiana* leaf disks the expression of *MpC002* and *Rack-1* were reduced by 30-40% on average compared to aphids fed dsGFP infiltrated *N. benthamiana* leaf disks. Plasmids from the *N. benthamiana* experiments were used to transform *Arabidopsis thaliana* plants. The F_3 generation of transformed plants was then subjected to aphid feeding. After 16 days of feeding transcript levels were determined using qRT-PCR. The target genes were down regulated by at least 50% compared to GFP controls.

This study was designed to generate transgenic Arabidopsis plants that express amiRNAs targeting an aphid gene to determine if aphid gene expression could be affected if the amiRNA is expressed in plants and introduced to the aphids while feeding on the transgenic plants.

4.2 MATERIALS AND METHODS

4.2.1 SELECTING TARGET GENE FRAGMENTS AND CONSTRUCTING AMIRNA PLASMIDS

Potential siRNA silencing fragments were selected by entering the *M. persicae* Cathepsin L gene sequence into siRNA finder

(http://www.ambion.com/techlib/misc/siRNA_finder.html). SiRNA finder is a free program offered by Ambion to help investigators dissect their target sequences into potential siRNA fragments. Candidate siRNA sequences were compared to the Arabidopsis genome using the basic local alignment search tool (BLAST) in The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org/). Potential siRNA fragment sequences that showed high sequence homology to important Arabidopsis genes were discarded. The remaining candidate siRNA sequences were then used to design and mimic the hairpin structure of the innate Arabidopsis miRNA precursor in the pRS300 plasmid (Schwab et al. 2006).

The pRS300 cloning vector contains the sequence encoding the native Arabidopsis MIR319a hairpin (Figure 4.1). Once the amiRNA sequence was determined, primers for mutating mature miRNA sequence were designed (Appendix 2). Maintaining gaps and bulges is important for RNAi machinery recognition and was carefully considered during primer design (Schwab et al. 2006).

Mutations in the pRS300 cloning vector were introduced by PCR. The template and primers were added to PCR Master Mix (Promega, catalog no. M7502), and PCR amplification performed as describe is Schwab et al. (2006). End point PCR was performed with Biorad DNA engine peltier thermo cycler. Primers started with high complementarity to the sequence being synthesized and lower complementarity in the middle where changes occurred to give gene specificity (Appendix 2).

PCR amplicons were cloned into pGEM T-easy and sequenced. Fragments of the correct sequence were enzymatically digested from pGEM T-easy, gel purified, and

ligated into the predigested CoYMV-GPTV and pBIB binary vectors. In both plasmids, the amiRNA fragment was inserted between the respective promoters and the nopaline synthase (NOS) transcriptional terminator (Figure 4.2). Binary plasmids were transformed into *Agrobacterium tumefaciens* GV3101 using electroporation. Cells were gently thawed on ice. Next, 40 µL of cell suspension was added to a prechilled Eppendorf tube, mixed with 2ng of DNA and incubated on ice 2 minutes. The mixture was transferred to a prechilled cuvette with a 2 mm gap. The cuvette was placed inside of the Eppendorf 2510 and pulsed at 1250 volts for 5 msec. Prechilled SOC medium (1 mL) was immediately added to the cuvette and used to resuspend the cells. The cells were then transferred to a sterile Eppendorf tubes and incubated at 28^{o}C for 1.5 hours in gently shaking tubes then pBIB was plated onto LB kanamycin (Kan) (50 µg/ml) selective medium and LB with no antibiotics while CoYMV-GPTV was plated onto LB ampicillin (Amp) (100 µg/ml) selective medium and LB with no antibiotics to incubate at 28^{o}C for 3 days. Selective medium plates were used to ensure colonies contained the inserted plasmid, while nonselective medium plates were used as a control to ensure cells were viable. Once colonies on selective medium plates were large enough to see, they were picked with a toothpick and grown in LB selective medium for floral dipping and infiltration experiments.

4.2.2 INFILTRATION ASSAY

Nicotiana benthamiana seeds were planted in ¾ potting soil mixed with ¼ vermiculite and placed inside of a growth chamber at 21^{o} C and 16:8 light:dark cycle. Plants were grown to approximately 4-6 weeks old before infiltration. A single colony of transformed *Agrobacterium tumefaciens* GV3101 was used to inoculate 1 mL LB

medium with antibiotic and grown overnight at 28°C. This overnight culture was then used to inoculate 10 mL of LB (pBIB in LB Kan and CoYMV-GPTV in LB Amp), 10mM MES and 20 µM acetosyringone and incubated 24 hours at 28° C. Cells were spun for 10 minutes at 5000 rpm and resuspended in 1 mL of infiltration medium (10 mM $MgCl_2$, 10 mM MES, 200 µM acetosyringone). 50 µL of resuspended cells was added to 950 µL of infiltration medium to measure OD_{600}. The target OD_{600} was 1.0 and dilution was modified accordingly. The ratio of cells to infiltration medium was then scaled up for the 150 mL volume needed to perform the experiment. *Agrobacteria* were incubated in the infiltration medium at room temperature for 2-3 hours. A 1 mL needleless syringe was used to infiltrate the *Agrobacteria*–infiltration solution into the leaf. The open end of the syringe was held against the abaxial side of the leaf, while the opposite hand supported the area to be infiltrated. The infiltration was completed by applying pressure to the syringe. The suspension going into the interstitial space between the cells made the leaf color appear darker. The boundaries of the infiltration in the leaves were marked and the infiltration was allowed to incubate for 3 days under normal growing conditions.

Tissue was collected by cutting out the marked area of the leaf. The leaf material was then placed in foil and immediately placed in liquid nitrogen. Total RNA was extracted using the TRIzol (Invitrogen, catalog no. 15596018) method.

4.2.3 RNA BLOT ANALYSES

The presence of processed amiRNA was tested using RNA gel blot and dot blot analyses. Twenty µg of total RNA was separated in a 15% polyacrylamide 8M urea gel (35 mL 15% acrylamide 8M urea solution + 250 µL 10% APS + 45 µL TEMED). Once

the gel was loaded with samples it was run at 225 V for 2.5 hours. Quality of total RNA was visualized by staining the gel with ethidium bromide and exposing to UV light. Samples were then transferred to Amerham Hybond-N+ membrane by running at 15 volts overnight. Membrane was UV cross linked using standard settings of Stratalinker (Stratagene UV Stratalinker 1800). The membrane was prehybridized with 5 mL PerfectHyb (Sigma-Aldrich, catalog no. H7033) for three hours in hybridization chamber at 37^0C.

Radio-labeled probes for the mature miRNA and U6 were made by incubating fresh P^{32} and the respective oligonucleotide with T4 kinase (New England BioLabs catalog # M0201S) at 37^0 C for 1 hour. Labeled probe was purified using G-25 resin column and activity determined by Geiger counter, pooling samples with highest activity. Purified probe (~200 µL) was denatured at 100^0C for 5 minutes quenched on ice for 5 minutes, added 5 mL of prehybridization buffer and allowed to hybridize at 37^0C overnight. Hybridization buffer containing the probe was decanted and the membrane was washed 3 times at 50^0C for 20 minutes with 20 mL wash buffer (2x SSC + 0.1% SDS). The hybridized membrane was wrapped with plastic wrap and exposed to a phosphoimager screen for 12 hours then viewed with a Typhoon scanner (GE Healthcare) (Appendix 2).

RNA dot blots were prepared by spotting 2 µg total RNA isolated from infiltrated *N. benthamiana* leaves on a 1 X 3 inch piece of Amerham Hybond-N+ membrane and 10 µM positive control DNA oligonucleotide of complementary sequence to the mature amiRNA (Appendix 2). The oligonucleotide was synthesized to serve as a positive hybridization control and spotted at the very end of the membrane as to not contaminate

other samples. The membrane was marked with a pencil to indicate where the RNA should be blotted. Once sample was placed on the membrane it was allowed to air dry. Membrane was then placed on blotting paper, presoaked with 2X SCC, with RNA blotted surface side up. The membrane was then UV cross-linked as described above, soaked in 2X SSC for 3 minutes, and transferred to the hybridization chamber for 2 hours with 5 mL of hybridization buffer at 37^0C. Hybridization solution was replaced after 2 hours with hybridization solution containing 200 µL of denatured probe, and allowed to hybridize to membrane over night at 37^0C. Hybridization buffer containing the probe was decanted and the membrane was washed 3 times at 50^0C for 20 minutes with 20 mL wash buffer (2XSSC + 0.1% SDS). The hybridized membrane was wrapped with plastic wrap and exposed to a phosphoimager screen for 12 hours then viewed with a Typhoon scanner (GE Healthcare).

4.2.4 LOW MOLECULAR WEIGHT RNA ENRICHMENT

Low molecular weight RNA was enriched from total RNA that was isolated from ~150 mg of infiltrated *N. benthamiana* leaves. The volume of the total RNA sample was increased to 2 mL with DEPC-treated water and added to 1mL of 25% PEG, 0.5 mL 5 M sodium chloride and 1.5 ml nuclease free water. The solution was incubated for 2 hours on ice and centrifuged for 30 minutes at 13,000g at 4^0C. The supernatant was collected and split between two tubes to which 0.875ml 3M NaOAc (pH 5.2) and 6.25 mL chilled absolute ethanol was added to each tube and incubated at -20^0C overnight. Precipitated low molecular weight RNA was collected by centrifugation at 4^0C for 30 minutes at 13,000g. The supernatant was discarded and the pellet was washed with 80% ethanol then centrifuged again at 4^0C for 10 minutes at 13,000g. Again the supernatant was

discarded and the pellet was air dried at room temperature for 10 minutes then resuspended in 50 µL of nuclease free water.

4.2.5 AMPLIFYING SMALL RNAS

Pulsed and stem-loop RT-PCR assays were completed as described in Varkonyi-Gasic et al. (2007). Briefly, dNTPs, nuclease-free water and stem-loop reverse transcription primer (final concentration 1 µM) are mixed then heated to 65^0C for 5 minutes, incubated on ice for 2 minutes, and centrifuged. To this solution, 5X first-Strand buffer, DTT, RNaseOUT and SuperScript II RT were added to create a master mix. One µL of template is added to 19 µLs of master mix and incubated at 16^0C for 30 minutes followed by pulsed reverse transcribed PCR [30^0C 30'> 42^0C 30'> 50^0C 1'] x 60 > 85^0C 5".

For real time PCR, 5× LightCycler FastStart SYBR Green I master mix (Roche Diagnostics) was combined with water, primers (1 µM final concentration) and 1 µL of template and replicated in triplicate. Samples were then subjected to standard RT-PCR amplification 95^0C 5"> [95^0C 5'>60^0C 10'>72^0C1'] x 40 (Applied Biosystems 7500). Data were analyzed using Pfaffl method (Pfaffl 2001).

4.2.6 ARABIDOPSIS TRANSFORMATION

Arabidopsis plants were grown until they produced stalks. The first stalk was clipped to promote proliferation of the inflorescences. Plants were ready to use when they had many immature flower clusters and a few siliques. *Agrobacterium tumefaciens* cultures containing the respective binary vectors were prepared by growing a 500 mL liquid culture at 28^oC in LB with antibiotics (pBIB in Kan and CoYMV-GPTV in Amp).

The bacteria were pelleted by centrifugation in a preparative rotor at 5000 RPM in a Beckman 2400 centrifuge for 10 minutes. The bacterial pellet was resuspended in freshly made 5% sucrose solution to an OD_{600} of ~0.8 and, silwet L-77 was added to a concentration of 0.05%. The aerial parts of plant were dipped in the *Agrobacterium* solution for 2-3 seconds gently agitating so that a film of bacterial suspension coated the plant. Dipped plants were placed under a plastic dome for 16-24 hours in high humidity and in low light. After 24 hours, the plants were watered and grown under normal conditions until siliques matured and dried. Seeds were harvested by cutting off the aerial portion of the plant, placing them inside an envelope, and dried at room temperature for 1 week. After drying, the seeds were collected and stored in open Eppendorf tubes with the lid open under a hood for 2-3 days. Tubes were shaken periodically to ensure seeds were dried completely.

Transgenic seeds were germinated *in vitro* on a solidified media containing selective antibiotics. Seeds were sterilized by soaking in 20% bleach solution for 10 minutes at 4^{o}C followed by five rinses with autoclaved distilled water. The sterilized seeds were plated in petri dishes containing MS macronutrient salts, 1% agar, and 50 µg/ml kanamycin for CoYMV-GPTV and 50 µg/mL hygromycin for pBIB. Plates were wrapped in aluminum foil and incubated at 4^{o}C for 2 days to stratify the seeds. After two days, the aluminum foil was removed and the plates were placed in a growth chamber with 16:8 light:dark cycles at 21^{o} C. Seedlings were allowed to grow until true leaves were prominent then transplanted to pots of presoaked soil, labeled, and return to normal growth conditions (23^{o}C 16:8 hours light:dark). Each plant represented an individual transformation event and was treated as an individual T_1 line.

T_1 plants were grown and self-fertilized, T_2 seeds were collected from mature plants. To confirm the T_2 transgenic plants, genomic DNA was isolated using Extract-N-Amp plant kit (Sigma-Aldrich catalog #XNAP2) following manufacturer's instructions. The presence of the T-DNA containing the amiRNA construct was confirmed by PCR using miRNA* Forward primer within the amiRNA and a reverse primer within the NOS terminator (Appendix 2). The insert was amplified from the genomic DNA by adding template and primers to PCR Master Mix (Promega, catalog no. M7502), and PCR amplification was performed following a standard program (94°C 5'> [94°C 15" > 50°C 30"> 68°C 40"] x 40 > 68°C 5') with Biorad DNA engine peltier thermo cycler. The PCR product was visualized in a 3% agarose gel. Seed from the positive transgenic plants was collected and preserved as described above. Seeds collected at the end of this step were the T_3 generation.

4.3 RESULTS AND DISCUSSION

The experimental approach in this part of the project is complex. The overall hypothesis proposes that an amiRNA designed to down-regulate an aphid gene that is expressed in the phloem of transgenic plants and consumed by a feeding aphid will indeed reduce expression of the targeted gene in the aphid. In choosing an aphid gene to target for down regulation, factors such as expression level, location of expression, and sequence homology to plant genes were considered. Selecting a gene expressed specifically in the gut of the aphid should give the highest chance of effective down-regulation as the amiRNA will not have to be transferred throughout the aphid. The epithelial cells in the gut are designed to readily absorb compounds from the diet and the

neutral pH of the gut provides some assurance the amiRNA will not be degraded before being taken up by the cells (Huvenne et al. 2010).

Cathepsin L was selected as a target gene in the aphid for amiRNA expressed in the phloem of transgenic Arabidopsis. Cathepsin L is a cysteine protease that is expressed in the epithelial layer in the gut of the aphid (Deraison et al. 2004) and was anticipated to have minimal sequence homology to *Arabidopsis thaliana* genes. However, plants contain numerous cysteine proteases some of which are abundant in the phloem sap (Hsien-Jung et al. 2010; Roberts et al. 2012). Candidate siRNA sequences for the *M. persicae* cathepsin L gene identified from the siRNA finder analysis were compared to the Arabidopsis genome and potential siRNA fragment sequences that showed high sequence homology to Arabidopsis genes were discarded. Out of the 100+ potential artificial miRNA sequences, only three were selected to construct the amiRNA. Several PCR primers were constructed to mutate innate miRNA sequence of the pRS300 plasmid to amiRNA sequence targeting the *cathepsin L* gene as in Schwab et al. (2006). The *cathepsin L* amiRNA was constructed in the pRS300 cloning vector. This vector contains the sequence encoding the native Arabidopsis MIR319a hairpin (Figure 4.1) and has been used by other investigators as a backbone on which to design and construct specific amiRNAs.

Two different promoters were employed to drive the expression of amiRNA constructs; the modified MAS super promoter and the Commelina Yellow Mottle Virus (CoYMV) promoter. The pBIB vector contains a MAS super promoter composed of a trimer of the octopine synthase (OCS)-upstream-activating sequence (UAS) to a mannopine synthase (MAS) promoter/activator region [*(OCS)*3*MAS*] to drive high levels

of constitutive transgene expression throughout the plant (Li et al. 2001; Ni et al. 1995). The CoYMV promoter cloned from pCoYMV89 is a phloem-specific promoter expressing in the phloem, phloem-associated cells, axial parenchyma of vegetative tissues, and flowers. The CoYMV promoter has at least 27% the activity of genes driven by the duplicated *(OCS)*3*MAS* promoter (Gittins et al. 2003; Medberry et al. 1992). Both promoters directed transgene expression to the phloem; however, "off target" effects that could impact plant growth and development are more likely to occur due to the high level of expression in other plant tissues that would be directed by the *(OCS)*3*MAS* promoter. The high level of amiRNA expression directed by the CoYMV promoter specifically to the phloem should decrease the opportunities for off target effects and allow accumulation of the amiRNA in the phloem sap affecting the aphids once they begin feeding on the phloem. The cathepsin L amiRNA was cloned between the respective promoters and a nopaline synthase terminator in binary vectors that were introduced into *Agrobacterium tumefaciens*. Despite being careful to avoid any sequence homology of the amiRNAs with Arabidopsis genes T3 generations did produce off-targets that were evident from their abnormal phenotype.

A transient expression assay was performed to confirm the functional integrity of the cathepsin L amiRNA construct. *N. benthamiana* leaves infiltrated with *Agrobacterium* harboring the pBIB-Cathepsin L amiRNA binary plasmid. Leaves were infiltrated then tissue was collected after two and three days of *Agrobacterium* incubation and total RNA was enriched for low molecular weight RNA. A very weak signal was detected for the mature amiRNA in RNA dot blots of low molecular weight enriched

RNA samples (Figure 4.3). This result suggests that *N. benthamiana* has processed the amiRNA from the pBIB-Cathepsin L amiRNA binary plasmid.

RNA gel blot analysis showed bands hybridizing to the *CatL* 1 probe (Lanes 2 & 3, Figure 4.4), from RNA isolated from two leaves that were infiltrated with *Agrobacterium* containing the amiRNA binary plasmid, but did not hybridize to RNA isolated from leaves infiltrated with infiltration media (IM) alone (Lane 1, Figure 4.3). The blot is difficult to interpret because although the positive control (Lane 8, Figure 4.3) showed abundant hybridization, both the blank lane and the negative control (Lanes 6 & 7, Figure 4.3) showed hybridization to the probe. Because these results were unclear qualitative real time PCR was performed using the stem-loop PCR protocol explained in Varonyi-Gasic et al. (2007) for detecting mature miRNA. Significant differences were not detected between samples infiltrated with *Agrobacterium* containing the amiRNA binary plasmid and IM only infiltrated samples enriched for low molecular weight RNAs (Table 4.1). There are two main explanations for not detecting the artificial miRNA with qRT-PCR. Either the infiltration experiment did not work or the plant was unable to process the artificial miRNA.

Transient expression assays are not always reliable, therefore transgenic Arabidopsis plants were generated for each of the promoter-cathepsin L amiRNA constructs. Selecting plant through the T_3 generation on selective media provided a very high probability that the plants had integrated the T-DNA from the binary plasmid into the Arabidopsis genome. Genomic PCR with insert-specific primers was conducted to confirm that plants were transformed. These primers amplified from the middle of the artificial miRNA precursor sequence through the NOS terminator. Figure 4.5 shows

amplicons from nine independent transformants. The amplicons were of the correct size and each contained the diagnostic *Kpn*I restriction enzyme site as verification that the correct sequence was amplified. All Arabidopsis lines that tested positive for the insert were grown and T4 seed collected for subsequent studies and aphid performance testing. Further tests on these lines will determine if mature miRNA is generated *in planta*, whether the mature miRNA are expressed in the phloem, and if there is an effect on the fitness of aphids feeding on the transgenic plants.

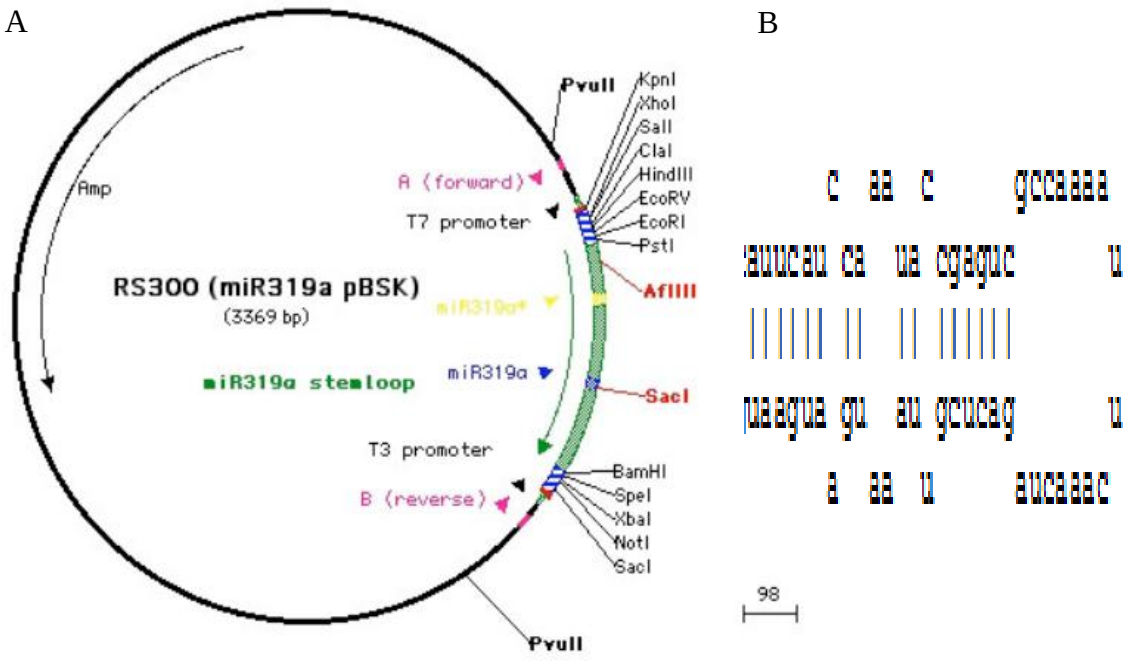


Figure 4.1 Construction of the *CatL* amiRNA. A) Plasmid map of pRS300. pRS300 is a plasmid containing an innate miRNA from *Arabidopsis thaliana*. This miRNA has been shown to efficiently express amiRNAs and provides excellent transformation results when compared to other Arabidopsis miRNA (Schwab et al. 2006) Panel B) The miR319a hairpin structure has a simple stem-loop fold with a few mismatches in the stem that were conserved when adding the mutations for cathepsin L targeting amiRNA.

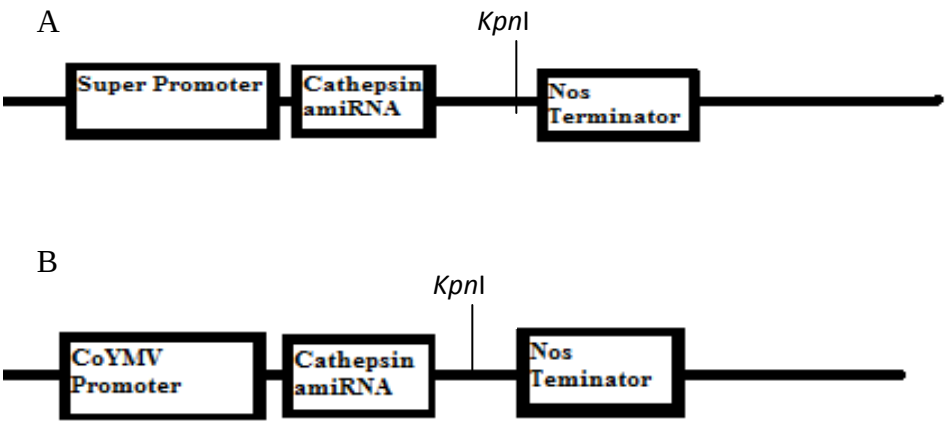


Figure 4.2 Maps of the amiRNA constructs. A) pBIB plasmid B) CoYMV-GPTV plasmid. A diagnostic *Kpn*1 restriction endonuclease site for each construct is shown.

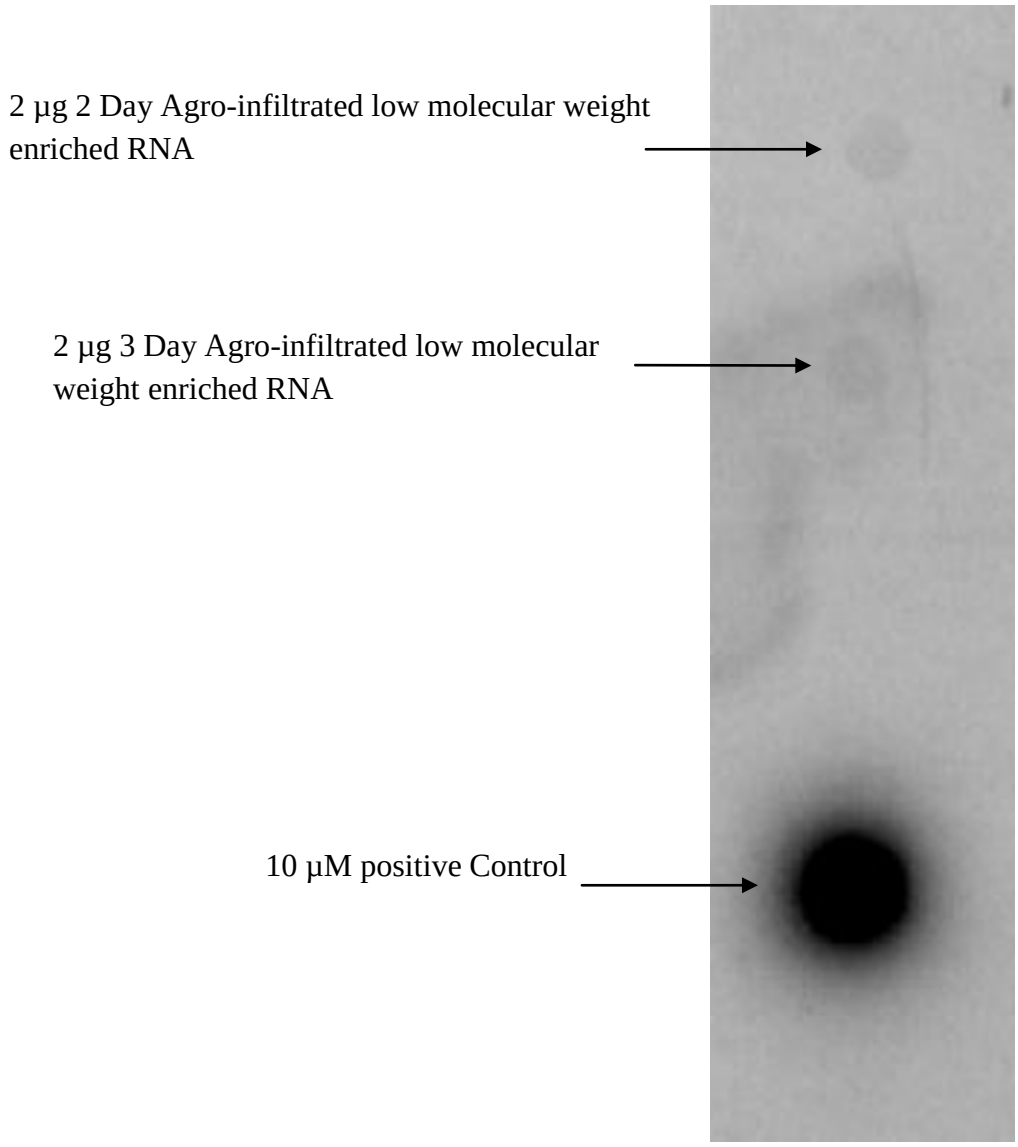


Figure 4.3 Dot blot analysis of *CatL* amiRNA. Autoradiogram of dot blot of low molecular weight RNA isolated from *N. benthamiana* leaves infiltrated with a plasmid designed to synthesize mature *CatL* artificial miRNA.

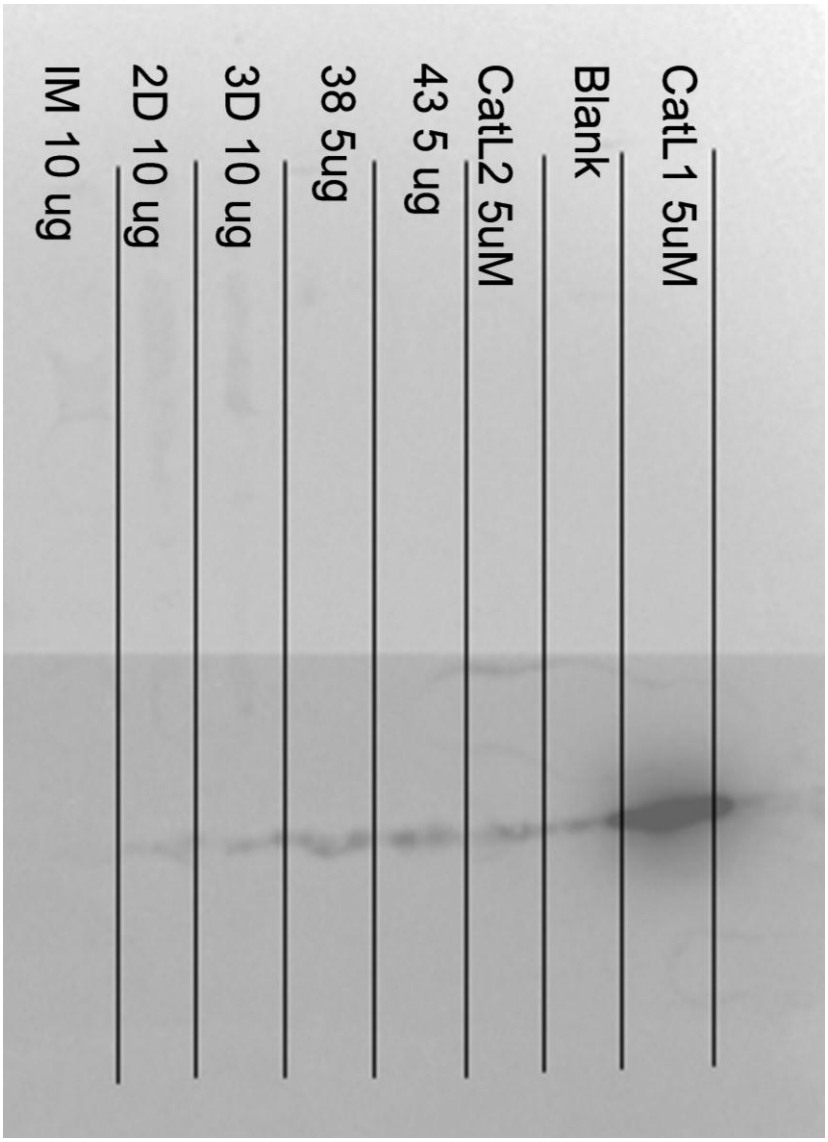


Figure 4.4 RNA blot from transient and stable transformations. Autoradiogram of RNA from infiltrated *N. benthamiana* leaves and two transgenic lines with amount of total RNA loaded indicated. From left to right: RNA extracted from *N. benthamiana* leaves infiltrated with only infiltration media, RNA extracted from *N. benthamiana* leaves that were allowed to incubate with *Agrobacterium* containing amiRNA for Cathepsin L for 2 days, incubated for 3 days, RNA from transgenic Arabidopsis plant line 38, Arabidopsis plant line 43, a negative control, blank lane and positive control.

Average CT values

	Cathepsin Ct	18s Ct	EF Ct
Control (no infiltration)	37.93873333	20.977	20.5733
pBIB promoter cathepsin infiltration	36.48365	22.6747	27.8922
CoYMV promoter cathepsin infiltration	38.2978	22.7617	26.63303

Table 4.1 Average Ct values of low molecular weight enriched RNA from *N. benthamiana* leaf tissue from infiltration studies.

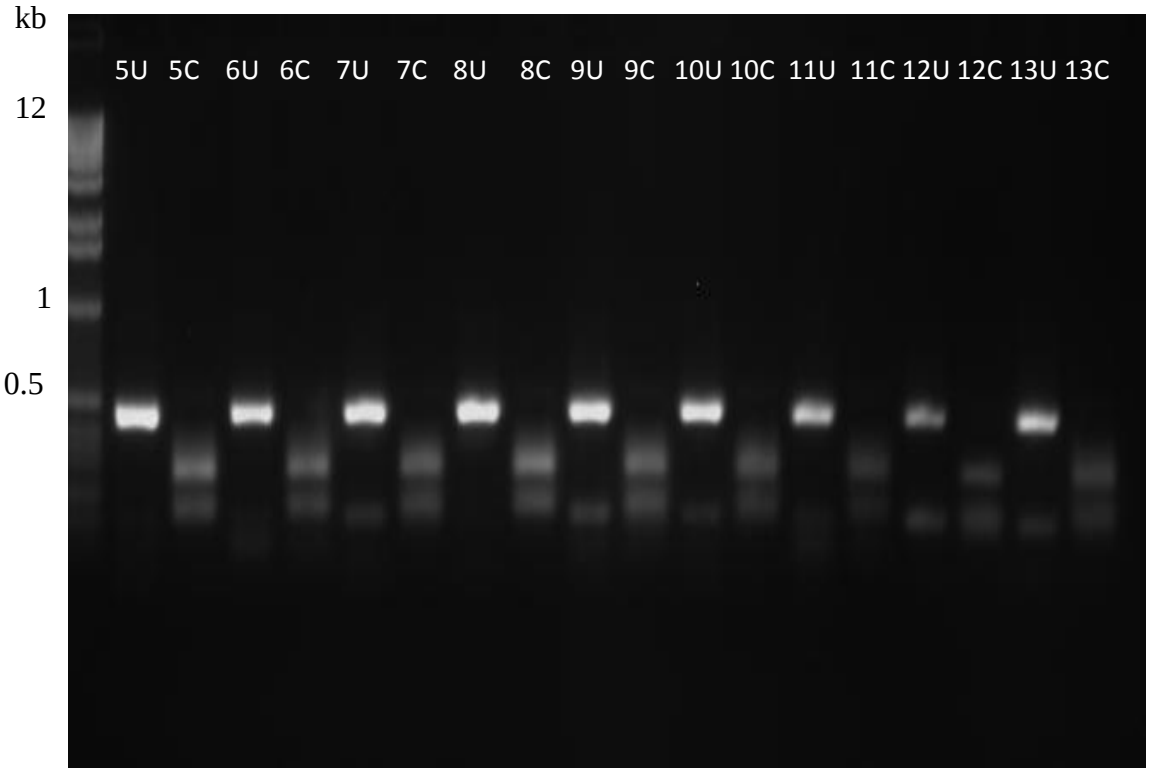


Figure 4.5 Confirmation of transgenic plants. Genomic DNA was obtained from each transgenic plant line and the insert sequence was amplified with specific primers. The amplified region was further verified by restriction digestion of a known restriction site in amplicon. Numbers at the top of each lane indicate transgenic line number. U= uncut C=cut enzymatically.

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APPENDICES

Appendix 1 Oligonucleotide primer sequences used for artificial diet experiments

Cloning and dsRNA Generation:

Cat L Gene Forward: 5'-ATGAAGGTAGTCATAGTTTTGGGATTGGTG-3'

Cat L Gene Reverse: 5'-TTAGACTAAAGGATAACTGGACTGGATGC-3'

Cat L forward: 5'-TAATACGACTCACTATAGGGatatgaagatgttaaagaa-3'

Cat L reverse: 5'-TAATACGACTCACTATAGGGctcaatgatactagcaca-3'

GFP forward: 5'-TAATACGACTCACTATAGGGccctcgtgaccaccct-3'

GFP reverse: 5'-TAATACGACTCACTATAGGG ttctcgttggggtctttgctc-3'

Primers for qRT-PCR for *M. persicae* Cathepsin L gene

Set 1: 5'-CATCAGCTCAGTTAGCTCGATA-3'

5'- CCAATGCATATGTTTCTTCGCCAG-3'

Set 2: 5'-TGTGGATCATGCTGGTCATTCAGTG-3'

5'-CCATAAGACCACCTTCACAACCATT-3'

Set 3: 5'-TGCTGTTGGTTTCCGGACTGACA-3'

5'-GGATAACTGGCACTGGAAGCAACA-3'

Primers for *M. persicae* housekeeping genes Actin and Elongation Factor:

MpActin Sense: 5'-CAAATCATGTTTGAAACCTTCA-3'

MpActin Antisense: 5'-AATGCATAACCTTCATAGATG-3'

MpEF-1a Sense: 5'-TTCGAAGAAATCAAGAAAGAAGTCAGCA-3'

MpEF-1a Antisense: 5'-TTTGAACCATGACATCTTTTCGGAA-3'

Appendix 2 Oligonucleotide primer sequences for amiRNA plasmids

amiRNA Mutation

miR*FOR: 5’-ttaggaatatatatgtagagaaacccaatactaagactacgacaggtcgtgatatgattca-3’

miR*REV: 5’-tcatatcacgacctgtcgtagtcttagtattgggtttctctacatatatattcctaaaacatc-3’

miR FOR: 5’-atcattgattctctttgaggtagtcatagttttgggattctctctttgtattccaattttc-3’

miR REV: 5’-aattggaatacaaaagagagaatcccaaaactatgactacctcaaagagaatcaatgatcca-3’

A: 5’-ctgcaaggcgattaagttgggtaac-3’

B: 5’-gcggataacaatttcacacaggaaacag-3’

Primers for Binary Vectors

CoYMV Forward: 5’-ggatccAAACCCAATACTAAGACTACGACAGGT-3’

CoYMV Reverse: 5’- ggtaccAATCCCAAAACTATGACTACCTCAA-3’

pBIB Forward : 5’-ggtaccAATCCCAAAACTATGACTACCTCAA-3’

pBIB Reverse: 5’-ggtaccAATCCCAAAACTATGACTACCTCAA-3’

Northern Probes

amiRNA Cat L 1: 5’-AATCCCAAAACTATGACTACC-3’

U6: 5’-TATGCGTGTCATCCTTGCGAG-3’

mature miRNA: 5’-GGTAGTCATAGTTTTGGGAT-3’

Verifying Transformed Arabidopsis

miR*Forward: 5’-ttaggaatatatatgtagagaaacccaatactaagactacgacaggtcgtgatatgattca-3’

NOS Reverse: 5’-GCGCGATAATTTATCCTAGTTTGCG-3’

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Scope and Method of Study: Aphids are economically important pest that have developed resistance to many known insecticides. In addition to the direct damage caused by aphid herbivory, aphids are vectors for pathogenic viruses. The goal of this study is to determine if small RNAs can play a role in plant resistance to aphids. RNAi has given rise to a potential new method of insect pest control for crop plants. To further develop understanding of how smRNAs may be used to control insect pests studies were designed to determine candidate genes for targeting using dsRNA in artificial diets and creating amiRNA expressing transgenic Arabidopsis plants.

Findings and Conclusions: Artificial diets demonstrated that Cathepsin dsRNA administered in complete artificial diet proved more toxic that GFP dsRNA. While transgenic Arabidopsis lines were not tested it was evident that the T-DNA containing the amiRNA was integrated into the plant genome. Further testing will be needed to validate the plants ability to process the amiRNA and effectively target the Cathepsin L gene of the aphid. Transient assays to determine *N. benthamiana*'s ability to process the amiRNA from *Agrobacterium* infiltration transient assays exhibited mixed results. Both RNA dot blot and RNA gel blot approaches to determining the plants processing ability showed some signs of a mature amiRNA; however, lanes that should have been negative showed hybridization. Also when testing infiltrated tissues with RT-PCR Ct values were much higher than those for housekeeping genes and again negative controls showed positive results.

ADVISER'S APPROVAL: Dr. Ramanjulu Sunkar