6-5-2018 9:00 AM

Evaluating plant root uptake of dsRNA for application in pest management

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Abstract

The greenhouse whitefly *Trialeurodes vaporariorum* is an agricultural pest that damages crops by feeding on plant sap and by vectoring plant viruses. Control of whiteflies has been managed through application of insecticides, but this strategy is not entirely effective and alternative control strategies are needed. In this thesis, I evaluated the efficacy of RNA interference as a means to control whiteflies on greenhouse-grown tomatoes. I found that root uptake of dsRNA synthesized from the *v-ATPase subunit A* gene caused significant gene knock-down and mortality in feeding whiteflies. This effect was, however, sensitive to the concentration of dsRNA delivery, and concentration was found to be negatively correlated with the plant's water content. In total, my results demonstrate the potential for gene knock-down technology in greenhouse pest management, particularly of tomato crops. I recommend that future work continue investigating plant management of dsRNA through this application to determine if this strategy can be effective.

Keywords

Trialeurodes vaporariorum, *Solanum lycopersicum*, RNA interference, dsRNA, greenhouse, pest management

Acknowledgments

I would like to start by thanking my supervisors, Dr. Ian Scott and Dr. Graham Thompson, for all their time, energy, and support. I would also like to thank my advisors, Dr. Anthony Percival-Smith, and Dr. Cam Donly for their help, guidance, and support during my project. Additionally, I would like to thank Dr. Donly and his team for welcoming me into their lab and allowing me access to their equipment and materials.

I am incredibly grateful for all the wonderful humans in my life who have been there for me during this project; I would like to thank Sophie Krolikowski, Lou Ann Verellen, Allan Humphrey, Grant Favell, Elizabeth Chen, Hanh Tran, Ren Na, and Arun Kumaran Anguraj Vadivel, and any other friends at Western and AAFC who have helped me, been there for me, or kept me company during those late night or early morning work days. In addition, I would like to extend my profound gratitude to Emine Kaplanoglu for all of her help, her friendship, and helping me stay positive. I greatly appreciate all the times you taught me new techniques and helped me troubleshoot problems. I would like to extend a large thank you to Jacqueline Menzies, Matthea Sanderson, and Geoff Sweatman for always being there and always willing to listen even after I had been a lab hermit for weeks. I would also like to thank Nicolle Latendresse for her friendship, for always making me laugh, and for helping me come up with clever and pun-filled titles for presentations.

Finally, I would like to thank my wonderful loving family for their constant support, understanding, encouragement, and perogies. I would like to especially thank my partner Samuel Schneider for his understanding, kindness, and love. Thank you for always trying to cheer me up when I'm down and being patient and kind. You are always there and have continually believed in me, even when I haven't believed in myself, and I am eternally grateful that you are a part of my life.

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List of Abbreviations

AAFC – Agriculture and Agri-food Canada

ANOVA – Analysis of variance

Bt – *Bacillus thuringiensis*

bp – Base pair

CAD – Canadian

Cry – Crystal

CTAB – Cetyl trimethyl ammonium bromide

cDNA – Complementary Deoxyribonucleic acid

CI – Confidence interval

DNA – Deoxyribonucleic acid

ddPCR – digital droplet PCR

dsRNA – double-stranded RNA

EF1a – Elongation factor 1-α

FAO – Food and Agriculture Organization of the United Nations

GLM – Generalized linear model

GLMM – Generalized linear mixed model

GMO – Genetically modified organism

GFP – Green Fluorescent protein

HRDC – Harrow Research and Development Centre

HSD – Honest significant difference

IHBT – Institute of Himalayan Bioresource Technology

IDT – Integrated DNA Technologies

IPM – Integrated pest management

LC_x – Lethal concentration required to kill x% of test subjects

LoRDC – London Research and Development Centre

mRNA – Messenger RNA

NTC – No template control

PCR – Polymerase chain reaction

qPCR – Quantitative PCR

RH – Relative humidity

RNA – Ribonucleic acid

RdRP – RNA-dependent RNA polymerase

RISC – RNA induced silencing complex

RNAi – RNA interference

rRNA – ribosomal RNA

siRNA – Small interfering RNA

SDS – Sodium dodecyl sulfate

SE – Standard error

SEM – Standard error of the mean

SID-1 – Systemic RNA interference defective

UBI – Ubiquitin

v-ATPaseA – *v-ATPase subunit A*

v:v – Volume:volume

w/v – Weight/volume

Chapter 1 Introduction

1.1 Greenhouse production and sap-feeding insects

Balancing environmental sustainability with the demands for global food security is a major mandate of modern agriculture. There is concern that with the overuse of water and loss of arable land, combined with growing agricultural pressure, the environment will plateau in its ability to sustain an expanding human population (Pimentel & Burgess, 2013). Agriculture is therefore both a solution to a demand for food, but also a problem, for agriculture itself contributes to the erosion, salinization, compaction, acidification, and chemical pollution of soil, and thus can contribute to the long-term degradation of farmable land (Food and Agriculture Organization of the United Nations [FAO], 2017). Conventional agriculture also demands heavy use of water, and farm expansion often transforms native habitats that naturally sustain biodiversity or that otherwise might buffer against pollution and climate change (Chakravarty et al., 2012).

Despite a steady increase in the global production of crops over the last fifty years (Figure 1.1), crop and agricultural success has not been consistent across regions, resulting in a reliance on food imports, and sometimes food aid, due to an inability to keep up with the population growth (Hazell & Wood, 2008; D'Odorico et al., 2014). Subsequently the availability of nutrient rich food in these regions is limited, and affected populations often suffer from malnutrition (Fita et al., 2015; FAO et al., 2017). For example, it is estimated 815 million people in the world are undernourished (FAO et al., 2017), and 150 million of those are children that are chronically undernourished (World Health Organization [WHO], 2017). In short, food insecurity is a global problem that is often linked to human conflict or to climate change, or both (FAO et al., 2017). As climate change can lead to drought or flooding, food insecurity may escalate, and thus exacerbate conflicts associated with primary productivity (Brown et al., 2007; Brown & Crawford, 2009; Burke et al., 2009).

Given the problems of land-use, water-use and with food availability, there is a clear need for solutions (Hazell & Wood, 2008; Godfray & Garnett, 2014). One major area of agricultural technology that may redress some of these issues is localized greenhouse

farming (Sardare et al., 2013). Unlike large-scale open agriculture, greenhouses can potentially operate under a smaller 'footprint' (Premanandh, 2011; Sardare et al., 2013), and with greater control of environmental variables that in turn can reduce exposure to pests, minimize the environmental variance associated with crop success, and more efficiently utilize water (Jensen, 1999; Czyzyk et al., 2014; Fiaz et al., 2016). Greenhouses and associated technologies can therefore potentially intensify farming to serve local demand (Bradley & Marulanda, 2001; Fita et al., 2015), including in regions not well-suited to open agriculture (Clark & Tillman, 2017; Benke & Tomkins, 2017).

The benefits of technology-assisted farming related to local demand and efficiency are, however, potentially offset by the distinctly un-natural ecologies that can arise within a greenhouse environment. For example, crops grown in greenhouses are more vulnerable to attack by insect pests. Insect pests, such as caterpillars (Lepidoptera), beetles (Coleoptera), and sap-feeding insects (Hemiptera), can thrive in greenhouses and be difficult to control once they become established (van Lenteren & Woets, 1988). Moreover, a primary invasion by pest insects can lead to secondary pathogens that are introduced and propagated by the insects, such as Fusarium wilt (Gillespie & Menzies, 1993), Lettuce mosaic virus (Fereres & Moreno, 2009) and Tomato spotted wilt virus (Fereres & Moreno, 2009), among others.

In the last 50 years, efforts to mitigate the impact of insect pests in greenhouses have heavily relied on the application of chemical insecticides (Zhang, 2003; Saravi & Shokrzadeh, 2011). However, intense and indiscriminate use of these chemicals over time has selected for insecticide-resistance (Heong, 2011; Maharaj, 2011), leading to pest insect populations that are ever more resistant to control. Additionally, improper use or application of insecticides in greenhouses can cause water and air contamination, thus exposing non-target organisms to these agents and allowing it to enter the food chain (Cox, 1993; Saravi & Shokrzadeh, 2011; Riche et al., 2017). Furthermore, broad-spectrum insecticides such as pyrethroids and neonicotinoids can be lethal to non-target insects, including pollinators such as bees (Apoidea), flies (Syrphidae) and wasps (Vespidae), and can decrease pollinator populations overall (Pimentel, et al. 2014). Resistant species are now known from at least eight different insect orders (Whalon et al.,

2008), including sap-feeding insects from the order Hemiptera such as aphids (Aphididae), whiteflies (Aleyrodidae) and leafhoppers or plant hoppers (Cicadellidae).

In addition to insecticide resistance, phloem-feeding insects are able to reproduce rapidly, and are able to vector plant pathogens (Karatolos et al., 2011; Koch et al., 2016). Moreover, the optimal conditions offered by greenhouses enable insects to develop rapidly and further increase reproduction, thereby increasing the pest population (Zhang, 2003; Bessin et al., 2007; Brissette et al., 2012). Due to their success in greenhouses, phloem-feeding insects are responsible for the majority of economic losses observed in the greenhouse industry (Ferguson & Murphy, 2002; Sudderth & Sudderth, 2013; Weintraub et al., 2017). For example, the estimated losses for greenhouse vegetables in 1996 were $115 900 (CAD) per hectare, and $50 000 (CAD) per hectare for a pepper crop in 1997. Likewise, a conservative estimate of losses in greenhouse ornamentals in 2002 was over $100 million per year (Ferguson & Murphy, 2002). Furthermore, the combination of plant pests and pathogens in some areas can result in total crop loss (Ferguson & Murphy, 2002).

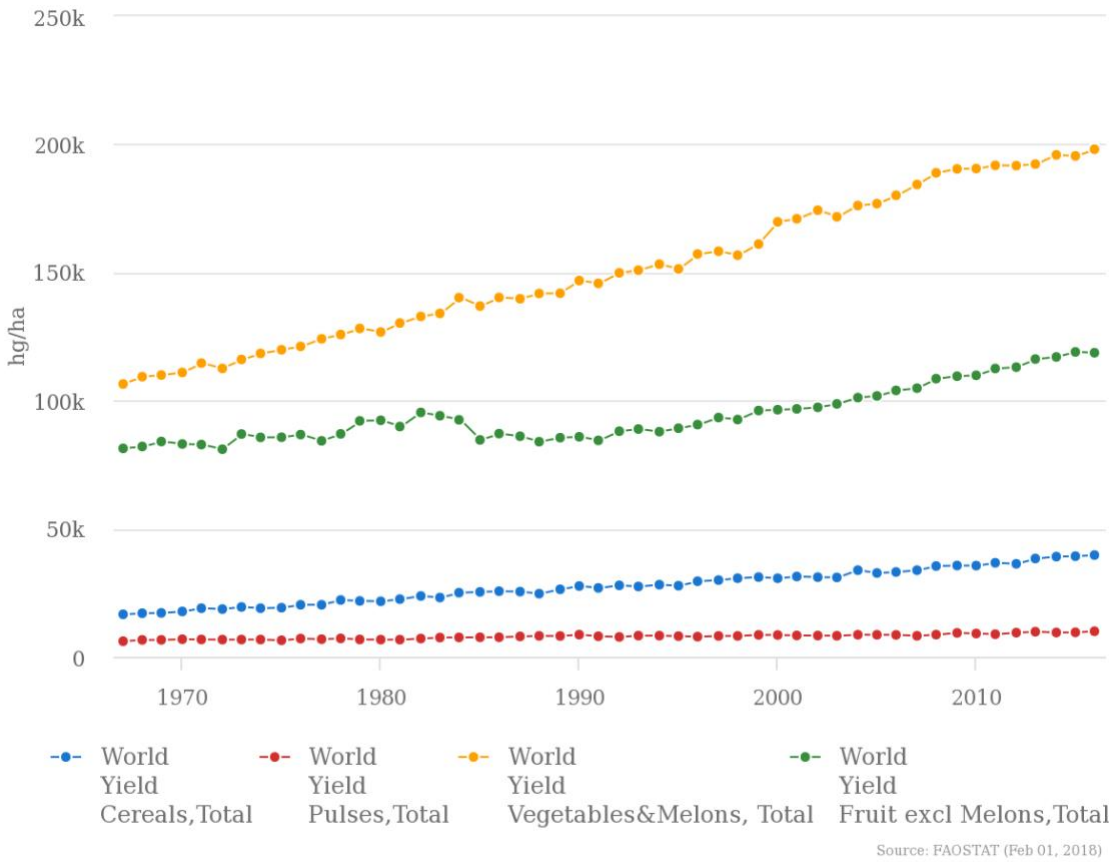

Figure 1. 1. World crop yield for cereals, pulses, vegetables, fruit, and melons from 1967 to 2016. All data taken from FAO-STAT (http://faostat3.fao.org/home/E). Figure produced using the FAO-STAT data comparison for production of crops (http://www.fao.org/faostat/en/#compare).

1.2 The greenhouse whitefly (*Trialeurodes vaporariorum*)

The greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), is so-named because of its association with the agricultural greenhouse environment (Capinera, 2008). Feeding by the whitefly can affect plant colouration, foliage set and overall plant growth and vitality (Ángeles-López et al., 2012). This single species of whitefly has a near worldwide distribution (Lourenção et al., 2008), is a vector of viral plant pathogens (Karatolos et al., 2011), and can promote the growth of black sooty mold (Antennulariellaceae, Capnodiaceae, Euantennariaceae and Metacapnodiaceae of Class Dothideomycetes; Chaetothyriaceae, Coccodiniaceae and Trichomeriaceae of Class Eurotiomycetes) (Chomnunti et al., 2014). Figure 1.2A shows how these secondary

infections reduce photosynthesis and crop quality (Jones 2003; Ferguson, 2014) (Figure 1.2A). In addition, the greenhouse whitefly has evolved resistance to the insect growth-inhibiting insecticides buprofezin (Gorman et al., 2002) and pyriproxyfen (Karatolos et al., 2012). The whitefly has also evolved resistance to the neonicotinoids class of insecticides (Karatolos et al., 2011), which mimic the action of the neurotransmitter acetylcholine (Goulson, 2013). By binding to the receptors, neurons are continuously stimulated and the receptors are blocked, resulting in insect paralysis and death (Goulson, 2013; Simon-Delso et al., 2015).

Figure 1. 2. Plant affected by whitefly infestation A) Sooty mold damage on *Nicotiana tabacum* (tobacco) caused by whitefly infestation **B)** Whitefly infestation on *Solanum lycopersicum* (tomato). White spots observed are individual whiteflies, with a large number visible on the underside of the leaf.

1.2.1 Biology and lifecycle of the greenhouse whitefly

An adult greenhouse whitefly is a small (1.5-2 mm long), winged insect from the order Hemiptera. As such, it is characterised by a piercing-sucking mouthpart, which whiteflies use to feed on phloem, and two pairs of wings. This species has a white powdery like appearance, and is diurnal. Figure 1.2B shows the underside of a leaf coated with over a hundred individual whiteflies; they prefer the underside of leaves and are often not spotted until the population is large. Female greenhouse whiteflies can reproduce without

mating and lay unfertilized eggs that develop into males (Roopa et al., 2012). Fertilized eggs, by contrast, will develop into females This mode of parthenogenic reproduction (arrhenotoky) allows individual female whiteflies to lay between 7-25 eggs per day depending on temperature and their host plant; at optimal temperatures, feeding on cucumber and tomato results in about 200 eggs overall (7 eggs/day x 30 days = 200 eggs/whitefly), while feeding on eggplant results in over 500 eggs overall (25 eggs/day x 30 days = 750 eggs/ whitefly) (Capinera, 2008; Reddy, 2016).

Figure 1.3 illustrates the major life stages of the greenhouse whitefly. The cigar-shaped eggs deposited on the underside of leaves by the greenhouse whitefly are initially whitish, and later turn dark brown to gray before they are ready to hatch (Stage 1). The first instar nymphs, referred to as 'crawlers', emerge five to ten days after eggs are laid, and resemble scale (Coccoidea) crawlers (Stage 2). During this stage, the crawler will search its immediate surroundings for a place to reside (Cranshaw & Shetlar, 2017). Once in place, the crawler will use its stylet (or piercing-sucking mouthpiece) to pierce into the leaf, and begin to feed. After about four days, the crawler will develop into a second and third instar nymph stage, where it becomes scale-like (Stage 3 and 4). These features allow the nymph to blend in well with the leaf underside. It continues to feed for about seven days. The last stage before emergence is the fourth instar 'pupae' (Stage 5 entering into pupae). This stage is an opaque yellow (Stage 6).

Finally, after about eleven days in the pupal stage, it will emerge as a winged adult (Stage 7) and is able to reproduce within 24 hours. The duration of the whitefly cycle varies with temperature, ranging from 18 days (at 30°C), to as long as 35 days (at 18°C) (Ferguson, 2014). Adult greenhouse whiteflies generally live between five to forty days post-emergence, but can survive for up to two months (Capinera, 2008). The thermal tolerance of this species is not well known, but it can survive year-round in areas with mild-winter climates (Lloyd, 1922; Capinera, 2008).

Figure 1. 3 Life cycle of *Trialeurodes vaporariorum*, the greenhouse whitefly. Regardless of fertilization, winged adult females lay pale green eggs on the underside of leaves, which turn brown as they mature and are ready to hatch **(1)**. The first instar is mobile, and will search its surroundings for a good place to settle and feed **(2)**, where it will move into its sessile second/third nymph instar stage **(3, 4)**. The second/third nymph instar continues to feed until reaching the pupal stage (fourth instar) **(5** entering pupal stage, **6)** after which it emerges as a winged adult **(7)**. If eggs are fertilized, the insect will be female and if the eggs are not fertilized, the insect will be male.

The greenhouse whitefly is a polyphagous herbivore, and will thus feed on, and damage, a broad range of plants including a variety of food and ornamental greenhouse crops (Russell, 1963). Despite its generalist-feeding behavior, the whitefly has shown general host preference for the following greenhouse crops: eggplant (Solanaceae), cucumber (Cucurbitaceae), gerbera (Asteraceae) and tomato (Solanaceae) (Lee et al., 2010). The

mechanisms by which whiteflies select hosts is based primarily on visual cues, with olfactory cues playing a relatively minor role (Vaishampayan et al., 1975). Colour in particular plays a critical role, with strong preference exhibited for yellow, green, red, orange, and purple (Van Lenteren et al., 1990).

1.2.2 Current greenhouse whitefly management strategies

Insect pest management over the past decade has largely focused on plant breeding or genetic technologies, or a combination of these techniques (Douglas, 2017). However, the application of these techniques mostly acts to mitigate the damage caused by leaf-chewing pest species. Phloem-feeding insects, like the greenhouse whitefly, require a different approach. Specifically, whitefly prevention presents some unique problems such that physical barriers to prevent whitefly entry into greenhouses, such as whitefly exclusion screens or sheets, are ineffective (Reddy, 2016); however, management strategies can be effective with early detection (Ferguson, 2014). This can be difficult as whiteflies are small and generally concealed under leaves (Mahr et al., 2012). Nonetheless, early detection can involve 'yellow sticky cards' that exploit the fly's normal motivations to home into yellow flowers, and traps them in place. When sticky traps are affixed throughout the greenhouse substructure they can serve as an early warning of an impending outbreak (Ferguson, 2014).

Alternatively, greenhouse growers can accept the presence of whiteflies and lure them to a decoy situated among the cash crops. The decoys can be crops themselves, which naturally exploit the visual cues of attraction of the whitefly. So-called 'trap crops' are typically host-preferred crops (i.e., eggplant), which can be paired with sticky traps, as a chemical-free pest management strategy; however, application of these chemical-free means has not shown a significant reduction on adult whitefly populations (Lee et al., 2010; Moreau et al., 2011). Biological control agents are another possible means of whitefly management. This strategy includes the application of: 1) predators, which feed on the whitefly at various stages; 2) parasitoids, which parasitize whitefly nymphal instars and 3) biopesticides, or entomopathogenic fungi, which invade the insect's body and release toxins. While whitefly predators, such as *Delphastus catalinae* (Le Conte) (Coleoptera: Coccinellidae) and *Delphastus pusillus* (Coleoptera: Coccinellidae), are able

consume large numbers of whiteflies at all stages, the most commonly used biocontrols are parasitoids, specifically *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae). Biopesticides, alternatively, are less frequently used due to the specific timing they must be applied at (Reddy, 2016).

E. formosa is a tiny parasitic wasp that can occur naturally in greenhouses within some regions or are available commercially for whitefly control (van Lenteren & Woets, 1988). Parasitic wasps can kill and affect whitefly populations in one of two ways: by laying their own eggs in the 3rd and 4th whitefly nymph instars and thus providing food for newly emerged *E. formosa*, killing the whitefly nymph (Reddy, 2016). Use of this agent in greenhouses can control small or fledgling populations of whitefly, but it is not well-suited for controlling heavy infestations (Bessin et al., 2007). As the whitefly population increases, control using *E. formosa* becomes more difficult until it can no longer impact the population (van Lenteren & Woets, 1988). Whitefly predators, *D. catalinae* and *D. pusillus*, on the other hand are more effective for large whitefly outbreaks and can ingest over 150 eggs or over 10 whitefly nymphs each day, and predator larvae can consume up to 1000 eggs (Reddy, 2016). Predators can also be deployed alongside *E. formosa* to further reduce whitefly populations (Ferguson, 2014). However, as temperatures decrease and in less humid conditions (< 70% humidity), *D. catalinae* and *D. pusillus* begin to slow down and become less effective, eventually succumbing to the cold (Simmons & Legaspi, 2004). Other agents, such as the entomopathogenic fungus *Beauveria bassiana*, can be slower acting and require close monitoring after application, as they are not necessarily host specific and can be lethal to pollinator insects (Ibrahim, 2015). *B. bassiana* additionally requires high humidity and specific timing of application to optimize effectiveness against the whitefly.

By combining a number of these whitefly control agents together, such as parasitoids and predators, and applying them when the insect is most susceptible, a successful integrated pest management (IPM) system can be developed. However, IPM requires close environmental and population monitoring for these to be successful (van Lenteren & Woets 1988; Simmons & Legaspi, 2004), and can be expensive overall (White, 2013).

1.3 Biotechnology in pest management

One of the longest continual practices in agriculture is plant breeding, which has been around for at least 10 000 years (Hallauer, 2011). One trait regularly under artificial selection is insect resistance (Maxwell, 1982). Selective breeding can, however, depend on the adaptability of pest insect populations, as genetic variability and sexual reproduction of insects can allow rapid resistance to plant defenses (Tripp, 2009). Furthermore, plant breeding is expensive, time-consuming (Luckett & Halloran, 2003), and can have significant consequences that affect crop yield or that increase plant susceptibility to insect pests not selected against (Tripp, 2009). By combining selective breeding with molecular techniques to genetically modify crops, precise changes to the genome can be made (Phillips, 2008). This specificity can hasten the development and production of insect resistant crops, limit yield and susceptibility trade-offs, and reduce insecticide use by controlling insect pests (Borlaug, 2000; Tripp, 2009; Douglas, 2017). Additionally, most crops can be genetically modified, including those with limited genetic variation due to breeding (Douglas, 2017).

One of the most recognized genetically modified organisms (GMOs) is *Bacillus thuringiensis* (Bt) maize, a hybrid corn designed through transgenic engineering to express the Bt protein. This protein synthesizes Crystal (Cry) toxins, which are toxic to a small range of related insects, particularly Lepidoptera larvae (caterpillars), some coleopterans, and a few dipterans. However, the mechanism through which Bt becomes toxic to hosts (Cry toxins become active under specific gut pH conditions; Hellmich, 2012; Palma et al., 2014) is not operational in all insects, and is particularly ineffective against hemipterans, including the greenhouse whitefly (Chougule & Bonning, 2012). Given the limitation of Bt transgenics, there is now a premium on technologies that are more widely adaptable, such as RNA interference, may be more suitable in selecting against the whitefly.

1.4 RNA interference

RNA interference (RNAi) is one technology that has become a focal point in agriculture. RNAi exploits the cellular mechanism for defense against viruses and transposable

elements (Obbard et al., 2009) of a living cell in order to suppress expression of a particular target gene (Fire et al., 1998). This mechanism is conserved and thus RNAi can be utilized in a broad range of organisms (Shabalina & Koonin, 2008). The RNAi pathway is often initialized by the introduction of double stranded RNA (dsRNA) into a cell where a dsRNA specific endonuclease (Dicer-2) cleaves it into smaller segments (21-24 bp). As Figure 1.3 shows, the small interfering RNAs (siRNAs) that result are assembled into an Argonaute-2 protein, to form the pre-RNA induced silencing complex (pre-RISC) (Kobayashi et al., 2016). During RISC maturation, two small RNA strands are separated within the complex: one is ejected and discarded from the complex, while the second is kept within the now-matured complex (Kobayashi et al., 2016). Using the remaining RNA strand as a guide strand, the RISC is able to bind to its endogenous complimentary messenger RNA (mRNA) and block its translation. In some cases, the blocked mRNA will begin to degrade, however; both the blocked mRNA and degraded mRNA prevent further expression of the target gene (Burand et al., 2013; Sheu-Gruttadauria et al., 2017). By targeting specific genes, we can disrupt and test gene function. RNAi therefore has widespread potential as a tool in applied agriculture and integrated pest management (Scott et al. 2013; Zamore 2001), including in a greenhouse setting (Thakur et al. 2014; Younis et al. 2014).

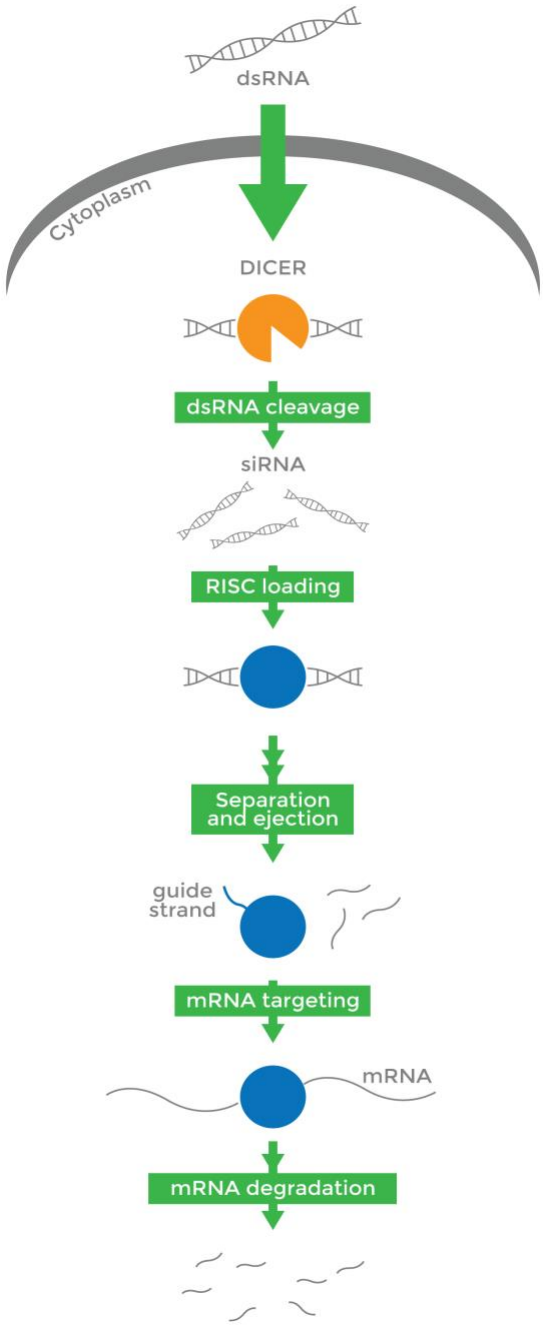

Figure 1. 4. RNA interference mechanism in eukaryotic cells. Once in the cell, double stranded RNA (dsRNA) is cleaved into small interfering RNA (siRNA) around 21-24 bp long. siRNA are then loaded into the RNA-induced silencing complex (RISC), where these pieces are unwound or separated; one of these strands is selected as the guide strand and the remaining pieces are ejected and discarded from the RISC. The guide strand is used to detect, locate, and bind RISC to corresponding mRNA, blocking translation and degrading mRNA.

1.4.1 dsRNA uptake in cells

Based on how dsRNA is introduced, the RNAi mechanism can be classified into two systems: cell-autonomous or non cell-autonomous (Whangbo and Hunger, 2008). Cell-autonomous RNAi restricts the mechanism effects, thereby occurring exclusively in cells that either express or have been directly exposed to dsRNA, whereas non cell-autonomous RNAi occurs when cells uptake exogenous dsRNA from their environment (environmental RNAi). In the latter case (environmental RNAi), the mechanism becomes systemic in multicellular organisms, and silencing signals derived from dsRNA are able to spread and transverse from cell to cell, away from the site of origin (Whangbo and Hunter, 2008). In order to become systemic, dsRNA should be constantly amplified or constantly acquired to maintain the response. However, RNA-dependent RNA polymerase (RdRP), which amplifies siRNA molecules used in the silencing signalling in plants, nematodes, and fungi, are absent in insects (Gordon and Waterhouse, 2007). The insect response should therefore be dependent on dsRNA quantity it intakes from its environment. Despite this, most insect species have displayed a strong systemic response to RNAi regardless of lacking homologs to RdRP. The systemic response observed in insects may be due to a currently unknown mechanism separate from those in nematodes (Huvenne and Smagghe, 2010; Mamta and Rajam, 2017). Alternatively, there are two dsRNA cell uptake mechanisms that may explain the systemic response and be related to the effectiveness of RNAi in insects: trans-membrane channel-mediated uptake and endocytosis-mediated uptake (Mamta and Rajam, 2017).

The trans-membrane channel protein is the best described mechanism involved in dsRNA uptake, and primarily uses the multi-transmembrane protein SID-1 (Systemic RNA interference Defective) (Winston et al., 2002). The SID-1 acts as a channel to passively transport dsRNA into cells, and has been observed in the nematode, *Caenorhabditis elegans* (Winston et al., 2002) and in most insect species (Katoch et al., 2013). However, presence of this protein is highly variable across insect orders. Sequence analysis in *Tribolium castaneum* (Coleoptera) has shown that *SID-1* like genes share more identity and may be orthologous to *C. elegans Tag-130* gene (not associated with systemic RNAi in nematodes) compared to *SID-1* (Tomoyasu et al., 2008). Likewise, *Aphis gossypii*

(Hemiptera) (Xu and Han, 2008), *Sitobion avenae* (Hemiptera) (Xu and Han, 2008), *Nilaparvata lugens* (Hemiptera) (Xu et al., 2013), *Leptinotarsa decemlineata* (Coleoptera) (Cappelle et al., 2016), *Bemisia tabaci* (Hemiptera) (Upadhyay et al., 2013) and *T. castaneum* (Tomoyasu et al., 2008) have exhibited dsRNA uptake using SID-1 like protein channels. Furthermore, while most insect orders have one *SID-1* like gene, beetles (Coleoptera) tend to have two to three (Tomoyasu et al., 2008; Cappelle et al., 2016), and Diptera lack *SID-1* genes and *SID-1* like genes altogether (Price and Gatehouse, 2008).

Although Diptera lack *SID-1* genes, they do have responsive RNAi machinery, and instead use the second dsRNA uptake mechanism: endocytosis-mediated uptake (Ulvila et al., 2006). Endocytosis uptake uses multiple scavenger-like pattern recognition receptors to take the silencing signal from the environment by receptor-mediated endocytosis (Ulvila et al., 2006; Saleh et al., 2006). Once this is achieved, the RNAi signal can be actively spread through a process involving vesicle-mediated intracellular trafficking (Saleh et al., 2006). Cappelle et al. (2016) showed that *L. decemlineata* (Coleoptera) uses both the SID-1 like proteins mechanism and the receptor-mediated endocytosis mechanism for dsRNA uptake. This illustrates the large variation between insect RNAi machinery, which can impact how RNAi needs to be applied and concentrations necessary to elicit a response.

1.4.2 dsRNA delivery methods in insects

There are presently three major dsRNA delivery methods: soaking, injection, and feeding. Soaking an organism in dsRNA solution, as for *C. elegans* (Tabara et al., 1998), is a simple procedure to deliver dsRNA into cells and trigger the knock-down response. Soaking is most often used with cell cultures. This process is less effective on insect cuticles that are less porous, which prevent dsRNA absorption during certain life stages (Katoch et al., 2013). Injection is another approach to dsRNA application and delivery to insects. Here, dsRNA is injected with a needle into target tissues or hemocoel (Fire et al., 1998). dsRNA delivery through injection additionally allows for specific concentration determination of dsRNA present in tissues. While injection is expensive, and methods can require time to optimize, it tends to be highly efficient in gene silencing, and is a

relatively popular method for laboratory testing, but it is ill suited for application in a pest management strategy (Zhang et al., 2013).

The most realistic method for application of RNAi in pest management, and the most popular method, is through oral delivery of dsRNA (Zhang et al., 2013). In addition to being less invasive and applicable to a number of insects at various life stages, oral delivery of dsRNA is versatile in its delivery method: it can be mixed and delivered within artificial diet (Whyard et al., 2009; Asokan et al., 2015), delivered in droplet form (Turner et al., 2006), delivered through leaflets either by soaking or taking up dsRNA through capillary action (Surakasi et al., 2011; Luan, et al., 2013), it can be sprayed onto leaves (Miguel and Scott, 2015), taken up by roots (Hunter et al., 2012; Li et al., 2015), or plants can be genetically modified to produce dsRNAs internally that are specific to pest insects (Zhang et al., 2015; Malik et al., 2016). However, despite its wide number of applications, oral delivery of dsRNA is less effective in inducing RNAi, and is not suitable for all species, with notable difficulties found in lepidopteran pests (Terenius et al., 2011), as was observed in *Heliothis virescens* (Shukla et al., 2016). In addition, it is difficult to establish how much dsRNA is accessible to and ingested by the insect during feeding (Surakasi et al., 2011). Furthermore, while plant production and delivery of dsRNA to insects appears to be an ideal application for pest management, it is even less effective than oral delivery itself, further limiting the number of insect species it can affect. This is due to the plants own RNAi pathway causing dsRNA to be processed within the plant into siRNA; siRNA are refractory to insect cell uptake in comparison to dsRNA (Bolognesi et al., 2012; Zhang et al., 2017). As well, plant production of siRNAs can cause off-target effects in non-target insects, silencing and affecting various genes in other insects as a result of possible sequence similarity (Lundgren and Duan, 2013).

1.4.3 Target gene selection

Selection of target gene to be silenced has a considerable impact on the RNAi effects. For example, Terenius et al. (2011) reviewed 130 possible gene targets for RNAi in Lepidoptera, but found only 38% of these were silenced at high levels, 14% at low levels, and 48% of genes failed to be silenced altogether; of these they noted immune-related genes are more sensitive to systemic RNAi, whereas genes from the protein binding

group are non-susceptible. In addition, the intended effect on the organism dictates target gene selection, as resulting effects of gene silencing can vary and have an impact on physiological, developmental, behavioral, or reproductive processes (Abdurakhmonov et al., 2016). Previously observed RNAi targets in insect pest management have ranged from genes related to detoxification of chemicals (such as insecticides) to essential genes that are critical for organism survival, and are chosen by desired outcome. Kaplanoglu et al. (2017) demonstrated that silencing *CYP4Q3* and *UGT2*, genes that code for targets important in detoxification in insecticide resistant *Leptinotarsa decemlineata* resulted in a significant increase of the beetles' susceptibility to the insecticide imidacloprid. By applying this two-step system (RNAi knock-down and then chemical insecticide), previously resistant insects can once more be made susceptible and, thus, killed. Alternatively, Thakur et al. (2014) transformed a tobacco plant to produce dsRNA for *Bemisia tabaci*, the sweet potato whitefly, that targets the gene *v-ATPase subunit A* (*v-ATPaseA)*, a subunit that acts as a catalytic site and is important in regulation within the v-ATPase complex, which is responsible for ATP hydrolysis. When exposed to the transgenic tobacco plant, the whiteflies were able to ingest ds-*v-ATPaseA* and gene silencing was significant within two days of feeding, and a significant increase in mortality was observed within five days of feeding (Thakur et al., 2014).

1.5 Research rationale and objective

The potential for application of RNAi in pest management as an alternative to insecticides, or as a tool to overcome insecticide resistance, is largely recognized in pest management research (Rodrigues & Figueira, 2016; Mamta & Rajam, 2017). Due to the sequence design capabilities, RNAi can be as broad (affecting all insects within a family) or as tailored (to a specific insect species or genotype) as desired. However, despite RNAi target and species specificity, the general public's attitude towards GMOs (specifically GMO crops) is reluctant and disapproving, with more frequent and larger movements against GMOs (Rohlinger & Gaulden, 2017; Rutjens et al., 2018). This results in a need for better communication with the public but also alternative methods of delivery and application of RNAi. Past studies have shown the potential for dsRNA uptake through plant roots as a means of delivery to sap-feeding insects (Hunter et al.,

2012; Li et al., 2015). There is, however, little information on the behaviour of the dsRNA molecule once it is inside the plant's cellular system. We therefore do not yet know how dsRNAs are sequestered and concentrated, if at all, throughout plants over time.

The goal of my thesis is two-fold. First, I seek to evaluate root uptake of dsRNA as one step towards a future RNAi-mediated mechanism for whitefly control, and second, to investigate the molecular 'behaviour' (its distribution within the plant, its concentration over-time, etc.) of dsRNA in aeroponically grown tomatoes. I achieved these two goals as follows: First, I evaluated the effects of different concentrations of *v-ATPaseA* dsRNA on the mortality of the greenhouse whitefly using a tomato leaflet delivery assay. In this experiment, my goal was to first measure dsRNA quantities in leaflets to determine the concentration of dsRNA putatively available for insect ingestion. Second, I estimated the lowest concentration necessary for significant whitefly mortality- i.e., the lowest lethal concentration. Finally, I evaluated whether submerging aeroponically grown *Solanum lycopersicum* L. cv. Micro-Tom roots in a *v-ATPaseA* dsRNA and water solution was an effective delivery mechanism, as measured by whitefly gene expression and mortality. During these experiments I measured *v-ATPaseA* dsRNA at three different plant heights (bottom, middle, and top), and conducted a second assay to measure dsRNA for this target over time.

Chapter 2 Materials and Methods

2.1 Tomato cultures

I obtained ~20 *Solanum lycopersicum* L. cv. Micro-Tom seeds from the London Research and Development Centre (LoRDC), Agriculture and Agri-food Canada (AAFC), London, Ontario. To surface sterilize seeds, I washed them in 70% (v:v) ethanol (1 min), rinsed them with distilled water (5 min), and then washed them again in 25% (v:v) bleach (30 min). To rinse, I washed the seeds in distilled water (15 min) and washed again (10 min) before air drying on a paper towel. Finally, I transferred a subset of seeds for immediate use into magenta jars (77 mm x 77 mm x 97 mm; W x L x H). I stored the remainder in centrifuge tubes (15 mL) at 4°C.

To germinate, I first transferred nine seeds into magenta boxes that contained 0.2% (w/v) Murashige and Skoog basal salt (Phyto Technology Laboratories, St. Lenexa, KS, USA) agar medium (supplemented with 3% (w/v) sucrose). To simulate germination I wrapped the magenta boxes in tin foil for five days and kept them at 22 ±1°C with a 16:8 h L:D photoperiod. After approximately 28-30 days, I transplanted the tomatoes into the Rainforest 318 aeroponic system (General Hydroponics, Santa Rosa, CA), which was kept in a growth cabinet at 24 ± 1°C for 16 h light and 20± 1°C for 8 h dark, and 60 ± 5% relative humidity (RH), and new plantlets were covered with clear plastic cups for 11 days to retain moisture as they developed a cuticle. From ripened tomatoes I harvested seeds and placed them into 10% (v:v) hydrochloric (HCl) acid (30 min) to remove the gel coating, and then rinsed them in water; this allowed me to select and grow Micro-Tom's that germinated faster and continually use those lines in experiments.

2.2 Whitefly cultures

I collected adult whiteflies from a continuous greenhouse colony maintained on tomato, *Solanum lycopersicum*, and tobacco, *Nicotiana tabacum*, at the Harrow Research and Development Centre, AAFC, Harrow, Ontario. At LoRDC, I acclimated whiteflies by transferring them to a Bug Dorm (BioQuip, Rancho Dominguez, CA, USA) containing a

tomato cutting in a vial of water, and held the insects in a cabinet (24 ± 1°C, 60 ± 5% RH) with a 16: 8 h L:D photoperiod for 24 h.

2.3 Primer design and *in vitro* transcription of dsRNA

I designed primers and probes (Table 2.1) with different parameters; while GC content (30-80%) and aversion to primer dimers (complements of no more than 4) were consistent among primers and probes, oligo length and melting temperature differed. During oligo design, I used Biosearch Tech on-line tool available at biosearchtech.com/support/tools/design-software and IDT Primer Quest, found at idtdna.com/Primerquest/Home/Index. The Operon oligo analysis tool, found at operon.com/tools/oligoanalysis-tool.aspx, was used to further evaluate and determine if primers/probes met parameters.

I amplified DNA template from synthesized gBlock sequences (linear, double-stranded nucleic acids) (Integrated DNA Technologies [IDT], Coralville, IA) for the essential whitefly gene *vATPaseA* (Thakur et al., 2014) and a GFP segment derived from GFP::L4440 plasmid (plasmid # 11335, Addgene, Cambridge, MA, USA). For this procedure, I followed the IDT gBlock amplification reaction mixture protocol, and used the PCR settings recommended by the Drosophila RNAi Screening Centre (DRSC; https://fgr.hms.harvard.edu/dsrna-synthesis; Kulkarni et al., 2006). To purify the template, I used a PCR purification kit (Qiagen, Hilden, Germany).

To synthesize single-stranded RNA I had T7 promoters attached to the 5' and 3' end of my template primers and used the T7 MEGAscript™ kit (Ambion, Huntingdon, UK) for *in-vitro* transcription. For synthesis of single-stranded RNA, I followed the manufacturer's protocol. To convert ssRNA into dsRNA suitable for my experiments, I annealed ssRNAs together by incubating at 75°C (5 min) before cooling to room temperature. To remove DNA template and purify dsRNAs, I followed the dsRNA Production by PCR Purification of RNA/dsRNA protocol of Kafatos (dsRNA Production by PCR; https://openwetware.org/wiki/Kafatos:dsRNA_Production_by_PCR).

2.4 Leaflet bioassays

I exposed whiteflies to ds-*v-ATPaseA* and dsGFP [negative control] at initial concentrations of 5, 3, and 1 µg/mL using leaf-mediated feeding, as described in Luan, et al. (2013). First, in order to eliminate bacteria and mold, I washed mature tomato leaflets in 2% (v:v) bleach solution (10 min), followed by a distilled water rinse (15 min). Second, one day (24 h) before introducing whiteflies to the feeding chambers, I placed cut ends of leaflet petioles into small Petri dishes (35 mm x 10 mm) that contained 4 mL of dsRNA solution (or water). Figure 2.1A shows the sterilized tomato leaflet after it's been placed in dsRNA or water.

After the leaflets were exposed to the dsRNA (or water) for 24 h, I gently transferred adult whiteflies into the leaflet feeding system. I followed Polston & Capobianco's (2013) recommendation for whitefly transfer to avoid static or damage to delicate whiteflies. As such, I gently tapped whiteflies from leaves or from the BugDorm into vials modified with soft mesh glued to the bottom. I then flipped vials onto each leaflet to form the feeding system. In order to isolate the feeding system, I fastened vials to the Parafilm covering each Petri dishes. Figure 2.1B shows how these feeding chambers appeared after whiteflies were introduced. Feeding systems were kept in growth cabinets set at 24 ± 1°C, 60 ± 5% RH, and 16: 8 h L:D photoperiod. I allowed whiteflies to feed for seven days, and estimated mortality by counting the number of dead whiteflies. In order to prevent leaflets from drying out, I injected water into the system through the Parafilm with a 27-gauge needle and a 10 mL syringe (Becton Dickinson, Franklin Lakes, NJ, USA).

To observe whitefly ingestion of dsRNA, I added food coloring (Club House Brand, London, ON, Canada) to treatments at a 0.4% (v:v) dilution (Wuriyanghan, et al., 2011). I assigned red to the *v-ATPaseA* treatment and green to the GFP treatment. I used water (no dye) as an experimental control. After whitefly mortality caused by dsRNA was confirmed, I established a dose-response curve by gradually decreasing the dsRNA concentration below 1 µg/mL- namely, 0.5 µg/mL, 0.2 µg/mL, 0.1 µg/mL, 0.05 µg/mL, 0.01 µg/mL, and 0 (water). Additionally, I examined leaflet concentrations of dsRNA

starting at 3 and 1 µg/mL. Here, I followed the same concentrations used for the dose-response leaflet assay.

I replicated initial concentrations (1, 3, and 5 µg/mL) of dsRNA treatments and control, as well as subsequent dose-response assays, four times for each concentration. I replicated leaflet concentrations, by contrast, only three times for each concentration. After counting the dead whiteflies on day seven, I froze the feeding chambers in a -20°C freezer for 24 h and counted the total number of adult whiteflies for each replicate.

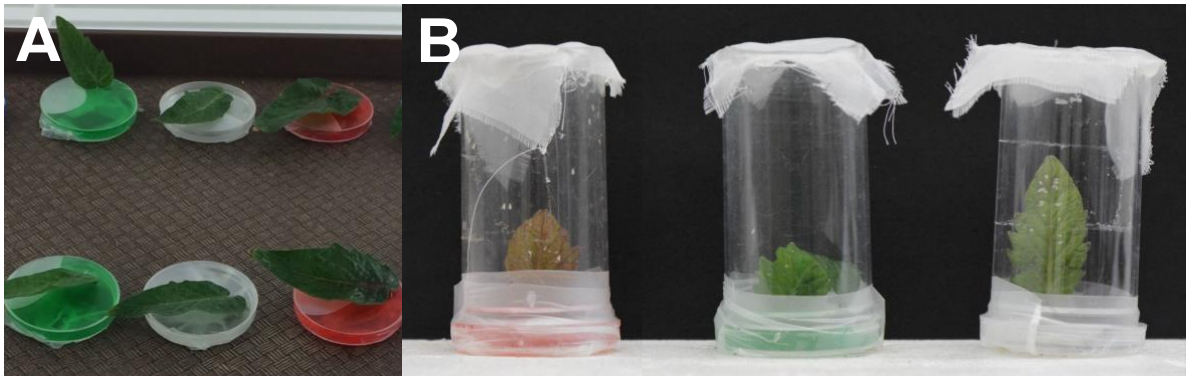

Figure 2. 1. Leaflet assay set-up. A) dsRNA solutions containing food dye and water for tomato leaflets to take up. **B)** dsRNA leaflet assay feeding system for the greenhouse whitefly (*Trialeurodes vaporariorum*). Green fluorescent protein dsRNA was green, *v-ATPaseA* dsRNA was red, and water remained uncolored.

2.5 *In planta* lethality assays and dsRNA consistency assays

I conducted *in planta* bioassays with whole tomato plants to test the lethality of 2 µg/mL of *v-ATPaseA* dsRNA taken up by the roots into plant tissues. I chose this concentration based on calculations that took into account the solute (dsRNA) distribution and plant size, and by scaling down the experimental design of Hunter et al. (2012) from a one metre tall tree to a ~11 cm tomato plant and increased transpiration rates expected in the absence of bark. In addition, I further adjusted dsRNA concentrations to account for 10% of the dsRNA moving into the mesophyll (Dr. W. Hunter, USDA, personal communication, 2016). With these modifications, I began my lethality and dsRNA consistency assays by placing flowering tomato plants into beakers containing 100 mL of 2 µg/mL ds-*v-ATPaseA*, as shown in Figure 2.2A. I set-up both assays 24 h before

placing plants into 30 cm x 30 cm x 30 cm polypropylene Bug Dorms, and the lethality assays also included a water treatment as a control. In both assays, I kept Micro-Toms and insects in growth cabinets (24 ± 1°C, 60 ± 5% RH, and 16 L: 8 h L:D).

The insect exposure in the lethality assays began when I released adult whiteflies from a plastic bag into each cage (Figure 2.2B and C). I left the bags for 24 h to ensure whiteflies migrated to plants, and then removed the bags from cages to avoid bacteria and mold development from dead whiteflies and wilted leaves from travel. I counted and removed whitefly cadavers each day for six days and collected live whitefly samples on day 6 to examine down-regulation of *v-ATPaseA*. I replicated each treatment three times and again, after post-count on day 6, cages were frozen and I counted the total number of adult whiteflies. To ensure accurate mortality counts, I secured red felt over the tops of the tomato pots. I lined the bottom of each dorm with black landscape fabric to ensure whitefly cadavers could be observed and removed.

To measure dsRNA concentrations throughout the plant, I collected 100 mg of leaf tissue from lethality assay plants on day 6 at three different heights (apex, middle, and bottom) of each plant. After removing all whiteflies from plants, I measured the height and length of the shoots and roots respectively, and the approximate diameter of the stem. As well, I weighed plants before and after I dried them at about 80°C for two days and calculated water content of each plant (fresh mass-dry mass/fresh mass). I calculated distribution of roots and shoots was also calculated and summed for each plant (wet mass/approx. plant volume), and was used, with water content, to calculate approximate water distribution for each plant (distribution of roots+shoots*plant water content).

For evaluation of dsRNA *in planta* over time, I conducted an exploratory assay using two plants. Here, I collected 100 mg of leaf tissue from the middle of each plant each day for six days, starting before dsRNA exposure. I replicated this assay using three new plants.

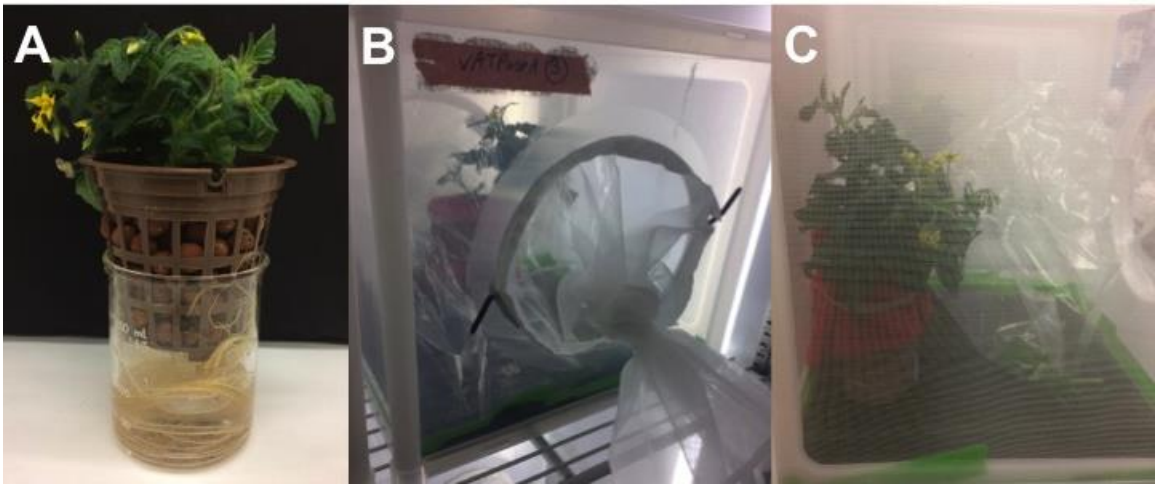

Figure 2. 2. *In planta* assays used aeroponically grown Micro-Tom tomatoes by exposing their roots to a *v-ATPase subunit A* dsRNA solution or water. A) Micro-Tom in aeroponic pot in a beaker filled with 75 mL of 2 µg/mL dsRNA. This set-up for root uptake of dsRNA was used for all *in planta* assays. **B)** *In planta* bioassay with whiteflies in a BugDorm; and **C)** *In planta* bioassay in BugDorm illustrating bag used to release whiteflies into the assay.

2.6 RNA extraction and cDNA synthesis

I extracted total RNA from 20 whiteflies per *in planta* cage using the RNeasy Mini Kit (Qiagen). I removed any residual genomic DNA using a Turbo DNA-free kit (Invitrogen). To generate whitefly cDNA, I used 0.625 µg of RNA with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Superscript III; Invitrogen) following the manufacturer's protocol. Due to the high polyphenols and polysaccharides content present in Micro-Toms, which bind to nucleic acids and contaminate RNA extractions respectively (Chan et al., 2007; Dash 2013), I needed to assess RNA extraction protocols. To extract tomato leaf tissue RNA, I assessed several RNA extraction methods including: RNeasy Plant Mini Kit (Qiagen), Complete DNA and RNA Purification Kit (MasterPure, Epicentre, Windsor, ON, CA), RNA isolation for secondary metabolite rich plants (Institute of Himalayan Bioresource Technology, Himachal Pradesh, India [IHBT], Ghawana et al., 2011), CTAB based extraction (original Li et al., 2014, modified by Cui et al., 2014), and a modified SDS acid phenol method, as well as a further modified version of this protocol (Hou et al. 2011; Deepa et

al., 2014). RNA integrity was evaluated using gel electrophoresis from these protocols and select methods (CTAB and further modified SDS acid-phenol) were further evaluated for extraction capability of dsRNA.

Based on its ability to extract dsRNA, I chose the Deepa et al., (2014) modified SDS acid-phenol protocol. For my extractions, I used 3 M sodium acetate throughout the extraction (instead of using 5 M sodium acetate). I also optimized this method for my plant tissue by inverting tubes 10X before precipitation, and I lengthened the precipitation time to 90 min (instead of 60 min). Additionally, I washed and centrifuged the pellet for 10 min. For dsRNA concentrations over time, I washed the pellet with 500 µL of 70% ethanol, twice, for purification. I then removed DNA from these samples using Turbo DNA-free kit (Invitrogen). For all other samples, I purified RNA and removed DNA on a spin column (Qiagen RNeasy kit) following an optimized protocol (W.M. Keck Foundation Biotechnology).

Before cDNA synthesis, I denatured Plant RNA template by incubating (95°C for 5 mins) before immediately cooling it. From denatured RNA, I synthesized cDNA for tomatoes containing dsRNA, and controls, using 1 µg of RNA from plant leaflets, 1 µg of RNA from various height levels of the lethality assay tomatoes, 1 µg of RNA for pilot dsRNA over time assays, and 0.65 µg of RNA for the consistency over time samples. I conducted cDNA synthesis with the same kit and protocol used for whitefly cDNA. For plant cDNA, however, I increased the incubation temperature from 50°C to 60°C. Finally, to evaluate RNA purity before and after DNA removal, I measured my samples on the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and assessed RNA quality and integrity using gel electrophoresis.

2.7 Quantitative PCR analysis

Expression of the target gene *v-ATPaseA* was quantified in adult whiteflies from *in planta* bioassays and exploratory analysis of dsRNA concentrations over time using qPCR. The 10 µL reaction mixture for all targets was comprised of 5 µL 2X SensiFAST SYBR No-ROX Mix (Bioline) and primer and water adjustments were as follows: 0.135 µL of 10 µM primers and 2.23 µL of water for whitefly *v-ATPaseA*, 0.125 µL of 10 µM

primers and 2.25 µL of water for ds-*v-ATPaseA* evaluation, and 0.9 µL of 10 µM primers and 0.7 µL of water for the reference genes, *Elongation factor 1-α* (*EF1a*) (Karatolos et al., 2012) and Ubiquitin (*UBI*) (Lovdal & Lillo, 2009) (Table 2.1); all mixtures used 2.5 µL of the respective cDNA template. For PCR amplifications, I used the Bio-Rad CFX96 thermal cycler (Bio-Rad) with the two-step cycling profile recommended by the kit: 1 cycle at 95°C for 2 min followed by 40 cycles of 95°C for 5 s, and 64°C for 30 s for whitefly *v-ATPaseA* expression, 64.5°C for 30 s for ds-*v-ATPaseA* evaluation, and 60°C for both reference genes. To check amplification product specificity and to ensure no template controls (NTCs) were contaminant free, I performed a melt curve analysis at the end of each run from 65 to 95°C, with an increase of 0.5°C every 5 s. I conducted three biological replicates per treatment in total (one from each *in planta* cage) for whitefly assays, and two biological replicates per day for dsRNA evaluation. I evaluated primer efficiencies by generating a standard curve (Taylor et al., 2010), and values for these can be found with qPCR primers in Table 2.1.

To analyze whitefly gene expression data, I normalized *v-ATPaseA* expression with the reference gene (*EF1a*) and used the $\Delta\Delta C_q$ method to calculate gene knockdown (Pfaffl, 2001; Haimes and Kelley, 2014). Similarly, to evaluate dsRNA concentration over time, I normalized ds-*v-ATPaseA* expression with *UBI* and used relative quantities to evaluate differences between days.

2.8 Absolute quantification (droplet digital RT-PCR)

To measure absolute quantities of ds-*v*-ATPaseA in leaf tissue from leaflets, lethality assays, and dsRNA concentration over time assays, I used droplet digital PCR. As mentioned in Methods 2.5, dsRNA was denatured prior to cDNA synthesis, thereby doubling the amount of template present in the sample. Thus, to account for this duplication, I diluted cDNA 2X before the reaction mixture was assembled. To avoid precision deterioration between technical replicates at high concentrations (Huggett et al., 2013), I diluted the starting cDNA template of samples by a factor of 20-600-fold (Table 2.2). Reaction mixtures for one well contained 2 µL cDNA template, 1.8 µL of 10 µM forward and reverse primer, 0.5 µL fluorescent probes specific for the target (*v-ATPaseA)* or reference gene (*UBI*), 3.9 µL water, and 10 µL of ddPCR Supermix for Probes (Bio-

Rad). Using a Droplet Generator (Bio-Rad), I generated droplets from 20 µL PCR reaction mixture and 70 µL Droplet Generator Oil (Bio-Rad). I transferred the droplets to a 96 well PCR plate (Eppendorf) and heat-sealed it. I PCR amplified cDNA using the Bio-Rad T100 Thermal-Cycler (Bio-Rad) with the following parameters: 1 cycle at 95°C for 10 min, accompanied by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 1 cycle of 98°C for 10 min. I then read these droplets using a Droplet Reader (Bio-Rad), and the number of positive and negative droplets was assessed using the Quantasoft software (Bio-rad). To normalize ds-*v-ATPaseA* concentration, I generated a normalization factor for each assay by dividing *UBI* concentrations by the largest *UBI* concentration present in the assay. I then divided ds-*v-ATPaseA* concentration by the normalization factor in order to get copies/µL of dsRNA. Primer and probe sequences, as well as primer efficiency (evaluated through qPCR), are listed in Table 2.1.

2.9 Statistical analysis

I analyzed mortality data from all whitefly lethality assays using a binomial generalized linear mixed model (GLMM), where mortality response acted as the binomial variable. I chose this model in order to retain the variation among whiteflies that occurs within the treatment blocks (leaflet vials, or BugDorms), thereby avoiding pseudoreplication and allowing me to accurately analyze whitefly mortality to dsRNA treatments. To do this, I identified replicates within treatments and treated them as a random effect, which can resolve the non-independence of whitefly mortality to leaflets or plants in vials or BugDorms, respectively. Depending on the analysis, fixed effects that evaluate mortality included treatment and dsRNA concentration. GLMM was additionally used to analyze ds-*v-ATPaseA* concentrations with a Gaussian distribution, and fixed effects included plant height level, plant water content, plant water distribution, and treatment. To generate these models, I used the lme4 package (Bates et al., 2015) in R. In the GLMM model for dsRNA concentrations per plant height, I denoted each plant as a random effect to account for the samples taken from one plant. For example, samples taken from the bottom, middle, and top of a single plant.

I further analyzed the initial leaflet mortality, *in planta* mortality, dsRNA concentration at different height levels and distribution of water in plant models using Tukey HSD with

the multcomp package (Hothorn et al., 2008). Initial leaflet mortality data was further evaluated using the Bonferroni correction with the multcomp package. Additionally, to compare concentration effects to the control (water) in dose response, I conducted a qualitative analysis on the dose response data using Dunnett contrasts within the multcomp package (Hothorn et al., 2008). To evaluate the LC_{50} for the dose-response leaflet mortality GLMM, I used a modified function of dose prediction (Pikounis, 2010; Bolker, 2017) in the MASS package (Venables et al., 2002).

In order to analyze dsRNA concentration data, concentrations were logarithmically transformed to satisfy the assumption of homoscedasticity (equal variances), and all results were back transformed to determine effects on the natural scale. For determining significant differences between measured leaflet concentrations of dsRNA, I used Dunnett contrasts to compare them to the control (0 µg/mL). As well, I also used R (R Core Team, 2017) to determine the Pearson correlation coefficient between concentration and whitefly mortality. To determine if there were significant changes in *v-ATPaseA* gene expression levels, I conducted a student's t-test using R (R Core Team, 2017). To compare *in planta* dsRNA between height levels, and dsRNA *in planta* over time for initial investigation and replicated assay, I used a one-way ANOVA and Tukey's HSD as necessary (R Core Team, 2017). I determined the Pearson correlation coefficient between plant water content and dsRNA using R (R Core Team, 2017). To evaluate possible interaction effects within the GLMM model for *in planta* dsRNA concentration per height level, I conducted a Type III ANOVA (Wald test) using the car package (Fox & Weisberg, 2011).

Table 2. 1. Primers used in template synthesis, qPCR analysis, and digital droplet PCR analysis.

Gene		Use	Forward and reverse primers and probes (5'-3')	Primer efficiency (%)[1]	Product size (bp)
v-ATPase subunit A	Thakur et al., 2014	Template synthesis	[2]CTGAAGCCTGAGAGAA	-	189
			[2]AGAAGTCACCACCAG		
	SRR066677.154485 (Karatolos et al., 2011)	Whitefly qPCR	TCGTTGGTGCAGTATCACCC	95.8	71
			AACGATACCAAGGGTGGCG		
		dsRNA qPCR	CCGGACGTTTGGCAGAGAT	91.4	143
			CGCGCCAACAATACTCACA		
		dsRNA ddPCR	CGGTTACCCTGCATATTTAG	95.7	107
			CAACAATACTCACAGAACCTTCT		
			[3]CTTCCTTCTACGAAAGAGCCGGTCGAATT		

[1] Target efficiency range was between 90-110% as per Taylor et al., 2010.

[2] Sequences containing T7 promoters (TAATACGACTCACTATAGGG) attached to the 5' end of each primer.

[3] Probe with 6-FAM dye on 5' end and ZEN-3' Iowa Black® FQ on the 3' end

Table 2.9. (continued) Primers used in template synthesis, qPCR analysis, and digital droplet PCR analysis.

Gene		Use	Forward and reverse primers and probes (5'-3')	Primer efficiency (%)[1]	Product size (bp)
Green fluorescent protein (GFP)	Addgene, plasmid #11335	Template synthesis	[2] TGTCAGTGGAGAGGGT [2] TGTCTTGTAGTTCCCG	-	292
Elongation factor 1-alpha (*EF1α*)	Karatolos et al., 2012	Whitefly qPCR reference	GATGGCACGGAGACAATATG TTGTCAGTGGGTCTGCTAG	98	~100
Ubiquitin (UBI)		Micro-Tom qPCR reference	ACGGACGTACTCTAGCTGAT AGCTTTCGACCTCAAGGGTA	103.6	132
	XM_010326024	Micro-Tom ddPCR reference	CACCCTTGCCGACTACAA TCTTGGATCTTGGCCTTGACATT [3]TCGACCCTTCACCTTGTCCTCCGTC	100.2	162

[1] Target efficiency range was between 90-110% as per Taylor et al., 2010.

[2] Sequences containing T7 promoters (TAATACGACTCACTATAGGG) attached to the 5' end of each primer.

[3] Probe with 6-FAM dye on 5' end and ZEN-3' Iowa Black® FQ on the 3' end.

Table 2. 2. Dilution factors for template cDNA prior to ddPCR amplification.

	Gene	
Concentration of ds-*v-ATPase subunit A* exposed to leaflet (µg/mL)	ds-*v-ATPase subunit A*	*Ubiquitin (UBI)*
0	-	10x
0.01	20x	20x
0.05	20x	20x
0.1	20x	20x
0.2	40x	20x
0.5	20x	20x
1	600x	20x
3	600x	20x

Chapter 3 Results

3.1 Tomato cultures

Tomato seed collection was largely successful. After 2-3 tomato harvests from aeroponically grown Micro-Toms, I collected enough seed to last for the remainder of the project. In total, the volume of seed I collected was equivalent to approximately 8 mL of a 15 mL Falcon tube. Seed germination and plantlet development were, however, highly variable between seeds and plants. On average, ~25% of tomato seeds would not germinate despite uniform appearance and seed treatment. As well, ~31% of the remaining seed would not develop a plantlet after the full period of 28-30 days. Despite this, a total of n = ~63 seeds generally germinated as expected and grew into well-developed plantlets. Once I transplanted plantlets into the aeroponic system, a high proportion (~72-83%) of them survived.

3.2 Leaflet assays

3.2.1 Whitefly response to dsRNA treatments

Table 3.1 provides a summary of whitefly mortality upon being fed dsRNA in the leaflet assay. Mortality was most pronounced at the highest concentration (5 µg/mL) of the target gene treatment. Specifically, consumption of *v-ATPaseA* dsRNA resulted in whitefly mortality of ~84 percent, relative to much lower estimates associated with the non-target gene (GFP; ~4.5 percent) and a non-genetic water control treatment (~4.85 percent). A GLMM analysis confirmed that the whitefly consumption of *v-ATPaseA* dsRNA at a high dose (5 µg/mL) in leaflets significantly increased whitefly mortality (~84 percent; $z = 8.67$, $P < 0.001$), as it did to a lesser degree at the medium (3 µg/mL) (~77 percent; $z = 8.09$, $P < 0.001$) and low-dose (1 µg/mL) concentrations (~66 percent; $z = 11.14$, $P < 0.001$).

Figure 3.1 shows the dose (on log scale) mortality in response to eight concentrations of *v-ATPaseA* dsRNA. This graph also extrapolates from this observed response to predict mortality for even higher doses of *v-ATPaseA* dsRNA that I did not test. The GLMM analysis revealed that concentration of dsRNA does have a significant effect on whitefly

mortality ($z = 6.55$, $P < 0.001$) and that this dose-effect is expected to plateau ~7-11 µg/mL.

I also evaluated the effect on mortality of the *v-ATPaseA* dsRNA concentration using a qualitative approach via Dunnett contrasts. Here, I set water (concentration = 0) as a treatment control. The lowest dose that I tested (0.01 µg/mL) had no significant effect on mortality (Table 3.2). I did find, however, that the full range of concentrations above the lowest dose, ranging from 0.05 to 5 µg/mL, did have a significant effect on whitefly mortality. Further, I noticed some behavioral differences between the water treatment and 0.01 µg/mL dsRNA exposed whiteflies: the treated whiteflies appeared to be lethargic and had fewer eggs and fewer first-instar larvae than did controls (data not recorded). Finally, the dose-mortality response for ds-*v-ATPaseA* with *T. vaporariorum* estimates an LC_{50} of 1.80 ± 0.27 µg/mL.

3.2.2 Quantification of dsRNA in leaflets

I quantified the amount of ds-*v-ATPaseA* in leaflets using ddPCR analysis. This allowed me to determine concentration more accurately. Figure 3.2 shows the measured ds-*v-ATPaseA* concentrations relative to a water control. Most concentrations tested (all except 0.05 µg/mL) have a measurably higher amount of dsRNA than do untreated controls. Overall this correlation between exposure concentration and measured concentration of dsRNA was significant ($F = 28.82$, $P < 0.001$), with the measured concentration showing strong correlation with whitefly mortality ($r = 0.74$).

Table 3. 1. Percent mortality of *T. vaporariorum* with 95% confidence intervals after seven days exposure to a high, medium, and low dose of ds-*v-ATPaseA* and dsGFP (green fluorescent protein) alongside water.

Treatment	Concentration (µg/mL)	Fitted estimated mortality (%)	Lower 95%	Upper 95%	Total whiteflies/treatment (N)
v-ATPaseA	5	84.0***	75.3	90.0	151
GFP	5	4.50	1.9	10.2	129
Water	0	4.85	1.9	11.7	100
v-ATPaseA	3	76.9***	67.0	84.5	298
GFP	3	11.3	6.5	18.8	211
Water	0	10.6	6.1	17.8	192
v-ATPaseA	1	65.7***	58.4	72.3	316
GFP	1	5.8	3.5	9.4	308
Water	0	7.1	4.6	11.0	315

***Bonferroni corrected *P*-value < 0.001; N= sample size pooled across four biological replicates. Mortality was estimated through a generalized linear mixed model (GLMM) followed by Tukey HSD.

Table 3. 2. Qualitative analysis of whitefly mortality in dose-response using Dunnett's method for multiple comparisons between concentrations of ds-*v-ATPaseA* and water.

Concentrations (µg/mL) compared	*z* value	*P*
0.01 – 0	0.39	1
0.05 – 0	5.18	< 0.001***
0.1 – 0	6.40	< 0.001***
0.2 – 0	6.05	< 0.001***
0.5 – 0	5.35	< 0.001***
1 – 0	8.87	< 0.001***
3 – 0	10.11	< 0.001***
5 – 0	10.33	< 0.001***

***Dunnett's test *P*-value < 0.001; Analysis was based on four biological replicates (feeding chambers). Water was defined as control.

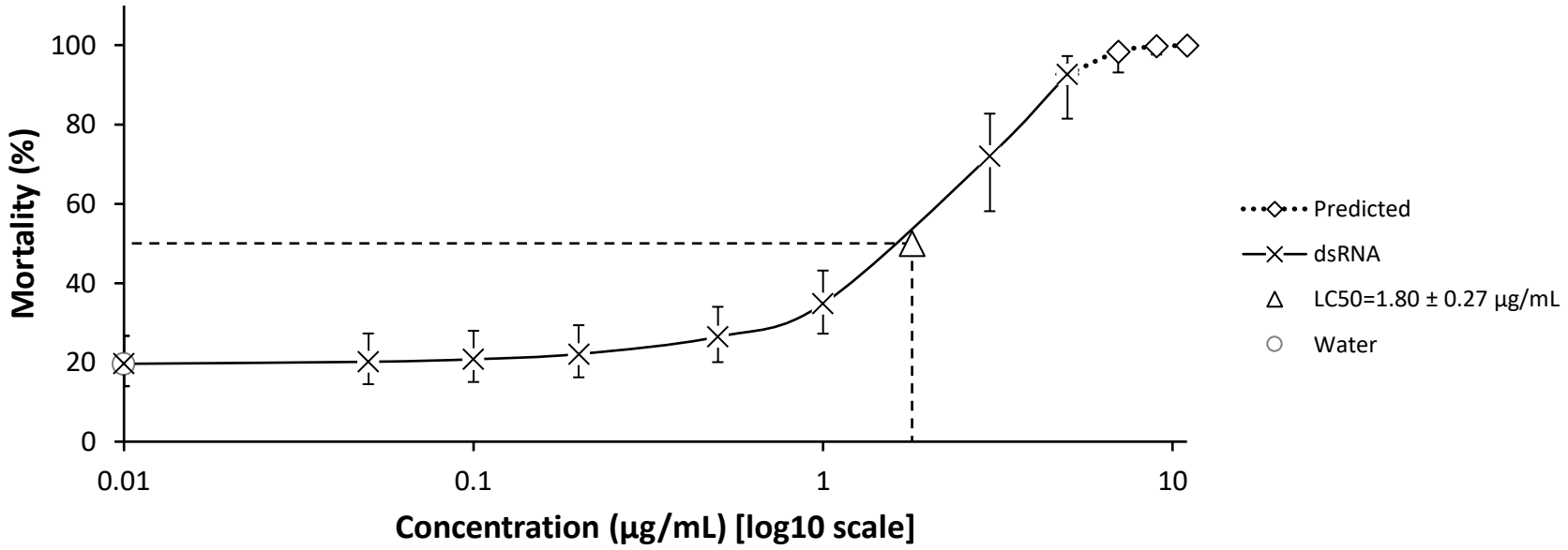

Figure 3. 1. Dose-mortality response of whiteflies following consumption of *v-ATPase subunit A* dsRNA. Adult whiteflies were exposed to dsRNA (or water) by feeding on *Solanum lycopersicum* cv. Micro-Tom leaflets that had taken-up the insect dsRNA. The observed dose-response across a range of experimental concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1, 3, and 5 µg/mL) was fit to a generalized linear mixed model (GLMM), and this same model was extended to predict mortality outside of this range (at 7, 9, and 11 µg/mL). The water control concentration ('zero') was offset to 0.01 µg/mL for plotting purposes. Data and predicted values are expressed as mean estimated mortality (± 95% confidence intervals) (n=4). The LC_{50} is estimated from the GLMM using modified dose prediction analysis.

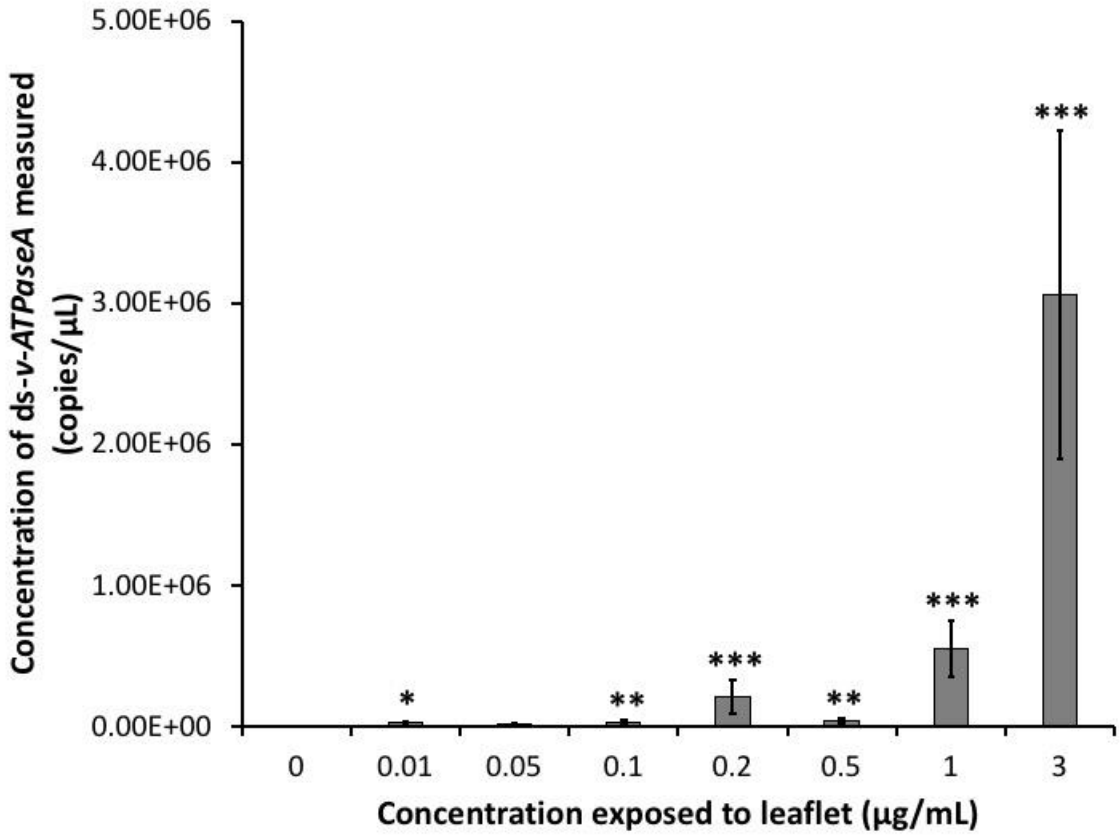

Figure 3. 2. Concentration of *v-ATPase subunit A* dsRNA measured in tomato leaflets with ddPCR. Data are expressed as normalized mean concentration (± SEM, n=3). Analysis was done on log-transformed data using a one-way ANOVA with Dunnett's test. I set the measured concentration of water-treated leaflets to 'zero' as a relative control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.3 *In planta* assays

3.3.1 Whitefly mortality and gene expression

Figure 3.3 shows the mortality of whiteflies feeding on ds-*v-ATPaseA*-treated plants relative to mortality of whiteflies that fed on water-treated controls. There was no significant difference in mortality ($z = -1.49$, $P = 0.14$). ddPCR analysis confirmed that whiteflies fed on ds-*v-ATPaseA* treated plants had lower *v-ATPaseA* expression than did control whiteflies fed un-treated plants ($F = 81.91$, d.f.=1, $P < 0.001$). Based on whitefly gene expression, shown in Figure 3.4, I estimate a three-fold decrease in *v-ATPaseA* gene

expression. Although there was no significant impact on mortality, whitefly gene expression is nonetheless reduced after feeding on leaves.

3.3.2 dsRNA localization in tomato

I estimated the number of ds-*v-ATPaseA* gene copies in the top, middle and bottom position of tomato plants from leaves using ddPCR (Figure 3.5A). There is a large amount of variation in these estimates, especially for samples taken from the middle of the plant. There was, however, no significant difference in dsRNA concentration as a function of plant sample height (F = 0.56, d.f.= 2, P = 0.58). Treated plants had more dsRNA than did controls (F = 4.99, d.f.=1, $P < 0.05$; Figure 3.5B). However, this was no longer significant when plant water content was added to the ANOVA model (F = 3.60, d.f. = 1, P = 0.08)

Table 3.4 indicates the water content and water distribution throughout dsRNA exposed plants, and each plant's normalized ds-*vATPaseA* concentration. Using a backward selection process of a generalized linear model, I determined there were no interaction effects between plant height level, treatment, and plant water content. However, it is important to note that plant water content is negatively correlated with ds-*v-ATPaseA* concentration (r = -0.39). In contrast, water distribution had no effect on, or correlation with, ds-*v-ATPaseA* (Wald= 0.02, d.f.= 1, P = 0.88; r = 0.06). Despite this, an interaction effect between water distribution, plant height level and treatment may exist, although results were insignificant (Wald= 5.76, d.f. = 2, P = 0.06).

Further, although there were no significant interactions, the model that best fit the concentrations observed (AIC= -2.63) was:

$$[\text{ds-}v\text{-}ATPaseA] = (\text{Plant Water Content} * \text{Plant Height Level} * \text{Treatment})^2 + \text{Random Effect[Plant]} + \text{Error}$$

3.3.3 Consistency of dsRNA in tomatoes over time

I used qPCR to evaluate the relative amount of dsRNA in tomato plants over a six-day period. Initial analysis using just two plants showed that Days had a significant effect on relative gene expression of dsRNA (F = 6.85, d.f.=6, P<0.05; Figure 3.6). I repeated the ds-*v-ATPaseA* over time experiments with three new tomato plants (Figure 3.7). This second set of experiments showed that there is no significant different between the concentrations for each day (F = 0.98, d.f.=6, P = 0.47).

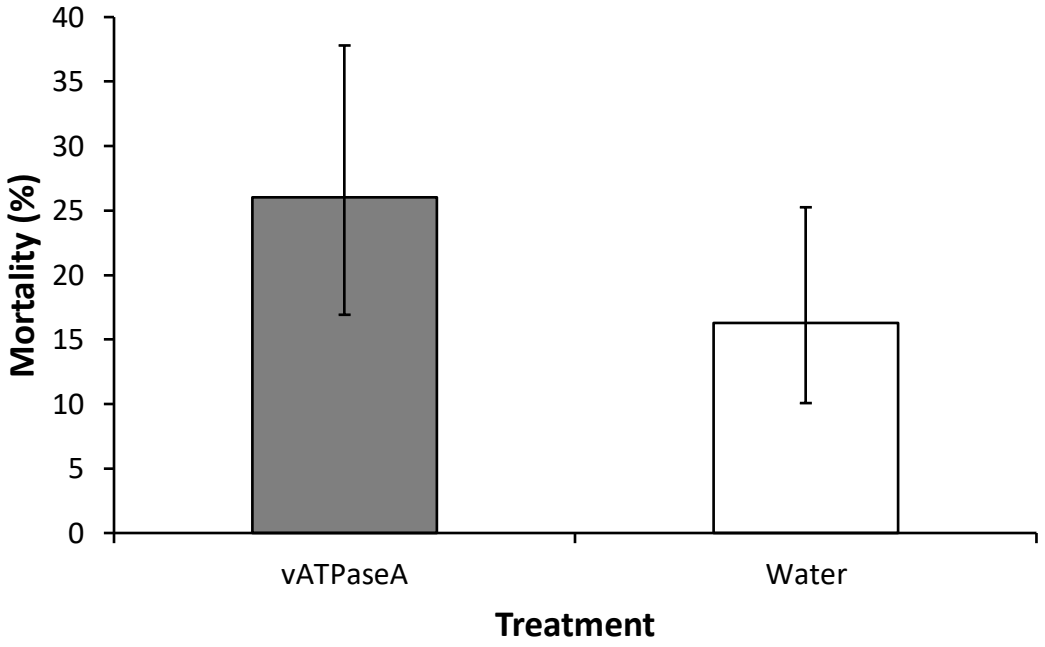

Figure 3. 3. Whitefly mortality when exposed to 2 µg/mL *vATPaseA* dsRNA delivered through tomato, relative to control plants (water). Data are fit to a generalized linear mixed model (GLMM) and expressed as fitted mean mortality with 95% confidence intervals (P = 0.14, n=3).

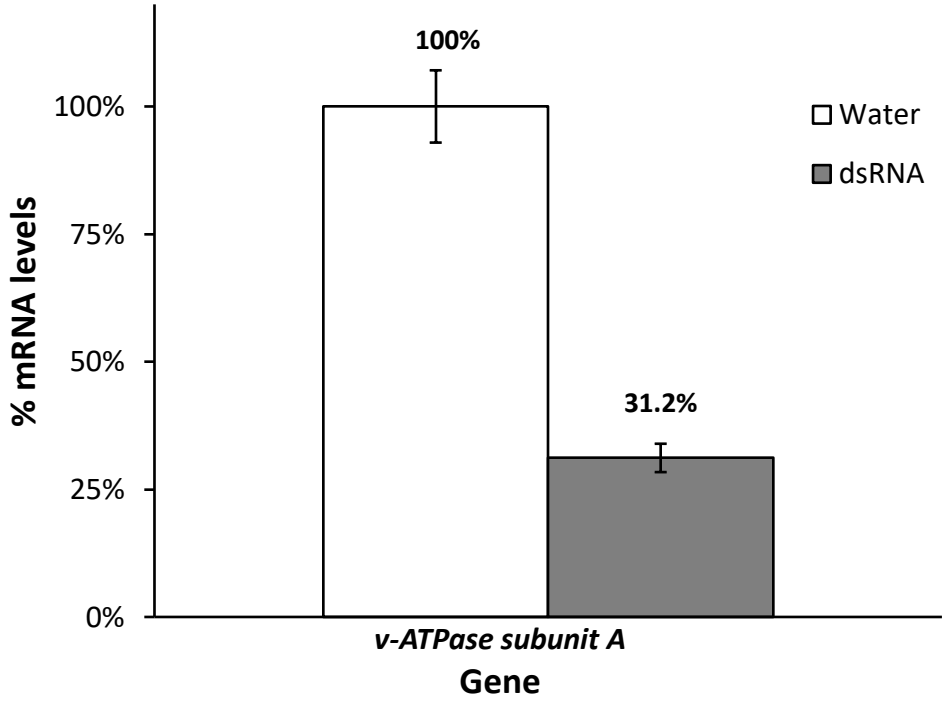

Figure 3. 4. Whitefly gene expression after feeding on ds-*v-ATPaseA*. Percent mRNA level was normalized using a stably-expressed endogenous reference gene (*EF1a*). For display purposes, I set mRNA quantity to 100% in the control (water). Percent mRNA level for dsRNA treatment was calculated relative to control using the ΔΔCq method (Pfaffl, 2001; Haimes and Kelley, 2014). Data are expressed as mean relative quantity (± SEM, n=3).

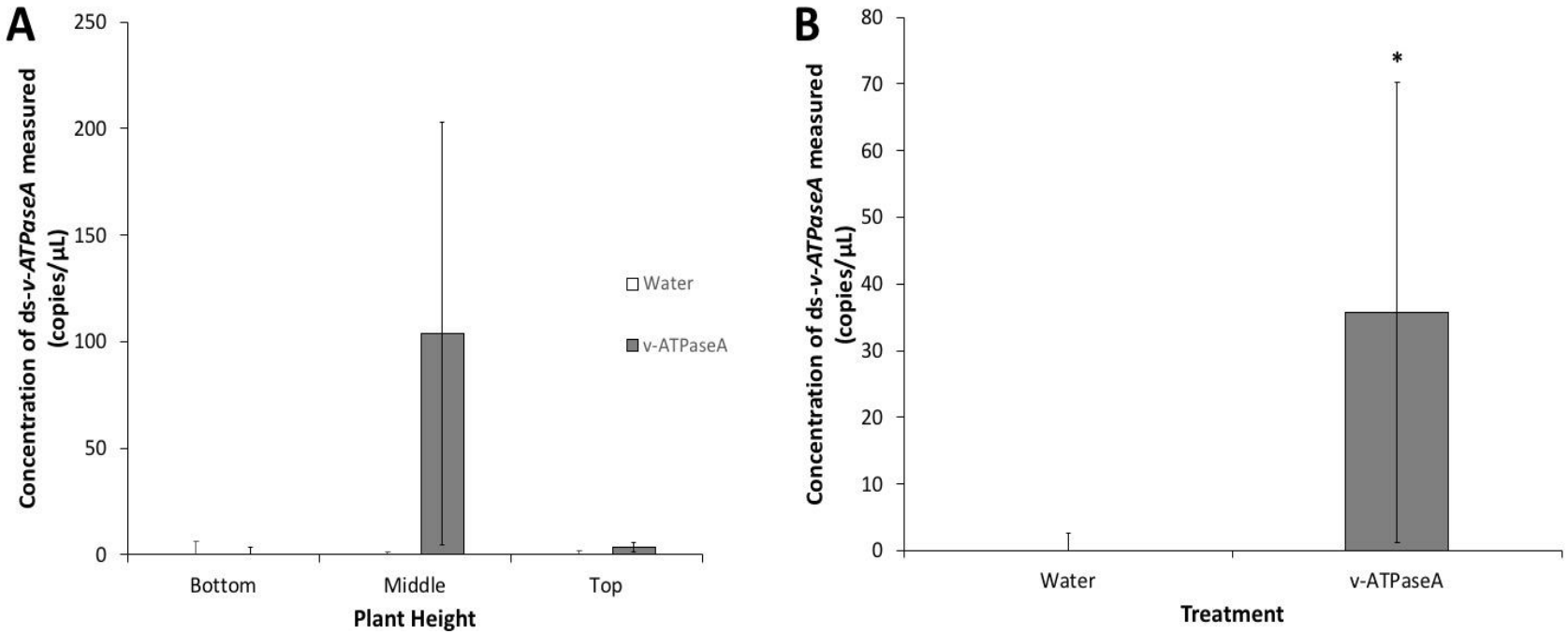

Figure 3. 5. Concentration of *v-ATPase subunit A* dsRNA in Micro-Tom leaves taken from *in planta* whitefly assays. (A) Measured concentration of ds-*v-ATPaseA* at varying plant height levels using ddPCR. **(B)** Average ds-*v-ATPaseA* concentration per treatment. Leaf samples were taken six days post initial root introduction. Data are expressed as mean concentration ± standard error mean (SEM), biological replicate (n)=3, and was normalized by UBI concentrations in leaf tissue. Statistical significance was determined using a one-way ANOVA on log-transformed data (*$P < 0.05$).

Table 3. 3. Distribution of water in each plant and *v-ATPase subunit A* dsRNA concentrations *in planta* at each height level on day 6.

Plant	Plant water content (%)	Plant water distribution	Plant height level	*v-ATPase subunit A* dsRNA concentration (copies/µL)
1	89.23	1.85	Bottom	5.94
			Middle	5.60
			Top	8.17
2	90.78	5.40	Bottom	1.69
			Middle	9.67
			Top	2.56
3	88.00	3.59	Bottom	13.15
			Middle	304.56
			Top	10.01

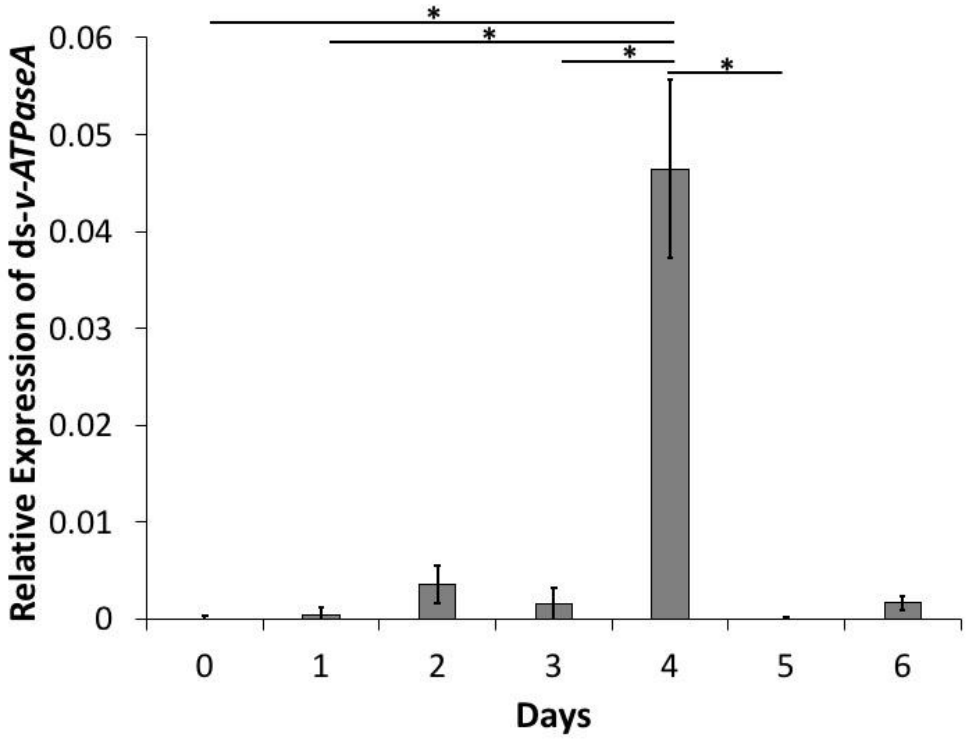

Figure 3. 6. Initial investigation of *v-ATPase subunit A* dsRNA quantities in Micro-Toms over six days. Data are measured using qPCR and expressed as normalized mean relative quantity ± standard error mean (SEM) (n=2). Statistical analysis was conducted with a one-way ANOVA and Tukey HSD on log-transformed data (*$P < 0.05$).

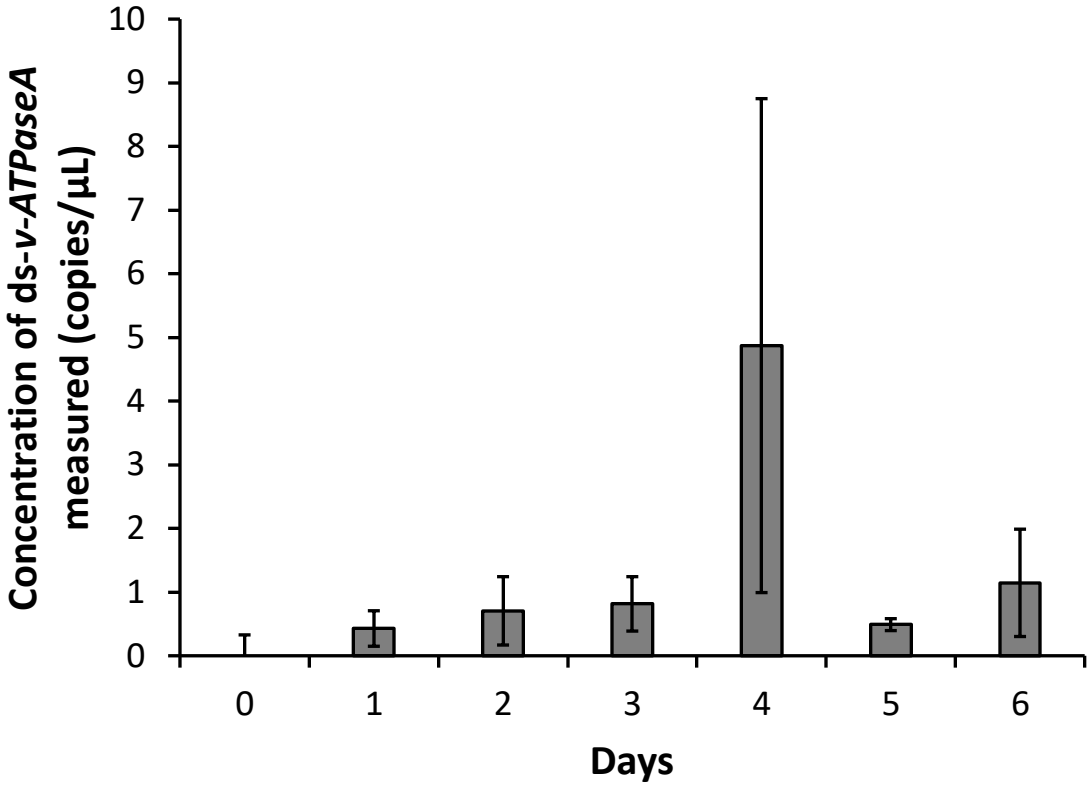

Figure 3. 7. Measured concentrations of *v-ATPase subunit A* dsRNA at the middle of Micro-Toms over six days. Absolute quantification was done using ddPCR and concentrations are expressed as normalized mean concentration ± SEM (n=3). Data are log-transformed for statistical analysis and no significant difference was seen using a one-way ANOVA (F = 0.99, d.f. =6, P = 0.47).

Chapter 4 Discussion

In this thesis I have evaluated the feasibility of root uptake of dsRNA for application of RNAi in pest management targeting hemipterans, specifically the greenhouse whitefly, an insect known for causing extensive crop loss and transmitting a considerable number of viral diseases (Gamarra et al., 2016). My results consist of two major findings. First, I confirmed that the *v-ATPaseA* dsRNA segment I produced can be incorporated into plant tissue and, when fed to whiteflies at high enough concentrations, significantly increased whitefly mortality. I can conclude therefore that the uptake is technically possible and that *v-ATPaseA* is a well-chosen gene to target for models in the development of future greenhouse-ready pest management strategies. Second, I showed that plant uptake will require further research and development as a technique prior to any future commercial application. Specifically, I found that whole Micro-Tom plants did uptake significant amounts of ds-*v-ATPaseA* for delivery to whiteflies, and I found that this delivery resulted in significant gene knock-down in living whiteflies, yet the effect on mortality in this system was inefficient and not yet comparable to the mortality observed for the leaflet assays. My results are therefore promising and should help to accelerate integrated pest management strategies of the future.

4.1 Whitefly response to dsRNA in leaflets

I chose to target a v-ATPase gene because previous work has shown that targeting the gene *v-ATPase subunit A* in whiteflies has an impact on the insect's mortality (Upadhyay et al., 2011, Thakur et al., 2014). My leaflet assays show that exposure to ds-*v-ATPaseA* greatly increases whitefly mortality at low dsRNA concentrations, confirming it as an essential gene and well-suited as a target in whitefly management models. My results with the leaflet assays further indicate that the whitefly itself is sensitive to (what I assume to be) the dysregulation of this gene's expression when exposed to very low (e.g., 0.05 µg/mL) concentrations of dsRNA in their host-plant leaf diet. The conserved nature and essential role of *v-ATPaseA* within insects (Wieczorek et al., 2000) may also attribute to the whiteflies' sensitivity, and although silencing this gene is effective in killing the whitefly, other insect species are likely to be affected by this same gene target sequence. Despite the demonstrated potential of this technique, however, it is currently limited in its

efficacy and needs further validation and troubleshooting that is beyond the scope of my dissertation. For instance, my estimate for an effective dose response of ds-*v-ATPase* on whitefly mortality (LC_{50}=1.80: Figure 3.1) was not different than a previously published estimate for si-*v-ATPaseA* (LC_{50}=3.08 µg/mL; Upadhyay et al., 2011). Because insect cell uptake of siRNA is refractory compared to uptake of dsRNA, the dsRNA treatment should be more effective than siRNA and thus yield a much lower LC_{50} compared to siRNA. siRNA can also result in off-target effects that may result in the silencing or down-regulation of other non-target genes in the organism. While it is difficult to directly compare LC_{50}'s estimated and obtained from different studies, the apparent lack-of-difference may be due to possible processing of dsRNA into siRNA within leaflets during the assays.

Some of the variation in mortality may be due to the sex of the individual insects, which is a factor that I did not control for. For example, because female whiteflies are able to reproduce asexually (arrhenotoky), they may feed, reproduce and respond differently than males to gene knock-down of *v-ATPaseA*. One example of how this may occur is in female whitefly nutrient acquisition (Xue, et al., 2012). The female whiteflies ability to reproduce asexually and nutrient acquisition is related to it's endosymbionts (Normark, 2003), which are present within the Malpighian tubules (Su, et al., 2014). The dsRNA target, *v-ATPaseA*, is part of the vacuolar (V-) ATPases and are also present in the Malpighian tubules. As well, v-ATPases function as transmembrane proton transporters and are best known for the acidification of organelles within insects, which is important in many processes and functions (Dow, 1995; Wieczorek et al., 2009). Thus, down-regulation of *v-ATPaseA* will affect v-ATPases, and is likely to inhibit or prevent localized processes, which may include nutrient acquisition. Therefore, silencing *v-ATPaseA* in female whiteflies may affect survivorship differently than in males.

4.2 dsRNA in leaflets

Leaflet assays have become a common means of dsRNA delivery via ingestion (Camargo et al., 2015; Galdeano et al., 2017). This approach can be effective but it remains difficult to determine the precise concentration ingested by the insect (Surakasi et al., 2011). In my leaflet assays I found that all exposure concentrations, with the exception of 0.05

µg/mL, resulted in a substantial amount of dsRNA uptaken into the leaflet, including at treatment concentrations as low as 0.01 µg/mL (but that had no effect on whitefly mortality). This highlights a peculiar difference between measured concentration and whitefly mortality. Additionally, as shown in Figure 3.2, there is a large difference in the measured amount of dsRNA in leaflets following exposure to 3 µg/mL dsRNA compared to 1 µg/mL or less. This variation in uptake (0.01-0.05 µg/mL and < 3 µg/mL; Figure 3.2) despite uniform treatment of leaflets may be explained by variation in stomata size. That is, because leaflet uptake of solution (water, dsRNA, etc.) is regulated by the stomata (Pearcy et al., 2000), and because stomata vary from leaf to leaf (Ambrose et al., 2010; Vialet-Chabrand et al., 2017), it follows that inter-plant variation in stomata-that I do not control for in my experiment- could impact dsRNA uptake.

Another possible source of variation in the amount of dsRNA uptake in leaflets is via the mechanism by which dsRNA is transported through the phloem. If the dsRNA travels across the cell membrane it is possible that some of this molecule is broken down into siRNA, creating variable concentration reads. The presence of siRNA would also explain the contrast in whitefly mortality and measured dsRNA concentration for two of my treatment concentrations (0.01 vs. 0.05 µg/mL): siRNA can produce varied mortality responses caused by off-target effects, which result in down-regulation of non-target genes in insects, which may include other essential genes (Nunes et al., 2013; Nandety et al., 2015). Further, because there are as many as three phloem pathways within the leaflet (the apoplastic pathway, the symplastic pathway, and the transcellular pathway; Figure 4.1), and because their transport routes vary and solute distribution (including dsRNA) may be uneven, I suggest that two of the three phloem pathways for dsRNA (the symplastic and transcellular pathways) lead to processing of dsRNA into siRNA (Figure 4.2A).

I speculate that the uneven distribution through the pathways may be affected when dsRNA is at relatively high concentrations (i.e. ≥ 3 µg/mL). The intracellular space may become saturated with ds-and si-RNA. Further, I suggest that this saturation could shift the osmotic potential causing dsRNA to move into and through the apoplastic (extracellular) pathway, thereby decreasing the likelihood of dsRNA processing (Figure

4.2B). Thus, when exposure concentrations are high there would be more un-processed dsRNA to measure compared to smaller concentrations where dsRNA is more likely processed.

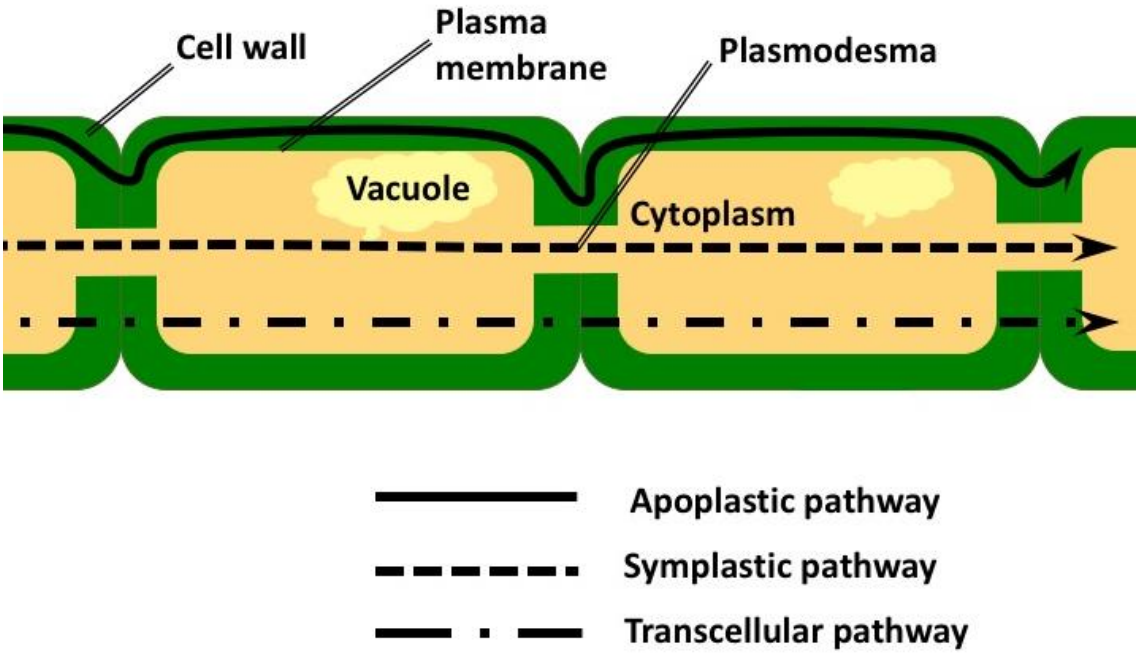

Figure 4. 1. The three phloem pathways within plants. The apoplastic where dsRNA would travel through the extracellular spaces and continuum of cell walls without crossing membranes or cytoplasm; the symplastic pathway, where dsRNA would travel within cells through the cytoplasm and between cells through the plasmodesmata, a microscopic channel enabling transport; and the transcellular pathway, where dsRNA would travel across cell membranes (reviewed in Holbrook & Zwieniecki, 2005).

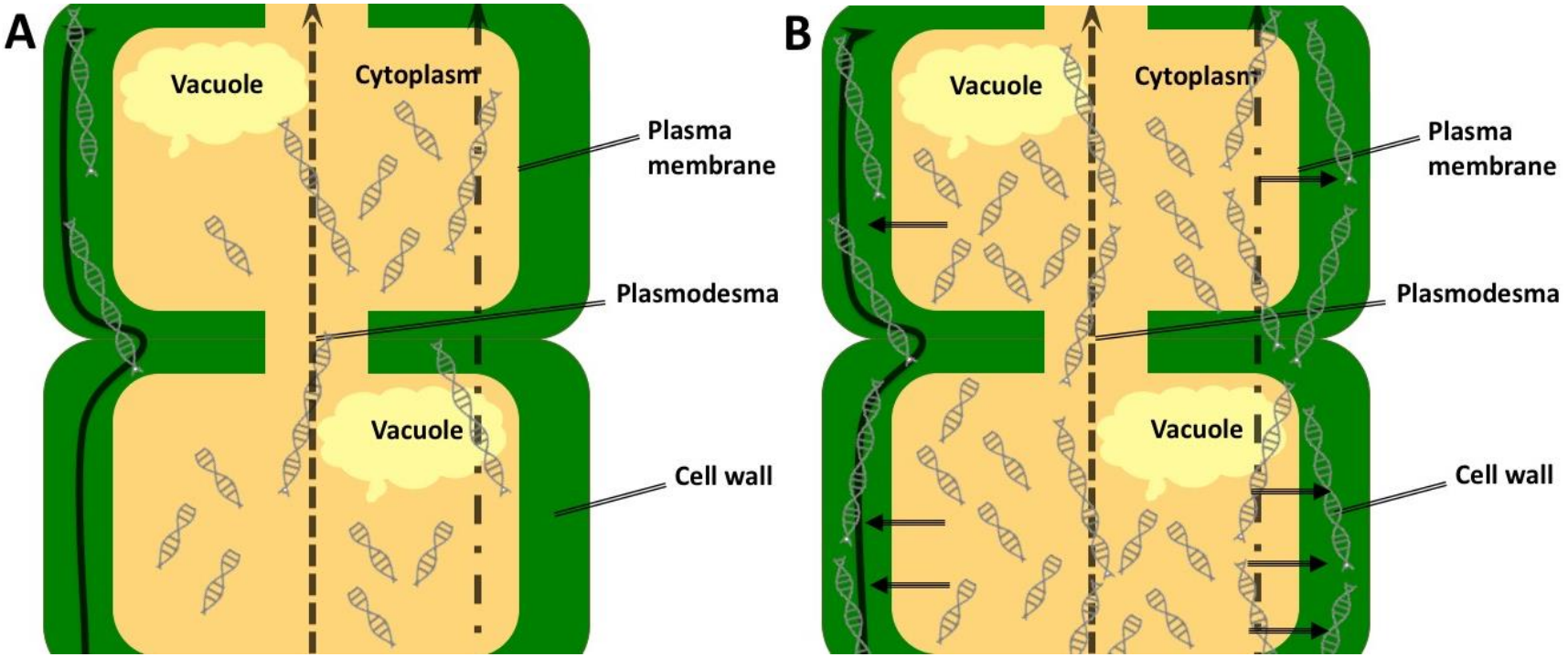

Figure 4. 2. Schematic of how dsRNA may travel through the phloem pathways within leaflets. (A) A leaflet exposed to relatively low concentrations of dsRNA, where dsRNA may primarily travel through the symplastic and transcellular pathways, and may be processed into siRNA within the cytoplasm. **(B)** A leaflet exposed to relatively high concentrations of dsRNA, where the intracellular space may become saturated with dsRNA and siRNA, changing the osmotic potential and forcing more dsRNA to travel through the apoplastic pathway.

4.3 Whitefly bioassays

During my *in planta* bioassays mortality in dsRNA treatment whiteflies was not different from water treatment whiteflies. The dsRNA-treated whiteflies did however appear to change their behaviour to become more sluggish, which is consistent with behavioural observations I made in leaflet assays. Specifically, I noticed this change when whiteflies were exposed to very low dsRNA concentrations i.e. 0.01 µg/mL. As well, in spite of a whitefly's tendency to position themselves on the top-most leaves of plants (Gamarra et al., 2016), whiteflies in ds-*v-ATPaseA* treatment cages tended to the middle area of the plant. I suggest this could be another behavioural response to the dsRNA caused by changes in organelle function within the Malpighian tubules as a result of v-ATPase irregular function. Finally, I confirmed that *v-ATPaseA* was down-regulated in the whiteflies exhibiting this strange behaviour. I suggest therefore that 1- the insects were able to ingest the dsRNA segment from the Micro-Toms, and 2-that the Micro-Toms were able to uptake dsRNA through the roots. Further, because whiteflies feed from the phloem (Walling, 2008; Moreau, 2010), the dsRNA appears to be taken-up through the roots into the xylem and then moved into the phloem.

4.4 dsRNA movement within tomatoes

My measurement of dsRNA in living plants showed that there was a significant amount of dsRNA overall in the treatment plants compared to the control as observed on day six of bioassay tomatoes. However, when I measured dsRNA over-time I observed a different pattern. In comparison to the bioassay (whitefly exposed) plants, where treated plants had a significant amount of dsRNA, dsRNA over-time treated plants showed no difference from control. While this difference in dsRNA concentration could be caused by plant variability, it may also be the result of changes in plant metabolism when under stress (i.e. when fed upon by whiteflies). Further, I observed an unusual (but non-significant) peak in dsRNA concentration on day four. This difference in dsRNA localization or 'behaviour' of dsRNA may be related to how the plant is transporting the dsRNA or to differences in plant stress i.e. stress (whitefly exposed) vs. non-stress (no whiteflies present).

As observed in changes in gene regulation from the whitefly bioassays, the dsRNA was able to move from the xylem and into the phloem of Micro-Toms. In flowering plants, such as my Micro-Toms, plant phloem is a channel for transporting hormones, proteins, nucleic acids, water, dissolved minerals, and other small molecules throughout plants (Brooker et al., 2008). Thus, it is possible the dsRNA may be treated as a nutrient and was transported through the phloem pathways (apoplastic, symplastic, and transcellular) (Joga et al., 2016). Transport methods and the pathway(s) used, however, remain unclear. If dsRNA movement occurs through the symplastic or transcellular pathway upon root uptake, the plant RNAi machinery should activate and begin cleaving dsRNA into siRNA, which is also observed when dsRNA is produced within plants through nucleic transformations (Eamens et al., 2008). The partial processing of dsRNA could result in variation in measured behaviour of dsRNA, as observed in my dsRNA throughout tomato plants and dsRNA over time.

4.4.1 Localization of dsRNA in tomato during predation

The amount of dsRNA did not vary with plant height. There was, however, a significant amount of dsRNA overall in dsRNA exposed plants compared to the controls. The significance in treatment was no longer present when plant water content was accounted for. The change in significance can be explained by the effects of collinearity (Yoo et al., 2014), as there is a negative correlation observed between ds-*v-ATPaseA* and plant water content, in which the dsRNA would travel. The negative correlation indicates that if plant water content were to decrease, by means of using a smaller plant or a plant that has less foliage, then dsRNA concentration would increase. The best explanation for dsRNA concentrations observed in my experiments takes into account plant height level, treatment, as well as plant water content. Although plant height level was not significant, it was found to contribute to the dsRNA concentration in each plant overall.

In this experiment, my measured ds-*v-ATPaseA* concentrations from the middle of the plant were the highest among the three areas measured (at least in two of the three plants examined). During these assays, Micro-Toms were no longer fertilized and were therefore deprived of nutrients from the moment of dsRNA exposure. Thus, the accumulation of dsRNA in the middle of the plant may be related to the plants nutrient

requirements, including breaking down dsRNA for nutrient acquisition (Paungfoo-Lonhienne et al., 2008), and the plants response to herbivory and need for defense. In support of this idea, previous work has shown that plants will re-allocate their energy towards gaining nutrients and break down what is available to meet these needs (Paungfoo-Lonhienne et al., 2010). Plant roots have the ability to then uptake and assimilate low molecular weight organic substances to meet their nutrient requirements (Lipson & Näsholm 2001; Jones et al., 2005; Näsholm et al., 2009), such as dsRNA. Hence, the Micro-Toms likely took up dsRNA and assimilated it in order to produce nutrients such as phosphorus or nitrogen. Further, Paungfoo-Lonhienne et al. (2010) has shown plants are able to re-model their cells walls by inducing gene expression for the purpose of taking up microbes for nutrition in hydroponically grown tomatoes.

While plants were exposed to the whiteflies, they would have allocated available nutrients to leaf tissue in order to activate defense mechanisms (Herms et al., 1992; War et al., 2012; Fürstenberg-Hägg et al., 2013). During a whitefly infestation, tomatoes will increase the amount of volatiles they produce in order to attract *Encarsia formosa* in a tri-trophic interaction (Tan & Liu, 2014; Cui et al., 2016), although these same volatiles are also attractive to whiteflies. Monoterpenes and sesquiterpenes are two volatile organic compounds that are associated with *E. formosa* (Cui et al., 2014), both of which include chemical compounds that require phosphate for synthesis (Singh & Sharma, 2015). Thus dsRNA, which, when broken down as a source for phosphorus and nitrogen, may have been utilized by the plant for nutrients in secondary metabolism to try and attract *E. formosa* in order to defend itself. This may also explain the tendency for whiteflies to locate to unusual regions of the plant during these assays.

4.4.2 dsRNA within plants over time

In measuring dsRNA over time, I observed significant differences in dsRNA concentration over a period of days. Figure 3.6 shows a substantial amount of dsRNA is sequestered on day four before disappearing on day five and six. These results may indicate that dsRNA is broken down into siRNA or into nucleic acids, which may explain the disappearance observed on day five. This result is tempered, however, by my inability to replicate the qPCR findings on a ddPCR platform. Specifically, there was no

significant difference between concentrations when I re-did the experiment and re-measured dsRNA using ddPCR. This difference between the assays may be due to the increased sensitivity of ddPCR relative to conventional qPCR, as well as to the increased level of replication, which should explain more of the statistical variation. In both cases, however, a consistent peak is present on day four with no build up before, or any notable peaks or fluctuations following that day. Again, this may have been the result of plant breakdown of dsRNA to quench nutrient requirements. Like bioassay plants, tomatoes used in assays over time were no longer given fertilizer once assays began and roots were exposed to dsRNA.

Figure 4.3 shows the initiation of the signaling cascade within a plant when exposed to insect herbivores and subsequent defense mechanism response. This can occur by any means that may wound the plant (Mithöf & Boland, 2008). Thus, it may be possible for a similar cascade to be activated when a shortage of plant nutrients is determined and survival is brought into question. Unlike those plants exposed to whiteflies, the tomatoes during this assay would not have experienced early wounding, for example on days one or two. This could have delayed the signaling initialization and resulted in a delayed utilization from the plant. As well, metabolic changes occur within plants between hours and days when wounded by an insect (Fürstenberg-Hägg et al., 2013), which could make sense of the unusual timeline for the dsRNA peak.

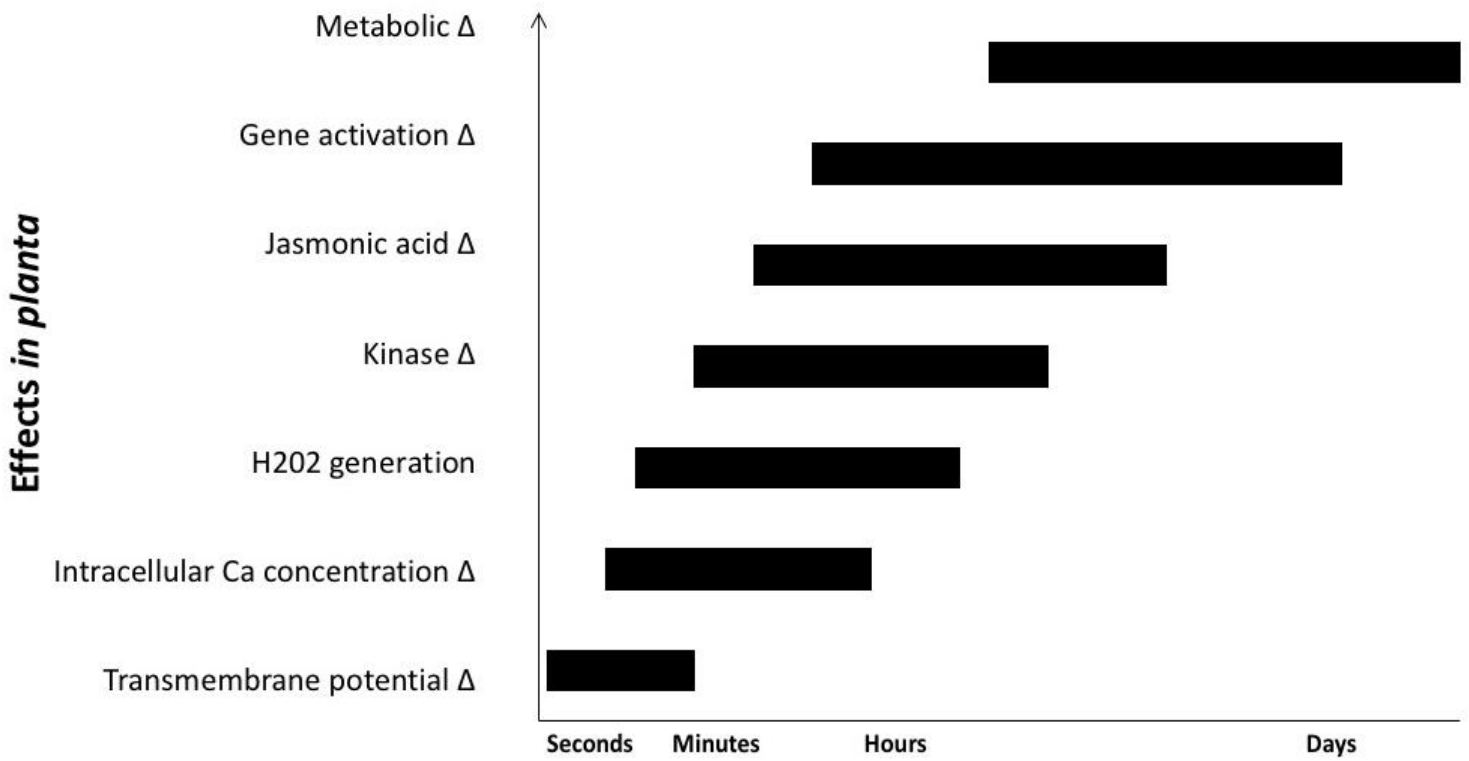

Figure 4. 3. The effects observed within plants in response to insect feeding (herbivory). Nearly instant changes are observed in the transmembrane potential when insects begin to feed on plants and are accompanied close behind by changes in intracellular calcium concentration. Increases in the cellular concentration of reactive oxygen species (caused by stress) result in hydrogen peroxide (H_2O_2) production, and is followed by changes in kinases (kinase cascades) and jasmonic acid. Within two hours gene activation can be observed and the plant begins to undergo metabolic changes to try and protect itself against the insect. Adapted from Fürstenberg-Hägg et al. (2013).

4.5 Conclusion and future directions

This project looked at better understanding and evaluating root uptake of dsRNA as a possible means of pest management application for phloem feeders, such as the greenhouse whitefly. I was able to confirm whitefly lethality to *v-ATPaseA* dsRNA, as well as determine the LC_{50} for this dsRNA target, and the lowest lethal concentration. This work was also the first use of absolute quantification using ddPCR of dsRNA in leaflets and *in planta*. As well, this study acts as the first evaluation, to my knowledge, of dsRNA behaviour *in planta* throughout the plant, or over time through root uptake delivery.

Although silencing *v-ATPaseA* can be effective against whiteflies, due to the level of gene conservation and the resulting high risk of affecting non-target organisms, I don't believe it is a suitable choice for pest management. Thus, more pest specific gene targets, such as *alpha glucosidase* or *aquaporin* in whiteflies (Raza et al., 2016), require further investigation and sequencing. Further, it may be of interest to examine possible differences in lethality between male and female whiteflies in response to gene silencing, however, this would likely require less whiteflies to ensure accurate sexing of insects. As transport of dsRNA via leaflets is largely used in RNAi studies, continued research into determining the behaviour of dsRNA in leaflets may prove valuable, especially when trying to evaluate potential off-target effects on other species.

To determine if using plant root uptake can result in mortality in phloem feeding insects, further research is required. Although I was able to successfully deliver dsRNA to whiteflies through this delivery method, I did not observe a change in mortality. Continued work on this model could increase the dsRNA concentration, however; I strongly believe that further research into dsRNA behaviour *in planta* is first required. Plant response mechanisms, including initiation and signal transduction, and changes *in planta* when utilizing or breaking down new nutrient sources, are not well understood. To determine this, evaluation of individual plant electrical signaling, evaluation of possible siRNAs *in planta*, using low concentrations of dsRNA, and continued efforts to evaluate dsRNA *in planta* while exposing plants to fertilizer should be explored. Although I

explored some possibilities of how dsRNA may behave within plants, future exploration may also investigate concentrations within the stem, the roots, and the flowers, to determine if there is risk of other insects being exposed to dsRNA.

Finally, although I was unable to measure this behaviour, whitefly demeanor was altered and this change was observed at low dsRNA concentrations, which, as observed in my *in planta* bioassays, may be able to indicate gene down regulation. In order to evaluate and quantify this response, videos should be taken of insects, controls and treatment, during assays. By adapting computer software, such as Noldus (Wageningen, The Netherlands), the type of behaviour may be quantified and compared to determine if there is a significant difference in response (Noldus et al., 2002). This could help in both observing effects on target insects, as well as off-target effects, in lab or environmental studies.

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Appendices

Appendix A: Transcriptome read from Karatolos et al., 2011 used to develop *v-ATPase subunit A* primers to measure whitefly gene expression

>SRR066677.154485 F19ZPOU02GQWOM

TCAGAGACGCACTCGTTTCTTATCCAAACCCCAGAAGACTTGAACGATACCA
AGGGTGGCGGATGTGACGGGATCCGAGAAGTCACCACCAGGGGGTGATACT
GCACCAACGATACTAACAGAGCCTTCACGGTCTGGGTTGCCAAGACATTTTA
CTCTACCGGCTCTTTCATAGAAGGACGCGAGACGAGCTCCCAGGTACGCAGG
ATAACCACTGTCAGCAGGCATTTCAGCTAAACGTCCAGAAATTTCTCTCAAG
GCCTCAGCCCAACGTGAGGTGGAATCAGCCATCATAGAAACATTGTAACCCA
TATCTCTGAAGTACTCAGATAGTGTAATACCTGTGTAAAATTGAGGCCTCTCG
AGCAGCTACAGGCATGTTGGATGTGTTAGCGACAAGAGCAGTACGCTTCATG
ATAGATTCAGTGACTCCATCTAATTCAATAGTTAACTCAGGGAAATCTCTCAA
TACCTCTGACATTTCGTTACCACGCTCTCCACACCCACGTACG

Appendix B: Mathematics used to approximate the concentration to expose to tomatoes.

250 mL (10 mg/mL) dsRNA in 18.93 L water exposed to 2.5 m tall tree (Hunter et al., 2010)

$$250\ mL\ \times\ 10mg/mL = 2500\ mg$$

$$2500\ mg \div 18.93\ L = 132.07\ mg/L = 132.07\ \mu g/mL$$

Will lose ~11% to mesophyll and vascular tissue (Hunter, W.B, personal communication, 2016)

$$132.07\ \mu g/mL \times\ (0.11) = 14.5277\ \mu g/mL\ likely\ gone$$

$$132.07\ \mu g/mL -\ 14.5277\ \mu g/mL = 117.54\ \mu g/mL\ left$$

For 2.5 m tall trees:

$$2.5\ m = 250\ cm$$

$$117.54\ \mu g/mL\text{: } 250\ cm$$

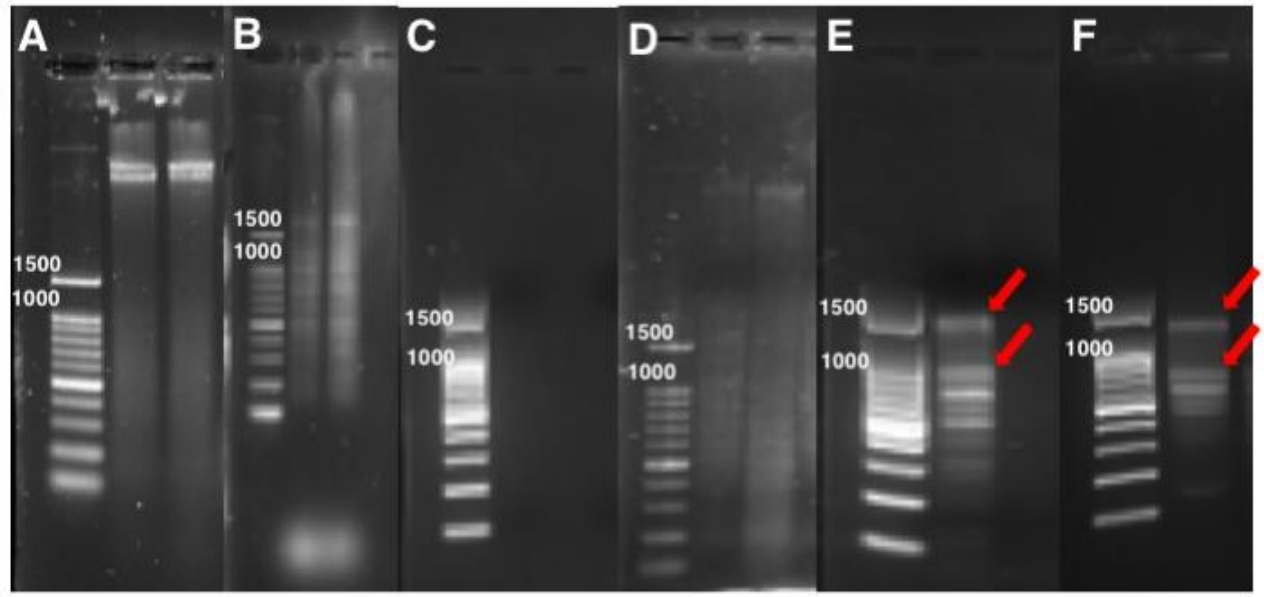
$$5.642\ \mu g/mL\text{: } 12\ cm^{*}$$

*assuming tomatoes reach roughly 12 cm

Rough calculation indicates 5.6 µg/mL is necessary to reach similar results in tomatoes, but because tomatoes do not have bark, transpiration is increased, therefore increasing uptake. Further, this work was conducted in soil so there is likely loss associated with it, whereas dsRNA in water should not be lost. Keeping this in mind I divided this amount in two and decreased it a little further to get 2 µg/mL.

Appendix C: Evaluation and optimization of RNA extraction protocols for RNA and dsRNA extraction from *Solanum lycopersicum* L. cv. Micro-Tom.

Evaluation of RNA integrity from various extraction methods of tomato leaf tissue. The sizes (in bp) are based on DNA, and the red arrows indicate the 28S (top) and 18S (bottom) ribosomal RNA (rRNA) bands.



A: MasterPure Complete DNA and RNA Purification Kit (Epicentre, Windsor, ON, CA)

B: RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

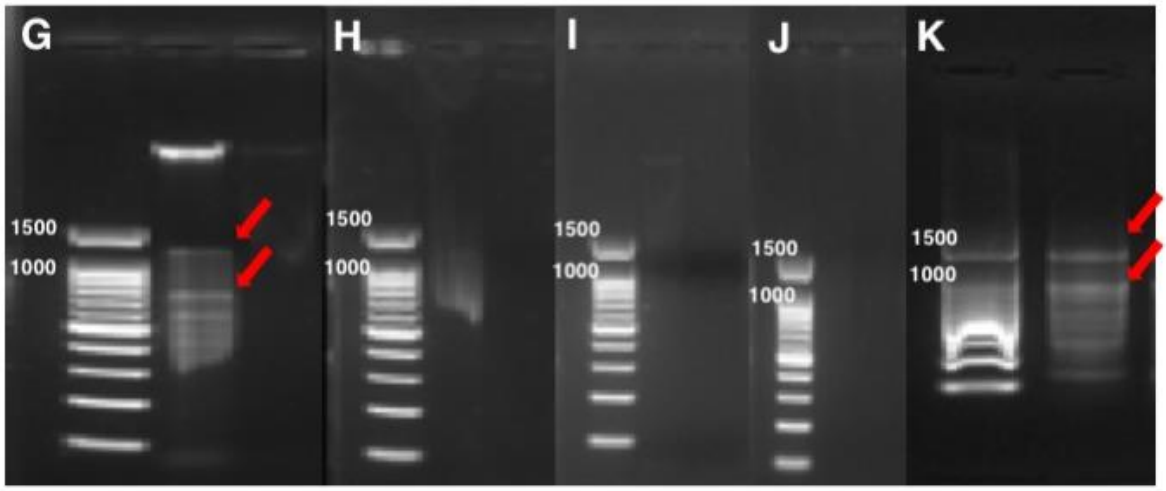
C: RNA isolation for secondary metabolite rich plants (IHBT, Ghawan et al., 2011)

D: Modified SDS acid phenol (Hou et al., 2011)

E: Modified CTAB method (Li et al., 2014, with modifications, Cui, H., Simkovich, A., Park, S., 2013)

F: Further modified SDS acid phenol (Deepa et al., 2014)

Evaluation of SDS carry over mitigation and RNA purification techniques.



G: Deepa et al., 2014 SDS acid phenol modified by replacing sodium acetate with sodium chloride

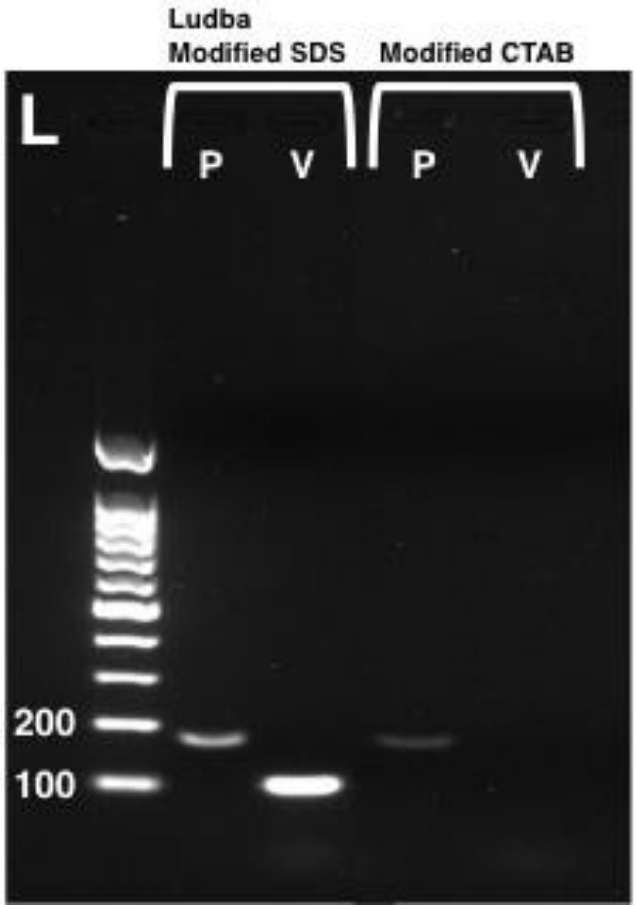
H: Post DNase of SDS acid phenol modified by replacing sodium acetate with sodium chloride

I: Chromaspin purification of RNA

J: RNeasy purification following manufacturers protocol

K: RNeasy purification following W.M. Keck Foundation RNA Purification with on-column DNase protocol

Comparison of dsRNA extraction efficiency from leaf tissue. cDNA synthesized from RNA extracted using my modified SDS phenol acid extraction, and the modified CTAB method, was amplified through PCR. P indicates amplification of the plant gene *Clath* (Forward: ATGCAATCACACCAGCAC, Reverse: ACTCAGCACAACAACAAAGG, amplicon: 200 bp) to verify cDNA synthesis worked, and V indicates amplification of the introduced *v-ATPase subunit A* dsRNA**.**



Appendix D: dsRNA concentration from ddPCR calculations.

Total sample concentration (for one biological replicate) is determined using the dilution factor for target and reference:

$$\text{Sample total [ds-}v\text{-}ATPaseA\text{]} = (\text{ddPCR[ds-}v\text{-}ATPaseA\text{]}) * \text{Dilution factor}$$

$$\text{Sample total } [UBI] = (\text{ddPCR}[UBI]) * \text{Dilution factor}$$

A normalizer factor for each biological replicate from the reference gene is determined by dividing each "Sample total $[UBI]$" by the highest Sample total $[UBI]$. This is done to retain units (copies/µL):

$$(\text{Normalizer factor/replicate}) = (\text{Sample total } [UBI])/(\text{Highest sample total } [UBI])$$

Normalization of ds-*v-ATPaseA* for one biological replicate:

$$\text{Normalized [ds-}v\text{-}ATPaseA\text{]or R} = (\text{Sample total [ds-}v\text{-}ATPaseA\text{]})/(\text{Normalizer factor/replicate})$$

Determining ds-*v-ATPaseA* concentration for treatment group (i.e. exposure concentration, plant height, day, etc.). "R" represents the normalized ds-*v-ATPaseA* concentration and the numeric represents each independent biological replicate (N):

$$\text{[ds-}v\text{-}ATPaseA\text{]} = (\text{R1} + \text{R2} + \text{R3} + \cdots \text{R}_\text{N})/(\text{N})$$

Curriculum Vitae

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Ludba, K., Donly, C., Thompson, G., Scott, I. 2016. Fatal attraction: the volatile influences that will lead whiteflies to deadly encounters and the dsRNA responsible. Ontario Pest Management Conference. Guelph,

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