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## Characterization of Acaricide Resistance, Plant-Mediated RNAi Against Two-Spotted Spider Mites (*Tetranychus urticae* Koch), and Assessing Off- and Non-Target Effects

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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## Abstract

The two-spotted spider mite (TSSM), *Tetranychus urticae* (Koch), is one of the most damaging agricultural pests in the world. It feeds on over 150 crops, causing considerable yield losses in greenhouses and agricultural fields. Currently, using synthetic acaricides is the main method to control TSSM. However, it can develop resistance to acaricides with repeated exposure, and typically resistance can occur within two to four years. To understand the underlying mechanisms of spider mite adaptation to acaricides is an essential part of resistance management strategy. The resistance ratio of the pyridaben-selected strain compared with the pre-selection strain was estimated at greater than 3000-fold. The results of synergism experiments indicated that detoxification by cytochrome P450 enzymes may be the major resistance mechanism to pyridaben by the spider mite population studied. Piperonyl butoxide (PBO), an inhibitor of cytochrome P450 monooxygenases, can be used as an effective synergist to control pyridaben resistant populations of spider mites. RNA interference (RNAi) can be applied as an alternative pest management strategy with less likelihood of developing resistance. Plant-derived *Vacuolar-type H<sup>+</sup>-ATPase* RNAi through dsRNA ingestion in spider mites resulted in a significant decrease of the level of *V-ATPase* transcripts by 36%, while fecundity was reduced by 28%. Unwanted non-target effects often hamper the application of plant-derived RNAi pest management. However, no non-target RNAi effect on mortality and gain weight of cabbage looper and green peach aphid exposed to dsRNAs targeting *Vacuolar-type H<sup>+</sup>-ATPase* RNAi through dsRNA ingestion was observed. These results represent a potential delivery method of dsRNA to control spider mites through transgenic plants.

## Keywords

*Tetranychus urticae*, Pyridaben, METI acaricides, Cytochrome P450, *Arabidopsis*, RNA interference, Transgenic plants, hpRNA, *Vacuolar-type H<sup>+</sup>-ATPase*, Off-Target effect, Non-target effect.

## Co-Authorship Statement

A part of Chapter 3 was published in PLoS One<sup>1</sup>, that was coauthored by Dr. Vojislava Grbić, Dr. Miodrag Grbić, Dr. Pierre Hilson, Dr. Vladimir Zhurov, Dr. Takeshi Suzuki, Dr. Maria Andreia Nunes, Dr. Maria Urizarna Espana, Dr. Tawhid Rahman, Nicolas Bensoussan, Pengyu Jin, and Rebecca De Clercq. The efficiency of RNAi knock-down in *Tetranychus urticae* using five methods of double-stranded RNA (dsRNA) delivery was compared. These methods include dsRNA ingestion through floating leaves on a dsRNA solution, dsRNA-expressing plants, artificial diet supplemented with dsRNA, dsRNA-coated leaves, and dsRNA soaking. Spider mite *TuVATPase* transgene was cloned and transformed into *Arabidopsis thaliana* plants by Dr. Pierre Hilson, and homozygous seed selection experiments were performed by Dr. Tawhid Rahman. I performed the rest of the plant derived RNAi experiments, analyzed data, and wrote the manuscript for the dsRNA-expressing plants delivery method, while my supervisor secured funding, wrote and polished the final manuscript, and submitted it to the journal.

---

<sup>1</sup>Suzuki T., Nunes M.A., España M.U., Namin H.H., Jin P., Bensoussan N., Zhurov V., Rahman T., De Clercq R., Hilson P., Grbić V. Grbić M. 2017. RNAi-based reverse genetics in the chelicerate model *Tetranychus urticae*: A comparative analysis of five methods for gene silencing. PLoS ONE, 12 (7), e0180654.

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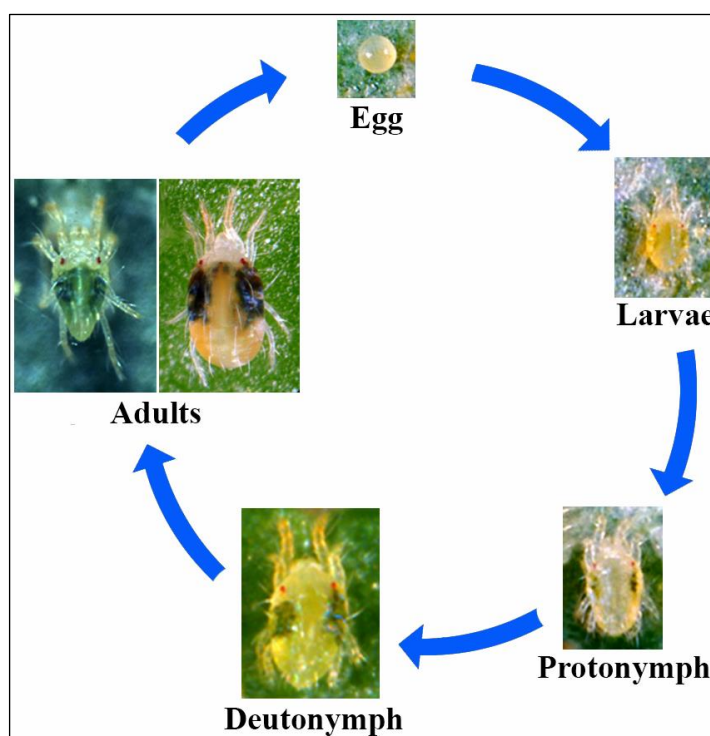
## Chapter 1

### 1 General Introduction

#### 1.1 *Tetranychus urticae* biology, life cycle and host plants

The two-spotted spider mite (TSSM), *Tetranychus urticae* (Koch) is an agricultural pest belonging to a group of web-spinning mites of the subphylum Chelicerata (mites, ticks, spiders, scorpions and horseshoe crabs) in the phylum Arthropoda. The life cycle of TSSM passes through five developmental stages of egg, larva, protonymph, deutonymph and adult (Figure 1.1.1). Two-spotted spider mite has a short generation time and can complete its life cycle, from egg to adult, in about seven days under favourable temperature (27 °C) and low humidity (55-60%) (Shih et al 1976). TSSM is a sexually dimorphic species; males are smaller with a tapered posterior end to their body, while females are larger and more rounded in shape. Males usually complete their nymph development earlier than females; upon emergence of the adult, males search for and wait beside a female deutonymph in the resting state (Penman and Cone 1972; Mitchell 1973). Copulation occurs soon after an adult female emerges (Helle and Sabelis 1985). Egg laying by females can begin as early as one or two days after adult female molting. Oviposition begins with an average of 5 or 6 eggs laid per day which can increase up to 12 eggs per day during the female's lifetime (Helle and Sabelis 1985). Eggs are translucent pearl-like spheres and are deposited singly. Eggs hatch in about 5 days in optimum conditions of 25-30 °C and 45-55% relative humidity, and each female may lay up to 100-150 eggs in her 30-day life span (Helle and Sabelis 1985). Females can switch to produce diapausing eggs that remain in a diapausing stage as the common overwintering response to unfavourable conditions such as short day lengths, cooling

temperatures or decline in the quality of the host plant (Mitchell 1973). Females that enter diapause stage can become brightly red in colour due to accumulation of carotenoid compounds in the body to hinder freezing and desiccation (Helle and Sabelis 1985). TSSM has arrhenotokous parthenogenetic reproduction, in which females are diploid while males are haploid. Fertilized eggs produce diploid females while unfertilized haploid eggs develop into males (Oliver 1971). Thus, unmated and unfertilized females can lay eggs that give rise exclusively to haploid males (Hebert 1981). This reproductive cycle is particularly useful in areas where bisexual reproduction may be hindered by unfavourable environmental conditions (Helle and Sabelis 1985). The sex ratio is approximately one male to three females which produces more offspring and a faster population expansion (Helle and Sabelis 1985).



**Figure 1.1.1.** Life cycle of TSSM consists of five developmental stages of egg, larva, protonymph, deutonymph and adult. TSSM can complete its life cycle, from egg to adult, in about seven days under favourable.

TSSM has a small genome (90 Mb) and belongs to the subphylum Chelicerata, the second largest group of animals next to the insects. It has a very short generation time and is easy to maintain and breed in a laboratory, which makes this species a good model organism to study. TSSM is an extreme polyphagous species and feeds on a wide range of over 1100 plant species from more than 140 plant families (Bolland et al 1998). It is a major pest of greenhouse and field crops, infesting many annual and perennial commercial crops such as tomatoes, peppers, cucumbers, strawberries, maize, soy, apples, grapes and citrus (Grbić et al 2011). Plants produce a wide variety of defense compounds which can affect herbivore fitness either by changing their behavior or by being directly toxic to them. The ability to metabolize and detoxify plant secondary metabolites is an important evolutionary trait of herbivores during their coevolution with plants. In general, polyphagous species such as TSSM have a greater capacity to detoxify plant phytochemicals and acaricides than specialist species (Krieger et al 1971; Dermauw et al 2013).

## 1.2 Crop damage of *Tetranychus urticae*

TSSM feeds from the lower epidermis cells by disrupting the leaf tissues to extract the cellular content, resulting in destruction of the individual palisade cells and spongy parenchyma cells (Campbell et al 1990). As a consequence, the rate of plant photosynthesis is reduced and tissue desiccation leads to stomatal closure (Freitas et al 2009). TSSM feeding causes necrotic spots, leaf bronzing, and even plant death in severe infestation. An adult TSSM consumes about 6 cells per hour (Bensoussan et al 2016). Yield losses caused by TSSM feeding approach 15 % for strawberries, 14 % for corn, 14-44 % for cotton, and 23 % for cucumber (Atanassov 1997; Powell and Lindquist 1997).

### 1.3 Acaricide as a predominant control strategy of TSSM

The reduction of yield losses caused by insect pests is a major challenge to agricultural crop production, and control of TSSM populations mostly relies on the use of acaricides or miticides. Acaricides are pesticides designed specifically to control the pest species of mites in the sub-class of Acari. However, frequent applications of acaricides can cause the microevolution of resistance development by TSSM. This arthropod pest has developed resistance to almost all registered acaricides. Selection for resistance in TSSM is accelerated by its high fecundity, its short life cycle and by arrhenotokous reproduction (Helle and Sabelis 1985; Croft and van de Baan 1988; Houck 1994). These specific life-history parameters facilitate early selection of potential recessive resistance genes and rapid spread of resistance to the offspring often after only a few applications.

Development of the resistance is even faster under greenhouse conditions due to the prolonged growing season, lack of natural enemies, and higher frequency of acaricide applications (Cranham and Helle 1985). Often when new acaricides with different modes of action become available, farmers will continue to use older products due to the lower cost, but this can result in the acceleration of the selection process (Vassiliou and Kitsis 2013). TSSM can develop resistance to a newly introduced acaricide within two to four years, or in some cases resistance to a new compound is already present because of cross-resistance with other older compounds from the same class or mode of action (Van Leeuwen et al 2010). Organotin compounds such as fenbutatin oxide and hexythiazox were among the earliest acaricides used to control TSSM from the early 1990s until the emergence of resistance in the mid-1990s (Mizutani et al 1998; Herron et al 1993; Yamamoto et al 1995). Mitochondrial Electron Transport Inhibitors (METIs) such as

pyridaben, tebufenpyrad and fenpyroximate were launched in the late-1990s as more species specific acaricides. However, resistance to METIs began to occur within 10 years after their introduction (Herron et al 1993; Goka et al 1998; Devine et al 2001; Nauen et al 2001). To date, 424 cases of resistance to 93 active ingredients of acaricides have been reported for TSSM (Van Leeuwen et al 2009) making it the most resistant arthropod pests globally in terms of resistance levels and the number of chemicals that have reduced susceptibility (Van Leeuwen et al 2009, 2010).

## 1.4 Mechanisms of insecticide resistance

According to the Insecticide Resistance Action Committee (IRAC), resistance to acaricides is defined as “a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species”. Acaricide resistance can result from the selection of one or more mechanisms including behavioral modification, integument alterations, sequestrations, metabolic resistance and molecular mutations. Behavioral changes involve minimizing or avoiding contact between the arthropod and the acaricide or insecticide. This type of resistance has been observed mainly with mosquitoes and cockroaches among the arthropods (Lockwood et al 1984; Wada-Katsumata et al 2013). Integument alterations or cuticle resistance includes certain alterations in the cuticle which may result in the reduction of acaricide penetration. Cuticle resistance is an unspecific modification and may not necessarily result in high levels of resistance alone. However, this mechanism can interact synergistically with other resistance mechanisms and cause resistance in arthropods to a wide range of different pesticides. Cuticle resistance can be associated with the increased expression of



cuticle genes, as seen in *Anopheles* sp. and *Cimex lectularius* (Awolola et al 2009; Vontas et al 2007; Koganemaru et al 2013). Sequestration is defined as “the deposition of secondary plant metabolites or chemicals into specialized tissues or glands of an insect” (Duffey 1980). This localization of molecules can mediate defense or communication against natural enemies, and has been reported in several herbivores (reviewed in Duffey, 1980). Sequestration of insecticide leads to storage of pesticides in fat bodies, ultimately preventing them from reaching the site of action.

Metabolic resistance, or enhanced detoxification of acaricides, occurs by increased enzymatic activity, for example esterases (ESTs), glutathione-S-transferases (GSTs) and P450 monooxygenases (P450s), caused by the up-regulation of detoxification enzyme genes or gene duplication (Oakeshott et al 2005; Feyereisen 2005; Enayati et al 2005; Li et al 2007; Demaeght et al 2013; Riga et al 2014). Modification of acaricides through enzymatic activity is one of the main mechanisms of resistance which may lead to cross-resistance among different classes of insecticides (Ranson et al 2011). Molecular mutations in the target site can result in insensitivity of the target site to acaricide binding (Knowles 1997; Horowitz and Denholm 2001; Liu et al 2006). High level of insecticide resistance can arise from a combination of multiple resistant mechanisms. For example, enhanced cytochrome P450 activity associated with insecticide resistance catalyzes cuticular hydrocarbon production has been reported in *Anopheles gambiae* (Balabanidou et al 2016; Yahouédo et al 2017).

## 1.5 The requirements for integrated pest management (IPM) of TSSM

IPM focuses on long-term prevention of pests and their damage through a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, use of resistant varieties, and minimizing the use of pesticides (Kos et al 2009). The main biological control methods of TSSM include utilization of predatory mites, entomopathogenic fungi, plant-extracts and essential oils. *Phytoseiulus persimilis* (Acari: Phytoseiidae) is a specialist predator of *Tetranychus* species that is used widely in many greenhouses to control mite species (Gerson and Weintraub 2012). Each adult *P. persimilis* is able to consume approximately 34 eggs or 5 TSSM females per day (McMurtry and Croft 1997). However, there are some limitations to use of this biological agent; for example, a relative humidity of 90% is necessary for their eggs to hatch. Similarly, *P. persimilis* is not effective on some specific plant crops such as tomatoes because it is hampered by triacylglycerols and some sesquiterpenes molecules released by the glandular trichomes (Kennedy 2003). A number of entomopathogenic fungi including *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii* and *Hirsutella thompsonii* have been evaluated for controlling TSSM (Zhang et al 2014; Shi and Feng 2009; Shah and Pell 2003; Chandler et al 2005; Ghosh et al 2007; Bugeme et al 2009). However, the fungi viability and ability to infect the host is highly reliant on the temperature and humidity, factors that should be monitored in any biological control program (Dimbi et al 2004; Tefera and Pringle 2003; Maniania et al 2008). The potential acaricidal activity of many plant extracts such as *Lolium perenne* L., *Anthemis vulgaris* L., *Chenopodium album* L., *Marjorana hortensis* L., *Chamomilla recutita* L. and *Eucalyptus* sp. have been shown to reduce egg hatchability and increase

adult mortality in TSSM in several studies (Yanar et al 2011; Abd El-Moneim et al 2012). However, adaptation to the plant metabolites by TSSM can occur after repeated applications (reviewed in Dermauw et al 2013). Currently, the control of mite species is mainly limited to the application of acaricides, resulting in several problems. Most of the acaricides have a more adverse effect on the predators of TSSM than on TSSM themselves, which may increase the risk of a mite outbreaks over time (Helle and Sabelis 1985). Acaricides also have undesirable effects on non-target organisms, such as beneficial pollinators, and can be hazardous to the environment and human health (Choi et al 2004; Van Leeuwen et al 2007, 2010). Finally, increased use of acaricides in intensified agricultural systems has caused escalating problems with the evolution of acaricide resistance in TSSM (Van Leeuwen et al 2009, 2010). This situation has created a need to develop alternative control strategies with a novel independent mode of action that has the least negative environmental effects.

## 1.6 Introduction to RNA interference

RNA interference (RNAi) is an example of a gene regulation mechanism via silencing individual genes. This mechanism initiates through formation of a population of small RNAs that bind to the target transcript and results in post-transcriptional gene silencing (Fire et al 1998). The RNAi pathway typically starts by the activity of a ribonuclease III enzyme (Dicer), that cleaves double stranded RNA molecules into small duplexes of short RNAs 20-30 bp in length (Burand and Hunter 2013). After unwinding RNA duplexes, one strand “antisense” is coupled to an endonuclease enzyme “Argonaute (AGO)” and is incorporated into a complex called RNA-induced silencing complex (RISC). The antisense strand guides the RISC complex to target the mRNA molecules

with the sequence complementary to itself thus resulting in post-transcriptional gene silencing. The full complementarity between the short interfering RNAs (siRNA) and the target mRNA results in degradation of mRNA while less complementarity results in translational repression or induction of chromatin modifications of the target gene. RNAi is an evolutionarily conserved mechanism of post-transcriptional gene silencing that is found across all eukaryotes and represents an active defense response against viral infection (Obbard et al 2009; Ding 2010). Because the core RNAi machinery is present in all arthropods, it is theoretically possible to apply this mechanism as a species-specific management strategy for virtually any pest species by disrupting the expression of essential genes (Baum et al 2007; Whyard et al 2009). RNAi can be developed as a potential method for controlling pest arthropods by silencing genes encoding proteins with essential functions for their survival and reproduction (Huvenne and Smaghe 2010; Burand and Hunter 2013).

## 1.7 Methods of dsRNA delivery

Double stranded RNA can be delivered to the insect body through three common methods of delivery including microinjection, ingestion, and soaking (Fire et al 1998; Tabara et al 1998; Maeda et al 2001; Turner et al 2006; Baum et al 2007). Microinjection was one of the first methods of dsRNA delivery into model organisms such as nematodes (*Caenorhabditis elegans*) and fruit flies (*Drosophila melanogaster*) (Fire et al 1998; Kennerdell and Carthew 1998). Microinjection is a highly efficient way to deliver the dsRNA as it can be used to deliver the accurate amounts of dsRNA into the specific target organ (Yu et al 2013). Most of the RNAi studies have applied it as a powerful reverse genetic tool for investigating gene function. RNAi silencing through dsRNA

microinjection into larvae among the chelicerates has been used to elucidate the functions of the *Distal-less* gene in TSSM and several ribosomal genes in the cattle tick (*Rhipicephalus microplus*) (Khila and Grbić 2007; Kurscheid et al 2009). However, microinjection is not an appropriate method of dsRNA delivery for the purpose of insect pest management. RNAi delivery by ingestion and soaking are of particular interest for insect control (Baum et al 2007). Efficient RNAi silencing through dsRNA ingestion in chelicerates was shown in deer tick (*Ixodes scapularis*), varroa mite (*Varroa destructor*), TSSM (*Tetranychus urticae*), and predatory mite (*Phytoseiulus persimilis*) (Soares et al 2005; Garbian et al 2012; Ozawa et al 2012; Kwon et al 2013). dsRNA soaking and spraying are easy and efficient methods of RNAi for pest management. The first demonstration of dsRNA spraying was carried out in the mosquito *Aedes aegypti*. In this study, RNAi silencing of an inhibitor of apoptosis protein 1 gene (*AaeIAP1*) by dsRNA spraying on the dorsal thorax of adult females resulted in significant mortality in these mosquitoes (Pridgeon et al 2008). Subsequently, further studies with dsRNA soaking of eggs and larvae of Asian corn borer (*Ostrinia furnacalis*) resulted in significantly decreased egg hatchability, stunting of larvae developmental and death (Wang et al 2011). For the soaking method, dsRNAs can be produced through *in vitro* enzymatic or chemical synthesis, but these methods may be costly to use in any IPM programs for agricultural field practices (Gu and Knipple 2013). Developing transgenic plants to initiate RNAi silencing in insect pests has attracted considerable attention in recent years. Transgenic plants can continuously produce adequate amount of dsRNA for delivering to herbivorous insect pests in a cost-effective manner for long-term management (Gu and Knipple 2013).

## 1.8 Plant-derived RNAi as a novel pest management approach

The idea of developing transgenic plants capable of expressing dsRNA offers an opportunity to control pests of economic importance in agriculture. The host plants are transformed via *Agrobacterium tumefaciens* carrying vectors with inverted repeats of the target pest gene sequences. After these inverted repeats are transcribed in the plants, they form hairpin RNAs (hpRNAs) that are functionally equivalent to linear dsRNAs. RNAi gene silencing is initiated in insect herbivores upon ingestion of expressed hpRNAs from the transgenic plants. Many studies have demonstrated the potential of transgenic plant-mediated RNAi to target the essential genes that encode important proteins in the insects' growth, development and survival. The potential of this RNAi-based pest management strategy was revealed for the first time in two articles in 2007. Baum and colleagues (2007) demonstrated that dsRNA expression of essential pest-specific genes in transgenic corn plants can trigger gene silencing in the western corn rootworm (WCR) *Diabrotica virgifera virgifera*. Expressed dsRNA hairpins in corn plants for targeting *Vacuolar-type H<sup>+</sup>-ATPase subunit A* in WCR caused severe rootworm stunting and mortality, and resulted in significant reduction of feeding damage by this pest (Baum et al 2007). In this study, out of the 290 screened target genes, only 14 were identified as efficient target genes that induced significant mortality or growth stunting in WCR through larval feeding on artificial diet supplemented with dsRNA (Baum et al 2007). However, the suppression of other gene transcripts did not result in any phenotypic response in WCR. This indicates that screening and identifying the effective target genes is the major challenge of developing RNAi-based pest management. Different subunits of *Vacuolar-type H<sup>+</sup>-ATPase* have been used as efficient target genes to control many other pest

species in subsequent RNAi-mediated silencing approaches with varying success (Kwon et al 2013; Li et al 2013, 2011a, 2011b; Upadhyay et al 2011). Another study that established the possibility of using transgenic plants to deliver dsRNA focused on engineering *Arabidopsis* plants that express a dsRNA hairpin targeting a *cytochrome P450 monooxygenase* gene (*CYP6AE14*) in the cotton bollworm (*Helicoverpa armigera*) (Mao et al 2007). *Cytochrome P450 monooxygenase* genes encode essential detoxification proteins for this pest to detoxify the cotton metabolite gossypol. RNAi silencing of *CYP6AE14* gene led to decreased bollworm tolerance to the cotton sesquiterpene aldehyde, and consequently resulted in reduced growth. However, reductions in transcript levels of this gene did not cause any significant mortality in cotton bollworm (Mao et al 2007). Developing transgenic plant-mediated RNAi as an alternative pest management method showed promising results in many pest species in the orders of Lepidoptera, Coleoptera and Hemiptera including cotton bollworm in cotton (Mao et al 2011; Mao et al 2015; Qi et al 2015; Chikate et al 2016) and tobacco (Zhu et al 2012; Xiong et al 2013; Mamta et al 2015; Tian et al 2015), tomato leaf-miner (*Tuta absoluta*) in tomato (Camargo et al 2016), Western corn rootworm (*Diabrotica virgifera virgifera*) in maize (Fishilevich et al 2016), brown plant-hopper (*Nilaparvata lugens*) in rice (Li et al 2011a; Zha et al 2011; Yu et al 2014; Qiu et al 2016), green peach aphid (*Myzus persicae*) in cotton (Mao et al 2015), tobacco (Pitino et al 2011; Khan et al 2013; Tzin et al 2015) and *Arabidopsis* (Coleman et al 2014; Li et al 2015) and English grain aphid (*Sitobion avenae*) in wheat (Xu et al 2014). Significant mortality and growth stunting after dsRNA ingestion, highlights the possible potential of RNAi-based pest control using dsRNA-derived transgenic plant technologies.

## 1.9 RNAi off-target and non-target effects

RNAi interference is based on sequence recognition of dsRNA to down-regulate the target genes. However, siRNAs can hybridize with unintended transcripts that have some sequence similarity, resulting in RNAi silencing of these genes (Scacheri et al 2004). This phenomenon is referred to as an off-target effect or cross-reaction, and can occur through two mechanisms: mRNA degradation or translational repression (Elbashir et al 2001; Meister and Tuschl 2004; Echeverri and Perrimon 2006; Davidson and McCray 2011). It has been suggested that if the guide strand of siRNA has three mismatches or less with any mRNAs, it would be silenced in addition to the target mRNA that shares 100% homology with this siRNA sequence (Naito et al 2004; Kim et al 2005). In the second scenario, nucleotide matches as short as six or seven nucleotides can act like the 'seed' region and mediate translational repression or induce chromatin modifications of several unintended targets (Lin et al 2005; Birmingham et al 2006; Jackson et al 2006). The hazards of siRNAs with the unintended target mRNAs through transgenic plants can be categorized as off-target gene silencing in the target organism, and non-target gene silencing in transgenic plants and non-target organisms. Although off-target silencing of the other genes rather than the target gene in the target organism would not appear to be a concern, non-target gene silencing in non-target organisms is a real hazard. Non-target effects for RNAi silencing of ecdysone receptor (*EcR*) in cotton bollworm (*Helicoverpa armigera*) by feeding on transgenic plants resulted in the knock-down of *HaEcR* and *SeEcR* genes in another lepidopteran, beet armyworm, *Spodoptera exigua* (Zhu et al 2012). In general, non-target effects of RNAi silencing are expected to be higher for conserved genes. For example, there is a possibility for non-target RNAi silencing of



highly conserved genes such as Ribosomal proteins (*RPL11*, *RPS2*, *RPL8*), and Proteasome 26S (*PROS26.4*) as was suggested by Wu and Hoy (2014) in a TSSM predator (*Metaseiulus occidentalis*). This suggests the potential for other predatory arthropods used in TSSM control to be negatively affected by RNAi, and should be examined as part of RNAi pest management against TSSM. In addition, non-target effects in transgenic plants can potentially result in unintended changes in plant physiology, fitness and biodiversity. Reduction of pollen viability is one of the examples of this type of non-target effects and has been reported in *Arabidopsis* RNAi transgenic plants (Xing and Zachgo 2007). Generally, using long dsRNAs for gene silencing increases the risk of co-suppression of closely related genes that are highly conserved. Thus, to minimize the possibility of off-target effects, it is necessary to design dsRNAs or siRNAs that have limited sequence similarities with the unrelated genes of non-target organisms or the respective host plant (Fairbairn et al 2007). This selectivity can be exploited to devise RNAi-based pest management strategies that have no or minimal effect to non-target species, and prevent potential problems to the environment.

## 1.10 Rationale and objectives

Evolution of acaricide resistance in pests that have many offsprings per generation can occur more rapidly after the initial outbreak. Therefore, the development of acaricide-resistance in greenhouse populations of TSSM is a growing concern for Canadian growers. Understanding the underlying mechanisms of TSSM adaptation to acaricides is an essential part of resistance management strategy in any IPM program. Pyridaben (Nexter) is a METI acaricides, commonly used to control mite populations in commercial greenhouses. However, development of high levels of resistance in several field

populations of TSSM have been reported (Cho et al 1995; Song et al 1995). The first objective of my thesis (Chapter 2) was to investigate the metabolic and molecular mechanisms that are involved in resistance to pyridaben in TSSM. The original field strain of *T. urticae* (FS) was collected from a cucumber production greenhouse in Southern Ontario. This strain showed reduced susceptibility to bifenthrin (Acramite), acequinocyl (Kanemite), pyridaben (Nexter), formetanate HCl (Carzol) and spiromesifen (Envidor) relative to an acaricide-susceptible laboratory strain through laboratory bioassays (Scott et al 2016). The pyridaben-selected TSSM strain (SFS) was further selected by successful repeated acaricide applications in the lab. Pyridaben sub-lethal concentration ( $LC_{30}$ ) and resistance ratio for FS and SFS was calculated using leaf disk bioassays. The involvement of detoxification enzymes of esterases (ESTs), glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (P450s) for FS and SFS was then evaluated. The mutations in the target site of the catalytic core of the ubiquinone oxidoreductase enzyme including subunits of TYKY, PSST, ND1, ND5 and the 49kDa are believed to be involved in METIs resistance mechanism (Van Pottelberge et al 2009, Schuler and Casida 2001). Therefore, the molecular mechanisms of target site insensitivity-mediated resistance in pyridaben resistant strain of TSSM was investigated by comparing the DNA sequence of TYKY, PSST, ND1, ND5 and the 49kDa subunits from SFS to FS. The results of this study are interpreted in terms of potential resistance diagnostics, to facilitate the choice of alternative acaricides to control the pyridaben resistant strain of TSSM in IPM programs.

The second objective (Chapter 3) was to evaluate plant-derived RNAi as delivery system of dsRNA to TSSM through ingestion, and to study the impact of plant-derived RNAi

constructs on spider mite fitness, reproduction and target gene expression after dsRNA ingestion. Developing RNAi-expressing plants involves generation of transgenic plants through *Agrobacterium*-mediated transformation that constitutively produce stable dsRNA. RNAi silencing of the genes involved in essential developmental processes or metabolism is assumed to provide a more efficient pest management strategy by causing high lethality in pests. In this study, five specific genes involved in essential developmental processes in spider mites including *Vacuolar-type H<sup>+</sup>-ATPase*, *ABC-transporters* subclass E, *Prospero*, *Octopamine receptor* and *Acetylcholinesterase* were chosen to be expressed in *Arabidopsis* plants. After transformation of *Arabidopsis* plants, selection of homozygous plants carrying a single or multiple copies of the transgene was carried out. The expression of hairpin RNA (hpRNA) in transgenic homozygous plants was confirmed by quantitative real time PCR (qRT-PCR). To avoid the TSSM escaping from the *Arabidopsis* plants, detached leaves were infested with synchronized larvae in specific cages. To confirm the continued expression of the transgene by the CaMV 35S (Cauliflower Mosaic Virus) promoter in transgenic leaves after detaching from the plants, the stability of *Vacuolar-type H<sup>+</sup>-ATPase* hpRNAs during 7 days was assessed. The impact of plant RNAi constructs on the spider mites' development, fecundity and mortality were evaluated through feeding of spider mites on transgenic detached leaves. Finally, the down-regulation of target genes in spider mites fed on RNAi plant lines compared to mites feeding on control plants was evaluated using qRT-PCR. The screening of the transgenic RNAi lines as a successful RNAi-pest management strategy against TSSM has been determined according to their effects on TSSM fitness parameters and down-regulation of the target gene.

A major challenge of developing plant-derived RNAi pest management against TSSM is to ensure minimal off- and non-target effects. My last objective (Chapter 4) was to evaluate the effects of plant RNAi constructs on non-target organisms including the *Arabidopsis* host plant, cabbage looper (*Trichoplusia ni*) and green peach aphid (*Myzus persicae*), and the off-target effects on TSSM. In general, off- and non-target effects can occur if the siRNAs have some sequence similarity with unintended gene or genes. To minimize this possibility, the sequence homologies between the transgenes and DNA sequence of *Arabidopsis*, cabbage looper, peach aphid and TSSM were examined based on DNA Basic Local Alignment Search Tool (BLAST) in NCBI and ORCAE databases. The siRNAs having 18 out of 21 identical nucleotides with any mRNAs would likely cause RNAi silencing of these mRNAs through off- or non-target gene silencing (Naito et al 2004; Kim et al 2005). The existence of similarity between the transgene sequence and the DNA sequences from *Arabidopsis*, cabbage looper, green peach aphid, and TSSM, represents the potential side effects of this pest control technology. After screening of the transgenic RNAi lines based on their effects on TSSM fitness parameters, the transgenic plants having the strongest effect on TSSM were used to examine their non-target effects on mortality of green peach aphid and the weight gain of cabbage looper larvae through dsRNA ingestion.

The findings of all 3 research chapters were integrated and summarized into Chapter 5 to provide conclusions and recommendations.

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## Chapter 2

### 2 Molecular and metabolic mechanisms of pyridaben resistance in TSSM

#### 2.1 Introduction

Pyridaben (Nexter) is used commonly to control mite pest species in tomatoes, cucumbers, apples, cherries, grapes, pears, strawberries and peach in greenhouses and orchards in Canada. However, TSSM control in greenhouses has become more challenging due to development of resistance to acaricides such as pyridaben by local TSSM populations. Pyridaben is harmful to certain beneficial insects such as predatory mites (*Phytoseiulus persimilis*). Using higher concentration of pyridaben to control the resistant population of TSSM in greenhouses often results in destruction of these biological agents and may intensify rather than reduce the TSSM infestation level. In addition, using products from the same class of acaricides such as cyenopyrafen and spirodiclofen to control the resistant strain of TSSM may also result in further selection of resistance in this population. Understanding the mechanisms underlying the pyridaben resistance in pyridaben-selected TSSM population is necessary to develop multiple chemical and non-chemical management strategies for these resistant populations.

##### 2.1.1 Mitochondrial Electron Transport Inhibitor (METI) acaricides

The METI acaricides target the electron transport in the mitochondrial respiratory chain which provides the driving force for ATP synthesis in mitochondria (Hollingworth and Ahammadsahib 1995, Wood et al 1996; Lümmer 2007; Karp 2008). METI-acaricides were developed in the 1990s and quickly gained popularity worldwide due to their quick knockdown effect, long residual activity and their high selectivity against all life stages of



several important pest mite species (Konno et al 1990; Kyomura et al 1990; Longhurst et al 1992; Hirata et al 1995). The inner membrane of the animal's mitochondria consists of five transmembrane complexes, including complex I to V (Schultz and Chan 2001). These transmembrane complexes mediate electron transport via several redox reactions from nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FADH<sub>2</sub>) to oxygen as a final electron acceptor. Generally, the METI acaricide modes of action can be categorized into two groups: 1) some are uncouplers preventing the formation of the proton gradient in the mitochondria; 2) others are specific inhibitors and probably bind to a subunit of the associated electron transport particles (Hollingworth and Ahammadsahib 1995). The METI acaricide family is divided into four classes, complex I, II, III and IV by the Insecticide Resistance Action Committee (IRAC) (Sparks and Nauen 2015).

### 2.1.2 METI acaricide classes

Complex I or NADH: ubiquinone oxidoreductase is the first and the most complex electron transport complex. It oxidizes NADH and transfers the electrons via a flavin mononucleotide cofactor and several iron-sulfur clusters to ubiquinone Q (Kühlbrandt 2015). Complex I METI acaricides include fenazaquin, fenpyroximate, flufenerim, pyrazoles, pyridaben, pyridazinones, pyrimidinamines, pyrimidifen, tebufenpyrad, tolfenpyrad, and quinazolines and are classified by IRAC as Group 21 insecticides (Hollingworth and Ahammadsahib, 1995; Hollingworth et al 1994). Among the Complex I METI acaricides, pyridaben has been used the most frequently for pest management due to its high efficacy against a broad number species of mites, whiteflies, aphids, leafhoppers, mealybugs, and thrips (Sparks and De Amicis 2007; van Leeuwen et al

2010). Additionally, pyridaben is compatible with many entomopathogenic fungi such as *Beauveria bassiana* when applied for the management of red mite, *Panonychus citri* (Kodandaram et al 2010). Complex II or succinate-quinone oxidoreductase, is a parallel electron transport pathway to complex I, that oxidizes succinate to fumarate in the Krebs cycle and reduces ubiquinone Q in the respiratory chain (Quinlan et al 2012). Complex II METI acaricides (IRAC Group 25) include beta-ketonitrile derivatives, cyenopyrafen and cyflumetofen, which have been commercialized specifically for spider mite control (Tomlin 2009; Nakahira 2011; Hayashi et al 2013). Complex III or coenzyme Q (cytochrome bc1), has an important role in biochemical generation of ATP by an asymmetric absorption and release of protons (Kühlbrandt 2015). Complex III METI acaricides (IRAC Group 20), including acequinocyl and bifenazate are inhibitors of cytochrome b at the Q-site (Van Leeuwen et al 2008; Van Nieuwenhuysse et al 2009). Complex IV or cytochrome c oxidase establishes a transmembrane difference of proton electrochemical potential to assist enzyme ATP synthase (Complex V) for the last step of ATP synthesis in mitochondria (Kühlbrandt 2015). Complex IV METI acaricides (IRAC Group 12) include organotin compounds, such as cyhexatin, diafenthiuron and fenbutatin oxide, that directly interfere with the process of ATP synthesis (Carbonaro et al 1986; Kadir and Knowles 1991; Miyasono et al 1992; Ruder and Kayser 1994).

### 2.1.3 Resistance mechanisms to METI acaricides

Target site mutations are one of the most common mechanisms associated with acaricide resistance in TSSM. For example, mutations in acetylcholinesterase (*AChE*), glutamate-gated chloride channel (*GluCl*), cytochrome *b* (*cytb*), voltage-gated sodium channel (*VGSC*), and chitin synthase (*Chs*) genes resulted in target site insensitivity to

organophosphate, abamectin, bifentazate, pyrethroid, etoxazole, hexythiazox and clofentezine respectively (Van Leeuwen et al 2008, 2012; Van Nieuwenhuysse et al 2009; Tsagkarakou et al 2009; Khajehali et al 2010; Kwon et al 2010a, 2010b; Demaeght et al 2014; Dermauw et al 2012). Target site mutations are also associated with resistance to METI acaricides, including pyridaben. Complex I of mitochondria in TSSM consists of 14 subunits, with TYKY, PSST, ND1, ND5 and the 49kDa subunits known as the catalytic core of the quinone reduction site of the enzyme (Lümmen 2007; Bajda et al 2017). Nuclear PSST and 49kDa subunits encode proteins to form a pocket located in the proximal half of the ubiquinone and inhibitor binding site, while the mitochondrial subunits ND1 and ND5 form the access path towards this site (Earley et al 1987; Murai et al 2009, 2011; Schuler and Casida, 2001). Pyridaben resistance can be both autosomally or maternally inherited (Stumpf and Nauen 2001). A mutation in ND1 or ND5 could result in maternal inherited resistance, while mutations in 49kDa and PSST subunits can result in autosomal inherited resistance (Goka 1998; Devine et al 2001; Stumpf and Nauen 2001; Sato et al 2004; Lümmen 2007; Bajda et al 2017). Heterozygous females of crosses between resistant males (R) and susceptible females (SS) have been shown to have lower levels of resistance to pyridaben and fenpyroximate than those heterozygotes resulting from crosses between RR females and S males (Stumpf and Nauen 2001). Thus, as only the maternally inherited METI resistance is fully dominant, mutations in subunits of ND1 and ND5 genes are likely responsible for the high level of resistance to pyridaben (Stumpf and Nauen 2001). However, the maternally inherited METI resistance has not yet been clearly supported (Sugimoto and Osakabe 2014).

Resistance to Complex I METIs acaricides in TSSM is commonly associated with cross-resistance to other METI acaricides, indicating that increased P450 activity is another important detoxification mechanism by TSSM (Hollingworth and Ahammadsahib 1995; Stumpf and Nauen 2001; Kim et al 2004, 2006; Van Pottelberge et al 2009). Although increased carboxyl esterase activity has also been reported for pyridaben resistance in TSSM by (Van Pottelberge et al 2009), detoxification by cytochrome P450 is the main mechanism of pyridaben resistance (Stumpf and Nauen 2001; Kim et al 2006; Sugimotoa and Osakabe 2014).

#### 2.1.4 Objectives

Today the major emphasis in resistance research lies in unravelling the underlying molecular mechanisms that control the development of resistant populations. To design an effective and sustainable TSSM resistance management strategy, it is important to reveal the mechanisms underlying the adaptation of TSSM populations to acaricides. A population of TSSM was originally collected from a cucumber production greenhouse (FS), and a pyridaben-selected resistant strain (SFS) was selected through exposure to pyridaben in the laboratory. The FS population had been exposed to bifenazate (Floramite) on cucumber in the greenhouse and laboratory bioassays with several acaricide classes determined there was reduced susceptibility to bifenazate (Acramite) relative to a susceptible laboratory strain (Scott et al 2016). The FS strain was also resistant to acequinocyl (Kanemite), pyridaben (Nexter), formetanate HCl (Carzol) and spiromesifen (Envidor) (Scott et al 2016). The resistance ratio of the pyridaben-selected strain (SFS)  $LC_{30}$  compared with the pre-selection strain (FS)  $LC_{30}$  was estimated to be greater than 3000-fold. The objective of this research was to determine the possible

resistance mechanism using synergists known to inhibit the major detoxification enzymes, esterases (ESTs), glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (CYPs) and to examine target site mutations in complex I subunits TYKY, PSST, ND1, ND5 and the 49kDa in the lab strain (LS), field strain (FS) and selected field strain (SFS). The results are interpreted in terms of potential resistance diagnostics and to facilitate the choice of alternative acaricides to control the pyridaben resistance strains of TSSM in IPM programs.

## 2.2 Material and methods

### 2.2.1 Acaricides and enzyme inhibitors

Nexter, a formulation of pyridaben (75%) was provided by Gowan Company (Arizona, USA). Enzyme inhibitors of cytochrome-P450, esterases, and glutathione S-transferase including piperonylbutoxide (PBO), S,S,S-tributyl-phosphorotrithioate (DEF) and diethylmaleate (DEM) respectively, were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

### 2.2.2 Spider mites

The laboratory strain (London, LS) is acaricide-susceptible and has been cultured at AAFC London for eleven years with no exposure to pesticides within the past 10 years. The field strain (FS) of TSSM was collected from cucumber plants in a Leamington Ontario commercial greenhouse in 2013, and reared on 3-week-old potted kidney bean plants (*Phaseolus vulgaris* L.) in climatically controlled conditions at  $26 \pm 2$  °C,  $60 \pm 5$  % relative humidity (RH) and 16:8h light: dark photoperiod with a cool-white fluorescent light intensity of  $100\text{-}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The selected field strain (SFS) of TSSM was

selected from the field strain (FS) by repeated applications of pyridaben at the LC<sub>90</sub> determined for the FS two times per year for 3 years in the laboratory.

### 2.2.3 Larval bioassays

To calculate the pyridaben LC<sub>30</sub> (the lethal concentration required to cause 30% mortality in a population), leaf disks (2 cm in diameter) were punched from three week-old kidney bean leaves and dipped into serial dilutions of pyridaben (10-1000 ppm). Two-centimeter diameter leaf disks were dipped into 3 ml solutions of the pyridaben concentrations for 5 seconds and air dried for 20 minutes. Leaf disks were then placed in plastic plates on a wet cotton pad with the upper leaf surface of the disc facing upward. Five age-synchronized, one-day old larvae were then transferred to each leaf disk using a fine brush. The plastic plates were kept in climatically controlled conditions at 26 ±2 °C, 60 ±5 % RH and 16:8 h light: dark photoperiod with a cool-white fluorescent light intensity of 100-150 μmol m<sup>-2</sup> s<sup>-1</sup> and the mortality was assessed on day 4 (n=100 from 4 replicates of 25).

### 2.2.4 Synergism studies

The enzyme inhibitor piperonylbutoxide (PBO), S,S,S-tributyl-phosphorotrithioate (DEF) and diethylmaleate (DEM) were dissolved in 1% ethanol. Based on preliminary tests, each inhibitor concentration (250 ppm for DEF, 2500 ppm for PBO and 8000 ppm for DEM) was chosen based on the highest dose that caused no significant mortality in the lab strain (LS) for 24 hours. Metabolic resistance was assessed by placing five age-synchronized larvae on an inhibitor treated leaf disk for 24 hours. Upon treatment, larvae were transferred to a set of pyridaben-treated leaf disks. The treated leaf disks were kept at 26 ±2 °C, 60 ±5 % RH and 16:8 h light: dark photoperiod with a cool-white fluorescent

light intensity of 100-150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Larval mortality was measured on day 4 and all treatments were replicated four times (n=100 from 4 replicates of 25).

### 2.2.5 Data analyses

The  $\text{LC}_{30}$  values were calculated by probit regression analysis using IBM SPSS Statistics 20.0 (SPSS Statistics 20.0, IBM Corp.). Resistance ratios (RRs) were calculated by dividing the  $\text{LC}_{30}$  value of the resistant strain (SFS) by that of the susceptible strain (FS). The interaction effects of ethanol and inhibitor concentrations on larval mortality were analyzed using the Kruskal-Wallis test in the case of more than two groups, or the Mann-Whitney U test when comparing two groups.

### 2.2.6 PCR amplifications and sequencing

Genomic DNA was extracted by homogenizing 100 females using DNeasy Blood and Tissue Kit (Toronto, ON, Canada). The nucleotide sequences of TYKY, PSST, 49KDa, ND1 and ND5 and their intragenic region were obtained from the ORCAE database (Sterck et al 2012). Primers for PCR amplification of these genes were designed by Primer 3 online software using the default settings and are listed in Table 2.2.6.1. Based on preliminary sequence analysis of these genes for the SFS and the LS TSSM, primer sets of ND13 and ND55 were designed later to amplify the regions with point mutations. The PCR reaction mixtures contained 1x Standard Taq Reaction Buffer, consisting of 10 mM Tris-HCl, 50 mM KCl, 2.5 mM  $\text{MgCl}_2$ , pH 8.3 (New England Biolabs, MA, USA), 200  $\mu\text{M}$  dNTP (Invitrogen, Carlsbad, CA, USA), 0.4  $\mu\text{M}$  of each primer, 1 unit Taq DNA polymerase (New England Biolabs), approximately 1-2  $\mu\text{g}$  of genomic DNA, and purified water to a final volume of 25  $\mu\text{L}$ . PCR was performed using a thermal cycler (Thermo Scientific, Pittsburgh, PA) under the following cycling parameters: 94 °C for 2

min, 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 45 sec, and a final extension for 5 min at 72 °C. The PCR products were visualized by ethidium bromide staining of an agarose gel (0.8%) after electrophoresis and purified using a QIAquick PCR Purification Kit (Toronto, ON, Canada) following the manufacturer's instructions. Sequencing was carried out using an Illumina MiSeq sequencer at London Regional Genomics Centre (Robarts Research Institute, London, ON, Canada). Sequences were assembled, trimmed and aligned using Geneious version 10.1.3 (<http://www.geneious.com>, Kearse et al 2012).



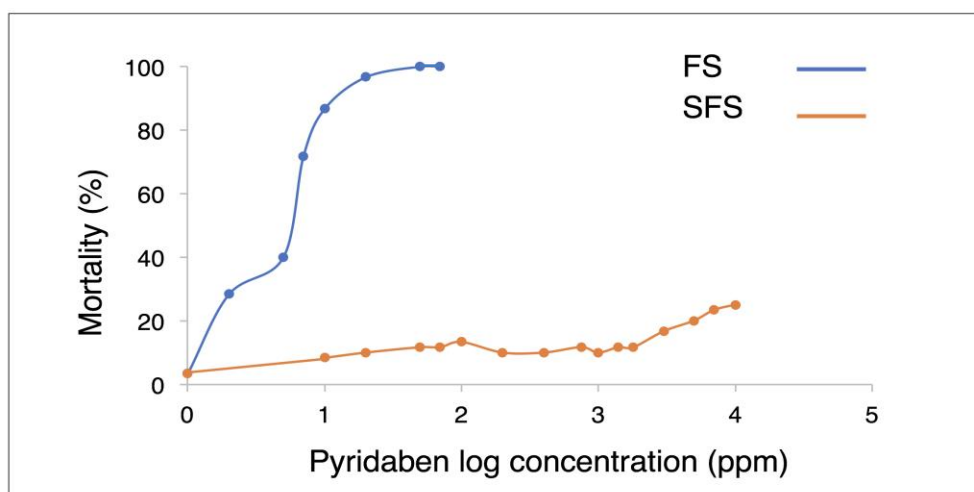
**Table 2.2.6.1. The list of primers, their sequence and PCR product used for amplification of TYKY, PSST, 49KDa, ND1 and ND5 mitochondrial subunits.**

Primer	Sequence	PCR product size
TYKY1 F	TGCAATCTTGGCTATTGTGG	489bp
TYKY1 R	CCATCAGGTCTCTCCTCAGC	
TYKY2 F	AGATTCAGGGGTGAACATGC	512bp
TYKY2 R	CATGATGAATGTTAATGAAGTGAGA	
PSST 1 F:	TGCGTTGTTGTTTATGTGAATTT	575bp
PSST 1 R:	TTGCCTAGGACTTGCTCTGAA	
PSST 2 F:	GTTGTGCCGTTGAAATGATG	564bp
PSST 2 R:	TGACCTTTGCTTTGTCATTCA	
49KDA 1 F:	TTGTTTTCTTTAATCTTTCTCTGCT	525bp
49KDA 1 R:	TCCATGTAGGGTAAGGCTTGA	
49KDA 2 F:	GGAACCGAAAACTGATGGA	526bp
49KDA 2 R:	GAAGCATTACCCCGCTGAAT	
49KDA 3 F:	GCTCGAACCGTTGATATTGG	639bp
49KDA 3 R:	AACTCCAAAAATATTGAAAAGTCCA	
ND1 1 F:	CCCCGTATATTCAATTTCCAA	577bp
ND1 1 R:	TCTCAATTTTATCAATATTTTCGAGTTT	
ND1 2 F:	TCAAGAAAGTCAAATGGTAAACG	749bp
ND1 2 R:	TCTTGATAGAATTATTTTTGTGTTGC	
ND1 3 F:	TCTCAATTTTATCAATATTTTCGAGTTT	255bp
ND1 3 R:	CAATGTAAATCCAGAACTAACTCA	
ND5 1 F:	TTTGTTAATATAGCAATACCTCCTTTT	659bp
ND5 1 R:	TTCTAACCCTTCTCAACCTAAA	
ND5 2 F:	TGTGTCAATTGTTGTTTTAATGTTTT	612bp
ND5 2 R:	CCAATTTGTCTTAATGTTGATAAAGC	
ND5 3 F:	TTTTTACAAAACGAATTCAAATCT	799bp
ND5 3 R:	GAAATCTTTTCCATCCATAATTCA	
ND5 4 F:	GATATTAATGAATTATGGATGGAAAAG	463bp
ND5 4 R:	AGGGGGAACCCCTATCTTTA	
ND5 5 F:	AGGGATAAGAAACATAAACAAATTTT	506bp
ND5 5 R:	TCCTAACCCTTCTCAACCTAAA	

## 2.3 Results

### 2.3.1 Pyridaben LC<sub>30</sub> and resistance ratio determination

The pyridaben dose-response curves for larval mortality of field strain (FS) and selected field strain (SFS) on day 4 are represented in Figure 2.3.1.1. The 30% lethal dose concentration (LC<sub>30</sub>) of the Pyridaben was 3.21 mg/L (95% CL 1.99 - 4.12) for the FS strain and more than 10000 mg/L (95% CL 7902.14 - 17113.21) for the SFS (Table 2.3.1.1). The RR of the resistant strain was more than 3000 times, indicating strong resistance in the selected field strain.



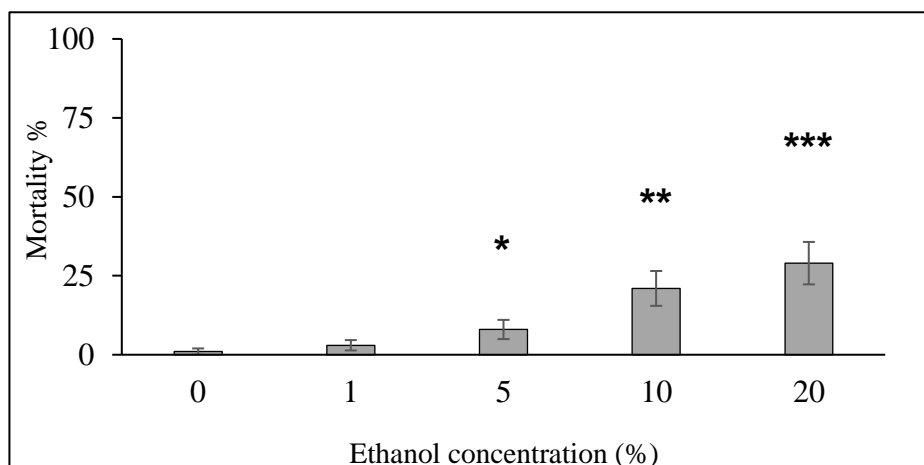
**Figure 2.3.1.1. Pyridaben dose-response curves for larval mortality of the TSSM in field strain (A) and selected field strain (B). The 30% lethal dose concentration (LC<sub>30s</sub>) of the Pyridaben for the FS strain and for the SFS was 3.21 and more than 10000 mg/L respectively (n=80 from 4 replicates of 20 larvae).**

**Table 2.3.1.1. The lethal dose concentration (LC<sub>30</sub>), 95% confidence limit (mg/L) and resistance ratio of pyridaben in field strain (FS) and selected field strain (SFS)**

Strain	Average LC <sub>30</sub> (mg/L)	95% Confidence Limit (mg/L)	Resistance Ratio
FS	3.21	1.99 - 4.12	-
SFS	>10000	7902.14 - 17113.22	>3000

### 2.3.2 Detoxification enzyme inhibition in pyridaben-susceptible (LS) and resistant strain (SFS) TSSM strains

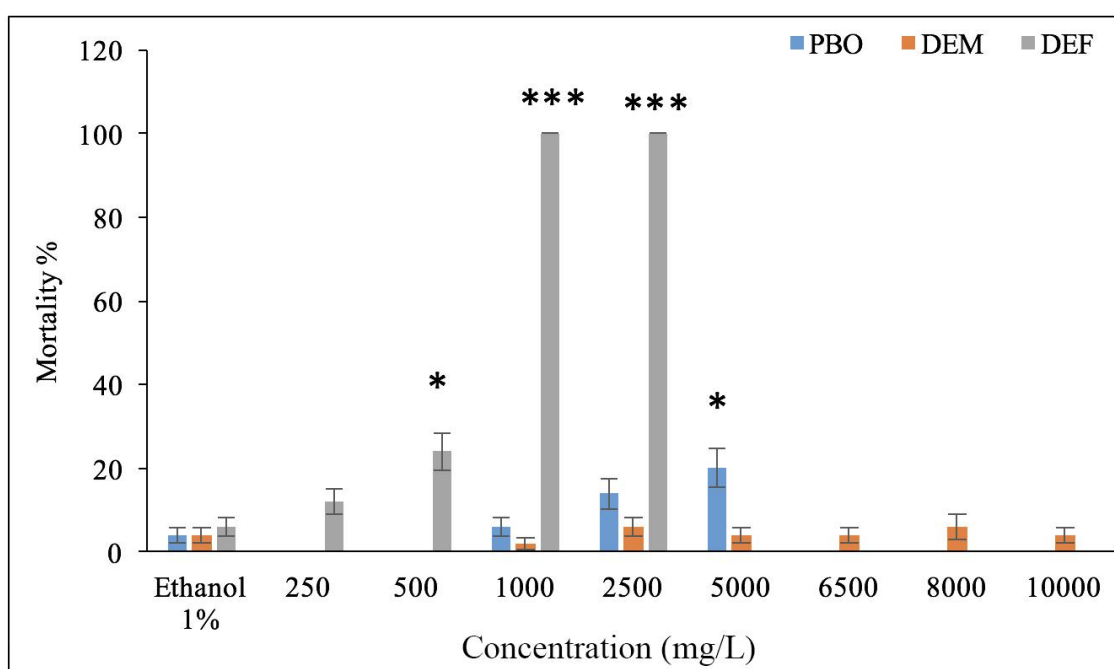
The three synergists piperonyl butoxide (PBO), diethylmaleate (DEM), and S,S,S,-tributyl phosphorotrithioate (DEF) were dissolved in 1% ethanol. This concentration of ethanol was determined to be the highest concentration that caused no larval mortality in the lab strain (LS) after four days (London) (Figure 2.3.2.1). The concentration of 5% ethanol or higher caused significant *T. urticae* larval mortality (Mann-Whitney U; no asterisk,  $P>0.05$ ; \*,  $P<0.05$ ; \*\*,  $P<0.005$ ; \*\*\*,  $P<0.0005$ ;  $n=100$  including 4 replicates of 25 larvae) (Figure 2.3.2.1).



**Figure 2.3.2.1.** The average percent mortality  $\pm$ SEM of TSSM exposed to 4 ethanol concentrations. Concentration of 1% ethanol caused no mortality in TSSM larvae after four days (Mann-Whitney U; no asterisk,  $P>0.05$ ; \*,  $P<0.05$ ; \*\*,  $P<0.005$ ; \*\*\*,  $P<0.0005$ ;  $n=100$  including 4 replicates of 25 larvae).

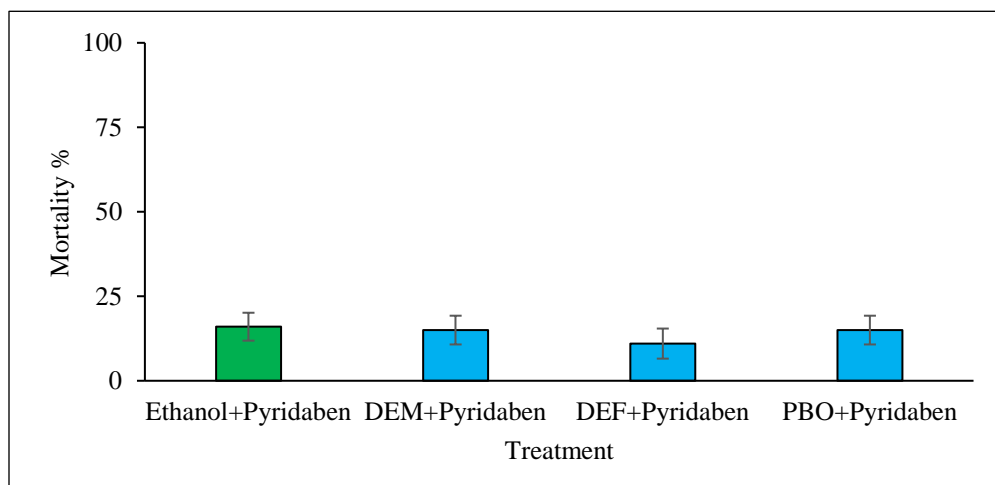
The concentrations of 2500, 8000 and 250 mg/L for PBO, DEM and DEF were used for testing the synergism effects of enzyme inhibitors and pyridaben. The concentrations of PBO and DEF were determined as the highest concentration of inhibitors that caused no larval mortality in LS by feeding on treated leaf disks for 24 hours (Figure 2.3.2.2). The concentrations of 5000, and 500 mg/L for PBO, and DEF caused significant larval

mortality in LS by feeding on treated leaf disks for 24 hours (Mann-Whitney U; no asterisk,  $P>0.05$ ; \*,  $P<0.05$ ; \*\*\*,  $P<0.0005$ ;  $n=100$  including 4 replicates) (Figure 2.3.2.2). Although the highest concentration of DEM (10000 mg/L) did not cause larval mortality, 8000 mg/L caused phytotoxicity to the bean leaf disks (Mann-Whitney U; no asterisk,  $P>0.05$ ,  $n=100$  including 4 replicates of 25 larvae).

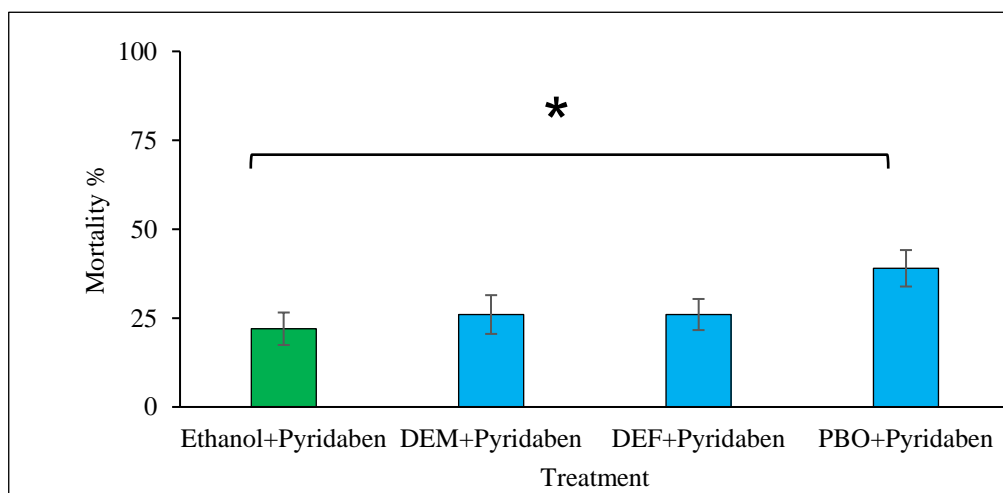


**Figure 2.3.2.2.** The average 24 hour percent mortality  $\pm$ SEM of TSSM exposed to PBO, DEM and DEF concentrations. The concentrations of 2500, 8000 and 250 mg/L for PBO, DEM and DEF were determined as the highest concentration of inhibitors that caused no larval mortality in TSSM after 24 hours (Mann-Whitney U; no asterisk,  $P>0.05$ ; \*,  $P<0.05$ ; \*\*\*,  $P<0.0005$ ;  $n=100$  including 4 replicates).

The combination of PBO and pyridaben caused a significant synergistic effect in the SFS but not FS, indicating the possible role of oxidative metabolism by cytochrome P450-dependent monooxygenases in the establishment of pyridaben-resistance in SFS strain (Mann-Whitney U; no asterisk,  $P > 0.05$ ; \*,  $P < 0.05$ ;  $n = 100$  including 4 replicates of 25 larvae, Figure 2.3.2.3 and Figure 2.3.2.4).



**Figure 2.3.2.3.** The average percent mortality  $\pm$ SEM of TSSM exposed to synergists and pyridaben compared to pyridaben alone in FS strains larvae on day 4 ( $n = 100$  including of 4 replicates of 20 larvae).



**Figure 2.3.2.4.** The average percent mortality  $\pm$ SEM of TSSM exposed to synergists and pyridaben compared to pyridaben alone in FSF strains. The combination of PBO and pyridaben caused a significant synergistic effect in the SFS (Mann-Whitney U; no asterisk,  $P > 0.05$ ;  $n = 100$  including 4 replicates).

### 2.3.3 Sequencing data

PCR products were aligned with the sequences of the reference genome strain (LS) (Grbić et al 2011). The list of mutations in NADH dehydrogenase subunits (49Kda, TYKY, PSST, ND1 and ND5) are given in Table 2.3.3.1. No exclusive mutation for DNA sequences for FS or SFS were detected. The gene encoding the NADH dehydrogenase subunit TYKY consists of 657 nucleotides and its protein consists of 219 amino acids. The DNA sequence of TYKY subunit in SFS was 100% identical to the DNA sequence of LS, and no mutations were detected in this gene sequence for SFS. The gene encoding the NADH-quinone oxidoreductase 49kDA subunit consists of 1443 nucleotides and its protein consists of 480 amino acids. DNA sequence of 49kDA subunit in FS and SFS were 99% identical to that of LS. Only one synonymous transition point mutation (T1281C) was detected for 49kDa subunit sequence in SFS. The gene encoding the NADH dehydrogenase PSST subunit consists of two exons including 687 nucleotides, and its protein consists of 229 amino acids. The DNA sequence of PSST subunit in FS and SFS was 99% identical to the DNA sequence of LS. Three synonymous transition point mutations (T60C, G489A and T672C) and one transversion point mutation (G168T) were identified in the PSST subunit sequence of both FS and SFS. The gene encoding the NADH dehydrogenase subunit 1 consists of 855 nucleotides and its protein consists of 285 amino acids. The DNA sequence of the ND1 subunit in FS and SFS was 99% identical to the DNA sequence of the LS. Two synonymous transversion point mutations (T556G and A301C) and 11 transition point mutations (C142T, C222T, T232C, C256T, G348A, A405G, C463T, T495C, A603G, A633G and A831C) were detected in the ND1 subunit sequence for both FS and SFS. In addition, 3 non-synonymous mutations

including A506G, T509C and C588T causing asparagine to serine (N169S), leucine to serine (L170S) and serine to alanine (S186A) were identified in the ND1 subunit sequence for both FS and SFS (Table 2.3.3.1). The gene encoding the NADH dehydrogenase subunit 5 consists of 1566 nucleotides and its protein consists of 285 amino acids. The DNA sequence of the ND5 subunit in FS and SFS was 99% identical to the DNA sequence of LS. Thirty-six synonymous mutations including 31 transition point mutations and 5 transversion point mutations were detected in ND5 subunit sequence for both FS and SFS. In addition, 14 non-synonymous mutations were identified in ND5 subunit sequence for both FS and SFS (Table 2.3.3.1).

**Table 2.3.3.1. The list of amino acid substitutions in mitochondrial NADH dehydrogenase subunits for lab strain (LS), field strain (FS) and selected field strain (SFS).**

Subunit	Position	TSSM strains		
		LS	FS	SFS
TYKY		-	-	-
49Kda		-	-	-
PSST		-	-	-
ND1	169	N	S	S
	170	L	S	S
	186	S	A	A
ND5	14	F	I	S
	16	M	S	I
	20	V	M	M
	48	V	M	M
	79	Y	S	S
	99	M	L	L
	241	I	V	V
	314	L	F	F
	321	N	I	I
	371	L	F	F
	386	Q	K	K
	418	L	F	F
	426	I	V	V
	442	M	T	T

## 2.4 Discussion

Many mitochondrial electron transport complex I inhibitors such as fenazaquin, fenproximate, pyridaben, and tebufenpyrad are used to control mite species. However, rapid evolution of resistance to METIs in phytophagous Tetranychids and especially in TSSM is a serious and increasing problem that has been reported in several geographical regions and many crops worldwide (Croft and van de Baan 1988, Van Leeuwen et al 2009). Two main resistance mechanisms have been suggested in arthropods. These include mechanisms that 1) decrease acaricide exposure through quantitative or qualitative changes caused by detoxification enzymes, and 2) decrease target site sensitivity by point mutations (Feyereisen et al 2015; Li et al 2007; Van Leeuwen and Dermauw 2016).



Piperonyl butoxide (PBO) synergized the activity of pyridaben and caused significantly greater TSSM larval mortality compared to pyridaben only in SFS (Figure 2.3.2.3). This suggests that metabolism by cytochrome P450 is an important resistance mechanism to pyridaben in TSSM, and confirms previous studies regarding the correlation of increased P450 activity to METIs complex I resistance in spider mites (Kim et al 2004, 2006; Stumpf and Nauen 2001; Van Pottelberge et al 2009). Although significant inhibition of carboxyl esterase by DEF in pyridaben resistance strain of TSSM has been reported (Van Pottelberge et al 2009), pretreatments with DEF did not show any significant effects on reduction of pyridaben resistance in the SFS in this study. Cytochrome P450s are a large family of multifunctional enzymes that play an important role in catalyzing xenobiotic compounds such as drugs, plant toxins and pesticides (Feyereisen 2005, 2006). It has been shown that cytochrome P450 genes are expressed differently at each insect developmental stage. For example, expression levels of *CYP392E10*, a CYP that metabolizes spirodiclofen, is very low in TSSM eggs compared to the other life stages (Demaeght et al 2014). Additionally, cytochrome P450 genes are associated with adaptation to the environment, and often results in development of insecticide resistance (Terriere 1984; Van Pottelberge et al 2008; Zhou et al 2010). Cytochrome P450s in arthropod have been divided into four major clades including CYP1, CYP2, CYP3 and CYP4 (Nelson 1998). Eighty-six cytochrome P450 (CYP) genes were identified in the TSSM genome with an expansion of the CYP2 clan (Grbić et al 2011). Several cytochrome P450 genes such as *CYP385C4*, *CYP389A1*, and *CYP392D8*, belong to the CYP3, CYP4, and CYP2 clans, respectively, have been linked to acaricide resistance such as abamectin, bifenthrin, and bifenthrin in TSSM (Piraneo et al 2015). *CYP392A11*

has also been involved in direct metabolism of METIs such as cyenopyrafen and fenpyroximate (Riga et al 2015). Evaluation of enzymatic activity and relative expressions of several detoxification-related P450 genes in the resistance and susceptible populations of TSSM in future studies can provide more clarification how P450s contribute to pyridaben metabolism in TSSM.

According to the earlier studies on complex I of mitochondria, it is believed that the catalytic core of the quinone reduction enzyme consists of five subunits including TYKY, PSST, ND1, ND5 and 49kDa subunit (Schuler and Casida 2001; Lümmer 2007; Vinothkumar et al 2014; Zickermann et al 2015; Fiedorczuk et al 2016). It has been shown that point mutations in 49kDa and PSST subunits can cause significantly decreased inhibitor sensitivity to METIs in yeast (Kashani-Poor et al 2001; Lümmer 2007). Additionally, the mutation of H92R in PSST subunit of complex I has been recently shown to be involved with METIs complex I resistance in TSSM (Bajda et al 2017). However, these mutations were not found for the gene sequence of PSST subunit in the pyridaben resistant strain (SFS) studied here. Moreover, no distinctive mutation at the protein level in any NADH dehydrogenase subunits were detected (49Kda, TYKY, PSST, ND1 and ND5) between the susceptible (FS) and resistant strains (SFS) used in this study. Three and fourteen non-synonymous mutations were identified for ND1 and ND5 subunits in both the pyridaben susceptible (FS) and resistant strains (SFS). As these mutations are common for both FS and SFS, non of them specifically associate with the pyridaben selection pressure, and thus, no correlation between these mutations and the mechanism of pyridaben resistance can be inferred.

Together, these results indicate that resistance to pyridaben in SFS is most likely not due to mutations in the target-sites of mitochondrial subunits, but most likely is because of metabolic detoxification of pyridaben by cytochrome P450 enzymes. Recent advances in genomic and molecular technologies have facilitated the development of RNAi-based pest management strategies. Therefore, a combination control strategy of pyridaben and RNAi silencing of *cytochrome P450 monooxygenase* genes can be applied against pyridaben resistant TSSM, while specificity of RNAi will also increase the safety to the beneficial insects.

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## Chapter 3

### 3 Plant derived RNA interference of TSSM

#### 3.1 Introduction

Although using chemical pesticides is still the most widely used method to control pest species, the toxicity to humans and the environment, and the development of resistance to pesticides have motivated the search for alternative pest control strategies. Transgenic RNAi plants can produce and deliver adequate dsRNA continually to pest species, and has been recognized as the most favourable dsRNA delivery method for RNAi-based pest management. This method involves generation of transgenic plant cells through *Agrobacterium*-mediated transformation that constitutively produce stable dsRNA. To achieve the RNA interference, a transgene DNA sequence homologous to the target gene is constructed typically as inverted repeats separated by an intron or non-functional sequence. Transcription of the transgene results in the formation of a RNA molecule with a hairpin loop structure (hpRNA) in plant tissues. hpRNAs generates populations of short interfering RNAs (siRNAs). Both hpRNAs and siRNAs are taken up by plant-feeding pest species, and initiates RNA interference in their bodies (Waterhouse et al 1998). The first application of RNAi-pest management was attempted by Baum and colleagues (2007) to target *Vacuolar-type H<sup>+</sup>-ATPase* gene in Western corn rootworm (*Diabrotica virgifera virgifera*) for reduction of root damage using transgenic corn plants. Subsequently, many plants including *Arabidopsis*, rice, tobacco, cotton, and tomato have been successfully transformed to control insect pests of Coleoptera, Hemiptera, and Lepidoptera (Mao et al 2007, 2011; Pinto et al 2011; Zha et al 2011; Mamta et al 2016). High mortality in the insects after ingesting dsRNAs highlights the potential of RNAi-based pest control using

dsRNA-derived transgenic plant technologies. However, the efficiency of this technique highly depends on various factors such as the concentration, length, stability, uptake of the dsRNA fragment, and most importantly choice of the target gene. Based on every target gene and organism, a minimal concentration of dsRNA to induce gene silencing is needed. The direct relationship between duration of dsRNA exposure and initiation of RNAi in the target organisms has been shown in previous studies (Meyering-Vos and Muller 2007; Shakesby et al 2009; Bolognesi et al 2012; Uryu et al 2013). The length of the dsRNA fragments has a great effect on dsRNA uptake in the gut, and consequently the success of gene silencing in the pest species. The required length of dsRNA to induce an effective RNAi response may vary depending on the pest species (Saleh et al 2006; Bolognesi et al 2012). Although efficient RNAi silencing using short interfering RNAs (siRNA) has been shown in some insects, it is understood that long dsRNAs can be more efficient resulting in down-regulation of the target gene, as a long dsRNA can provide a greater diversity of siRNAs for RNAi knockdown (Saleh et al 2006; Kumar et al 2009; Bolognesi et al 2012; Upadhyay et al 2011). Generally, using dsRNA that range from 140 to 500 nucleotides in length can produce a successful RNAi response in the target organism (reviewed in Joga 2016). Expressed dsRNAs in transgenic plants must be imported into the cells of a herbivore to initiate the RNAi response, but the stability and up-take of dsRNAs can be affected during this delivery phase. For example, high dsRNA-degrading activity of the dsRNAs by salivary nucleases has been observed in tarnished plant bug (*Lygus lineolaris*) and pea aphid (*Acyrtosiphon pisum*) (Allen and Walker 2012; Christiaens et al 2014). The stability of the dsRNA in the midgut can also be affected by the insect's gut pH. For example, the ability of dsRNA degradation



through a strongly alkaline gut (up to pH 10.5) has been shown previously in some lepidopteran species (reviewed in Katoch et al 2012). Moreover, upon the ingestion of dsRNA and its arrival to the gut, dsRNA molecules must be efficiently taken up by the gut's epithelial cells in order to be processed by the intracellular RNAi machinery to initiate systemic RNAi response. Despite these obstacles, the main challenge for RNAi-mediated pest management is the target gene selection. For example, out of 290 potential targets selected by Baum and colleagues (2007), downregulation of only 14 genes resulted in larval stunting and mortality in Western corn rootworm. Generally, silencing the genes involved in essential developmental processes or metabolism provides more efficient pest management strategy by causing high rates of lethality in pests. For this reason, five specific genes involved in essential developmental processes in spider mites including *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, *ABC-Transporter* subclass E, *Prospero*, *Acetylcholinestrace (AChE1)* and *Octapamine receptor*, were chosen to be expressed in *Arabidopsis* plants. I investigated the effects of each transgenic line on TSSM in order to determine which target would be the best one to proceed forward with as a pest management tool.

The Vacuolar-type H<sup>+</sup>-ATPase is a multi-subunit proton pump, involved in many important physiological processes such as intracellular trafficking, endocytosis, and protein degradation (Nelson 1992). Additionally, this protein acts as a proton pump to establish the pH gradient within the gut lumen of many animal cells (Nelson 1992). dsRNAs targeting several subunits of *Vacuolar-type H<sup>+</sup>-ATPase* have been shown to cause significant larval mortality in a wide range of insects such as Western corn rootworm, Southern corn rootworm (*Diabrotica undecimpunctata howardii*), Colorado

potato beetle (*Leptinotarsa decemlineata*), cotton boll weevil (*Anthonomus grandis* Boheman), fruit flies (*Drosophila melanogaster*), flour beetles (*Tribolium castaneum*), pea aphids (*Acyrtosiphon pisum*), and tobacco hornworms (*Manduca sexta*) (Baum et al 2007; Price and Gatehouse 2008; Whyard et al 2009). RNAi silencing of this gene also resulted in significant mortality in TSSM (Kwon et al 2013). However, this is the first work to target *Vacuolar-type H<sup>+</sup>-ATPase* through plant derived hpRNA to control TSSM.

The ATP-binding cassette (ABC) transporter superfamily are ATP-dependent proteins that transport various substrates such ions, sugars, amino acids, lipids, lipopolysaccharides, peptides, metals, xenobiotics and chemotherapeutic drugs across biological membranes (Higgins 1992). The ABC transporter superfamily can be divided into eight subfamilies of ABCA to ABCH (Dean and Annilo 2005). ABC transporters mediate tolerance to xenobiotics and detoxification of pesticides in many insects.

Consequently, increased insecticide sensitivity to combination of insecticides and ABC transporter inhibitors have been shown in several studies (Borycz et al 2008; Richardo and Lehmann 2009; Labbe et al 2011). Moreover, the correlation between insecticide resistance and elevated levels of *ABC transporters* transcripts have been reported in tobacco budworm (*Heliothis virescens*), bed bug (*Cimex lectularius*), diamondback moth (*Plutella xylostella*) and TSSM previously (Lanning et al 1996; Mamidala et al 2012, You et al 2013; Dermauw and Van Leeuwen 2014). In the TSSM genome, 103 *ABC transporters* genes have been identified, which is the highest number of *ABC transporter* genes discovered in a metazoan species (Dermauw et al 2013). *ABC transporters* can be considered as potential targets for RNAi-based control of the acaricide resistant strains of TSSM.

*Prospero* is involved in neuronal precursor cells, cell fate decision, axonal and dendritic outgrowth, glial development and is a regulator of mitotic activity in embryos (Doe et al 1991; Vaessin et al 1991; Manning and Doe 1999; Freeman and Doe 2001). Loss of *Prospero* function resulted in mis-regulation of a subset of genes involved in growth, autophagy and function of the olfactory system in fruit fly (*Drosophila melanogaster*) (Guenin 2010). RNAi silencing of *Prospero* for pest management of insects or TSSM has not been reported yet. Therefore, the RNAi silencing effect of *Prospero* on fitness parameters in TSSM will be the first attempt to silence this gene in TSSM.

Acetylcholinesterase is encoded by two genes, *Ace1* and *Ace2*, and hydrolyzes the neurotransmitter acetylcholine into acetyl-coenzyme A and acetate as components for termination of the neurotransmission in postsynaptic membrane (Fournier and Mutero 1994). This enzymatic activity is necessary for clearing the residual neurotransmitter molecules from the synaptic cleft that regulate normal behavior (Taylor and Radic 1994; Oakeshott et al 2005). Inhibition of Acetylcholinesterase in whitefly (*Bemisia tabaci*) leads to increased acetylcholine levels, continuous stimulation of muscles and glands, muscular dysfunction, paralysis, and death (Malik et al 2016). Acetylcholinesterase can be inhibited by some insecticides, e.g. organophosphate and carbamate, thus RNAi silencing of *Ace* genes can be an effective strategy to control pest species (Matsumura 1985; Weill et al 2003). RNAi silencing of the *Ace* gene resulted in increased mortality and growth inhibition in some insect pests including cotton bollworm (*Helicoverpa armigera*), diamondback moth (*Plutella xylostella*) and whitefly *Bemisia tabaci* (Kumar et al 2009; Gong et al 2013; Malik et al 2016). RNAi silencing of *Acetylcholinesterase*

has not been carried out in TSSM yet, and this is the first attempt to silence this gene in TSSM.

Octopamine is a neurotransmitter in many invertebrates, and an equivalent of norepinephrine in insects (David and Coulon 1985; Roeder 1999; Scheiner et al 2003). It mediates many behaviors including rhythms, feeding, flying, courtship, aggression, learning and memory in insects (Long and Murdock 1983; O'Dell 1993; Orchard 1993; Hammer and Menzel 1998; Cohen et al 2002; Pophof 2002; Mentel et al 2003; Monastiriotti 2003; Schwaerzel et al 2003; Zhou et al 2008). Application of octopamine receptor antagonists such as epinastine and mianserin resulted in impaired appetitive and learning in crickets (Awata et al 2016). Potential application of *octopamine receptor* as a target gene for RNAi silencing in pest management has not been evaluated yet, and this is the first attempt to silence this gene in TSSM.

The objectives of the research described in this chapter were to evaluate *Arabidopsis* transgenic plants that overexpress hpRNAs targeting the above-described genes, and to test the effect of plant produced dsRNA on developmental time, fecundity and survival of spider mites. The impact of plant RNAi constructs on spider mites' developmental time, fecundity and mortality was evaluated through feeding assays with spider mites on the RNAi plants to determine if RNAi of target genes in spider mites has occurred.

## 3.2 Materials and Methods

### 3.2.1 *Arabidopsis* transformation

DNA fragment sequences for targeting five genes including *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, *ABC-Transporter* subclass E, *Prospero*, *Acetylcholinesterase (AChE1)* and

*Octopamine receptor* from spider mite genome were cloned and transformed as inverted copies into *Arabidopsis thaliana* plants using pAGRIKOLA binary vector carrying Basta selection gene in Dr. Pierre Hilson's lab at VIB, Ghent, Belgium. Kondara accession was used as the wild type for all the analyses since it was the most susceptible ecotype of *A. thaliana* to spider mites feeding damage among 20 tested *Arabidopsis* strains (Zhurov et al 2014). Wild type Kondara plants were transformed via *Agrobacterium tumefaciens*-mediated floral-dip method and multiple independent transgenic lines were recovered for each target gene (Clough and Bent 1998). The information regarding the identifications and the fragment size of the transgenes are provided in Table 3.2.1.1.

**Table 3.2.1.1. The five transgenes and fragment sizes in the transformed *Arabidopsis thaliana* (Kondara) plants.**

Gene ID	Gene name	Transgene size
Tetur09g04140	<i>Vacuolar-type H<sup>+</sup>-ATPase</i> subunit A	254bp
Tetur30g01400	<i>ABC transporter</i> subclass E	216bp
Tetur02g12740	<i>Prospero</i>	248bp
Tetur19g00850	<i>Acetylcholinesterase (AChE1)</i>	470bp
Tetur11g01270	<i>Octopamine receptor</i>	232bp

### 3.2.2 Plant growth conditions and homozygous plant selection

Plants were grown with a cool-white fluorescent light intensity of 100-150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under a short-day photoperiod (10 h light: 14 h dark) at  $24 \pm 2$  °C and humidity of  $55 \pm 5$  RH %. Seedlings carrying T-DNA insertional alleles were selected in T1 progeny of the infiltrated plants by spraying the herbicide Basta (glufosinate-ammonium, 10 mg/L) for 14 days (1 time/day) at the seedling stage. Six resistant seedlings per independent line

were self-pollinated and set seeds (T2). The T2 seeds were treated with Basta in order to identify the number of T-DNA insertions. Progeny of T2 seedlings with Basta resistant:susceptible ratios of 3:1 were identified as segregating for a single T-DNA insertion. Six hemizygote T2 seedlings were self-pollinated and T3 seeds were collected. T3 seedlings were subjected to Basta treatment to identify populations that showed 100% resistant phenotype and to identify homozygous seed stocks for each transgenic plant expressing an RNAi construct.

### 3.2.3 Plant gene expression analysis by quantitative RT-PCR

To check the transcript level of the transgene in RNAi plants, total RNA was extracted from approximately 100 µl of crushed leaves of three week old *A. thaliana* using the RNeasy Plant Mini Kit, including DNase treatment (Qiagen, Toronto, ON, Canada). To assess the stability of transgene expression, RNA samples were collected from 3 leaves at 1, 4 and 7 days after they were detached from the original plant. Two µg of total RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Toronto, ON, Canada). Reactions were performed in three replicates for each genotype using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Toronto, ON, Canada). The RT-qPCR was performed using an Agilent Mx3005P qPCR instrument (Agilent Technologies, Santa Clara, CA). The reference plant gene *PEX4* (AT5G25760), an ubiquitin conjugating enzyme was used as a reference gene (Czechowski et al 2005). The information regarding the primers used in this study is provided in Table 3.2.3.1. Cycle Threshold (Ct) values of three technical replicates were averaged to generate a biological replicate Ct value. For plotting, the expression value for each target gene (T) was normalized to the reference gene (R) and a

normalized relative quantity (NRQ) was calculated as follows:  $NRQ = \frac{(1+E_R)^{CtR}}{(1+E_T)^{CtT}}$ . The NRQ values were then normalized to a mean of those in the control. Differences in the mean of normalized NRQ values between the control and treatments were analyzed by one-way ANOVA (IBM SPSS Statistics 20.0 IBM Corp.).

**Table 3.2.3.1. Transgenic plant RT-qPCR assay primers, sequence and primer efficiency. Primer efficiency above 100% may be indication of contamination.**

Primer names	Oligonucleotide sequence (5' to 3')	Primer efficiency
PEX4 (AT5G25760) F	GCTCTTATCAAAGGACCTTCGG	102.4%
PEX4 (AT5G25760) R	CGAACTTGAGGAGGTTGCAAAG	
VATPase qPCR F	GAGGGAAAAAGTCCCATTTGA	109.4%
VATPase qPCR R	CAAAGGCCTCTCAAAGATATTCA	

### 3.2.4 Spider mite rearing conditions and larva synchronization

To examine the effect of *Arabidopsis*-produced dsRNA, detached leaves of four-week-old *Arabidopsis* RNAi plants were inoculated with *Arabidopsis* adapted spider mites (Ratlamwala 2014). *Arabidopsis*-adapted spider mites were used for these experiments in order to enhance spider mite feeding on *Arabidopsis* plants. The spider mites were adapted by rearing TSSM London strain on Columbia *Arabidopsis* plants for 12 months (Ratlamwala 2014). The *Arabidopsis* adapted TSSM strain was maintained on 3 to 4 week old Columbia *Arabidopsis* plants in a climate controlled chamber at L:D 16:8 with a cool-white fluorescent light intensity of 100-150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $26 \pm 2^\circ\text{C}$ , and  $50 \pm 5\%$  RH. For larval synchronization, females were allowed to produce eggs for 24 hours on the detached leaves under the same conditions in a plastic petri plate. The adult females were

then removed, and the day-old eggs were submerged in water. Three days later water was removed from the petri plate and the four day-old eggs were dried. The synchronized hatching of larvae occurred within 3 hours (Suzuki et al 2017). These larvae were used in the *Arabidopsis* and TSSM bioassays.

### 3.2.5 Spider mite performance assays on *Arabidopsis* plants

The youngest fully-expanded leaves were detached from the 3-week-old transgenic and control (untransformed) *Arabidopsis* plants. For determination of mite developmental time and mortality, a detached *Arabidopsis* leaf was infested with 10 synchronized larvae using a fine hair brush in 10 replicates. Developmental mite stages were recorded over a 10-day period. For determination of mite fecundity, a detached *Arabidopsis* leaf was infested with one mated newly-emerged adult female mite and the total numbers of eggs were measured over a 10-day period. Detached leaves were replaced every other day and, these experiments were conducted in 3 independent experimental repeats. The total numbers of individuals or eggs were compared to those on Kondara wild type using one way ANOVA and Tukey's multiple comparison at means test in the case of more than two groups. An independent sample t-test test was used when comparing two groups (IBM SPSS Statistics 20.0 IBM Corp.). To analyze non-normally distributed data, a Kruskal-Wallis test was used in the case of more than two groups, or a Mann-Whitney U test when comparing two groups.

### 3.2.6 Spider mite gene expression analysis by quantitative RT-PCR

Detached *Arabidopsis* leaves were collected from either transgenic or control plants and were infested with about 250 newly-hatched larvae. About 150 adult spider mites



(collected in 3 independent batches of approximately 50 mites) were recovered from a detached leaf. Mites were flash frozen in liquid nitrogen and stored in  $-80^{\circ}\text{C}$  until RNA extraction. cDNA synthesis, RT-qPCR and data analysis were performed as described in the plant gene expression section, except that *RP4914* (tetur18g03590), a ribosomal protein, was used as the reference gene for spider mite gene expression analysis (Demaeght et al 2013). The information regarding the primers used in this study is provided in Table 3.2.6.1.

**Table 3.2.6.1. The RT-qPCR primers, sequences and primer efficiency used for analyzing TSSM target gene expression. Primer efficiency above 100% may be indication of contamination.**

Primer names	Oligonucleotide sequence (5' to 3')	Primer efficiency
RP49 (tetur18g03590) F	CTTCAAGCGGCATCAGAGC	99.9%
RP49 (tetur18g03590) R	CGCATCTGACCCTTGA ACTTC	
V-ATPase qPCR F	GGGTACCATCACATTCCTCG	104.1%
V-ATPase qPCR R	AATCGGTCTGGTTTGACGAAC	
ABC transporter qPCR F	TCTTTCTGGTGGTGAAGCTCA	99%
ABC transporter qPCR R	CTGAGTCAAGATGAGCGGAAG	
Octopamine qPCR F	TTCACAGAGGCGAGAAGGTG	99.9%
Octopamine qPCR R	CCTTGACTTCCACCACCACT	
Prospero qPCR F	GGTCCATCTCCATTTGCCTT	92.1%
Prospero qPCR R	GCA TGCAATAGATTTGGTGGA	
Acetylcholine qPCR F	GGCCATCTCTGATCGAAAAA	101.7%
Acetylcholine qPCR R	CAACCAATTCAGAGGCAGGT	

### 3.3 Results

#### 3.3.1 The selection of RNAi plant lines

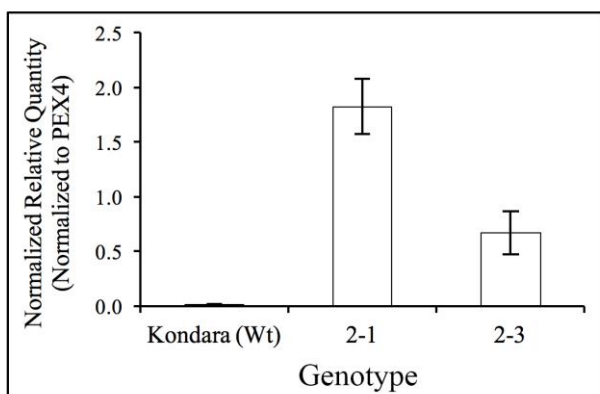
Fifteen single homozygous and one multiple T-DNA insertion seed stocks belonging to five transgenic RNAi lines were identified by Basta treatments (Rahman unpublished data). The information regarding the number of single and multiple T-DNA insertions in transgenic *Arabidopsis* plants is provided in Table 3.3.1.1.

**Table 3.3.1.1. The single or multiple T-DNA insertion lines identified for each independent RNAi plant. Plant transgenic line 2-1 was revealed as a multiple insertion line.**

Target gene	Lines	T-DNA insertion
<i>Vacuolar-type H<sup>+</sup>-ATPase</i> subunit A	2-1	Multiple insertion
	2-3	Single insertion/homozygous
<i>ABC-Transporter</i> subclass E	23-1	Single insertion/homozygous
	23-5	Single insertion/homozygous
<i>Prospero</i>	11-1	Single insertion/homozygous
	11-3	Single insertion/homozygous
	11-4	Single insertion/homozygous
	11-5	Single insertion/homozygous
<i>Octopamine receptor</i>	21-1	Single insertion/homozygous
	21-2	Single insertion/homozygous
	21-4	Single insertion/homozygous
	21-5	Single insertion/homozygous
<i>Acetylcholinestrerase</i>	7-2	Single insertion/homozygous
	7-3	Single insertion/homozygous
	7-4	Single insertion/homozygous
	7-5	Single insertion/homozygous

### 3.3.2 Transgene expression in selected RNAi plant lines

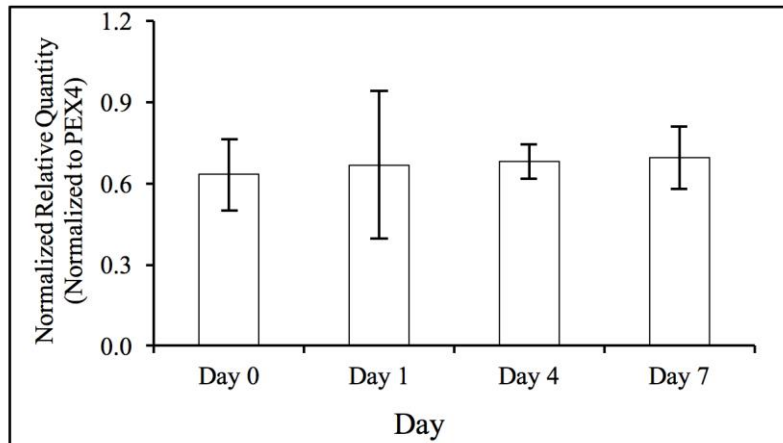
The expression level of hairpin RNA (hpRNA) for *ABC-Transporter* subclass E, *Prospero*, *Octopamine receptor*, and *Acetylcholinestrase* in transgenic RNAi and Kondara wild type plants were checked previously (Rahman unpublished data). The expression level of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A hpRNA in three-week old *Arabidopsis* transgenic plants was checked in a multiple (2-1) and a single insertion line (2-3) (Figure 3.3.2.1). As expected, hpRNA was transcribed in lines 2-1 and 2-3, but not in untransformed *Arabidopsis* control plants (Figure 3.3.2.1).



**Figure 3.3.2.1.** The expression levels of *Vacuolar-type H<sup>+</sup>-ATPase* hpRNA in wild type *Arabidopsis* (Kondara), RNAi lines 2-1 and 2-3. Mean fold changes detected by qRT-PCR (n = 3). Error bars are  $\pm 1$  SEM.

### 3.3.3 Transgene stability in *Arabidopsis* detached leaves

Since mite performance assays were carried out in detached leaves, I initially verified that the expression of the hpRNA transcripts was not affected by the leaf detachment in the course of the experiment. *Vacuolar-type H<sup>+</sup>-ATPase* transcripts from a fresh, one, four and seven-day old detached leaves from three-week old plants of line 2-3 (a single insertion line) is shown in Figure 3.3.3.1. No significant degradation of *Vacuolar-type H<sup>+</sup>-ATPase* hpRNA transcripts was detected in seven day old detached leaves compared to fresh leaves (One-way ANOVA; no asterisk,  $P > 0.05$ ).

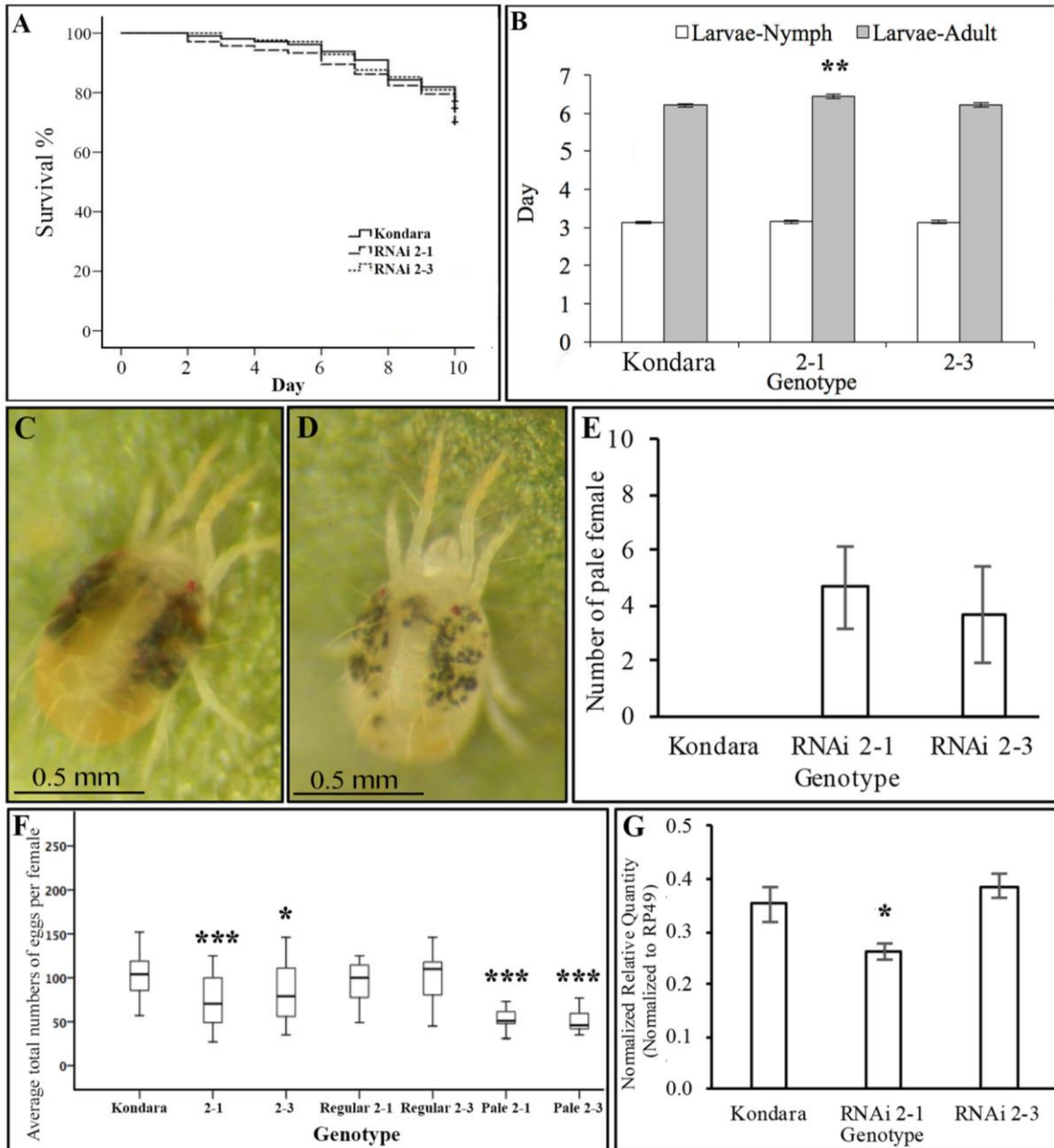


**Figure 3.3.3.1. Stability of *Vacuolar-type H<sup>+</sup>-ATPase* hpRNA transcripts in detached leaves of a single insertion line (2-3) over seven days. Mean fold changes detected by qRT-PCR (n = 3). Error bars are ± 1 SEM.**

### 3.3.4 Spider mite performance on *Vacuolar-type H<sup>+</sup>-ATPase* RNAi lines

Survival of TSSM fed on the three genotypes was not significantly different over a 10-day period (Kaplan-Meier,  $P > 0.05$ , Figure 3.3.4.1A). However, the larvae to adult developmental time of spider mites on transgenic RNAi line 2-1 was significantly longer than those on Kondara (Mann-Whitney U, \*\*,  $P < 0.005$ ; Figure 3.3.4.1B). A specific pale phenotype with a frequency of approximately 50% and 30% was observed for the females fed on both transgenic lines 2-1 and 2-3, respectively (Figure 3.3.4.1C, D and E). The pale females showed stopped oviposition after developing this phenotype. These females were detected visually by comparing their colour to those fed on wild type plants,

and by checking the numbers of eggs, they laid daily. An average fecundity of females displaying the pale phenotype fed on lines 2-1 and 2-3 was significantly lower compared to those fed on Kondara control plants (Mann-Whitney U, \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$ , Figure 3.3.4.1F). In addition, *Vacuolar-type  $H^+$ -ATPase* transcripts levels were significantly lower in spider mites fed on the multiple insertion line (2-1) compared to the control (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.4.1G).

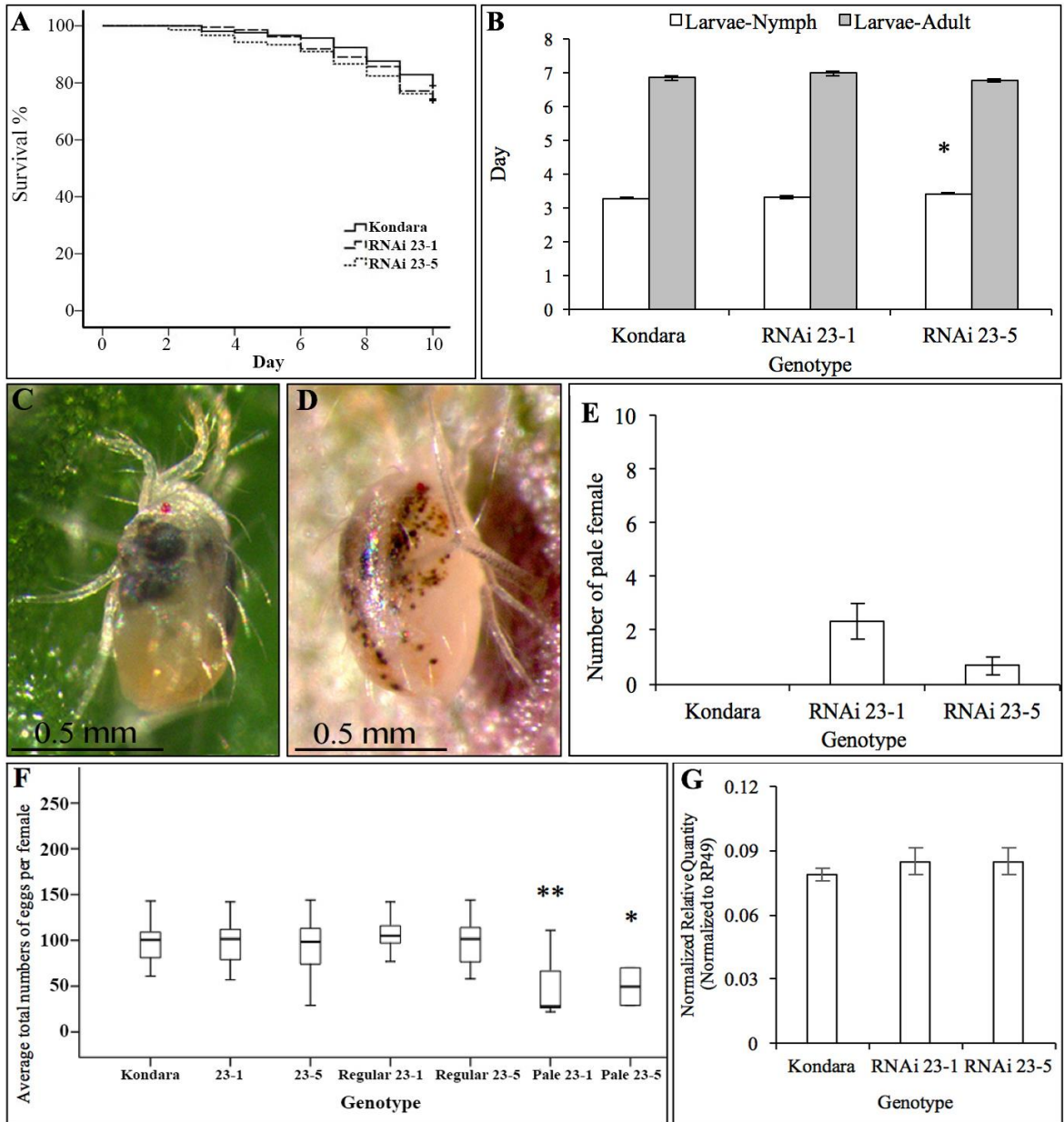


**Figure 3.3.4.1. The effect of *Vacuolar-type H<sup>+</sup>-ATPase* hpRNA ingestion on TSSM fitness parameters, fecundity and down-regulation of the target gene over a 10-day period. A) Survival of spider mites fed on RNAi lines and Kondara wild type plants over a 10-day period (Kaplan-Meier,  $P > 0.05$ ,  $n = 210$  from 3 independent replicates of 70). B) Developmental time of larvae to nymph and larvae to adult for the spider mites on RNAi lines and Kondara (Mann-Whitney U, \*\*,  $P < 0.005$ ,  $n = 210$  from 3 independent replicates of 70). C and D) A normal and pale phenotype female. E) Number of pale females on RNAi lines ( $n = 30$  from 3 independent replicates of 10). F) The average fecundity of females on lines 2-1 and 2-3 compared to Kondara control plants at 17 days (Mann-Whitney U, no asterisk,  $P > 0.05$ , \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$ ,  $n = 30$  females from 3 independent replicates). G) The expression level of *Vacuolar-type H<sup>+</sup>-ATPase* in adult TSSM on the multiple insertion line (2-1), the single insertion line (2-3), and wild type Kondara. Mean fold changes detected by qRT-PCR ( $n = 3$ ). Error bars are  $\pm 1$  SEM.**



### 3.3.5 Spider mite performance on *ABC-Transporter* RNAi lines

Survival of *T. utricae* was not significantly different over a 10-day period irrespective of the leaf genotype on which they fed (Kaplan-Meier,  $P > 0.05$ , Figure 3.3.5.1A). The developmental time of larvae to nymphs on transgenic RNAi line 23-5 was significantly longer than that for nymphs on wild type plants (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.5.1B). However, no significant difference for the developmental time from larvae to adult between the spider mites fed on RNAi lines to the wild type was found. A specific pale phenotype with the frequency of approximately 20% and 6% was detected for the females on both transgenic RNAi lines 23-1 and 23-5 respectively (Figure 3.3.5.1C, D and E). The pale females showed stopped oviposition after developing the phenotype, and were detected visually by comparing their colour to those fed on wild type plants. The fecundity of pale females decreased over time, and their cumulative fecundity over 10 days was significantly lower from those fed on wild type plants (Mann-Whitney U, \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; Figure 3.3.5.1F). Additionally, no significant difference in *ABC-Transporter* subclass E transcripts level was found in spider mites fed on transgenic RNAi lines compared to those on control wild type (Mann-Whitney U, no asterisk,  $P > 0.05$ , Figure 3.3.5.1G).

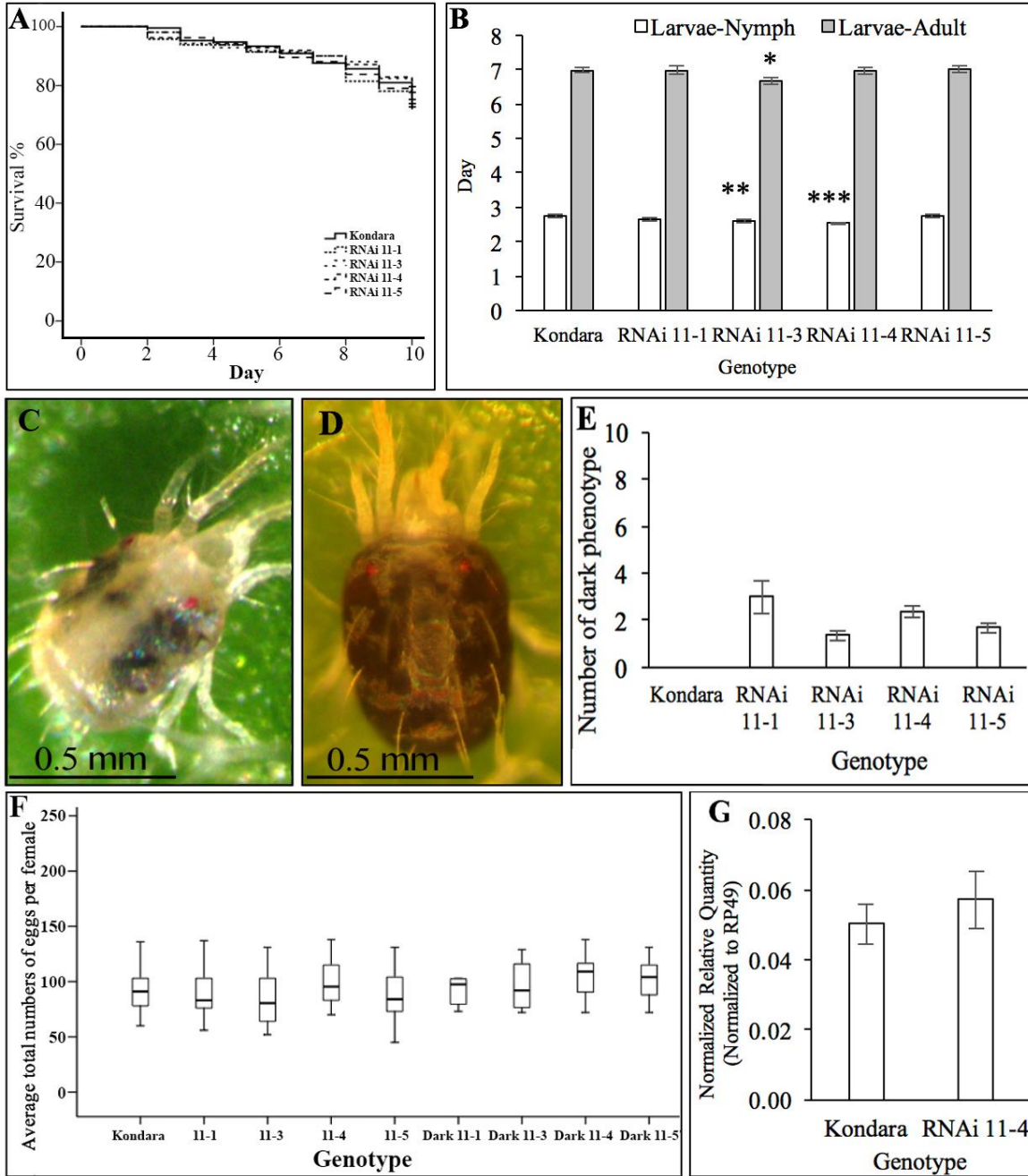


**Figure 3.3.5.1. The effect of *ABC-Transporter* subclass E hpRNA ingestion on TSSM fitness parameters, fecundity and down-regulation of the target gene over a 10-day period. A) Survival of spider mites fed on RNAi lines and control plants over a 10-day period (Kaplan-Meier, no asterisk,  $P>0.05$ ,  $n=210$  from 3 independent replicates of 70). B) Developmental time of larvae to nymph and larvae to adult for the spider mites on RNAi lines 23-1, 23-5 and wild type plants. C and D) Normal and pale female phenotype. E) Number of pale females on RNAi lines ( $n=30$  from 3 independent replicates of 10). F) Fecundity of spider mites fed on RNAi lines over a 10-day period (Mann-Whitney U; no asterisk,  $P>0.05$ ; \*,  $P<0.05$ ; \*\*,  $P<0.005$ ). G) The expression level of *ABC-Transporter* subclass E for the spider mites fed on transgenic RNAi lines 23-1, 23-5, and Kondara wild plants (Mann-Whitney U, no asterisk,  $P>0.05$ ,  $n=9$  from 3 independent replicates). Mean fold changes detected by qRT-PCR ( $n = 3$ ). Error bars are  $\pm 1$  SEM.**

### 3.3.6 Spider mite performance on *Prospero* RNAi lines

No significant difference was detected for the survival of TSSM over a 10-day period between those fed on transgenic *prospero* RNAi plants compared to those on wild type (Kaplan-Meier,  $P > 0.05$ , Figure 3.3.6.1A). The developmental time of larvae to nymphs on transgenic RNAi lines 11-3 and 11-4 was significantly shorter than that for nymphs on wild type plants (Mann-Whitney U, \*\*,  $P < 0.005$ , \*\*\*,  $P < 0.0005$ , Figure 3.3.6.1B).

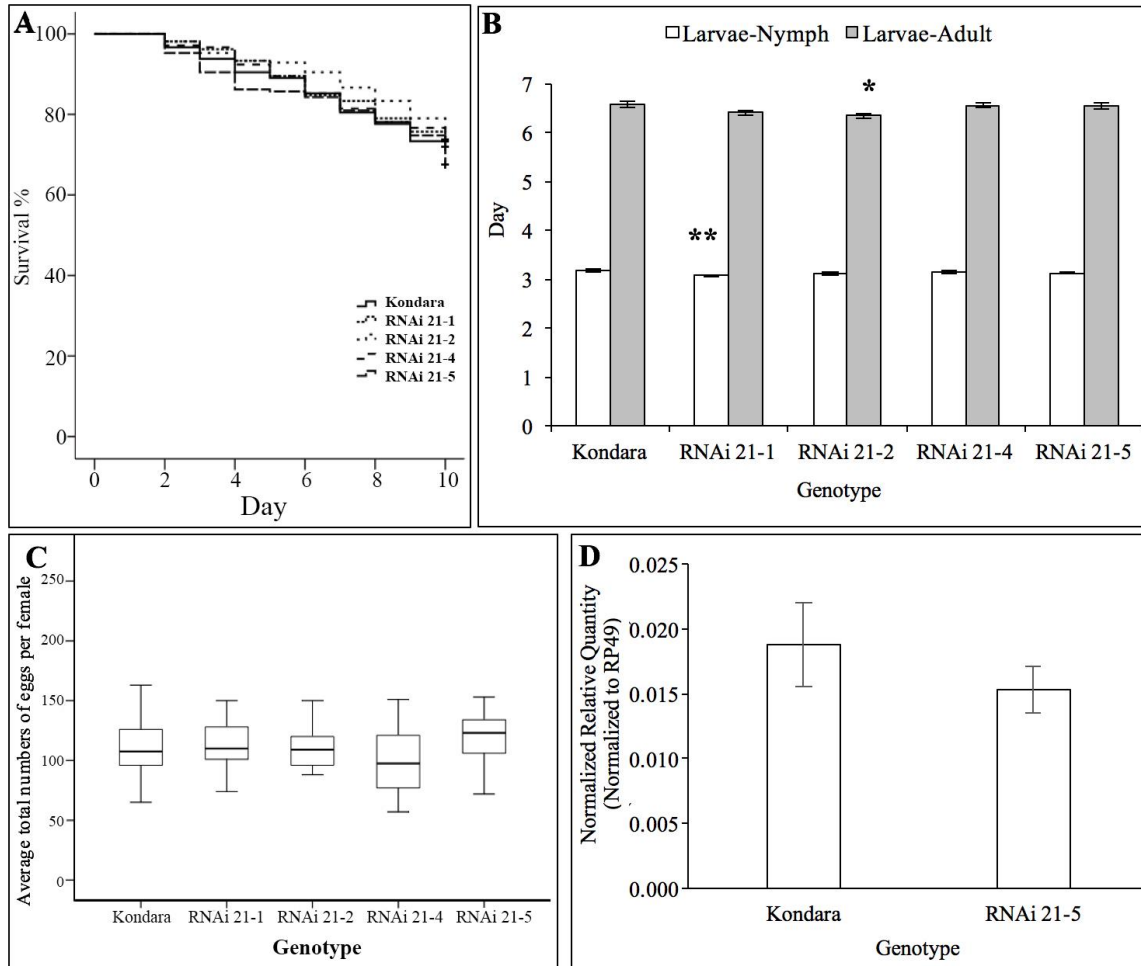
Additionally, larvae to adult developmental time of spider mites fed on transgenic RNAi line 11-3 was significantly shorter than those on Kondara (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.6.1B). A specific dark phenotype with a frequency of approximately 10 to 30% for the females on all transgenic RNAi lines was observed (Figure 3.3.6.1C, D and E). The dark females were detected visually by comparing their colour to those fed on wild type plants. No significant difference was observed in the total fecundity of dark females compared to the females feeding on control plants (Kruskal-Wallis, no asterisk,  $P > 0.05$ , Figure 3.3.6.1F). Moreover, the expression level of *Prospero* was not significantly different in the spider mites fed on transgenic RNAi line 11-4 compared to those fed on wild plants (Mann-Whitney U, no asterisk,  $P > 0.05$ , 3.3.6.1G).



**Figure 3.3.6.1. The effect of *Prospero* hpRNA ingestion on TSSM fitness parameters, fecundity and down-regulation of the target gene over a 10-day period. A) Survival of spider mites fed on RNAi lines and control plants over a 10-day period (Kaplan-Meier, no asterisk,  $P>0.05$ ,  $n=210$  from 3 independent replicates of 70). B) Developmental time of larvae to nymph, and larvae to adult for the larvae fed on RNAi lines and Kondara wild type plants (Mann-Whitney U, no asterisk,  $P>0.05$ , \*\*,  $P<0.005$ , \*\*\*,  $P<0.0005$ ,  $n=210$  from 3 independent replicates of 70). C and D) Normal and dark female. E) Number of dark females on RNAi lines and Kondara ( $n=30$  from 3 independent replicates of 10). F) Average fecundity of females fed on *Prospero* RNAi lines and Kondara plants (Kruskal-Wallis U; no asterisk,  $P>0.05$ ). G) The expression level of *Prospero* for the spider mites fed on transgenic RNAi line 11-4 and those fed on Kondara wild type plants (Independent sample t-test, no asterisk,  $P>0.05$ ,  $n=6$  from 2 independent replicates). Mean fold changes detected by qRT-PCR ( $n = 3$ ). Error bars are  $\pm 1$  SEM.**

### 3.3.7 Spider mite performance on *Octopamine receptor* RNAi lines

Survival of *T. uritace* was not significantly different over a 10-day period irrespective of the leaf genotype on which they fed (Kaplan-Meier,  $P > 0.05$ , Figure 3.3.7.1A) The developmental time of larvae to nymphs on transgenic RNAi line 21-1 was significantly shorter than that for nymphs fed on wild type plants (Mann-Whitney U, \*\*,  $P < 0.005$ , Figure 3.3.7.1B). The developmental time of larvae to adult on transgenic RNAi line 21-2 is significantly shorter than that for adults on wild type plants (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.7.1B). No significant difference for the fecundity of females fed on transgenic RNAi lines and those fed on wild type plants was detected (One-way ANOVA,  $P > 0.05$ , Figure 3.3.7.1C). Additionally, no significant difference for *Octopamine receptor* transcripts was found in spider mites fed on transgenic RNAi line 21-5 compared to those fed on wild type (Mann-Whitney U,  $P > 0.05$ , Figure 3.3.7.1D).

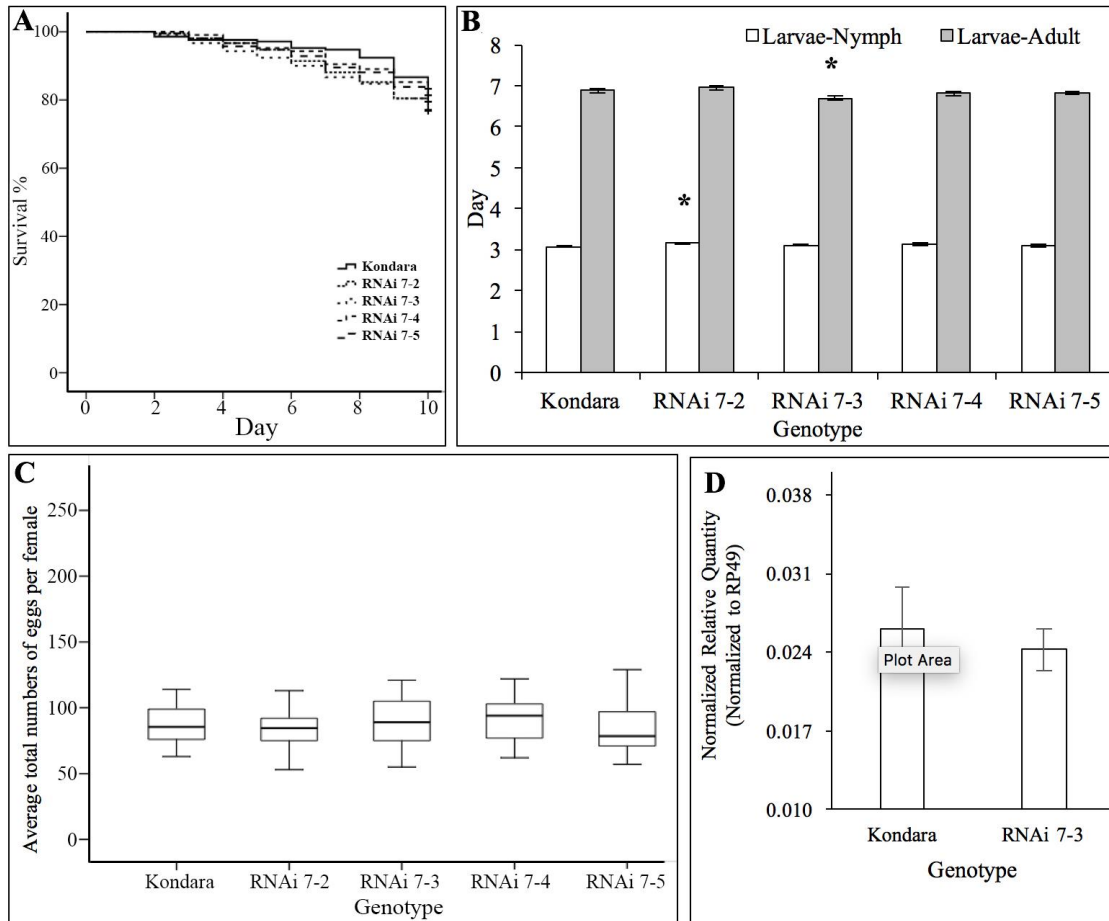




**Figure 3.3.7.1. The effect of *Octopamine receptor* hpRNA ingestion on TSSM fitness parameters, fecundity and down-regulation of the target gene over a 10-day period. A) Survival of spider mites fed on RNAi lines and controls over a 10-day period (Kaplan-Meier, no asterisk,  $P > 0.05$ ,  $n = 210$  from 3 independent replicates of 70). B) Developmental time of larvae to nymph, and larvae to adult for the larvae fed on RNAi lines and Kondara wild type plants (Mann-Whitney U, \*,  $P < 0.05$ , \*\*,  $P < 0.005$ ,  $n = 210$  from 3 independent replicates of 70). C) Average fecundity of females fed on *Prospero* RNAi lines and Kondara (One-way ANOVA; no asterisk,  $P > 0.05$ ). D) The expression level of *Octopamine receptor* for the spider mites fed on transgenic RNAi line 21-5 and those fed on wild plants (Mann-Whitney U, no asterisk,  $P > 0.05$ ,  $n = 6$  from 2 independent replicates). Mean fold changes detected by qRT-PCR ( $n = 3$ ). Error bars are  $\pm 1$  SEM.**

### 3.3.8 Spider mite performance on *Acetylcholinesterase* RNAi lines

Survival of TSSM was not significantly different over a 10-day period irrespective of the leaf genotype on which they fed (Kaplan-Meier,  $P > 0.05$ , Figure 3.3.8.1A). The developmental time of larvae to nymphs on transgenic RNAi line 7-2 was significantly shorter than that for nymphs developing on wild type plants (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.8.1B). Moreover, the developmental time of larvae to adult on transgenic RNAi line 7-3 was significantly shorter than that for adults on wild type plants (Mann-Whitney U, \*,  $P < 0.05$ , Figure 3.3.8.1B). No significant differences for the fecundity of females fed on transgenic RNAi lines and those on wild type plants was detected (One-Way ANOVA, no asterisk,  $P > 0.05$ , Figure 3.3.8.1C). Additionally, no significant difference for *Ace1* hpRNA transcripts was found in spider mites fed on transgenic RNAi line compared to those on control wild type (Independent samples t-test, no asterisk,  $P > 0.05$ , Figure 3.3.8.1D).



**Figure 3.3.8.1. The effect of *AChEI* hpRNA ingestion on TSSM fitness parameters, fecundity and down-regulation of the target gene over a 10-day period. A) Survival of spider mites fed on RNAi lines and control plants over a 10-day period (Kaplan-Meier, no asterisk,  $P>0.05$ ,  $n=210$  from 3 independent replicates of 70). B) Developmental time of larvae to nymph, and larvae to adult for spider mites fed on RNAi lines and wild type Kondara plants (Mann-Whitney U, \*,  $P<0.05$ ,  $n=210$  from 3 independent replicates of 70). C) The average fecundity of females fed on RNAi lines, and wild type Kondara plants over a 10-day period (One-Way ANOVA, no asterisk,  $P>0.05$ ). D) The expression level of *AChEI* for the spider mites fed on transgenic RNAi line 7-3, and those on wild plants (Independent samples t-test, no asterisk,  $P>0.05$ ,  $n=6$  from 2 independent replicates). Mean fold changes detected by qRT-PCR ( $n = 3$ ). Error bars are  $\pm 1$  SEM.**

### 3.3.9 Summary

The RNAi effects of the spider mites' fitness parameters and expression of the target genes for the spider mites fed on all the studied transgenic RNAi plant lines and wild type Kondara are shown in Table 3.3.9.1.

**Table 3.3.9.1. The summary of RNAi effects on TSSM fitness parameters, fecundity and the expression of target genes after hpRNA ingestion.**

Target gene	Phenotype frequency	Gene down-regulation	Mortality	Fecundity	Developmental time	
					Larvae - Nymph	Larvae - Adult
<i>V-ATPase (2-1)</i>	47%	P=0.02	P=0.10	P=0.0003	P=0.89	P=0.003
<i>V-ATPase (2-3)</i>	37%	P=0.46	P=0.58	P=0.008	P=0.90	P=0.88
<i>ABCE (23-1)</i>	23%	P=0.46	P=0.24	P=0.97	P=0.67	P=0.10
<i>ABCE (23-5)</i>	7%	P=0.33	P=0.16	P=0.60	P=0.02	P=0.43
<i>Pros (11-1)</i>	30%	-	P=0.36	P=0.76	P=0.11	P=0.68
<i>Pros (11-3)</i>	13%	-	P=0.63	P=0.16	P=0.002	P=0.01
<i>Pros (11-4)</i>	23%	P=0.44	P=0.64	P=0.16	P=0.0001	P=0.61
<i>Pros (11-5)</i>	16%	-	P=0.28	P=0.38	P=0.72	P=0.85
<i>Oct receptor (21-1)</i>	0%	-	P=0.84	P=0.98	P=0.004	P=0.11
<i>Oct receptor (21-2)</i>	0%	-	P=0.73	P=1.0	P=0.10	P=0.02
<i>Oct receptor (21-3)</i>	0%	-	P=0.94	P=0.40	P=0.34	P=0.76
<i>Oct receptor (21-5)</i>	0%	P=0.52	P=0.24	P=0.53	P=0.19	P=0.73
<i>Acel (7-2)</i>	0%	-	P=0.07	P=0.88	P=0.01	P=0.34
<i>Acel (7-3)</i>	0%	P=0.37	P=0.08	P=0.99	P=0.36	P=0.01
<i>Acel (7-4)</i>	0%	-	P=0.57	P=0.90	P=0.08	P=0.26
<i>Acel (7-5)</i>	0%	-	P=0.29	P=0.82	P=0.39	P=0.40

### 3.4 Discussion

The *Vacuolar-type H<sup>+</sup>-ATPase* is involved in important processes in eukaryotic cells such as receptor-mediated endocytosis, intracellular membrane trafficking, protein degradation and coupled transport (Forgac 1998). Previous RNAi silencing experiments of several subunits of *V-ATPase* were shown to cause significant mortality in a wide range of insects such as two-spotted spider mites, western corn rootworm, southern corn rootworm, Colorado potato beetle, cotton boll weevil, fruit flies, western flower thrips, flour beetles, pea aphids, and tobacco hornworms (Baum et al 2007; Price and Gatehouse 2008; Whyard et al 2009; Zhu et al 2011; Kwon et al 2013; Thakur et al 2014; Badillo-Vargas et al 2015). RNAi silencing of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A resulted in significant larval mortality and reduction of fecundity in TSSM through delivery of dsRNA via leaf coating and soaking (Suzuki et al 2017a). The RNAi silencing of TSSM through ingestion of plant-derived hpRNAs did not affect survival, but resulted in reduction of fecundity by 21-28% in females fed on transgenic RNAi 2-1 and 2-3 plants compared to wild type plants (Figure 3.3.4.1F). The limited effect of RNAi through feeding on plant-derived hpRNAs in spider mites compared to the other tested delivery methods may be due to the lower concentration of expressed dsRNA present in transgenic plant tissues relative to the effective concentrations delivered via leaf coating and soaking methods (Suzuki et al 2017a). The developmental time of larvae to adult spider mites when fed on transgenic RNAi line 2-1 was also significantly longer compared to those fed on wild type plants (Figure 3.3.4.1B). However, the delay in developmental time and moderate reduction in fecundity that resulted from RNAi silencing of *Vacuolar-type H<sup>+</sup>-ATPase* would need to be improved to be considered in

IPM programs for TSSM control. A stronger effect on TSSM performance parameters such as fecundity reduction, longer developmental time and down-regulation of the target gene was observed in spider mites fed on the multiple insertion line 2-1 compared to the single insertion line (2-3) (Figure 3.3.4.1. F and G). This may be due to the higher expression of hpRNAs in a multiple insertion line (2-1) compared to single insertion line (2-3) (Figure 3.3.2.3). The transcript level of *Vacuolar-type H<sup>+</sup>-ATPase* in TSSM was assessed using the RNA extracted from the whole bodies of adults, and repeating this experiment for checking the level of transcripts from the gut's cells only, may reveal the down-regulation of this gene for those fed on the single insertion plant line.

Approximately 30 to 50 percent of the females fed on transgenic lines 2-3 and 2-1 developed a pale body phenotype. This incomplete phenotype may be due to the variable ability of spider mites to absorb dsRNA molecules from the gut that can lead to down-regulation of the target gene (Suzuki et al 2017a, b). In addition, the presence of nucleases, variable expression levels, and sequence variability of the RNAi targeted gene region may result in the observed phenotypic variability (Suzuki et al 2017a). The pale phenotype females treated with dsRNA against *Vacuolar-type H<sup>+</sup>-ATPase* showed very low fecundity. This specific phenotype in females may correlate with down-regulation of *Vacuolar-type H<sup>+</sup>-ATPase* in gut's lumen cells, and consequently resulted in disruption of digestion process in the gut, and ultimately affecting female production. However, more evidence for the enzymatic activity using assays with *Vacuolar-type H<sup>+</sup>-ATPase* subunit A protein to measure its abundance in gut's cells in pale females after inducing RNAi is still required.

ABC transporter proteins play vital roles in metabolism and development in insects, and are known to contribute to insecticide resistance as they protect against xenobiotics (Labbe et al 2011; Dermauw and Van Leeuwen 2014). This gene family has been suggested as the potential targets for RNAi-based control of the acaricide resistant strains of TSSM (Dermauw and Van Leeuwen 2014). RNAi silencing of several *ABC transporter* subunits in red flour beetle (*Tribolium castaneum*) through dsRNA injection resulted in increased mortality due to abortive molting and desiccation (Broehan et al 2013). Similarly, increased larval mortality, was also reported for RNAi silencing of these genes through both injection and oral delivery of dsRNA into diamondback moth larvae, (*Plutella xylostella*) (Guo 2015). However, dsRNA targeting *ABC transporter* subclass E through transgenic plants did not result in significant larval mortality in TSSM (Figure 3.3.5.1A). Although the developmental time of larvae to nymphs was significantly longer for the spider mites feeding on transgenic RNAi line 23-5, the developmental time of larvae to adults for the spider mites fed on transgenic RNAi lines was not affected (Figure 3.3.5.1B). A similar pale phenotype with the frequency of 6 to 20 percent was detected for the females on both transgenic RNAi lines (Figure 3.3.5.1E). All the pale females had lower fecundity, which may be a sign of malnutrition or stress in these females. The expression level of *ABC-Transporter* subclass E was not significantly different for the spider mites fed on transgenic RNAi lines compared to those on wild plants (Figure 3.3.5.1G). However, the expression level of the target gene may not be always the best parameter to judge the efficiency of RNAi knock-down, due to the mRNA turnover and protein stability (reviewed in Scott et al 2013). For example, the weak phenotypic response after RNAi silencing of *Dα6* (nicotinic acetylcholine receptor subunit)



in *Drosophila melanogaster* and *Tribolium castaneum* is believed to be due to its long protein half-life (Lomazzo et al 2011; Rinkevich and Scott 2013). Thus, analyzing the protein level of ABC-Transporter from the pale females compared to the those fed on wild type plants may provide more support regarding the RNAi silencing of this gene and its involvement to this specific phenotype in TSSM.

The role of the *Prospero* gene in cell fate, neuronal cells growth and development, and regulation of mitotic activity in fruit fly and nematode embryos have been reported previously (Doe et al 1991; Vaessin et al 1991; Manning and Doe 1999; Freeman and Doe 2001). The developmental time of larvae to nymphs, and larvae to adult was significantly shorter for the spider mites fed on transgenic RNAi lines 11-3 and 11-4 compared to those on Kondara (Figure 3.3.6.1B). Generally, the developmental time of TSSM can be faster under favourable environmental conditions, such as optimal temperature, humidity and host plants (Helle and Sabelis 1985). However, no significant effect of RNAi on the expression level of target gene or life parameters such as survival and oviposition of spider mites was detected (Figure 3.3.6.1A, F and G). For RNAi silencing of *Prospero* gene through systemic RNAi response, dsRNA molecules must be efficiently taken up by gut's epithelial cells and delivered to neural cells to initiate the RNAi response. The stability of the dsRNA in the midgut can be affected by nucleases and gut pH. Moreover, midgut epithelial membrane may act as a physical barrier to the delivery of dsRNA, which makes RNAi silencing of *Prospero* gene in neuronal cells more challenging. A specific dark phenotype with the frequency of 10 to 30 percent for the females on all transgenic RNAi lines was detected (Figure 3.3.5.1E). This phenotype

can be observed in naturally aging females in lab populations, indicating that *Prospero* RNAi may have caused a stress-phenotype in the part of the treated population.

Except for differences in the developmental time for spider mites that fed on transgenic RNAi lines targeting *Acetylcholinestrerase* and *Ocotopamine receptor* compared to the wild type plants, no significant effect of RNAi silencing on TSSM fitness parameters such as survival, fecundity and expression level of target gene was observed. This limited effect of RNAi in spider mites may be due to the low concentration of expressed dsRNA in the single insertion lines, high degradation of dsRNA during delivery, and physical barriers for dsRNA delivery to the target tissues. The plant's own RNAi machinery for producing short interfering RNAs can prevent the accumulation of high amounts of hairpin RNA transcripts, and results in production of insufficient hpRNAs in plant tissues (reviewed in Zhang et al 2015). Thus, successful application of plant-derived dsRNA requires production of hairpin RNAs at rates faster than they can be diced inside the transgenic plants. To overcome these limitations and to further characterize the importance and potential applications of RNAi silencing of these genes in IPM programs, RNAi silencing of *Acetylcholinestrerase* and *Ocotopamine* in TSSM through other delivery methods such as dsRNA coating or soaking is suggested.

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## Chapter 4

### 4 RNAi non-target effects

#### 4.1 Introduction

The main goal of RNAi silencing-based strategies in crop protection is to specifically and efficiently control pest species. This goal can be achieved properly by limiting the effects to the target genes without affecting the expression of other genes or homologues of the target gene in other organisms. RNAi off- and non-target effects occur when unintended genes are down-regulated by siRNAs due to an unintended high sequence similarity between siRNA and these genes (Nawal et al 2016). Silencing of unintended genes by transgene can happen either directly or indirectly (Qui et al 2005). In direct off- and non-target gene silencing, sufficient high sequence similarity between the short interfering RNA (siRNA) and the unintended genes can induce RNAi silencing of these gene by the RNA-Induced Silencing Complex (RISC) (Qui et al 2005). The indirect off-target effects can happen through silencing a gene that regulates the expression of other genes (Qui et al 2005). The off- and non-target effects of transgenic RNAi plants can be categorized as non-target silencing of unintended genes in the host transgenic plants, off-target silencing of unintended genes in the target organism, and non-target RNAi silencing of the target genes in non-target organisms.

Off-target effects were first described through genome-wide microarray profiling in cultured human cells to study the specificity of siRNAs for gene silencing (Jackson et al 2003). Non-target effects occurring in related species to the target organism have been described in several studies (Huang et al 2006; Baum et al 2007; Whangbo and Hunter

2008; Sindhu et al 2009). Possible non-target effects of transgenic RNAi plants were demonstrated for the first time through the ingestion of plant derived hpRNAs targeting root-knot nematodes (*Meloidogyne incognita*) that also caused mortality and reproductive failure in three other *Meloidogyne* species (Huang et al 2006). In addition, studies of transgenic RNAi corn plants developed to control western corn rootworm (*Diabrotica virgifera*) also caused significant mortalities in other non-target coleopteran species including spotted cucumber beetle (*Diabrotica undecimpunctata*) and Colorado potato beetle (*Leptinotarsa decemlineata*), (Baum et al 2007). RNAi knock-down of both cyst nematodes species *Heterodera glycines* and *Heterodera schachtii* through feeding on transgenic *Arabidopsis* plants also revealed non-target effects of RNAi for related species (Sindhu et al 2009). Expressing dsRNA of a particular target gene in transgenic plants may also result in emergence of unexpected pleiotropic effects, such as reduced pollen viability in the host plants (Xing and Zachgo 2007). Thus, to minimize non-target effects that cause unpredictable physiological effects in the host plant and non-target organisms, it is necessary to improve the specificity of RNAi based crop protection before commercialization of RNAi-based transgenic plants.

One of the strategies to minimize the potential of non-target effects in transgenic plants is to design hpRNAs with limited sequence similarities to unrelated genes by searching for homologies between the transgene, and the genome of the respective host plant (Fairbairn et al. 2007). RNAi specificity can be enhanced by expressing the hpRNA constructs that target less conserved genes or less conserved non-coding untranslated regions (Van der Velden and Thomas 1999; Bashirullah et al 2001; Jansen 2001; Mignone et al 2002). In addition, it has been shown that due to the presence of a limited number of RISCs in a



cell, high levels of exogenous siRNAs can saturate a cell's RNAi machinery leading to an increase in the off- and non-target effects (Agrawal et al 2003; Dillin 2003). Direct correlation between the off-target effects and siRNA concentration was also confirmed in several other studies (Jackson et al 2003; Sledz et al 2003; Semizarov et al 2003; Hannon and Rossi 2004; Jackson et al 2006; Kahn et al 2009). Although the control of the expression levels of transgene hpRNA in transgenic plants is difficult, the production of low levels of the transgene can reduce the non-target effects, but also diminish its effectiveness in inducing RNAi of the target gene (Baum et al 2007). Genomic databases are very useful for determining the likelihood of off- and non-target effects. Currently, the most feasible tools for risk assessment of off-target effects by siRNA fragments is to BLAST (Basic Local Alignment Search Tool) search the designed dsRNA sequences in genome databases. However, as genome sequences are still limited to a small number of organisms, the assessment of off-target effects is restricted, requiring their experimental determination.

Based on the results of Chapter 3, the strongest effect of RNAi silencing through ingestion of transgenic *Arabidopsis* plants was associated with the increased expression of hpRNAs targeting *Vacuolar-type H<sup>+</sup>-ATPase* subunit A in TSSM. The effects of the RNAi silencing of this gene were observed as significant down-regulation of target gene in TSSM and the induction of pale phenotype, longer developmental time and reduction of fecundity (Figure 3.3.4.1). Therefore, among all the developed transgenic RNAi plant lines, the potential off- and non-target effects of *Vacuolar-type H<sup>+</sup>-ATPase* transgenic plants were selected for further evaluation. The non-target effects of these RNAi transgenic plants on two *Arabidopsis* plant feeding insects, the cabbage looper

(*Trichoplusia ni*) and the green peach aphid (*Myzus persicae*) was evaluated. The sequence homology of transferred *Vacuolar-type H<sup>+</sup>-ATPase* subunit A fragment, used for the creation of dsRNA, was compared against the genome of *Arabidopsis*, spider mite, cabbage looper and green peach aphid using the BLAST to search NCBI and the ORCAE databases for homologous sequences. The non-target effects of the RNAi transgenic plants on cabbage looper and green peach aphid then were determined by analyzing the weight gain and mortality of these insects after feeding on transgenic plants.

## 4.2 Materials and Methods

### 4.2.1 DNA sequence analysis

The transgene sequence of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A was used as query sequence and searched by BLAST program against NCBI reference sequence (RefSeq) of the *Arabidopsis*, cabbage looper and green peach aphid genomes. The guide strands of siRNA having three mismatches or less with any mRNAs (3 out of 21 base pairs) would likely cause RNAi silencing of these mRNAs by RNA-induced silencing complex (RISC) (Naito et al 2004; Kim et al 2005). It was determined that the reference sequences had high homologies to the query sequence, so DNA fragments having 18 identical nucleotides in possible fragment lengths of 21 base pairs were checked manually in the query and reference sequences. The existence of these shared DNA fragments between the transgene sequence and the genome of target organism would increase the potential RNAi silencing of non-target mRNAs. The same protocol was used to find possible off-target effects in *T. urticae* by BLAST search the sequence of transgene in ORCAE database (Sterck et al 2012).

#### 4.2.2 *Trichoplusia ni* rearing conditions and performance assays on *Arabidopsis* plants

Cabbage looper eggs were obtained from a continuously cultured colony maintained at the London Research and Development Centre (LoRDC), Agriculture and Agri-Food Canada, in London (AAFC), Ontario, Canada. Hatched larvae were reared following a standard protocol (Guy et al 1985). Three-day old second instar larvae of cabbage looper were transferred onto detached *Arabidopsis* leaves held in 4.5 cm diameter petri dishes (Gelman) enclosed in separate screened, plexiglass cages (25 cm x 25 cm x 25 cm) for each genotype and held in a growth room at  $25\pm 2$  °C,  $50\pm 5\%$  RH and 16:8 h light:dark photoperiod with a cool-white fluorescent light intensity of  $100\text{-}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

Detached leaves were replaced every other day until the pre-pupal stage. These experiments were replicated in 3 independent experiments for a total number of 30 larvae per RNAi line. The weight gain of larvae was determined after 7 days. The survival after 7 days, at pupation and adult emergence was determined. Survival curves were calculated by the Kaplan-Meier method with comparisons based on the log-rank test using IBM SPSS Statistics 20.0 (IBM Corp.). ANOVA and Tukey tests were used to determine if the weight gain of cabbage looper larvae fed on transgenic plant RNAi lines was significantly different from those fed on Kondara (wild type) plants using IBM SPSS Statistics 20.0 (IBM Corp.).

#### 4.2.3 *Myzus persicae* rearing conditions and performance assays on *Arabidopsis* plants

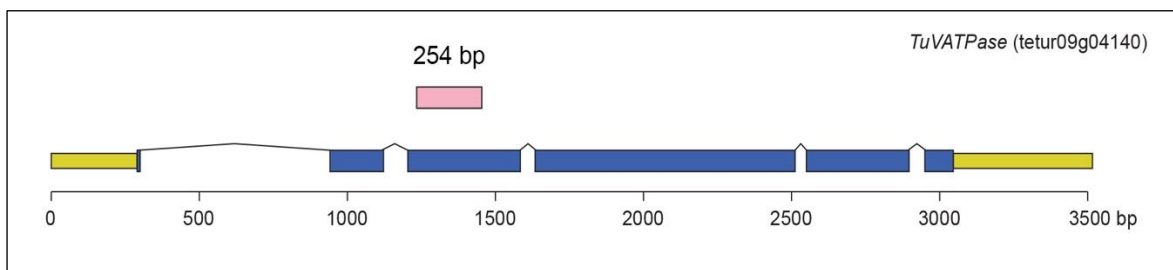
Approximately 20 to 30 wingless female aphids were transferred onto two to three Columbia *Arabidopsis* plants and were kept under environmental conditions described in section 4.2.2. for cabbage looper. Since aphids have parthenogenic embryogenesis the

resulting nymphs were collected after one day. Each Kondara or transgenic RNAi plant pot was infested with 10 nymphs, and two pots of each genotype were kept in a separate screened plexiglass cage for 10 days. The number of nymphs and adults from each cage were counted on day 10. ANOVA and Tukey tests were used to determine if the numbers of nymphs or adults found on transgenic plant RNAi lines were significantly different from those found on Kondara (wild type) plants using IBM SPSS Statistics 20.0 (IBM Corp.).

## 4.3 Results

### 4.3.1 Gene sequence analysis

The gene encoding the *Vacuolar-type H<sup>+</sup>-ATPase* subunit A in TSSM consists of 1866 nucleotides and its protein consists of 622 amino acids. The gene contains 6 exons. The length of the sequence of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A used to construct the dsRNA is 254 base pair that targets the 3rd exon of this gene in TSSM (Figure 4.3.1.1).



**Figure 4.3.1.1. Schematic of the TSSM *TuVATPase* locus. DNA sequence used for the generation of hpRNA-*TuVATPase* is located in the 3rd exon of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A. Untranslated region (UTR) and coding sequences are shown in yellow and blue, respectively.**

The DNA sequence of transgene has 76% identity with the *Vacuolar-type H<sup>+</sup>-ATPase* subunit A in *Arabidopsis* genome, and some identical sequence fragments of 18 nucleotides were identified (Figure 4.3.1.2). The DNA sequence of transgene has 75% identity with the *Vacuolar-type H<sup>+</sup>-ATPase* subunit A in green peach aphid genome, and some identical sequence fragments of 18 nucleotides were identified. However, no identical sequence over the 18 base pairs in length was found when *Vacuolar-type H<sup>+</sup>-ATPase* subunit A transgene sequence against the available gene sequences of cabbage looper. The transgene sequence against TSSM genome database had a similarity of 25 out of 29 nucleotides in nuclear pore complex protein *Nup98-Nup96* gene (tetur01g06540).

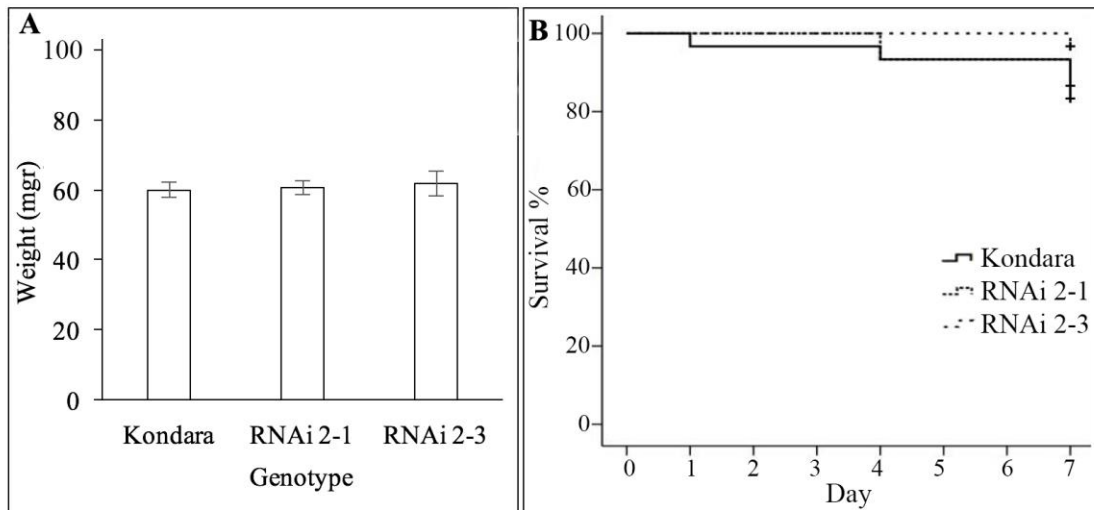
Arabidopsis thaliana vacuolar type ATPase subunit A (At1g78900) mRNA, complete cds					
Sequence ID: <a href="#">BT002589.1</a> Length: 1927 Number of Matches: 1					
Range 1: 221 to 324 <a href="#">GenBank</a> <a href="#">Graphics</a> <span style="float: right;">▼ Next Match ▲ Previous Match</span>					
Score	Expect	Identities	Gaps	Strand	
75.2 bits(82)	3e-12	79/104(76%)	0/104(0%)	Plus/Minus	
Query	151	AGGCCTTTGAATACCATCAAAAATAGATCCCATAATACCTGGTCCGAGCTCAACAGATAA			210
Sbjct	324	AGGCCTCTGAATTCATCAAAAGATATTTCCAATATTCCTGGCCCGAGCTCCACAGAAAG			265
Query	211	TGGTTTACCGGTTTCGAGAACTGGATCACCAACAGTTACACCAG			254
Sbjct	264	TGGCTTGTGTGTTTCGAAGAACGGGATCATTAACGTCAATCCAG			221

**Figure 4.3.1.2.** Blast search results of spider mite *Vacuolar-type H<sup>+</sup>-ATPase* subunit A transgene against *Arabidopsis* genome. One of the possible siRNA fragment as a potential non-target effect in *Arabidopsis* is indicated with a red rectangle.

#### 4.3.2 Cabbage looper weight gain and mortality assays

The average weight gain of cabbage looper larvae was not significantly different between those fed on the two RNAi plant lines compared to Kondara over 7 days (One-way ANOVA,  $P > 0.05$ , Figure 4.3.2.1A). Similarly, the average mortality of cabbage looper

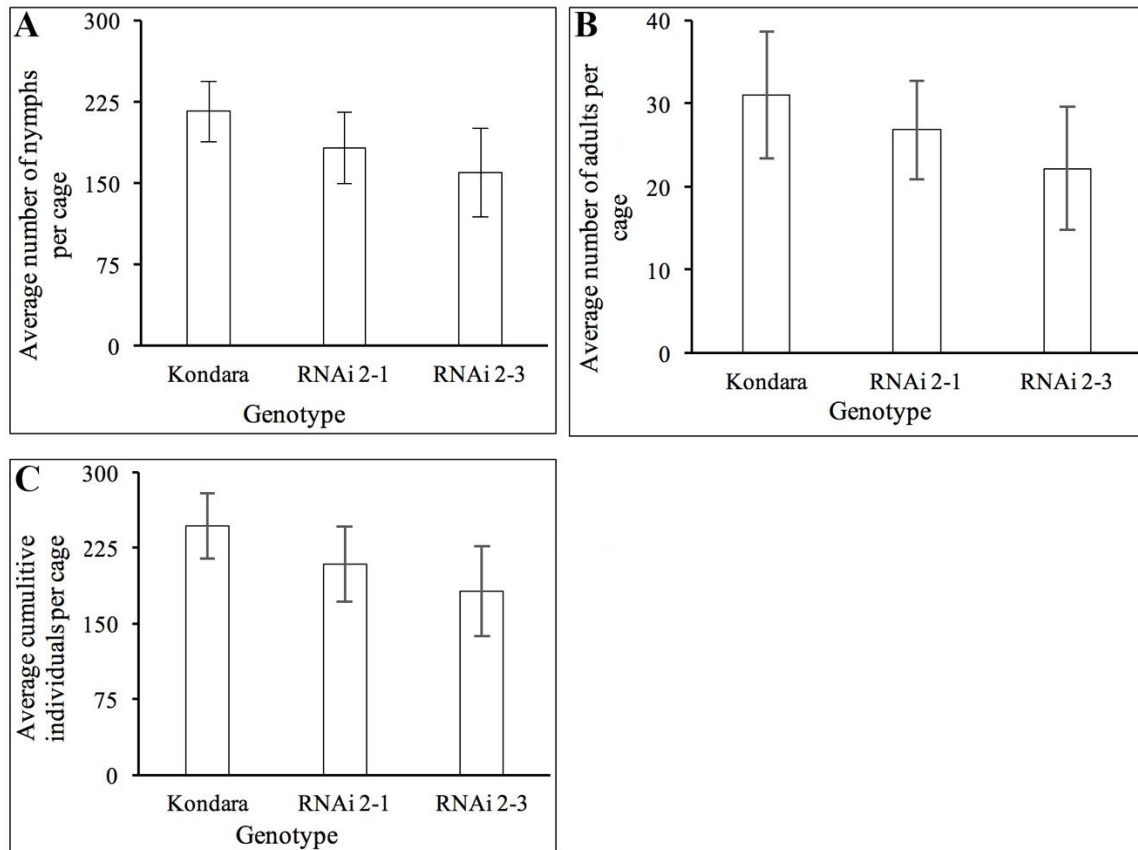
larvae was not significantly different between those fed on RNAi plant lines compared to Kondara (Figure 4.3.2.1B.  $P>0.05$ ).



**Figure 4.3.2.1.** The average weight gain  $\pm$ SEM and mortality of cabbage looper larvae fed on RNAi plant lines compared to Kondara plants over 7 days. **A)** The average weight gain of cabbage looper larvae fed on RNAi plant lines and Kondara (One-way ANOVA, no asterisk,  $P>0.05$ ). **B)** The mortality of cabbage looper larvae fed on RNAi plant lines compared to Kondara in 7 days (Kaplan-Meier,  $P>0.05$ ). Data for both experiments were collected from 3 independent replicate experiments of 10 larvae.

### 4.3.3 Green peach aphid populations assay

The average numbers of aphid nymphs, adults and cumulative number of nymphs and adults after 10 days were not significantly different for those fed on RNAi plant lines compared to Kondara (Figure 4.3.3.1, One-way ANOVA, no asterisk,  $P>0.05$ ).



**Figure 4.3.3.1. Average total number of green peach aphid larvae and nymphs ±SEM from 2 pots on one cage after 10 days feeding on RNAi and Kondara plants. A) Average number of green peach aphid nymphs from 2 pots in one cage after 10 days. B) Average number of green peach aphid adults ±SEM from 2 pots in one cage after 10 days. C) Average total number of green peach aphid nymphs and adults ±SEM from 2 pots in one cage after 10 days. The average numbers of green peach aphid nymphs, adults and cumulative number of nymphs and adults fed on RNAi plant lines and Kondara after 10 days (One-way ANOVA, no asterisk,  $P > 0.05$ ). Data for all experiments were collected from 5 independent replicates of 20 nymphs.**

## 4.4 Discussion

Off-and non-target effects can occur either because of a full or partial complementarity between siRNA and any unintended target. BLAST search of spider mite *Vacuolar-type H<sup>+</sup>-ATPase* subunit A transgene against *Arabidopsis* genome indicated the existence of some similarities between this gene and its *Arabidopsis* homologue (Figure 4.3.1.2). This indicates the potential non-target effects of RNAi silencing of this gene by the expressed transgene hpRNAs in the transgenic RNAi lines. Plants' vacuolar-type H<sup>+</sup>-ATPase is a large multimeric enzyme complex that is responsible for acidification and expansion of vacuoles (Taiz 1992; Barkla and Pantoja 1996; Liittge and Ratajczak 1997; Marty 1999). Subunit A of vacuolar-type H<sup>+</sup>-ATPase is about 70 kDa, and is the catalytic subunit of this protein. This subunit contains a nucleotide binding motif that binds and hydrolyzes ATP (Randall and Sze 1987). Vacuolar-type H<sup>+</sup>-ATPase protein is involved in many important metabolic functions such as the maintenance of homeostasis and endocytosis, and RNAi silencing of this gene may induce lethal phenotypes in plant species. However, no observable off-target effects such as reduced pollen viability or reduced fitness of transformed *Arabidopsis* plants after their transformation and selections was observed. Gene expression analysis of *Vacuolar-type H<sup>+</sup>-ATPase* in transgenic RNAi lines compared to Kondara plants using quantitative reverse transcription PCR (RT-qPCR) would provide more evidence regarding the occurrence of any RNAi silencing of this gene as a potential part of the non-target effects in developed transgenic plants.

The lack of genome annotation, and especially sequence data for *Vacuolar-type H<sup>+</sup>-ATPase* genes for cabbage looper, makes sequence homology through BLAST search



difficult and indicates the importance of assessing the non-target effects through feeding bioassays. Among the fitness parameters examined, such as mortality and weight gain in cabbage looper and green peach aphid, no significant non-target effects of RNAi silencing of *Vacuolar-type H<sup>+</sup>-ATPase* was observed (Figure 4.3.1.2 and 4.3.3.1). Since there is a limited effect of RNAi through feeding on plant-derived hpRNAs compared to other dsRNA delivery methods for spider mites, *in planta* RNAi represents the lowest concentration of dsRNA among delivery methods compared by Suzuki et al (2017). The expressed dsRNAs in transgenic plants must be efficiently taken up from the gut to initiate the RNAi response, while the stability of the dsRNA in the gut can be affected by insect nucleases and gut pH. dsRNA degradation by a strongly alkaline gut (up to pH 10.5) common in lepidopteran species, and salivary nucleases in aphids, has been shown (Allen and Walker 2012; Christiaens et al 2014). Thus, to prove the potential non-target RNAi silencing of spider mites' *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, and its dsRNA concentration dose response in cabbage looper and green peach aphid, RNAi silencing of these organisms through dsRNA soaking or leaf coating is suggested.

BLAST search of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A against TSSM genome database indicates a similarity of 25 out of 29 nucleotides in nuclear pore complex protein *Nup98-Nup96* gene (tetur01g06540). Nup98 and Nup96 are two nucleoporins involved in nuclear pore complex (Rout et al 2000; Ryan and Wentz 2000; Vasu and Forbes 2001; Cronshaw et al 2002). The nuclear pore complex is a massive multiprotein structure in eukaryotic cells that is responsible for RNA and protein trafficking between the nucleus and cytoplasm (Fontoura et al 2001; Griffis et al 2003). Both Nup98 and Nup96 proteins have been found on the nucleo-plasmic and cyto-plasmic sides of nuclear pore complex

(Griffis et al 2003). While Nup96 seems to be a stable protein, having a structural role in nuclear pore complex, Nup98 is a mobile protein that shuttles on and off the nuclear pore complex (Belgareh et al 2001; Fontoura et al 2001; Griffis et al 2002, 2003). Since the aim of engineered transgenic plants is to express TSSM dsRNAs for inducing changes in life fitness parameters such as increasing mortality or reduction of fecundity in spider mites, off-target silencing of this gene in this target pest would not appear to be a concern. However, analyzing the expression level of *Nup98-Nup96* transcripts after RNAi silencing of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A using dsRNA delivery by soaking and leaf coating will provide more information regarding any possible involvement of this gene knock-down to mortality or reduced fecundity in TSSM.

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## Chapter 5

### 5 Summary

#### 5.1 *Tetranychus urticae* adaptation to host plants and chemical controls

The two-spotted spider mites (TSSM) are generalist arthropod herbivore that feed on a wide range of over 1100 plant species from more than 140 plant families (Bolland et al 1998). Spider mites threaten greenhouse, field, orchard, and vine production and infest many annual and perennial commercial crops such as tomatoes, peppers, cucumbers, strawberries, corn, soy, apples, grapes, hops and citrus (Jeppson et al 1975). TSSM female produce up to 150 eggs per life time, with many generations per year due to their short life cycle, allowing for dramatic population increases making it a significant crop pest worldwide (Cranham and Helle 1985). Control of TSSM mainly relies on acaricides leading to the development of resistance to almost all of the acaricides used for its control (Van Leeuwen et al 2009). Metabolic resistance and molecular mutations have been identified as main mechanisms of developing resistance and cross-resistance to several acaricides in the same class.

#### 5.2 Metabolic and molecular resistance to pyridaben

Among the Mitochondrial Electron Transport Inhibitor (METI) acaricides, pyridaben was developed and first used in Korea in 1992 (KCPA 2004). However, high levels of resistance developed shortly afterwards in several field populations of TSSM (Cho et al 1995; Song et al 1995). As ND1 or ND5 subunits are encoded by mitochondria, mutations in these subunits may be involved for the maternal inherited resistance, while

mutations in 49kDa or PSST subunits as they encode by nuclear genome may be involved in autosomal inherited resistance to METIs (Goka 1998; Devine et al 2001; Stumpf and Nauen 2001; Sato et al 2004; Lümmer 2007; Bajda et al 2017). In addition, detoxification by cytochrome P450 is a recognized mechanism of pyridaben resistance in TSSM and is often involved in cross-resistance to other METI acaricides (Stumpf and Nauen 2001; Kim et al 2006; Sugimotoa and Osakabe 2014).

The development of acaricide resistance in TSSM populations in Canadian vegetable greenhouses is a growing concern for growers and the processing industry. My research revealed that metabolic resistance mechanisms were likely underlying the adaptation of TSSM to pyridaben in one Ontario greenhouse population. The contribution of detoxification enzymes, esterases (ESTs), glutathione-*S*-transferases (GSTs) and cytochrome P450 monooxygenases (P450s), were assessed using enzyme inhibitors in the pyridaben-selected strain of TSSM, that indicated the resistant ratio of 3300-fold relative to the unselected strain. Combination of PBO and pyridaben showed a significant synergistic effect indicating the possible role of oxidative metabolism by cytochrome P450-dependent monooxygenases in the pyradiben-selected strain but not the greenhouse collected or lab strain (Figure 2.3.2.3). This result confirms previous studies regarding the correlation of increased P450 activity to METI complex I resistance in spider mites (Kim et al 2004, 2006; Stumpf and Nauen 2001; Van Pottelberge et al 2009). Moreover, multiple-resistance of this strain to abamectin, bifenazate, acequinocyl and spirodiclofen reported by Scott et al (2016), also indicates the potential involvement of cytochrome P450 as the main mechanism of resistance. Several cytochrome P450 genes such as *CYP392E10*, *CYP385C4*, *CYP389A1*, *CYP392D8*, and *CYP392A11* have been shown to

be linked to acaricide resistance such as spirodiclofen, abamectin, bifenazate, bifenthrin, cyenopyrafen and fenpyroximate in TSSM (Demaeght et al 2014; Riga et al 2015; Piraneo et al 2015).

Point mutations in 49kDa and PSST subunits caused significantly decreased inhibitor sensitivity to METIs in yeast (Kashani-Poor et al 2001; Lümmer 2007). H92R mutation in the PSST subunit of complex I has been implicated in the TSSM resistance against METIs complex I resistance in TSSM (Bajda et al 2017). However, this mutation was not observed in the gene sequences of the pyridaben-selected field strain studied here. Three and fourteen non-synonymous mutations in ND1 and ND5 subunits for both field and selected field strains were identified here, but none of these mutations occurred due to the added pyridaben selection pressure. Therefore, based on these results no correlation between these mutations and the mechanism of pyridaben resistance can be inferred. Together, these results indicate that resistance to pyridaben in the selected field strain is most likely not due to molecular mutations in the target-sites of mitochondrial subunits, but most likely occurred due to the altered expression of cytochrome P450. Thus, evaluation of relative expression of these detoxification-related P450 genes in the resistant and susceptible populations of TSSM in future studies can provide more clarification how P450s contribute to pyridaben metabolism. Even though the observed mutations in ND1 and ND5 subunits did not contribute to the resistance of the pyridaben-selected strain, the contribution of these nucleotide changes in the establishment of the initial lower level resistance observed in the field strain remains unknown.

### 5.3 Plant derived RNA interference against TSSM

Increasing use of acaricides in intensified agricultural field practices has resulted in several problems, such as the previously described resistance, as well as environmental pollution and secondary pest outbreaks (Kos et al 2009). One solution is to develop alternative control strategies that can reduce selection pressure typical of chemical acaricides, while having the reduced negative effects to the environment. RNA interference (RNAi) is as such alternative control strategy. It has been investigated for several crop pests and has shown promise as a selective pest control strategy that has reduced likelihood of resistance developing (reviewed in Price and Gatehouse 2008). The potential of transgenic plant-mediated RNAi to cause significant mortality or developmental effects through silencing of genes that encode important proteins for insects' growth, development and survival, has been well documented (Baum et al 2007; Khila and Grbić 2007; Mao et al 2011; Li et al 2011, 2015; Pitino et al 2011; Zha et al 2011; Zhu et al 2012; Khan et al 2013; Kwon et al 2013; Xiong et al 2013; Coleman et al 2014; Yu et al 2014; Xu et al 2014; Mamta et al 2015; Mao et al 2015; Qi et al 2015; Tian et al 2015; Tzin et al 2015; Chikate et al 2016; Camargo et al 2016; Fishilevich et al 2016; Qiu et al 2016). These studies utilized several dsRNA delivery methods including injection, soaking and through *in planta* exposure. The important findings of the current research is that *Arabidopsis* plant-derived transgenes targeting *Vacuolar-type H<sup>+</sup>-ATPase* subunit A affected the spider mite's fitness and reproduction through dsRNA ingestion. Other important findings were that *Vacuolar-type H<sup>+</sup>-ATPase* hpRNA transcripts did not degrade over a seven day period (Figure 3.3.3.1) and the level of transcripts were significantly down-regulated in spider mites fed on the multiple insertion line but not the

single insertion line (Figure 3.3.4.1G). RNAi silencing of *Vacuolar-type H<sup>+</sup>ATPase* subunit A using other methods of dsRNA delivery such as leaf coating and soaking resulted in significant larval mortality and reduction of fecundity in TSSM (Suzuki et al 2017). However, ingestion of plant-derived hpRNAs only resulted in reduction of fecundity by 21 to 28% in TSSM females fed on transgenic RNAi lines compared to wild type plants (Figure 3.3.4.1F). This limited effect of RNAi through feeding on plant-derived hpRNAs in spider mites may be due to the lower concentration of dsRNA in transgenic plant tissues compared to dsRNA concentrations delivered by other methods. A pale phenotype of females feeding on transgenic RNAi lines targeting *Vacuolar-type H<sup>+</sup>ATPase* and *ABC-Transporter* subclass E was observed (Figures 3.3.4.1D and 3.3.5.1D). The pale females had reduced fecundity compared to normal phenotype females, a possible sign of malnutrition or stress in these females. Incomplete penetrance of RNAi, reflected in development of the pale phenotype only in a portion of the treated population, indicates that there is a polymorphism in TSSM populations that affect either degradation of dsRNA during delivery route, physical barriers for dsRNA delivery to the target tissues and processivity of dsRNA, or its effectiveness. To partially overcome these limitations, greater expression of hpRNA transgene can be developed, possibly through chloroplast transformation, where higher expression levels of foreign genes over nuclear transformation has been observed (Daniell 2007; Verma and Daniell 2007; Verma et al 2008). Highly effective RNAi mediated pest control via chloroplast transformation against cotton bollworm and Colorado potato beetle has been shown recently (Jin et al 2015; Zhang et al 2015). Future work should therefore focus on developing new RNAi plant lines by chloroplast transformation of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A to

increase the expression of dsRNA in plant tissues and to improve the efficiency of RNAi knock-down in spider mites.

The remaining four *in planta*-tested RNAi constructs that targeted *ABC-transporters* subclass E, *Prospero*, *Octopamine receptor* and *Acetylcholinesterase* genes indicated that there was no significant down-regulation of their corresponding target genes, nor was there a change in mortality or other fitness parameters such as fecundity. This differential effectiveness of RNAi over a range of gene targets has been previously demonstrated (e.g. Baum et al 2007), indicating that target selection is going to be the major bottleneck in developing RNAi technology for the control of any pest.

#### 5.4 Assessment of the off-target and non-target effects of RNAi plants

Understanding the mechanism underlying off- and non-target effects, and identifying the risk associated with developing plant-derived RNAi as reliable molecular based pest managements is necessary. Genomic databases may be very useful in determining the likelihood of such off- and non-target effects, and currently BLAST searches can be used to design dsRNA sequences. However, as genome sequences have been obtained for only a small number of organisms so far, and there is a lack of the complete genomic sequences for most reported genomes, the assessment of the sequence uniqueness of dsRNA cannot be guaranteed. Thus, in order to evaluate the risk of siRNAs fragments causing off- and non-target effects, bioassays with the specific organisms should be conducted. Since the strongest effect of RNAi silencing on several fitness parameters of spider mites was by hpRNAs ingestion of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, it was important to evaluate the potential non-target effects on herbivores that would consume

*Arabidopsis*, e.g. cabbage looper (*Trichoplusia ni*) and green peach aphid (*Myzus persicae*). No similarity or identical DNA fragments with a consecutive 18 base pair length was found by querying *Vacuolar-type H<sup>+</sup>-ATPase* subunit A transgene sequence against the available genome database for cabbage looper. However, the lack of complete genomic databases for this non-target organism studied here presents a challenge to predict the likelihood of non-target effect. In addition, limited expression of hpRNAs in the transgenic RNAi lines along with high dsRNA degradation through insect delivery route may diminish the off-target effects on these organisms. It was therefore necessary to measure the non-target effects of the RNAi transgenic plants on cabbage looper and green peach aphid by measuring fitness parameters after feeding on these plants. Since no differences were found between the insects fed on RNAi plants compared to wild type, the potential non-target RNAi silencing of spider mite *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, should be evaluated through dsRNA soaking or leaf coating as previously described.

Some similarity between transgene of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A, and *Vacuolar-type H<sup>+</sup>-ATPase* subunit A of *Arabidopsis*, and green peach aphid, and *Nup98-Nup96* gene of TSSM has been found which indicates the risk of off-target effects in these organisms. However, no unexpected phenotypic effects such as reduced pollen viability or developmental defects in *Arabidopsis* plants, or fitness parameters in green peach aphid was observed. Gene expression analysis of *vacuolar-type H<sup>+</sup>-ATPase* subunit A in RNAi lines (2-1 and 2-3) compared to Kondara, and the expression of this gene in green peach aphid after feeding, using quantitative reverse transcription PCR (RT-qPCR) will reveal any down-regulation of this gene as a potential non-target effect in these non-target organisms. In addition, analyzing the expression level of *Nup98-*

*Nup96* transcripts after RNAi silencing of *Vacuolar-type H<sup>+</sup>-ATPase* subunit A using dsRNA soaking and leaf coating will provide more evidence about the involvement of this gene in fecundity reduction that has been observed in TSSM.

## 5.5 Conclusion

This research revealed the metabolic resistance through enhanced detoxification of cytochrome P450 as the underlying mechanism for the adaptation of TSSM to pyridaben. Understanding the mechanism of resistance to acaricides in TSSM will enable growers to avoid applying acaricides with the same mode of action, and will facilitate the choice of more effective acaricides to control the acaricide-resistant strains of TSSM in the greenhouses. Targeting the expression of genes that contribute to acaricide resistance such as *CYP450s*, in combination with acaricides can also help to overcome the TSSM resistance against acaricides such as pyridaben. The current research determined that *Arabidopsis* plant-derived transgenes targeting *Vacuolar-type H<sup>+</sup>-ATPase* subunit A affected the spider mite's fitness and reproduction through dsRNA ingestion. Thus, transgenic plants represent a potential delivery method of dsRNA to control TSSM. Among several methods of RNAi delivery by ingestion, RNAi-expressing plants produce a continuous supply of dsRNA against leaf-feeding pests, and may eventually replace chemical insecticides in future plant protection programs. Developing new RNAi plant lines by chloroplast transformation will increase the expression of dsRNA in plant tissues and will improve the efficiency of this method to control TSSM. However, to improve the specificity of plant derived-RNAi pest management, it is necessary to evaluate the potential non-target effects on other plant feeding organisms that would consume the same plants, and the beneficial insects such as predatory mites and pollinators.



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