An effective method to screen dsRNA as a foliar-applied acaricide against the two-spotted spider mite, *Tetranychus urticae* Koch

PhD Dissertation

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Chapter 1. General introduction

1.1. *Tetranychus urticae* Koch

Polyphytophagous arthropods are generally distributed worldwide by utilizing different habitat patches in response to changes in environments. Spider mites (Acari: Tetranychidae) belong to the subphylum Chelicerata that is the second largest group in arthropods and their extreme polyphytophagy has caused the severe economic damage on agricultural crops in the world (Bolland et al. 1998; Kennedy and Storer 2000).

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch, is one of the most serious polyphytophagous pest arthropods. TSSM had been originally described from a specimens collected in Europe by Koch (1836) but was found in the subtropical regions as well as a temperature zone species (Tuttle and Baker 1968; Jeppson et al. 1975). TSSM can feed on more than 1,100 plant species including economically important crops (Migeon and Dorkeld 2018). The life cycle of TSSM starts as the egg (≈0.1 mm in diameter) that hatches into the larva followed by two nymphal stages (protonymph and deutonymph) and then emerges into the adult (≈0.5 mm in body length). The larval, protonymphal, and deutonymphal stages contain quiescent (resting) periods named as protochrysalis, deutochrysalis, and teleiochrysalis, respectively, just before molting into each next stage. TSSM has a wide range of temperature for development and reproduction; 27°C is the optimum temperature for development completed for 6.6 to 12.2 days (Fasulo and Denmark 2016; Abo-Shnaf 2017).

TSSM has a pair of gutter-shaped stylets (≈150 μm in length) which combine with each other to form a single tube (≈2 μm in inner diameter); the stylet penetrates the leaf either in between epidermal pavement cells or through a stomatal opening, without damaging the

epidermal cellular layer and sucking the cell leaf contents and causing the chlorotic spots that are not immediately but eventually formed on infested leaves (Bensoussan et al. 2016). TSSM infestation reduced stomatal conductance in soybean leaves and consequently reduce the photosynthetic rate (Bueno et al. 2009), which causes economic losses in crop production.

1.2. Chemical control

Pesticides are chemical compounds and, in most cases, primary used for controlling pests. Acaricides are pesticides that are effective to control the members belonging to the subclass Acari (i.e. mites and ticks). The worldwide acaricide market was around 900 million Euro (M€) which was 7% of the worldwide insecticide market in 2013 (Van Leeuwen et al. 2015). Almost 80% of the total acaricide market in 2008 was occupied by targeting on spider mites including *Tetranychus* spp. (372 M€) in which TSSM is the main species followed by *Panonychus* spp. (100 M€) in which the citrus red mite *P. citri* (McGregor) and the fruit tree red spider mite *P. ulmi* (Koch) are the main pest species in orchards. In the sales figures of acaricides targeting on spider mites in 2010, spirodiclofen, an acetyl CoA carboxylase inhibitor, was the most sold active ingredient (79 M€) followed by bifenazate (43 M€), a mitochondrial complex III electron transport inhibitor, and propargite (39 M€), an ATP synthase inhibitor.

However, resistance to 96 different active ingredients has been reported in TSSM so far and the number is the highest in arthropod pest species, followed by 95 in the diamond-back moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) and 80 in the green peach aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae) (Mota-Sanchez and Wise 2018). Table 1 shows the list of the pesticides to which TSSMs have developed resistance and the molecular mechanisms of resistance, which has been summarized by the Insecticide Resistance Action Committee (IRAC 2018). These suggest that novel measures rather than pesticides are urgently needed to be developed to avoid the resistance problem in TSSM control strategy.

Table 1. List of the pesticides to which TSSMs showed resistance reported by IRAC.

Chemical class	IRAC group	Distribution	Mechanism
Pyrethroids, Pyrethrins	3A	Worldwide	Target site point mutation (main) P450 monooxygenase (secondary)
Clofentezine, Hexythiazox, Diflovidazin	10A	Worldwide	cytochrome P450 and esterase
Acequinocyl	20B	Worldwide	Esterases (but probably not GST and P450 enzymes)
METI acaricides and insecticides	21A	Worldwide	Oxidative metabolsim (MFOs)
Carbamates	1A	Worldwide	Metabolic: Esterases
Orgaophosphates	1B	Worldwide	AChE point mutations
Avermectins, Milbemycins	6	Worldwide	Metabolic: Glutamate S Trans-ferase (GST) inhibition Target site: point mutations on GluCl1 and GluCl3 channels
Organotin miticides	12B	Worldwide	Oligomycin-sensitive Mg ATPase, Esterase isozymes, Oxidases
Tetronic and Tetramic acid derivatives	23	n/a	Oxidative metabolism possible but field resistance not validated
Amitraz	19	Australia and USA	Oxidative metabolism possible but field resistance not validated

1.3. Biological control

Although acaricides are still the primary method for controlling TSSMs, they have developed resistance to almost all types of active ingredients (Table 1) due to their high reproductive potential based on the short life-cycle and metabolic detoxification mainly mediated by cytochrome P450 monooxygenases (CYPs) that catalyze mono-oxygenation of xenobiotics (Dermauw et al. 2013; Piraneo et al. 2015). Therefore, great attention has been paid to biological control using natural enemies against TSSMs as the measures alternative to chemical control. For example, the predatory mite *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) and the predatory insects *Stethorus punctillum* Weise (Coleoptera: Coccinellidae), *Conwentzia psociformis* (Curtis) (Neuroptera: Coniopterygidae) and others significantly decreased the damage levels caused by TSSMs in strawberries (Garćia-Mar and Gonzalez-Zamora 1999). The predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) is one of the most widely used biological agents against TSSMs, whereas this species can consume only 2 or 3 adults or several dozen egg in a day (Nachman 1987).

The entomopathogenic fungus, *Isaria cateniannulata* (strain 08XS - 1) increased mortalities to 100%, 100%, and 70% in TSSM eggs, larvae and adult females, respectively, at 25°C and 100% RH (Zhang et al. 2016). The entomopathogenic fungi, *Metarhizium anisopliae*, *Paecilomycis fumosoroseus*, and *Verticillium lecanii* decreased the hatchability of TSSM eggs, and *P. fumosoroseus* was most effective to increase the mortality of adult female TSSMs (Amjad et al. 2012). High mortality in TSSMs was induced by *Beauveria bassiana* (Jeyarani et al. 2011); the hatchability decreased to 25% and the adult mortality reached to 89% (Hassan et al. 2017).

However, biological control using natural enemies such as predatory mites not always has the satisfied suppression effect on the pest populations, which is mainly due to the

fluctuation of prey-predator population densities (Ehler and Kinsey 1995). For the efficiency in control of the spruce spider mite *Oligonychus ununguis* (Jacobi) (Acari: Tetranychidae), using the predatory mites *Neoseiulus fallacis* (Garman) and *Metaseiulus* (*Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) (Acari: Phytoseiidae) was 2.5~7 times higher than using chemical pesticides when the initial pest population was high (Shrewsbury and Hardin 2003). In addition, entomopathogenic fungi can be affected adversely by hash environments such as ultraviolet radiation (UV), but the sensitivity to UV varies among different fungi species and strains (Fargues et al. 1996). Taken together, biological control alone still remains unsatisfied to maintain the TSSM population at less than economic injury levels on crops. Thus, development of alternative control measures to enhance integrated pest management (IPM), “an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides (FAO 2019)”.

1.4. RNA interference (RNAi)

RNA interference (RNAi)-mediated gene silencing is one of the promising approaches for pest control (Meister and Tuschl 2004). RNAi was first discovered in the nematode worm *Caenorhabditis elegans* Maupas (Nematoda: Rhabditida) in which the destruction of messenger RNA (mRNA) is induced by the double-stranded RNA (dsRNA) fragments that have complementary nucleotide sequences to the mRNA (Fire et al. 1998). Fire was awarded to the 2006 Nobel Prize for Physiology or Medicine with Carig Mello for their first discovery RNAi. The mode of action of RNAi is 1) dsRNA is processed into short interfering RNA (siRNA) consisting of 21 to 25 nucleotides long by the RNase III enzyme Dicer-2 (Lee et al. 2004). In the second step, siRNAs are loaded into the RNase H enzyme Argonaute to form the effector complex RNA-induced silencing complex (RISC). The siRNA is unwound during RISC assembly and the single-stranded RNA hybridizes with target mRNA (Agrawal et al. 2003).

RNAi in TSSM was first conducted with direct injection of dsRNA or siRNA targeting on *Distal-less*, a conserved gene involved in appendage development, to adult females and the maternal effect was observed in their progeny (Khila and Grbic 2007). However, handling for the injection into a tiny animal such as TSSMs is difficult and time-consuming, and thus is not suitable for the RNAi screen using a large sample size. Therefore, leaf disc-mediated oral administration of dsRNA has been conducted for RNAi-mediated gene silencing in TSSMs (Kwon et al. 2013, 2016). This method was effective to test the potential gene targets for controlling TSSMs, whereas it consumed a huge volume of dsRNA. In another method, dsRNA was successfully delivered into TSSMs through ingestion of leaf discs coated with a small volume of dsRNA (Suzuki et al. 2017b). However, the dsRNA solution must be spread manually over the surface of leaf discs, which is also time-consuming.

Vacuolar-type H^+-ATPase (V-ATPase) has been a common target gene for RNAi-based control of pest insects (Baum et al. 2007; Whyard et al. 2009; Upadhyay et al. 2011; Zhu et al. 2011). *V-ATPase* is a conserved gene encoding a constitutively expressed ATP-driven proton pump though the membranes (Finbow and Harrison 1997). RNAi targeted on the *V-ATPase* in TSSMs (*TuVATPase*) induced the mortality (Kwon et al. 2016; Suzuki et al. 2017b), the reductions of fecundity and digestive cell function (Bensoussan et al. 2018) resulting an indigestion-related phenotype which showed a dark color in the almost whole digestive system (Suzuki et al. 2017b). Other potential target genes for controlling TSSMs are 1) the *coatomer protein subunit E* (*TuCOPE*) gene encoding a vesicle transporter protein between the endoplasmic reticulum and Golgi, 2) the *ribosomal protein S4* (*TuRPS4*) gene encoding a protein that functions in post-translational modifications, 3) the major intrinsic protein (*TuMIP*) gene encoding a protein that functions as a transmembrane channel, and 5) the *aquaporin 9* (*TuAQP9*) gene encoding a protein that function as a water channel, because of the high mortality after RNAi with the leaf disc-mediated oral delivery of dsRNA (Kwon et al. 2016).

1.5. Surfactants

Surfactants are required for the foliar application of many sprayable pesticides. Surfactants decrease the surface tension of aqueous solutions and act as a wetting agent (Fig. 1). Enhanced distribution of the aqueous solution of pesticides can improves pest control performance on sprayed plants. Based on the dissociation in water, surfactants are classified into anionic, nonionic, and cationic groups (Venezuela 2002; Tu et al. 2001).

Silwet L-77 is a non-ionic organosilicone surfactant that has a remarkable ability to reduce the surface tension of aqueous solutions. The application of Silwet L-77 allows aqueous solutions to interact with hydrophobic or waxy surfaces and consequently enhances the spreading over leaves (Witco 1997; Pollicello et al. 1995). Silwet L-77 has often used to improve the efficiency of sprayable pesticides. The efficiency of abamectin was improved using Silwet L-77 against eggs and nymphs of the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) whereas the improvement did not appear in adults (Srinivasan et al. 2008). Silwet L-77 has also used to increase the efficiency of the entomopathogenic fungi such as *Beauveria bassiana* and *Metarhiziopsis microspore* to control different arthropod pests (Leland 2004; Gouli et al. 2013).

The Silwet hydrostable products Silwet L-77, Silwet 408, and Silwet 806 were toxic to adult female TSSMs with the lethal dose for 50% mortality (LC_{50}) was 8.6, 5.5, and 8.9 ppm (v/v), respectively (Cowles et al. 2000). On the other hand, Silwet L-7200 and Silwet L-7607 were less toxic whereas the reduction of surface tension was lower than Silwet L-77, Silwet 408, and Silwet 806 (Cowles et al. 2000). The toxicity of Silwet L-77 at the concentrations 0.1% to 0.5% (v/v) has also reported in other arthropods such as the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae), Western flower thrips *Frankliniella occidentalis* (Pergande)

(Thysanoptera: Thripidae), and Pacific spider mite *Tetranychus pacificus* (McGregor) (Acari: Tetranychidae) (Tipping et al. 2003).

Tween 20 [polyoxyethylene (20) sorbitan monolaurate], a nonionic surfactant, is widely used in the fields of cell and molecular biology to decrease the surface tension of aqueous solutions because of its less toxicity. The US Food and Drug Administration (FDA) has been approved Tween 20 as an additive in food and pharmaceutical preparations (Ravichandran et al. 2018). So far, there are no reports showing the negative effects of Tween 20 on arthropods. In addition to its less toxicity, Tween 20 can improve drug passage across the cell membrane by changing the physical properties (Hua et al. 2018). Although there are no reports on the practical use of Tween 20 for the foliar application as a pesticide spreader, it may enhance the RNAi efficiency because the high membrane permeability has a potential to improve the cell uptaking of dsRNA in TSSMs.

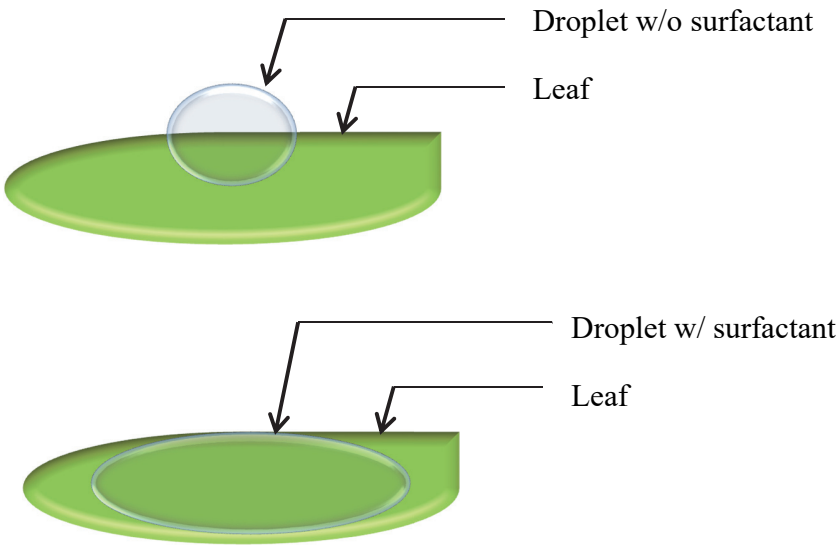

Fig. 1. Effect of surfactants on distribution of an aqueous solution on leaf surface.

1.6. Mission and objectives

TSSMs have rapidly developed the resistance to all acaricides. To control this pest species, development of technologies that are alternative to chemical measures and enhancing the IPM program are urgently needed. RNAi is a post-transcriptional gene silencing and has the potential for pest species-specific control because of the target sequence-specific manner, which may be established as environment-friendly measures for controlling pest but conserving beneficial organism and introduced to the IPM program. To achieve this goal, first oral delivery of small molecules such as dsRNA to TSSMs via leaves needs to be developed. In the previous study, the organosilicone surfactant Silwet L-77 was used for enhancing distribution of the aqueous solution of test compounds over the surface of leaf discs (Suzuki et al. 2017a) but toxic for TSSMs (Cowles et al. 2000).

In the Chapter 2, I developed new method that can prepare leaf disc coated with test compounds in the absence of surfactants.

In the Chapter 3, I examined the RNAi efficiency with this delivery method when changing the concentration of dsRNA targeting on *TuVATPase* which encodes a protein that functions as a proton pump located in membranes to maintain the acidic condition in vesicles.

In the Chapter 4, I examined RNAi effects on most potential four target genes *TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9* with my method.

Chapter 2. Leaf disc-mediate oral delivery of small molecules in the absent of surfactant to the two-spotted spider mite, *Tetranychus urticae*

2.1. Abstract

The two-spotted spider mite (TSSM), *Tetranychus urticae*, is a chelicerate herbivore with a wide host range and strong ability to develop pesticide resistance. In addition to easy maintenance of experimental TSSM populations, the recent completion of the TSSM genome sequence and development of RNA interference–based reverse genetics protocols make TSSM an ideal chelicerate model for the study of pesticide resistance and plant–herbivore interactions. In such studies, leaf discs treated with a test compound are often used as a means for oral delivery. When preparing the leaf discs, the organosilicone surfactant Silwet L-77 is frequently used to promote wetting of the leaf surface and to ease the distribution of a test compound across the leaf surface. Here, I tested the toxicity of Silwet L-77 to TSSM and found that it is toxic. Hence, I developed a novel method of preparing leaf discs in which a polypropylene sheet rather than a surfactant was used to ensure sufficient distribution of a tracer dye across the leaf surface. These leaf discs were then successfully used to deliver the tracer dye into the midgut of TSSMs. In addition, no significant differences were observed in the survival, fecundity, or feeding activity of TSSMs fed on leaf discs treated with water via my novel method compared with those fed on untreated leaf discs. Thus, my novel method of preparing leaf discs eliminates concerns regarding the bioactivity of surfactants in TSSMs, and I anticipate that this method will be useful for improving oral delivery–based bioassays for TSSMs.

2.2. Introduction

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae), is a major agricultural pest. Due to its short life-cycle and the high reproductive potential accompanied with an enhanced metabolic detoxification system (Dermauw et al. 2013; Piraneo et al. 2015), TSSM is capable of rapidly developing resistance to almost all types of pesticides (Van Leeuwen et al. 2010; Mota-Sanchez and Wise 2017), making it difficult to control.

RNA interference (RNAi) is a type of post-transcriptional gene silencing that is induced by the delivery of double-stranded RNA (dsRNA) into a cell's cytoplasm (Fire et al. 1998). In arthropods, RNAi can be induced by injecting either dsRNA or small interfering RNA (siRNA) into eggs or body cavities of test animals. RNAi in TSSMs was first reported by Khila and Grbić (2007), who injected dsRNA and siRNA into adult female TSSMs. This work targeted *Distal-less* (*Dill*), an evolutionally conserved gene involved in appendage development in metazoans and found that mite offspring embryos showed truncated or fused appendage phenotypes. No success in using direct injection to TSSM eggs has been reported (Bryon et al. 2017) because of their small size (approx. 0.1 mm) and susceptibility to damage resulting from the injection.

Compared with dsRNA injection, oral administration of dsRNA is less invasive and has the potential to be used in high-throughput RNAi screens. For the oral administration of dsRNA to TSSMs, leaf disc–mediated delivery methods have been used recently (Kwon et al. 2013, 2016, 2017; Shi et al. 2015, 2016; Liao et al. 2016; Xia et al. 2016; Li et al. 2017; Suzuki et al. 2017b; Xu et al. 2017a, b). Leaf disc–mediated delivery of dsRNA has drawn intense interest because these methods mimic a potential application of dsRNA as a sprayable pesticide (San Miguel and Scott 2015; Suzuki et al. 2017b). Among the different leaf disc–mediated delivery methods reported, the use of dsRNA-coated leaf discs (Suzuki et al. 2017b) requires the smallest

amount of dsRNA but manual painting to spread dsRNA over the leaf surface is generally time-consuming.

The organosilicone surfactant Silwet L-77 that is commonly used in *Agrobacterium*-mediated plant transformation to increase the infiltration into plant tissues (Clough and Bent 1998), could be also used to ensure the distribution of a small volume of the aqueous solution over the leaf surface (Suzuki et al. 2017a). However, it has been shown that Silwet L-77 is toxic to TSSMs with a concentration for 50% mortality (LC_{50}) ranging from 8.6 to 84.2 ppm (v/v) depending on the host plant species (Cowles et al. 2000). Thus, avoiding the use of Silwet L-77 when preparing leaf discs would provide improved bioassays with greater reliability. Here, I developed a method of preparing a test compound–infiltrated leaf discs with a small volume of the aqueous solution that does not include the use of a surfactant.

2.3. Material and methods

2.3.1. Mite preparation

The TSSM population collected in London (Ontario) that was previously used for whole-genome sequencing (Grbic et al. 2011) was used in this study. The population was maintained on kidney beans (*Phaseolus vulgaris* L.) at 25°C and 50% relative humidity (RH) under a light period of 16 h d^{-1}. By following the methods of Suzuki et al. (2017a) with some modifications, I synchronized the molting of teleiochrysalis females to obtain newly emerged adult females. Briefly, teleiochrysalis females were collected with an air pump–based sucking system (Cazaux et al. 2014) and transferred to a Petri dish (diameter, 35 mm). The Petri dish was placed inside a polystyrene cup (diameter, 94 mm), the bottom of which was lined with water-soaked cotton. The cup was sealed with a lid to keep 100% RH inside and then incubated at 20°C for 2 days. After incubation, the lid was removed and the teleiochrysalis females were incubated at 25°C and 50% RH for 30 to 60 min to initiate their molting. After molting, newly emerged adults were used in the following experiments.

2.3.2. Sandwich method

A droplet (6 μL) of the aqueous solution of interest was added onto the adaxial surface of kidney bean leaf discs (diameter, 10 mm; 1 droplet/disc). Then, the leaf discs were covered with a polypropylene sheet (AZ-540; Sekisei, Tokyo, Japan) which is generally available as transparent card sleeves, so that the droplet of aqueous solution was sandwiched between the adaxial surface and the polypropylene sheet. Formation of this sandwich structure caused the droplet of aqueous solution to immediately spread over the adaxial surface of the leaf discs. The leaf discs were then maintained at 25°C and 50% RH for approximately 8 min to allow the aqueous solution to

infiltrate the leaf disc. After infiltration, the leaf discs were gently removed from the polypropylene sheet and the adaxial surface was allowed to dry at 25°C and 50% RH for up to 1 min (Fig. 2).

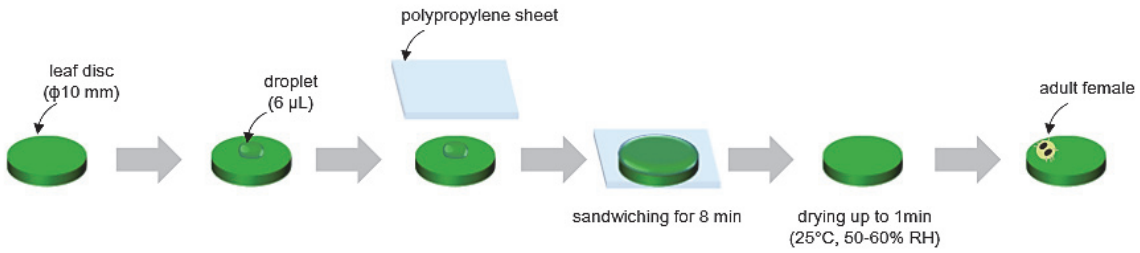

Fig. 2. Sandwich method. A 6-μL droplet of test compound solution was added to the adaxial surface of a ϕ10-mm bean leaf disc. A piece of polyethylene sheet was then placed over the leaf disc to spread the solution on the adaxial surface. The sandwiching condition was kept to infiltrate the solution into the leaf disc up to 8 min and then the polyethylene sheet was removed to completely dry the adaxial surface of the leaf disc. Finally, a test mite was inoculated onto the leaf disc to conduct a bioassay.

2.3.3. Oral delivery test

First, I compared the effectiveness of the coating method for preparing leaf discs (Suzuki et al. 2017a) and my novel sandwich method for the oral delivery of a small molecule to the midgut of TSSMs by using a fluorescent tracer (Alexa Fluor 488; Thermo Fisher Scientific, Waltham, MA). Fluorescent signals are visible in the TSSM digestive tract because they have a semi-transparent body (Suzuki et al. 2017a). In the coating method of preparing leaf discs, the adaxial surface of a bean leaf disc (diameter, 10 mm) was coated with a droplet (6 μL) of an aqueous solution containing 100 ng μL^{-1} fluorescent tracer and 250 ppm (v/v) Silwet L-77 as described by Suzuki et al. (2017a). In the sandwich method of preparing leaf discs, the adaxial surface of a bean leaf disc was coated with a droplet (6 μL) of an aqueous solution containing 100 ng μL^{-1} of the fluorescent tracer alone. Uncoated leaf discs served as controls. For all treatments, the leaf discs were placed on water-soaked cotton at the bottom of separate polystyrene cup. Newly emerged adult females were inoculated onto the leaf discs (1 female/disc) by using a tapered brush (Interlon 1026-3/0; Maruzen Artist Materials & Works, Tokyo, Japan). The polystyrene cup was then maintained at 25°C and 50% RH under a light period of 16 h d^{-1} for 4 days. For all treatments, fresh leaf discs were provided at the beginning of day 3. At the end of day 4, the mites were mounted in 50% (v/v) glycerol and 0.1% (v/v) Tween 20 in phosphate-buffered saline solution, and fluorescent signals from the digestive tract were observed with a fluorescence stereoscopic microscope (M205 FA; Leica Microsystems, Wetzlar, Germany). RGB images under visible light and fluorescence excitation light were recorded with a digital camera (EOS Kiss X7; Canon, Tokyo, Japan) installed on the microscope. The oral delivery tests were repeated three times.

2.3.4. Mite performance test

To examine the effects of Silwet L-77 and the method of preparing leaf discs on TSSM survivorship and fecundity, leaf discs (diameter, 10 mm) were prepared by using the coating method and a droplet (6 μL) of an aqueous solution of Silwet L-77 (250 ppm [v/v]) or the sandwich method and a droplet of water, as described in the previous sections. Untreated leaf discs were used as controls. Newly emerged adult females were inoculated onto the leaf discs (1 female/disc) and allowed to feed at 25°C and 50% RH under a light period of 16 h d^{-1}. All leaf discs were replaced with fresh ones every 2 days up to day 6. Survivorship and fecundity were recorded every day, and the test was repeated three times.

A separate experiment was used to examine feeding activity. After adult females had been left to feed for 2 days on leaf discs prepared by using either the coating method and Silwet L-77 or the sandwich method and water, or on untreated control leaf discs (5 females/disc), as described above for the survivorship and fecundity experiment, RGB images of the adaxial surface of each leaf disc were obtained with an image scanner (GT-X980; Seiko Epson, Suwa, Japan). The mite performance tests were repeated five times.

2.3.5. Data analysis

The RGB images of the TSSMs in the oral delivery tests were split into red, green, and blue component images and converted to 8-bit (0 to 255) grayscale by using ImageJ 1.51j8 (ImageJ command: ‘Split Channels’). Fluorescence signals from the digestive tract of treated mites were defined as pixels exhibiting a grayscale value from 37 to 255 in the green component images (ImageJ command: ‘Threshold’). In the range of grayscale values, no positive pixels were counted from the green component images of TSSMs fed on control leaf discs. The area of

fluorescence in the digestive tract was calculated from the ratio of the number of positive pixels to the total number of pixels in the image and relativized to the maximum value within the individual data (ImageJ command: 'Analyze Particles'). Arcsine square-root transformation was applied to normalize the relative area of fluorescence. Differences in the normalized relative area of fluorescence among the treatments were statistically analyzed with Turkey's honestly significant difference test after one-way ANOVA in R 3.3.2 (R Core Team 2016; R function: 'glht', package: 'multcomp'). Survival curves were plotted with the Kaplan–Maier method (R function: 'survfit', package: 'survival'). Differences in the survival curves among treatments were statistically analyzed by using the log-rank test (R function: 'survdiff', package: 'survival'). Differences in TSSM fecundity among treatments were statistically analyzed by using the Wilcoxon–Mann–Whitney test with Bonferroni correction (R function: 'pairwise.wilcox.test'). To quantify TSSM feeding activity, five leaf discs in each treatment group were randomly sampled 2 days after treatment and the area of leaf disc damage caused by the five adult females was determined by using a digital imaging method (Cazaux et al. 2014) using Adobe Photoshop CC 19.1.5 (San Jose, CA). Differences in the area of leaf disc damage among the treatments were statistically analyzed by using Tukey's honestly significant difference test after one-way ANOVA. Results for the relative area of fluorescence in the TSSM digestive tract, the fecundity of treated mites, and the damaged area of leaf discs are presented as overlaid bee-swarm (R function: 'beeswarm', package: 'beeswarm') and box-and-whisker plots (R function: 'boxplot').

2.4. Results

2.4.1. Oral delivery test

Irrespective of whether the coating or sandwich method was used to prepare the leaf discs, the fluorescent tracer was distributed over the entire adaxial surface of the leaf discs (data not shown) and was detected in the digestive tracts of TSSMs fed on the leaf discs (Fig. 3A). Although the relative areas of fluorescence between TSSMs fed on leaf discs prepared with the coating or sandwich methods were comparable, the relative area of fluorescence in TSSMs fed on leaf discs prepared with either method was significantly higher than that in TSSMs fed on control leaf discs (Fig. 3B).

2.4.2. Mite performance test

No significant differences were observed among the survival curves obtained for TSSMs fed on the three types of leaf discs (Fig. 4A). Fecundity in TSSMs fed on leaf discs prepared via the sandwich method was comparable with that in TSSMs fed on control leaf discs, whereas it was significantly lower in TSSMs fed on leaf discs prepared via the coating method compared with that in TSSMs fed on control leaf discs or leaf discs prepared by the sandwich method (Fig. 4B). The same trend was observed for the area of leaf disc damage after feeding (Fig. 5); a significantly lower area of leaf disc damage was observed for leaf discs prepared by the coating method compared with control leaf discs or leaf discs prepared by the sandwich method.

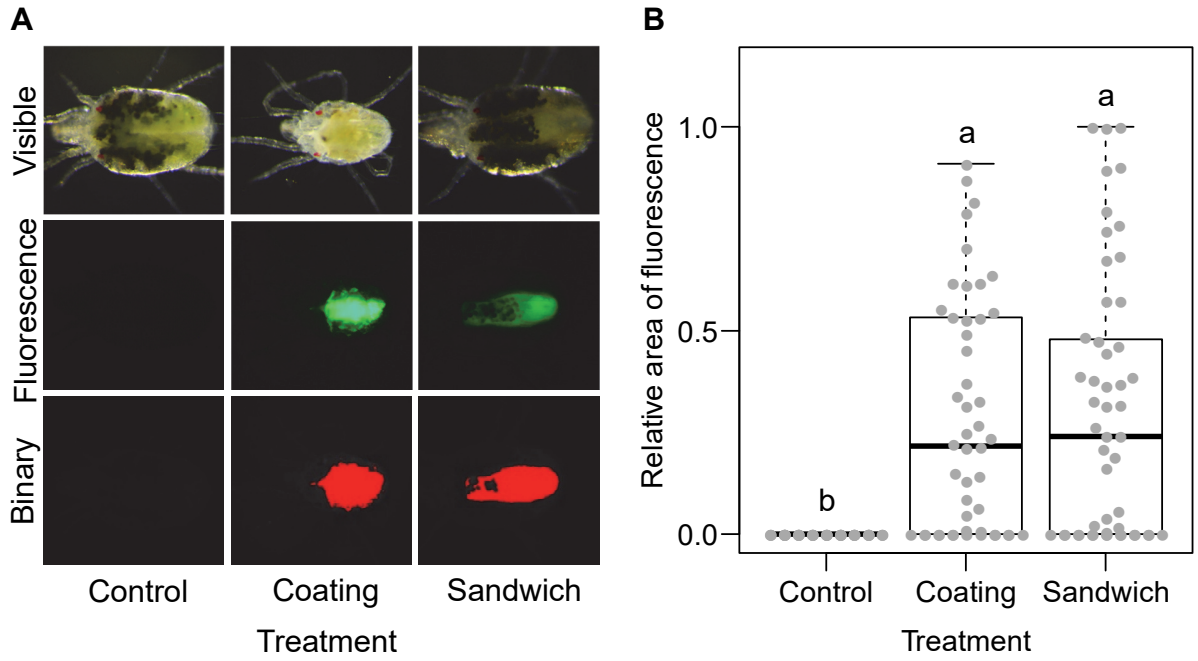

Fig. 3. Adult female two-spotted spider mites (*Tetranychus urticae*) after feeding for 4 days on untreated bean leaf discs (control) or bean leaf discs treated with a green-fluorescent tracer (Alexa Fluor 488, 100 ng μL^{-1}). Leaf discs were replaced with fresh ones at the beginning of day 3. (A) Accumulation of the fluorescent tracer in mites fed on leaf discs prepared via a coating method (Suzuki et al. 2017a) using 250 ppm Silwet L-77 or my sandwich method without surfactant. (B) Relative area of fluorescence based on the number of pixels exhibiting a grayscale value between 37 and 255 in 8-bit green-component images of the mites (A, binary). Arcsine square-root transformation was applied to normalize the relative area of fluorescence before statistical analysis. Different letters indicate significant differences at $p < 0.05$ (Tukey's honestly significant difference test after one-way ANOVA). Individual data are displayed as a bee swarm. In the box-and-whisker plots, the central line (second quartile, Q2) indicates the median, the distance between the box bottom (first quartile, Q1) and top (third quartile, Q3) indicates the interquartile range (IQR), and the whisker bottom and top indicate the minimum and maximum values, respectively.

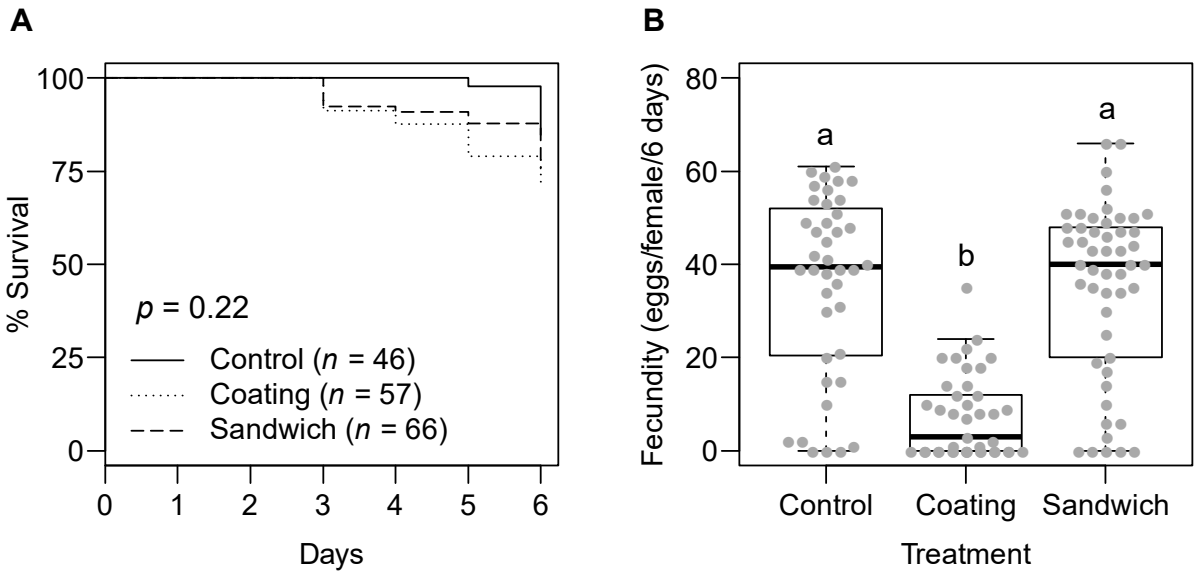

Fig. 4. Survival and fecundity of adult female two-spotted spider mites (*Tetranychus urticae*) after feeding on untreated bean leaf discs (control) or bean leaf discs prepared with the coating method and 250 ppm (v/v) Silwet L-77 or with the sandwich method and water. Leaf discs were replaced with fresh ones every other day. (A) Survival of mites during a 6-day period feeding on untreated or treated leaf discs. Survival curves were plotted by using the Kaplan–Maier method and compared by using the log-rank test. (B) Fecundity of mites during a 6-day period feeding on untreated or treated leaf discs. (See the caption to Fig. 5 for explanations of the bee swarm and box-and-whisker plots. Outliers that are outside the range between the lower (Q1 − 1.5 × IQR) and upper limits (Q3 + 1.5 × IQR) are plotted outside of the IQR.) Different letters indicate significant differences at $p < 0.05$ (Wilcoxon–Mann–Whitney test with Bonferroni correction).

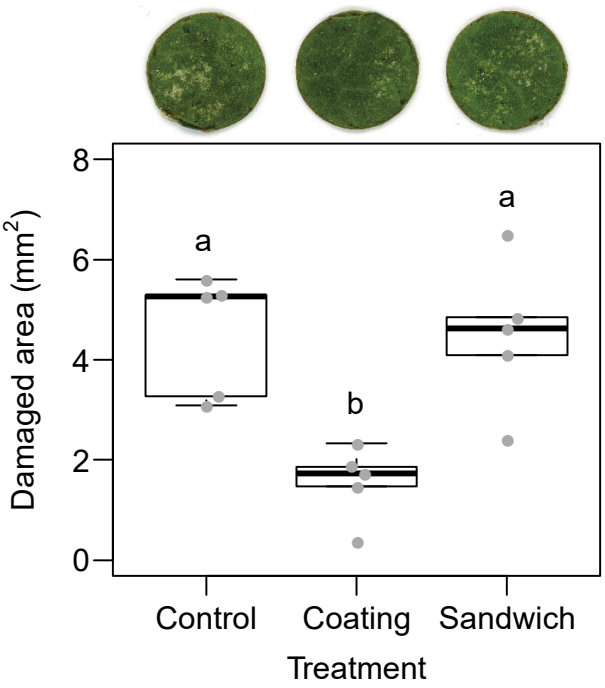

Fig. 5. Leaf disc damage after a 2-day feeding period by adult female two-spotted spider mites (*Tetranychus urticae*) on untreated bean leaf discs (control) or bean leaf discs prepared with the coating method and 250 ppm (v/v) Silwet L-77 or with the sandwich method and water. (See the captions to Figs. 5 and 6 for explanations of the bee swarm, box-and-whisker plots, and outliers.) The area of leaf disc damage caused by a single female was determined by using a digital imaging method (Cazaux et al. 2014). Different letters indicate significant differences at $p < 0.05$ (Tukey's honestly significant difference test after one-way ANOVA).

2.5. Discussion

For the oral delivery of small molecules, a system in which TSSMs feed on a solution of the xenobiotic sealed behind a stretched artificial membrane (usually Parafilm M; Bemis NA, Neenah, WI) by piercing the membrane with their stylet was developed decades ago (Walling et al. 1968; Salama and Rasmy 1971; Kantaratanakul and Rodriguez 1979; Van Der Geest et al. 1983; Hare and Bethke 1988). This membrane-based feeding system has enabled researchers to use spider mites in nutrition and oral toxicity assays (e.g., Bosse and Veerman 1996; Gotoh et al. 2008) or to collect spider mite saliva (Jonckheere et al. 2016; Zhu et al. 2018). Recently, assays for gene function have also been conducted by using the membrane-based feeding system for oral administration of dsRNA for RNAi-mediated gene silencing in TSSMs (Suzuki et al. 2017b). However, success in TSSM development from the egg to adult stages has been limited in the membrane-based feeding system because of insufficient nutrition or uncomfortable environments for TSSMs (Van Der Geest et al. 1983; Suzuki et al. 2017b); thus, leaf discs still remain to be used as a main material for laboratory bioassays using TSSMs.

Recently, several leaf disc–mediated feeding systems were developed for the oral administration of dsRNA to TSSMs for RNAi-based screening of novel pesticides (Kwon et al. 2013, 2016, 2017; Shi et al. 2015, 2016; Liao et al. 2016; Xia et al. 2016; Li et al. 2017; Suzuki et al. 2017b; Xu et al. 2017a, b). These feeding systems can be divided into three types: (1) feeding on leaf discs floating on a dsRNA solution (Kwon et al. 2013, 2016, 2017), (2) feeding on dehydrated leaf discs that are subsequently infiltrated with dsRNA solution (Shi et al. 2015, 2016; Liao et al. 2016; Xia et al. 2016; Li et al. 2017; Xu et al. 2017a, b), and (3) feeding on leaf discs coated with dsRNA (Suzuki et al. 2017b). Of the three systems, the leaf disc coating method requires the smallest amount of dsRNA. Although no detailed procedure was mentioned

for the leaf disc coating method (Suzuki et al. 2017b), manual painting of the dsRNA solution with a fine material (e.g. pipette tip) is required to spread it over the adaxial surface and this step is time consuming. To improve the coating method, Suzuki et al. (2017a) proposed the use of a surfactant such as Silwet L-77 which reduces the surface tension of water. The coating method using Silwet L-77 enhanced the spreading of the aqueous solution containing the test compound across the epicuticular wax that covers the leaf adaxial surface and did not require the painting step in the previous method (Suzuki et al. 2017b). In the present study, although no significant reduction in survival was observed in TSSMs fed on leaf discs prepared by using this coating method (Fig. 4A), TSSMs fed on such leaf discs developed a small body and the caecum and ventriculus contained few fully developed digestive cells (Fig. 3A) (Bensoussan et al. 2018). I also found that TSSMs fed on such leaf discs had significantly reduced fecundity (Fig. 4B) and feeding activity (Fig. 5) compared with TSSMs fed on control leaf discs. The toxicity of Silwet L-77 has been reported not only in TSSMs but also in other insect pests such as aphids, armyworms, fruit flies, leafminers, thrips, mealybugs, and psyllids (Imai et al. 1995; Chandler 1995; Purcell and Schroeder 1996; Shapiro et al. 1998; Cowles et al. 2000; Tipping et al. 2003; Srinivasan et al. 2008). Compared with the toxicity of dipping TSSMs directly in Silwet L-77, which in that context has a reported LC_{50} value ranging from 8.6 to 84.2 ppm (Cowles et al. 2000), the oral toxicity of leaf discs prepared by using the coating method and Silwet L-77 was moderate even at 250 ppm. In contrast, no negative effects on survival and fecundity were observed in TSSMs fed on leaf discs prepared by using the coating method that used Silwet L-77 (Suzuki et al. 2017a). The effect of Silwet L-77 on fecundity of TSSM differed despite the same concentration (250 ppm) and mite populations were used in both studies. At present, the basis of this discrepancy is not clear, however, it may be worth noting that different cultivars of the host

bean plants were used. Regardless, because I observed negative effects on fecundity (Fig. 4B) and feeding (Fig. 5), and the lethality of Silwet L-77 has been reported by Cowles et al. (2000), the use of Silwet L-77 should be avoided to provide more precise bioassays.

In the present study, I used a polypropylene sheet instead of Silwet L-77 (Suzuki et al. 2017a) or time-consuming painting (Suzuki et al. 2017b) to ensure that the test solution coated the entire leaf surface. By using this method of preparing leaf discs, I was able to deliver a fluorescent tracer to the midgut of TSSMs (Fig. 3). Because TSSMs feed on the contents of palisade and spongy mesophyll cells beneath the leaf epidermis by pushing their stylet either in between pavement cells or through stomatal openings (Bensoussan et al. 2016), my results suggest that the fluorescent tracer was not only spread over the leaf surface but was able to infiltrate into leaf mesophyll cells despite the absence of a surfactant. An equivalent level of dye uptake between the coating and sandwich methods was observed (Fig. 3); however, there were significant differences in fecundity (Fig. 4B) and leaf damage inflicted by mite feeding (Fig. 5). This suggests that Silwet L-77 delivered to the midgut of TSSMs inhibits feeding such that normal fecundity is prevented. No significant reductions in survival, fecundity, or feeding activity were observed in TSSMs fed on leaf discs prepared with the sandwich method and water (Figs. 4 and 5).

Thus, my novel sandwich method may be useful for the delivery of small molecules to the midgut of TSSMs without inducing negative effects on their biology. Further studies are warranted to examine whether this method can be used for the oral administration of small molecules to other arthropods that feed on leaf cell contents. I anticipate that my method will be useful for preparing leaf discs of oral delivery of small molecules to TSSMs for use in a wide

range of bioassays such as in RNAi silencing, examining the efficacy and biological effects of pesticides, and elucidating the interactions of herbivores with plants, fungi, bacteria, and viruses.

2.6. Conclusion

Delivery method is an important element to determine the efficiency of dsRNA, therefore, to focus on how to deliver dsRNA for different genes to pests is the key to successful control application. Sandwich method showed high efficiency to deliver the fluorescent dye without any surfactant when allow the TSSMs fed on treated leaf discs when they showed high fluorescent signals, which lead to a successful method can be an easy and simple method to examine the target genes in future.

Chapter 3: Dose-response effect of dsRNA targeting *Vacuolar-type H^+-ATPase* in the two-spotted spider mite, *Tetranychus urticae*

3.1. Abstract

RNA interference (RNAi)-mediated gene silencing by oral delivery of double-stranded RNA (dsRNA) has potential for new method of controlling pest arthropods. The two-spotted spider mite (TSSM), *Tetranychus urticae*, is a cosmopolitan herbivore that feeds on >1100 plant species including >150 agricultural crops. TSSM is one of the pest species that has motivated the development of RNAi-based control strategy because its whole genome was the first among chelicerates to be completely sequenced and annotated. In addition, several methods for oral delivery of dsRNA to TSSMs have been preparing for the RNAi-based platform for functional gene screens in this species. Here I examined the efficacy of a leaf disc-mediated method currently developed for the oral delivery of test compounds to TSSMs via their feeding. The Vacuolar-type H^+-ATPase (TuVATPase) was used as a reference baseline gene because the silencing effect is obvious from the body color change (yellow-green to dark) and decreases in survival and fecundity. Almost 40% of TSSMs showed dark body color after feeding on leaf discs treated with dsRNA designed for *TuVATPase* (dsRNA-*TuVATPase*) at 0.1 or 1 μg μL^{-1}, whereas the phenotype was not observed in the treatment with dsRNA-*TuVATPase*) at 0.01 μg μL^{-1}. This trend was commonly observed in decreases of fecundity and survival. Tween 20 has no improving the efficiency of dsRNA neither in survival rate nor in fecundity. Taken together, dsRNA-*TuVATPase* at lowest 0.1 or 1 μg μL^{-1} has a potential for foliar application to control TSSMs.

3.2. Introduction

In the two-spotted spider mite (TSSM) *Tetranychus urticae* Koch (Acari: Tetranychidae), the whole genome had been sequenced as the first complete chelicerate genome and was the smallest sequenced arthropod genome at 90 megabases (Grbic et al. 2011) which help the scientists to find a new a potential target genes to be silencing to control this pest. Based on this genome, one of the most potential target genes for TSSM control is *Vacuolar-type H^+-ATPase* (*V-ATPase*) encoding a protein that is an ancient enzyme in all eukaryotes. It was first proven that V-ATPase functions as a proton pump operated by the ATP hydrolysis in tonoplasts of vacuoles (Kirshner 1962). V-ATPases locate in organelles membranes, where they mediate proton transport (Pedersen and Carafoli 1987), and it is a primarily responsible for the establishment and maintenance the acidity condition in both endocytic and secretory organelles by pumping cytosolic protons into the lumen vacuoles using the energy generated from ATP hydrolysis (Nelson et al. 2000; Forgac 2007; Nishi and Forgac 2002). V-ATPase has two domains; peripheral V_1 domain composed of eight subunits (A to H) of molecular mass 70~10 kDa, and responsible for ATP hydrolysis; but integral V_0 domain composed of six different subunits (a, d, c, c', c'', e) of molecular mass 100–9 kDa and carries out proton translocation (Arai et al. 1988; Forgac 1998; Ohira et al. 2006; Kitagawa et al. 2008).

In the silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), oral administration of dsRNA targeting on *V-ATPase* caused a high mortality and the LC_{50} was 3.08 ng μL^{-1} (Upadhyay et al. 2011). Oral administrations of bacteria expressing dsRNA targeting on *V-ATPase* or *in vitro* synthesized dsRNA of the same target induced a mortality in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Zhu et al. 2010). Direct injection of dsRNA targeting on *V-ATPase* subunits A and E dramatically decreased the

survival and fecundity in the bed bug *Cimex lectularius* L., (Hemiptera: Cimicidae) (Basnet and Kamble 2018). The *V-ATPase* gene in TSSMs (*TuVATPase*) was downregulated and caused 21% corrected mortality at 120 h after feeding on leaf disc-infiltrated with 160 ng μL^{-1} dsRNA targeting on *TuVATPase* (dsRNA-*TuVATPase*) (Kwon et al. 2013).

It was revealed that 160 ng μL^{-1} dsRNA-*TuVATPase* induced dark-body phenotype and decreased the survival and fecundity in TSSMs with five different methods for oral delivery of dsRNA (socking, leaf disc coating, leaf disc floating, artificial diet, and transgenic plants expressing a hairpin dsRNA) (Suzuki et al. 2017b). Therefore, *TuVATPase* is highly potential target gene can be used in the foliar application of dsRNA for RNAi-based TSSM control. However, the dose-response relationship between dsRNA-*TuVATPase* and RNAi effects in TSSMs has remained unclarified.

In this Chapter, I used the sandwich method (Abouelmaaty et al. 2019) to orally deliver different concentrations of dsRNA-*TuVATPase* with or without the nonionic surfactant, Tween 20 [polyoxyethylene (20) sorbitan monolaurate]. It has been known that Tween 20 causes a change in the properties of cell membrane and improves drag permeability (Hua et al. 2018). Therefore, it is expected that Tween 20 may improve the permeability of dsRNA into cells in TSSMs and enhance the RNAi effects

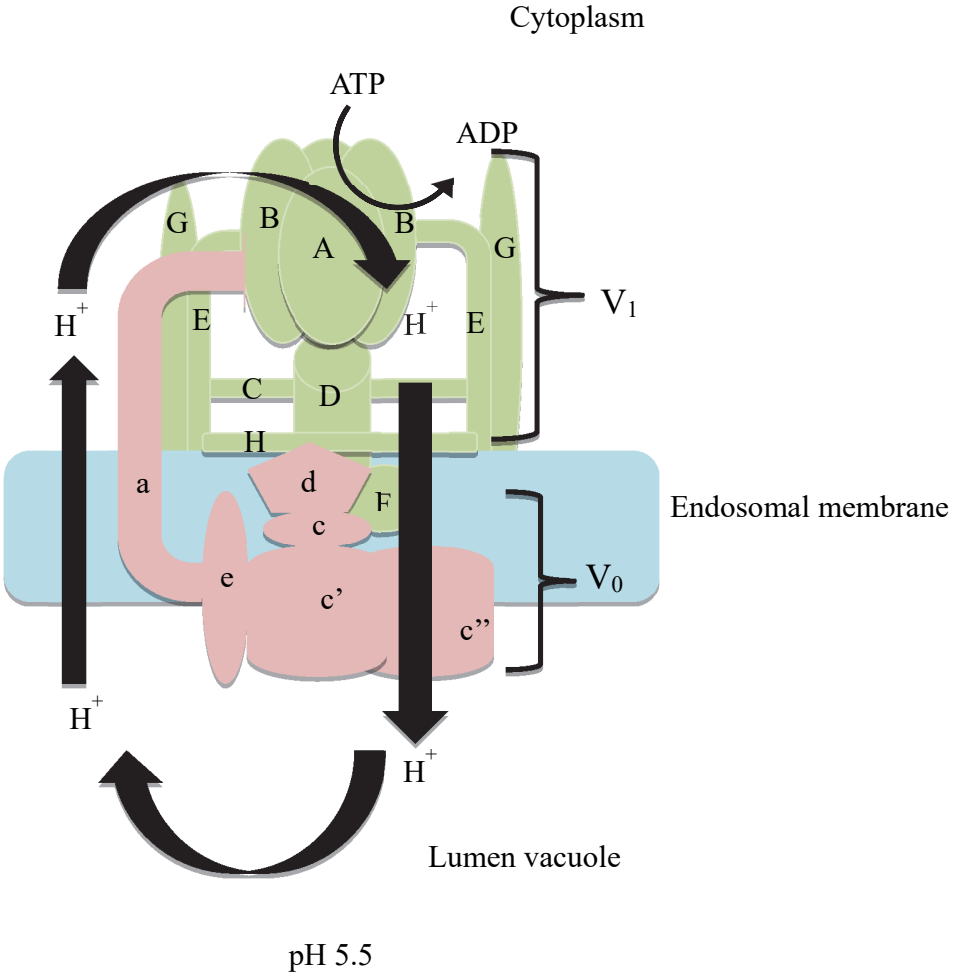

Fig. 6 Schematic diagram of the structure of *Vacuolar-type H^+-ATPase* in eukaryotic cellular membranes. V_1 complex consists of eight different subunits A-H and is responsible for ATP hydrolysis. V_0 complex consists of at least four different subunits a, d, c-c'', and e, and is responsible for proton translocation.

3.3. Material and methods

3.3.1. Mite preparation

The stock population of TSSM which used in this study was collected in London (Ontario) that was previously used for whole-genome sequencing (Grbic et al. 2011). The population was maintained on kidney beans (*Phaseolus vulgaris* L.) at 25°C and 50% relative humidity (RH) under a light period of 16 h d^{-1}. The teleiochrysalis females were synchronized to obtain newly emerged adult females by following the methods of Suzuki et al. (2017a) with some modifications. Briefly, by air pump–based sucking system (Cazaux et al. 2014) teleiochrysalis females were collected and transferred to a Petri dish (diameter, 35 mm). The Petri dish was placed inside a polystyrene cup (diameter, 94 mm), the bottom of which was lined with water-soaked cotton. The cup was sealed with a lid to keep 100% RH inside and then incubated at 20°C for 2 days. After incubation, the lid was removed and the teleiochrysalis females were incubated at 25°C and 50% RH, after 30 to 60 min teleiochrysalis initiated their molting to the newly emerged adults which used in the following experiments.

3.3.2. dsRNA fragments

Primer sets (Table 2) were used for amplifying the 600-bp fragment within the 4th exon of *TuVATPase* (Fig. 7) as a template for the preparation of dsRNA (dsRNA-*TuVATPase*). In addition, a 382-bp fragment locating the region 1690614–1690995 between tetur12g03500 and tetur12g03520 in the scaffold 12 was chosen as a template for the preparation of a negative control dsRNA (dsRNA-NC), which was previously used by Suzuki et al. (2017b).

Table 2. Primers used in the PCR amplification and RT-qPCR assay (RP49: tetyr18g03590).

PCR amplification primers	Oligonucleotides sequence (5' to 3')	Fragment size (bp)
TuVATP-T7-F	TAATACGACTCACTATAGGGGTTGCGGTGAGAGAGGTAATG	600 bp
TuVATP-T7-R	TAATACGACTCACTATAGGGGAAGAGGTACGAAATCTGGG	
TuSC12-T7-F	TAATACGACTCACTATAGGGCGACCCCATCAGGCTATTGA	382 bp
TuSC12-T7-R	TAATACGACTCACTATAGGGGCCCTCTCCTGGTTGTAAACTT	
RT-qPCR assay primers	Oligonucleotides sequence (5' to 3')	Primer efficiency (%)
TuVATP-qPCR-F	GGGTACCATCACATTCCTCG	90%
TuVATP-qPCR-R	AATCGGTCTGGTTTGACGAAC	
TuRP49-qPCR-	CTTCAAGCGGCATCAGAGC	91%
TuRP49-qPCR-R	CGCATCTGACCCTTGAACTTC	

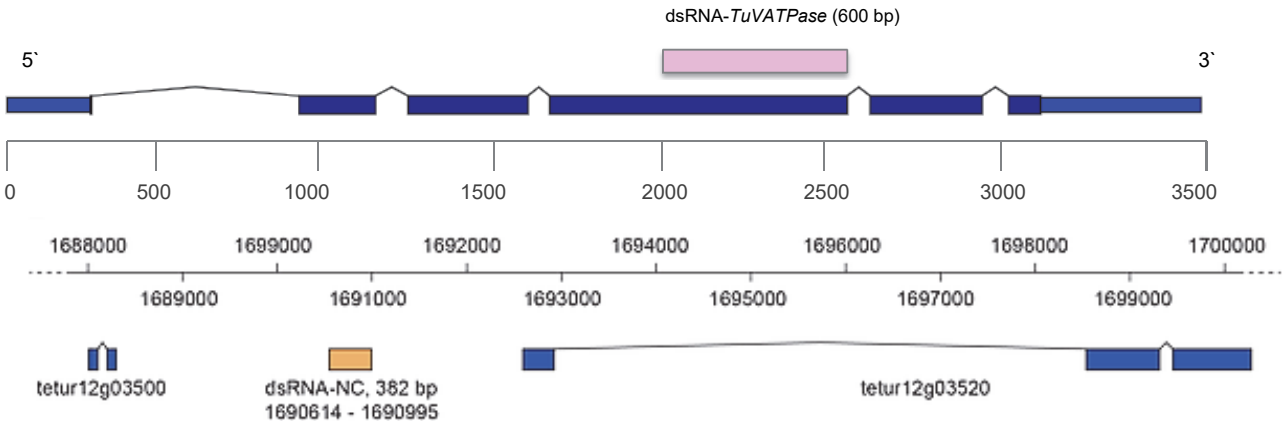

Fig. 7. DNA fragments used for the synthesis of dsRNA-*TuVATPase* and dsRNA-NC. Schematic diagram of *TuVATPase* (tetur09g04140) and the intergenic region in scaffold 12 (between tetur12g03500 and tetur12g03520). The intergenic region was used for synthesizing the negative control dsRNA (dsRNA-NC) (Suzuki et al. 2017b).

3.3.3. dsRNA preparation

Total RNA were extracted from 100 adult females frozen in liquid N_2 with a spin column kit (NucleoSpin RNA Plus; Macherey–Nagel, Düren, Germany) and quantified using a spectrophotometer (NanoPhotometer N60; Implen, Munich, Germany). cDNA was synthesized from 3 μg of the extracted total RNA with a reverse transcriptase (SuperScript II Reverse Transcriptase; Thermo Fisher Scientific, Waltham, MA) and an oligo $(dT)_{12–18}$ primer (Thermo Fisher Scientific) according to the manufacturer's protocol, and stored at −30°C. Genomic DNA (gDNA) was extracted with a spin column kit (NucleoSpin Tissue; Macherey–Nagel) and stored at −30°C. Using cDNA or gDNA as a template, the DNA fragment of *TuVATPase* or a negative control sequence (NC) in an intergenic region (Fig. 7) was PCR amplified with their specific primers (Table 2) containing the T7 polymerase promoter sequence (5'-TAATACGACTCACTATAGGG-3'), a DNA polymerase (Phusion High-Fidelity DNA Polymerase; New England Biolabs, Hitchin, UK), and a PCR thermal cycler (TaKaRa PCR Thermal Cycler Dice® Touch; Takara Bio, Shiga, Japan). The amplified DNA fragments were then purified with a spin column kit (NucleoSpin® Gel and PCR Clean-up; Macherey–Nagel). After purification, DNA fragments were confirmed with 2% (w/v) agarose gel electrophoresis and quantified with the spectrophotometer, and stored at −30°C. RNA fragment for *TuVATPase* or NC was synthesized from 100 ng μL^{-1} of the each DNA fragment as a template with a transcription kit (*in vitro* Transcription T7 Kit; Takara Bio) using as the following conditions at 42°C for 2 h, followed by adding 2 μL of RNase free DNase I and incubated for 30 min at 37°C, then denaturated at 95°C and slow cool-down to room temperature for 5 hours to facilitate formation of dsRNA. dsRNA was purified by phenol-chloroform extraction followed by ethanol precipitation as described in Kirby (1965). After purification, dsRNA dissolved in RNase free

water was confirmed with 2% (w/v) agarose gel electrophoresis and quantified using the spectrophotometer.

3.3.4. Dose-response RNAi test

Solutions of dsRNA-*TuVATPase* or dsRNA-NC were prepared at three different concentrations (0.01, 0.1, or 1 μg μL^{-1}) with or without 0.1% (v/v) Tween 20 by sandwich method (Abouelmaaty et al. 2019) as described in the Chapter 2. The leaf discs were treated with 6 μL of dsRNA with different concentrations and one newly emerged adult female was allowed to feed on the treated leaf discs. Every two days leaf discs replaced with new treated leaf discs with dsRNA. All experiments were conducted three replicates time and incubated at (25±1°C, 50 to 60% RH). Survival and fecundity were recorded for 6 days and taken for statistical analysis.

3.3.5. RT-qPCR analysis

30 adults females allowed to feed on leaf discs treated with dsRNA-*TuVATPase* or dsRNA-NC for 4 days at 25±1°C and 50 to 60% RH and then collected in a single 1.5-mL tubes and freeze them in liquid N_2 until use, total RNA were extracted from them and cDNA were synthesized (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). The RT-qPCR was performed with three technical replicates using PowerUp™ SYBR® Green Master Mix (Applied Biosystems), and a real-time PCR system (StepOnePlus™; Applied Biosystems). The ribosomal protein gene *RP49* (tetur18g03590) was used as a reference gene (Demaeght et al. 2013). Primers sequences and efficiency (*E*) are shown in Table 2. Gene expression values were plotting based on the average of cycle threshold (*Ct*) values (Livak and

Schmittgen 2001). The normalized relative quantity (*NRQ*) values were calculated by following formula:

$$NRQ = \frac{(1 + E_{\mathrm{R}})^{Ct_{\mathrm{R}}}}{(1 + E_{\mathrm{T}})^{Ct_{\mathrm{T}}}}$$

where, T and R indicate target and reference genes, respectively.

3.3.6. Data analysis

Normalized NRQ values between the control and treatments in gene expression levels were analyzed with the Dunnett's test (R function 'glht', package 'multcomp'). Survival curves were plotted with the Kaplan–Maier method (R function 'survfit', package 'survival'). The significance of differences in the survival curves among treatments was analyzed by using the log-rank test (R function 'survdiff', package 'survival'). The significance of differences in fecundity of mites among treatments was analyzed by using the Wilcoxon–Mann–Whitney test with Bonferroni correction (R function 'pairwise.wilcox.test'). Abbott's formula was applied to correct the mortality (Henderson and Tilton 1955). From the corrected mortality, LC_{50} values were then calculated by using the corrected mortality and the four-parameter log-logistic analysis (R function 'LL.4', package 'drc'). Statistical analyses were performed and graphics were generated with R 3.3.2 (R Core Team 2016).

3.4. Results

3.4.1. Gene expression

The expression level of *TuVATPase* was determined by qPCR for the concentration of 0.1 µg µL^{-1} dsRNA with or without 0.1% Tween 20 in day four after feeding. Result showed in both treatments the dsRNA inhibit the expression of *TuVATPase* gene ($p < 0.05$) (Fig. 8).

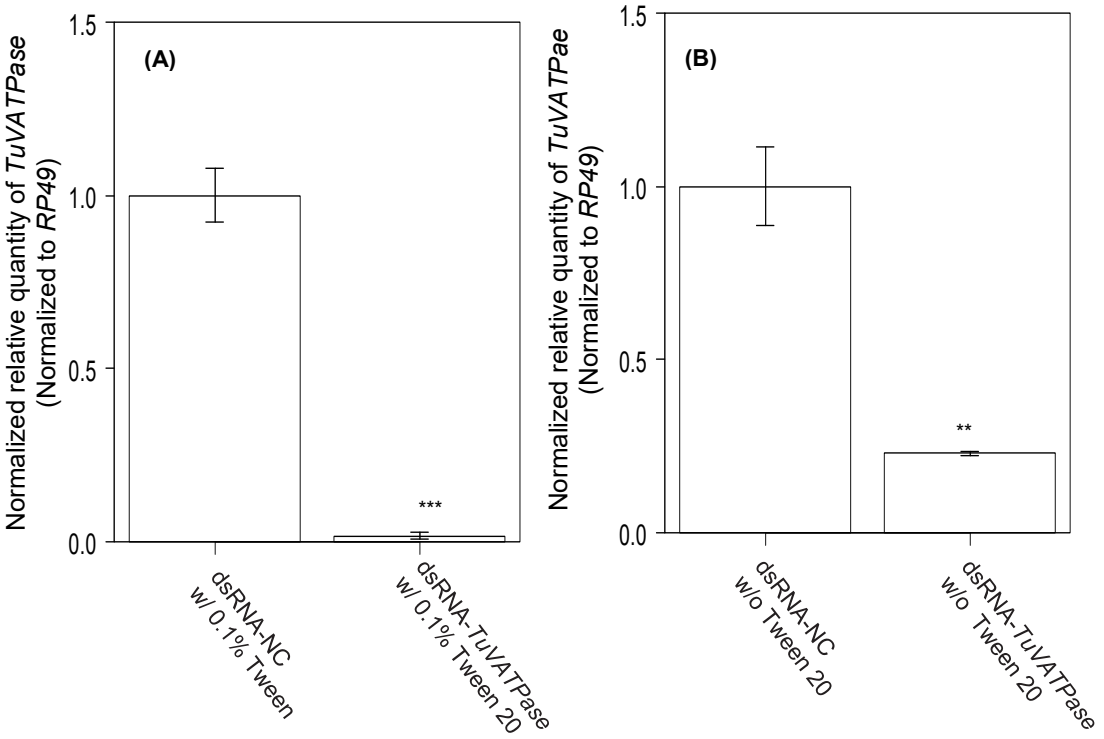

Fig. 8. *TuVATPase* gene expression relative to the expression of *RP49* reference gene in adult female mites after 4-day feeding on leaf discs coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC at 0.1 μg μL^{-1} with (A) or without 0.1% (v/v) Tween 20 (B). All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs (n = 20 to 25, each) and were compared using the Dunnett's test (**, $p < 0.01$, ***, $p < 0.0001$). Data are represented as mean±SE.

3.4.2. Body color phenotype

Almost 40% of TSSM females showed black body color after feeding on dsRNA-*TuVATP*ase at 0.1 and 1 μg μL^{-1} (Figs. 9 and 10). In contrast, no TSSM females showed the color phenotype after feeding on dsRNA-NC.

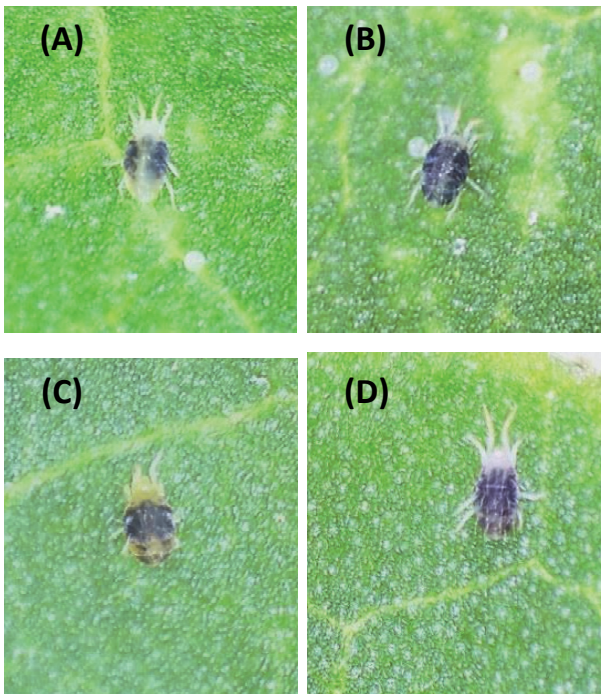

Fig. 9. The effect of dsRNA-*TuVATPase* delivered by the sandwich method (Abouelmaaty et al. 2019). (A) Adult female mite fed on leaf discs coated with the solution of dsRNA-NC at 1µg μL^{-1}. (B) Dark-body survived mite or (C, D) dead mites after feeding on leaf discs coated with the solution of dsRNA-*TuVATPase* at 1 µg μL^{-1}.

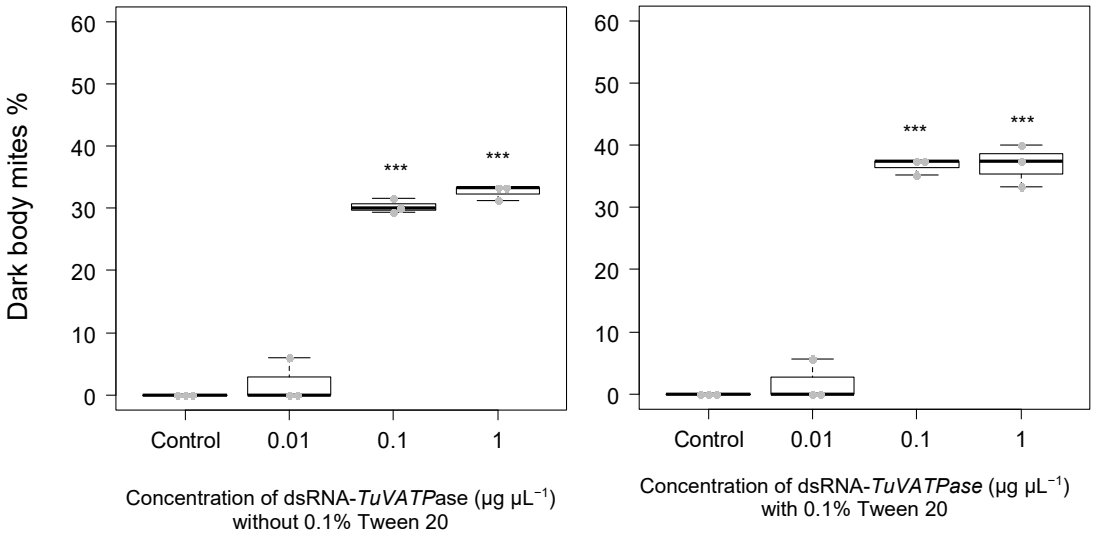

Fig. 10. Proportion of dark-body mites observed after 6-day feeing on leaf disc coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC (control) at 0.01, 0.1, or 1 µg µL^{-1} with or without 0.1% (v/v) Tween 20. All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs (n = 25, each) and were compared using the Wilcoxon–Mann–Whitney test with Bonferroni correction (***, corrected p < 0.001).

3.4.3. Survival

Mites fed on dsRNA-*TuVATPase* at 0.01 and 0.1 µg µL^{-1} without Tween 20 showed no significant reduction in their survival (p = 0.83 and 0.124, respectively) when compared with dsRNA-NC (Fig. 11). Survival was significantly reduced when mites fed on dsRNA-*TuVATPase* at 1 µg µL^{-1} (p = 0.010).

No significant reduction in survival was observed in mites fed on dsRNA-*TuVATPase* at the concentrations of 0.01 and 0.1 µg µL^{-1} when mixed with Tween 20 (p > 0.05), whereas the survival was significantly reduced when the concentration was as high as 1 µg µL^{-1} (p = 0.002) (Fig. 12).

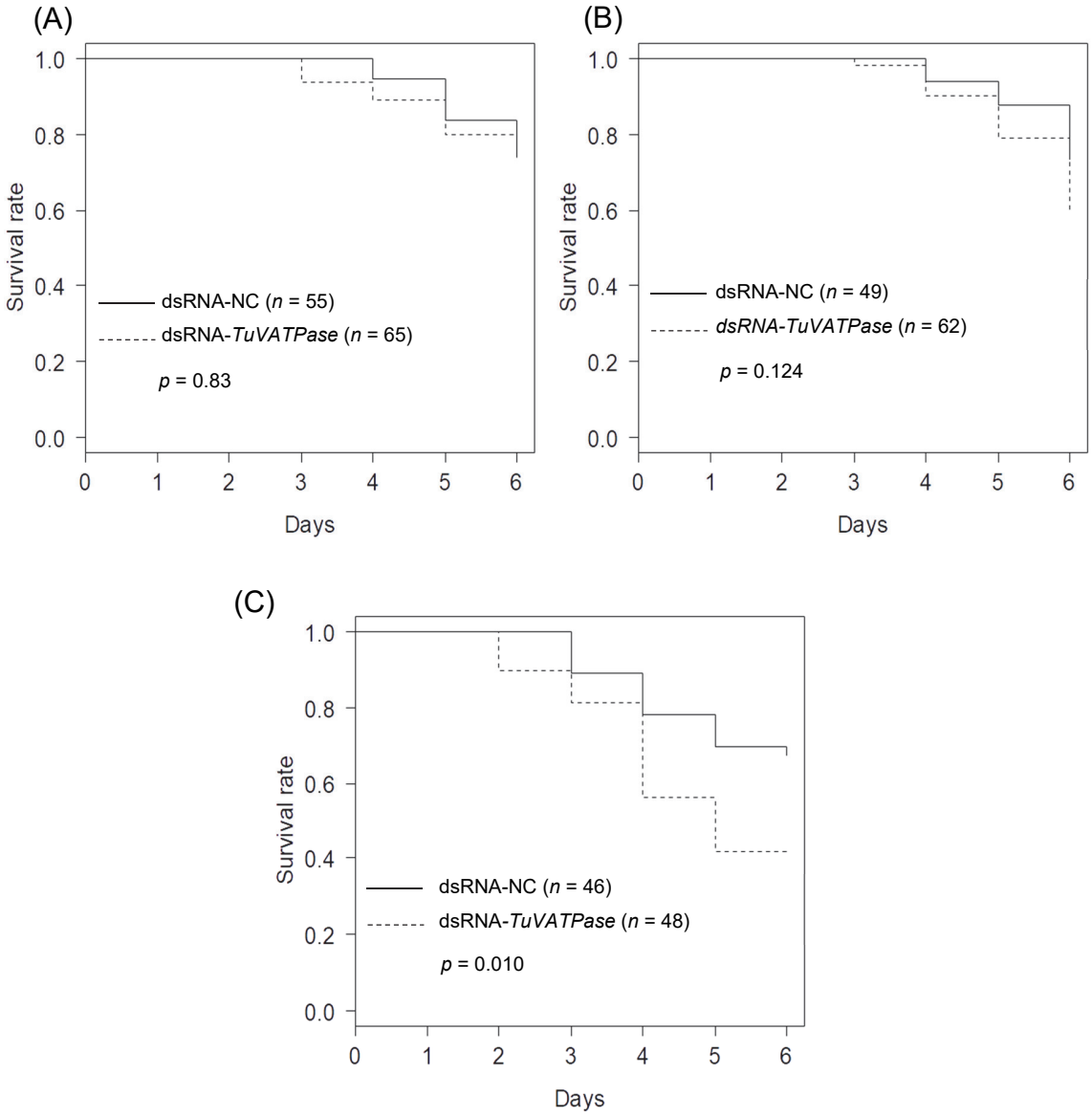

Fig. 11. Survival of adult female mites when feeding on leaf discs coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC at (A) 0.01, (B) 0.1, or (C) 1 μg μL^{-1}. All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs. Survival curves were plotted using the Kaplan–Meier method and compared using the log rank test.

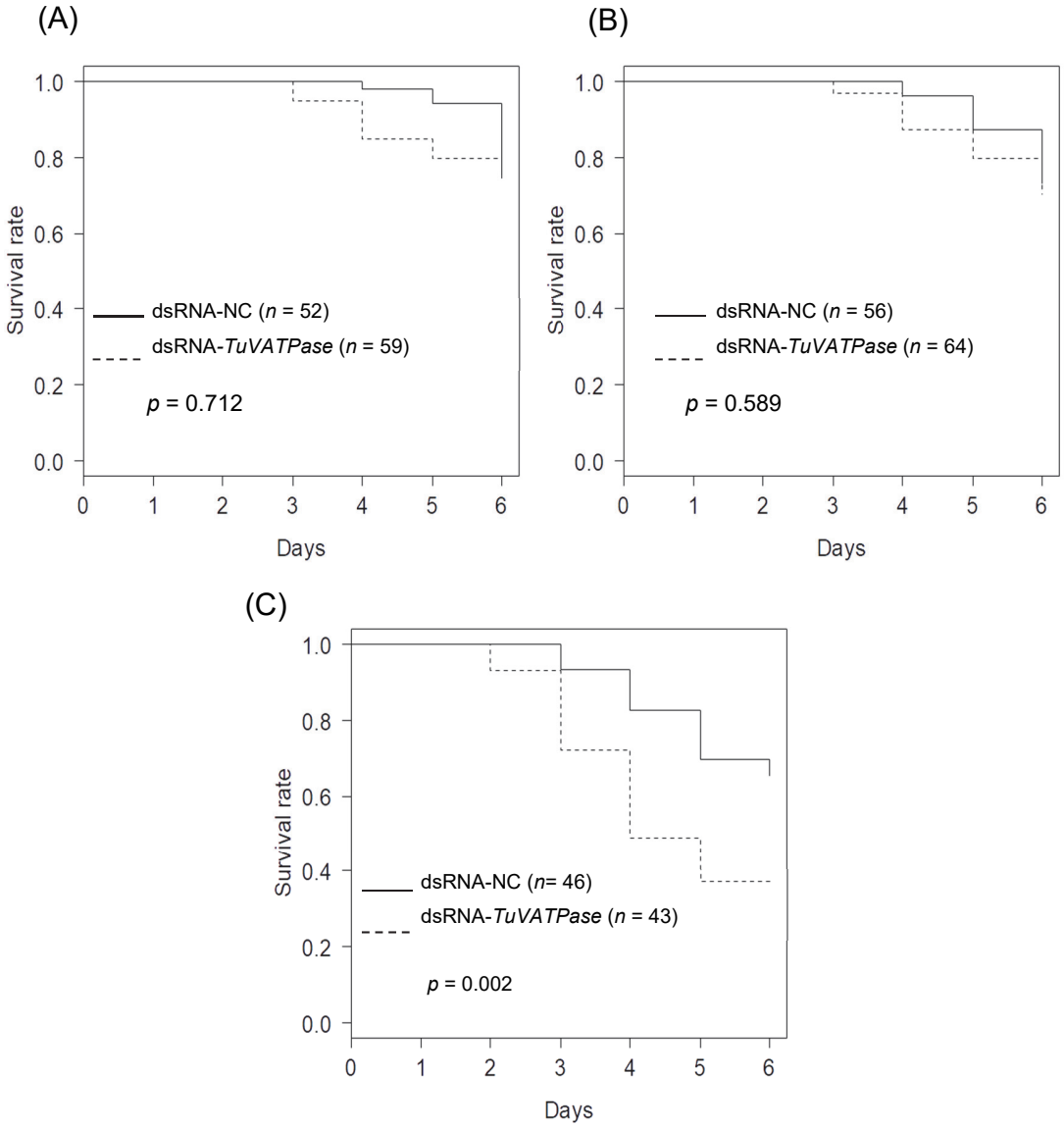

Fig. 12. Survival of adult female mites when feeding on leaf discs coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC at (A) 0.01, (B) 0.1, or (C) 1 μg μL^{-1} with 0.1% (v/v) Tween 20. All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs. Survival curves were plotted using the Kaplan–Meier method and compared using the log rank test.

3.4.4. Fecundity

Mite fecundity was significantly reduced by the RNAi treatment without Tween 20 even when the concentration of dsRNA-*TuVATPase* was as lower as 0.01 μg μL^{-1} ($p = 0.009$) (Fig. 13). The reduction of fecundity was more obvious when the concentration of dsRNA-*TuVATPase* increased to 0.1 and 1 μg μL^{-1} ($p < 0.0001$). On the other hand, the RNAi treatment with Tween 20 significantly reduced mite fecundity only when the concentration of dsRNA-*TuVATPase* was 0.1 ($p = 0.0005$) or 1 μg μL^{-1} ($p = 0.0002$) (Fig. 14).

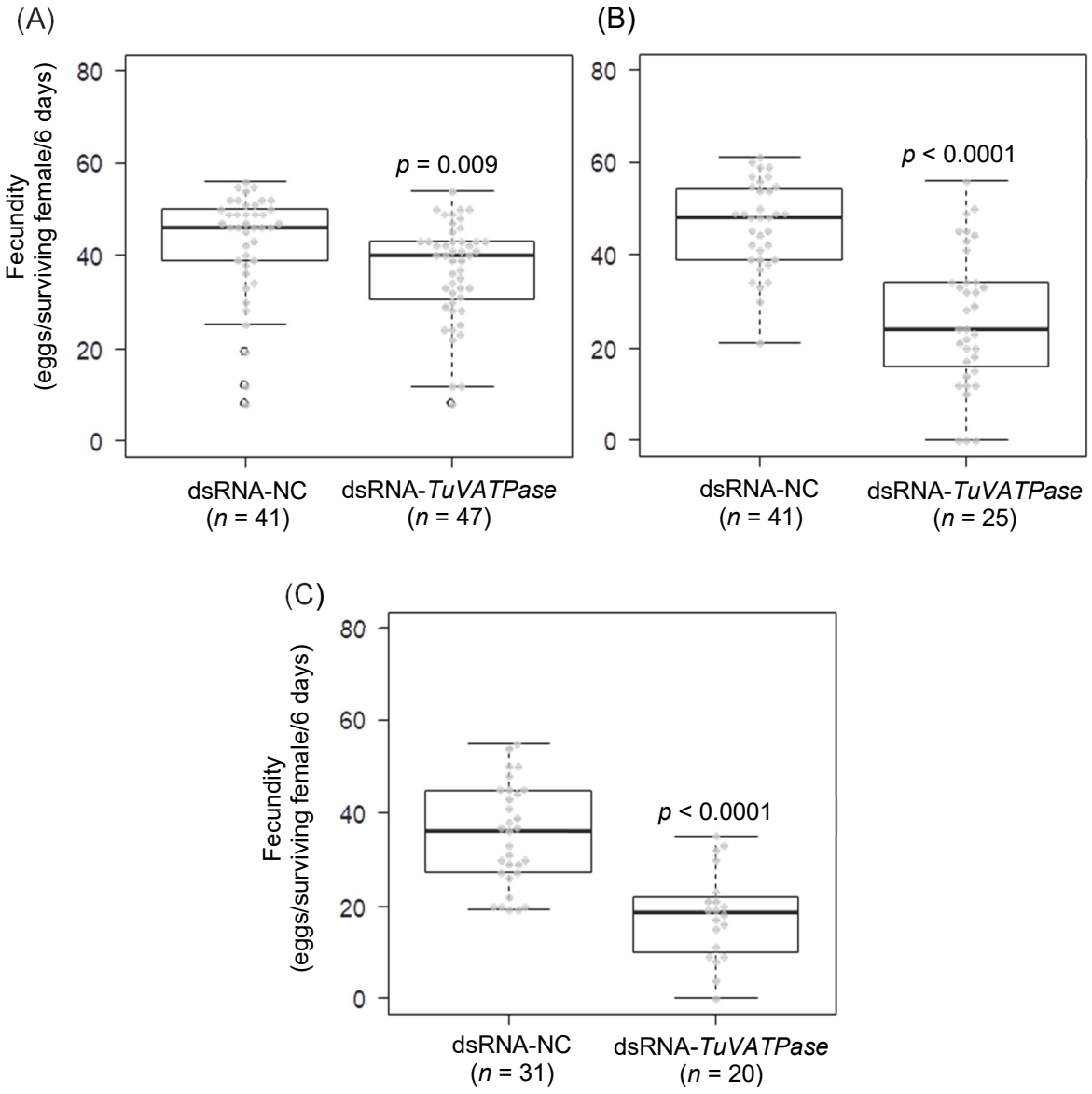

Fig. 13. Fecundity of adult female mites survived after 6-day feeding on leaf discs coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC at (A) 0.01, (B) 0.1 or (C) 1 μg μL^{-1}. All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs and were compared using the Wilcoxon–Mann–Whitney test.

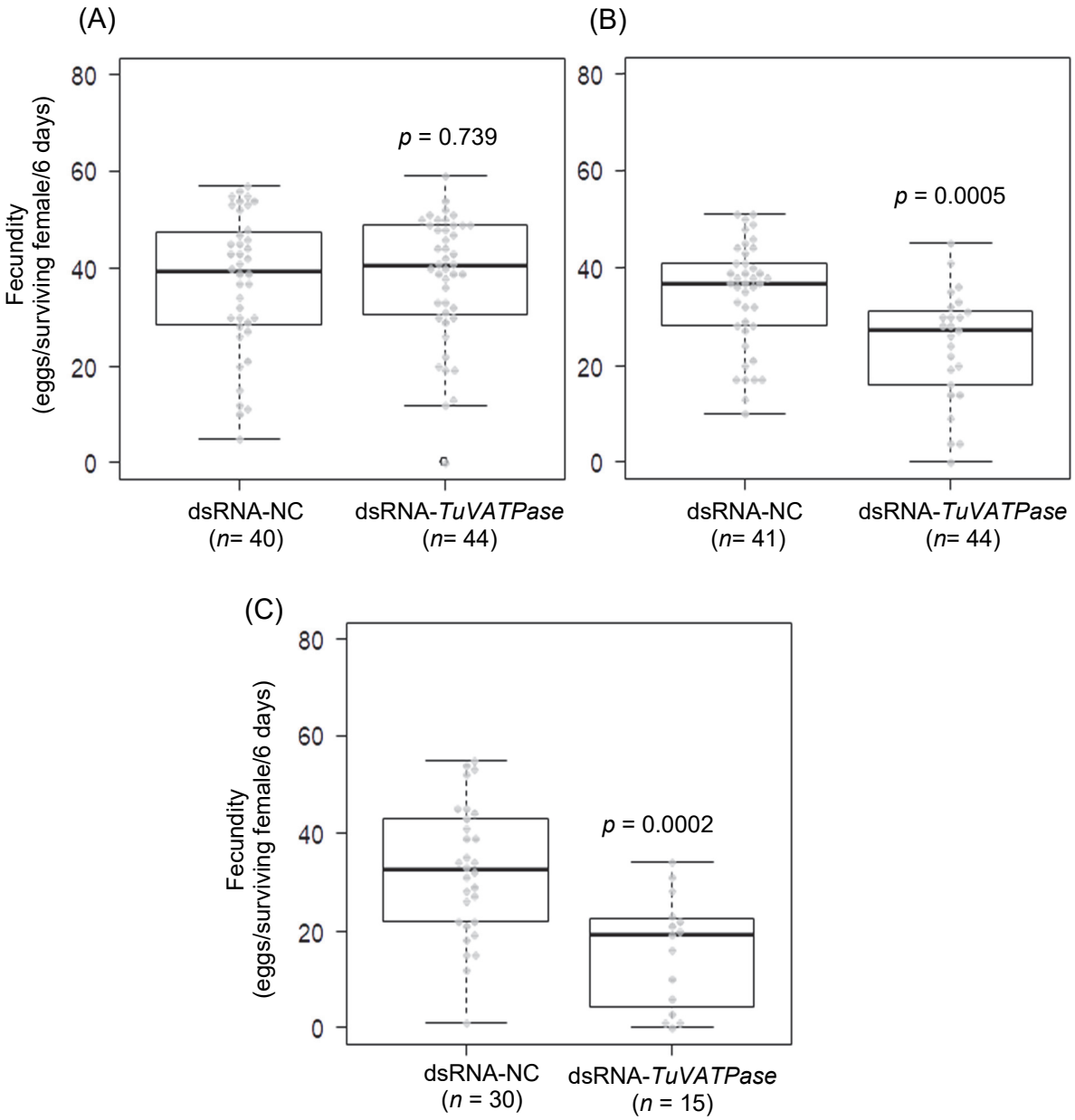

Fig. 14. Fecundity of adult female mites survived after 6-day feeding on bean leaf discs coated with the solution of dsRNA-*TuVATPase* or dsRNA-NC at (A) 0.01, (B) 0.1 or (C) 1 μg μL^{-1} with 0.1% (v/v) Tween 20. All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs and were compared using the Wilcoxon–Mann–Whitney test.

3.5. Discussion

RNAi technology has been considered as one of the most potential measures for controlling agricultural pests, because a pest species-specific effect can be achieved due to its sequence-specific manner and it can cope with pest's development of resistance by redesigning dsRNA that skip a mutation site in the target sequence. The TSSM genome was the first among chelicerates to be completely sequenced and annotated (Grbic et al. 2011). This is considerably helpful for designing dsRNA for the gene of interest and has opened the door to reverse genetics in this species.

In all eukaryotes, *V-ATPase* is an essential gene encoding a protein that maintains the acidic condition in the lumen of lysosomes and vacuoles by pumping the protons from the cytoplasm into the lumen vacuoles with energy released from the ATP hydrolysis (Wagner et al. 2004; Forgac 2007; Mauvezin et al. 2015). In previous studies, 80 and 160 ng μL^{-1} of dsRNA-*TuVATPase* were orally delivered into larvae and adult females to induce RNAi-mediated silencing of the *TuVATPase* gene, and consequently the reductions of survival and fecundity, and dark pigmentation of body were observed (Kwon et al. 2013; Suzuki et al. 2017b). However, dose-response relationships between dsRNA-*TuVATPase* and each RNAi-induced phenotype have remained unclarified. In the present study, I evaluated the dose-response effect of dsRNA-*TuVATPase* (0.01, 0.1, and 1 ng μL^{-1}) and the improving effect of Tween 20 on RNAi-induced phenotype as well as RNAi-mediated gene silencing. Downregulation of the *TuVATPase* expression was observed in TSSMs fed on leaf discs coated with dsRNA-*TuVATPase* after 4 days (Fig. 8). This result is comparable with the previous study (Suzuki et al. 2017b) in which a significant downregulation of the *TuVATPase* expression was observed at 5 days post-treatment. In addition, significant reductions of survival and fecundity were observed particularly at the

high concentrations (0.1 and 1 ng μL^{-1}) of dsRNA-*TuVATPase* (Figs. 11-14). Furthermore, the black body mites (Fig. 9) were observed also in the high concentrations of dsRNA-*TuVATPase* (Fig. 10). In the fruits fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), loss of the *V-ATPase* gene caused a disruption of the acidity in lysosomes, which resulted in an accumulation of large non-functional autolysosomes (Mauvezin et al. 2015). Therefore, RNAi of *TuVATPase* might disturb maintaining the acidity in the digestive cells that and float in the midgut lumen and have lysosome-like spherical vesicles (Bensoussan et al. 2018). The vesicles take up the midgut lumen contents and fuse each other, and the volume of each digestive cell is finally occupied by a single dark-colored large vesicle (Bensoussan et al. 2018). This suggests that the volume of midgut lumen might be occupied by functionally-disrupted digestive cells at the final stage in TSSMs fed on leaf discs coated with dsRNA-*TuVATPase*, which resulted in the distinct dark body color (Fig. 9).

Contrary to my expectation, no significant effects of Tween 20 were observed on the RNAi-mediated silencing of *TuVATPase* and the consequent RNAi-induced phenotypes in the present study. This indicates that just a simple aqueous solution of dsRNA can be effective for feeding RNAi in TSSMs when the sandwich method is applied.

3.6. Conclusion

Since RNAi has been becoming an alternative method to control the agricultural pest instead of chemical pesticides, and luckily the whole genome of TSSM already sequenced; a great number of genes can be tested and used to control pests. Leaf disc-mediated delivery of dsRNA-*TuVATPase* was successful in suppressing *TuVATPase* expression, survival, and fecundity even without any adjuvants. This indicates that *TuVATPase* is a promising target for RNAi-based control of TSSMs.

Chapter 4. Effects of dsRNA targeting four potential candidate genes for RNAi-based control of the two-spotted spider mite, *Tetranychus urticae*

4.1. Abstract

The two-spotted spider mite (TSSM), *Tetranychus urticae*, is a worldwide-distributed agricultural pest that causes serious economic damage to agriculture. TSSM has a short life cycle and high reproductive potential, which enhance its ability to develop resistance to the majority of known chemical pesticides. RNA interference (RNAi) has shown to be a potential strategy that is alternative to chemical pesticides for controlling TSSMs. Albeit the efficiency of RNAi-based TSSM control will depend on identifying candidate target gene(s) that is essential for the development, survival, and reproduction. In the present study, I assessed the toxicity of double-stranded RNAs (dsRNAs) targeting on four TSSM genes: *TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9*. Significant downregulations of the genes were observed in adult female mites fed on leaf discs coated with 80-ng μL^{-1} dsRNAs of *TuRPS4*, *TuMIP* and *TuAQP9*. These three dsRNAs caused ≥50% mortality in the mites at 6 days after treatment. Fecundity was significantly reduced in mites fed on the dsRNAs of all tested genes. These genes will be useful for developing reliable and effective strategy for the RNAi-based TSSM control.

4.2. Introduction

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae), is a serious arthropod pest to a vast array of agricultural crops. TSSMs are conventionally controlled with synthetic pesticides. However, due to their accelerated development and the high reproductive rate, TSSMs rapidly develop resistance to pesticides particularly by long-term and repeated applications of them, in which chemical control has been no longer effective (Van Leeuwen et al. 2006). RNA interference (RNAi) has proved itself as a powerful tool for gene functional analysis. In addition, great attentions have been devoted to RNAi-mediated gene silencing as an alternative to pesticides for pest control. Evidently, TSSMs as well as several insect pest species are amenable to RNAi by delivery of double-stranded RNA (dsRNA) with direct injection or oral administration (Khila and Grbic 2007; Kwon et al. 2013, 2016; Suzuki et al. 2017b; Sanscrainte et al. 2018). While successful RNAi have been attained in most cases, accumulation of data particularly with practical methods for dsRNA delivery is needed.

Leaf disc-mediated oral delivery of dsRNA is considered as a less invasive technique for TSSMs and mimics its foliar application as a sprayable RNAi-based pesticide. Yet, there are still several technical issues that include a high consumption of dsRNA and delicate handling for preparing leaf discs, which is less efficient and labor intensive especially for the high-throughput RNAi screen of target gene(s). Such difficulties were mitigated by developing the “sandwich method” in which dsRNA is simply distributed on the entire surface of leaf discs with a polypropylene sheet rather than surfactants (Abouelmaaty et al. 2019). This method greatly reduces the volume of dsRNA solution and the overall time for preparation of lead discs. In addition, pure effects of tested dsRNA can be investigated without unwanted effects of surfactants. In this Chapter, the sandwich method was applied for the further functional analysis

of RNAi target genes: coatomer subunit epsilon (*TuCOPE*; tetur08g03990), ribosomal protein S4 (*TuRPS4*; tetur24g01600), major intrinsic protein (*TuMIP*; tetur05g03630), and aquaporin-9 (*TuAQP9*; tetur35g00530). These genes were previously screened in TSSMs with mite feeding on leaf discs floating on dsRNA solution (Kwon et al. 2016), but the knockdown efficiency and effect on fecundity still remain uninvestigated.

The coatomer is a protein complex and divided into two types, COPI and COPII, which are responsible for vesicle transport between the endoplasmic reticulum (ER) and the Golgi apparatus retrogradely (Golgi to ER) and anterogradely (ER to Golgi), respectively (Rothman and Wieland 1996; Duden and Kajikawa 1998; Sato 2007; Kirchhausen 2009). The cytosolic protein COPI consists of seven subunits α, β, β', γ, δ, ε and ζ (Waters et al. 1991; Goldberg 2000). Injection of dsRNA targeting on the subunit γ of COPI induced nearly 90% mortality within 3 days in the Yellow Fever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae) (Isoe et al 2011). In addition, Kwon et al. (2016) reported that TSSMs showed a substantial mortality with 50% lethal time (LT_{50}) of 81 and 96 h when they allowed to feed on leaf discs floating on the solution of dsRNA (80 ng μL^{-1}) targeting on the subunits ε (*TuCOPE*) and β' (*TuCOPB2*), respectively.

Ribosomes in eukaryotes consist of small (40S) and large (60S) subunits; each subunit consists of one or more ribosomal RNA (rRNA) and several ribosomal proteins (RP) (Kressler et al. 2010). Ribosomes have an evolutionally conserved function which is not only translation but also post-translational modifications, including phosphorylation, acetylation, methylation, *O*-linked β-*N*-acetyl-glucosaminylation, and ubiquitylation (Xue and Barna 2012). In recent studies, RNAi-mediated silencing of two RP genes (*RPS6* and *RPL26*) in adult females of the housefly *Musca domestica* L. (Diptera: Muscidae) decreased the fecundity and induced a delay in the

ovary devolvement upon direct injection of 5-µg dsRNA (Sanscrainte et al. 2018). Similarly, direct injection of dsRNA targeting on *RPS6* and *RPL26* into adult females of *A. aegypti* decreased the fecundity by more than 96% and 91%, respectively (Estep et al. 2016). In addition, Kwon et al. (2016) reported that TSSMs showed a substantial mortality with LT_{50} of 105 h when they allowed to feed on leaf discs floating on the solution of dsRNA (80 ng μL^{-1}) targeting on *TuRPS4*.

The MIP family is a specific membranous channels group and consists of more than 350 members (Heymann and Engel 2000). The MIP family includes aquaporins (AQPs) which function as a channel to transfer water and small molecules across biological membranes (Duchesne et al. 2001). Kwon et al. (2016) reported that TSSMs showed a substantial mortality with LT_{50} of 110 and 98 h when they allowed to feed on leaf discs floating on the solution of dsRNA (80 ng μL^{-1}) targeting on *TuMIP* and *TuAQP9*, respectively.

In this Chapter, dsRNAs targeting on the four genes (*TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9*) were examined in depth with the sandwich method (Abouelmaaty et al. 2019) for further accumulation of the detailed biological data on these candidate targets and finding the potential for the foliar application of dsRNA for RNAi-based TSSM control.

4.3. Material and methods

4.3.1. Mite preparation

The TSSM population used in this study was collected in London (Ontario, Canada) which had been used for whole-genome sequencing by Grbic et al. (2011). The population was maintained on kidney beans (*Phaseolus vulgaris* L.) at 25°C and 50% relative humidity (RH) under a light period of 16 h d^{-1}. The teleiochrysalis females were collected and synchronized to obtain newly-emerged adult females according to Suzuki et al. (2017a) with some modifications. Briefly, The teleiochrysalis females were collected by using an air pump–based sucking system (Cazaux et al. 2014) and then transferred to a Petri dish (diameter, 35 mm). The Petri dish was placed inside a polystyrene cup (diameter, 94 mm) in which water-soaked cotton was laid out on the bottom. The cup was then sealed with a lid to keep 100% RH inside and incubated at 20°C for 2 days. After incubation, the lid was removed and the teleiochrysalis females were incubated at 25°C and 50% RH. Within 30 to 60 min the teleiochrysalis molting was initiated in this condition. The newly-emerged adults were used in the following experiments.

4.3.2. dsRNA fragments

The primer sets used in Kwon et al. (2016) were used for PCR amplifying the DNA fragment specific to each of the four genes: *TuCOPE* after the 4th exon, *TuRPS4* within 3rd and 4th exons, *TuMIP* on the 3rd exon, and *TuAQP9* within 2nd and 3rd exons (Table 3 and Fig. 15). Each DNA fragment was then used as a template for dsRNA synthesis. In addition, a 382-bp fragment locating the region 1690614–1690995 between tetur12g03500 and tetur12g03520 in the scaffold 12 was chosen as a template for the preparation of a negative control dsRNA (dsRNA-NC), which was previously used by Suzuki et al. (2017b).

Table 3. Primers used in the PCR amplification and RT-qPCR assay (RP49: tetyr18g03590).

PCR amplification Primers	Oligonucleotides sequence (5' to 3')	Fragment size (bp)
TuCOPE-T7-F	TAATACGACTCACTATAGGGATCTATCTGATGCTGGATCGA	455 bp
TuCOPE-T7-R	TAATACGACTCACTATAGGGAAAGTGTATTGCCTGGTCAAC	
TuRPS4-T7-F	TAATACGACTCACTATAGGGGACCATTGATCGTACTAAGGA	510 bp
TuRPS4-T7-R	TAATACGACTCACTATAGGGCGCTTAAGAGCCAAACGCTTA	
TuMIP-T7-F	TAATACGACTCACTATAGGGTCAAAGGGCCTGGTTCCATT	340 bp
TuMIP-T7-R	TAATACGACTCACTATAGGGGCACGCGATTCGGTGTTTTA	
TuAQP9-T7-F	TAATACGACTCACTATAGGGACAAACTGAGGGTGCAAATGC	420 bp
TuAQP9-T7-R	TAATACGACTCACTATAGGGTTTTGAGCCGGCCAATGAAG	
TuSC12-T7-F	TAATACGACTCACTATAGGGCGACCCCATCAGGCTATTGA	382 bp
TuSC12-T7 -R	TAATACGACTCACTATAGGGGCCCTCTCCTGGTTGTAAACTT	
RT-qPCR assay primers	**Oligonucleotides sequence (5' to 3')**	**Primer efficiency (%)**
TuCOPE-qPCR-F F	ACCCGATGCTGGACTGCGA	97%
TuCOPE-qPCR-R	ATCCATGCCTGAGCAAGCTGA	
TuRPS4-qPCR-F F	TGCATGGTTACTGGAGGACA	91%
TuRPS4-qPCR-R	ACCACGACCCTTAGGAAGTG	
TuMIP-qPCR-F F	CTGCGGAGCAAATCAATCCA	95%
TuMIP-qPCR-R	ACGATACGTCCAAGAACCCA	
TuAQP9-qPCR-F F	CGAGACTTTGGTCCGAGACT	90%
TuAQP9-qPCR-R	ATGAGGAGCAACCAGTGGAA	
TuRP49-qPCR-	CTTCAAGCGGCATCAGAGC	91%
TuRP49-qPCR-R	CGCATCTGACCCTTGAACTTC	

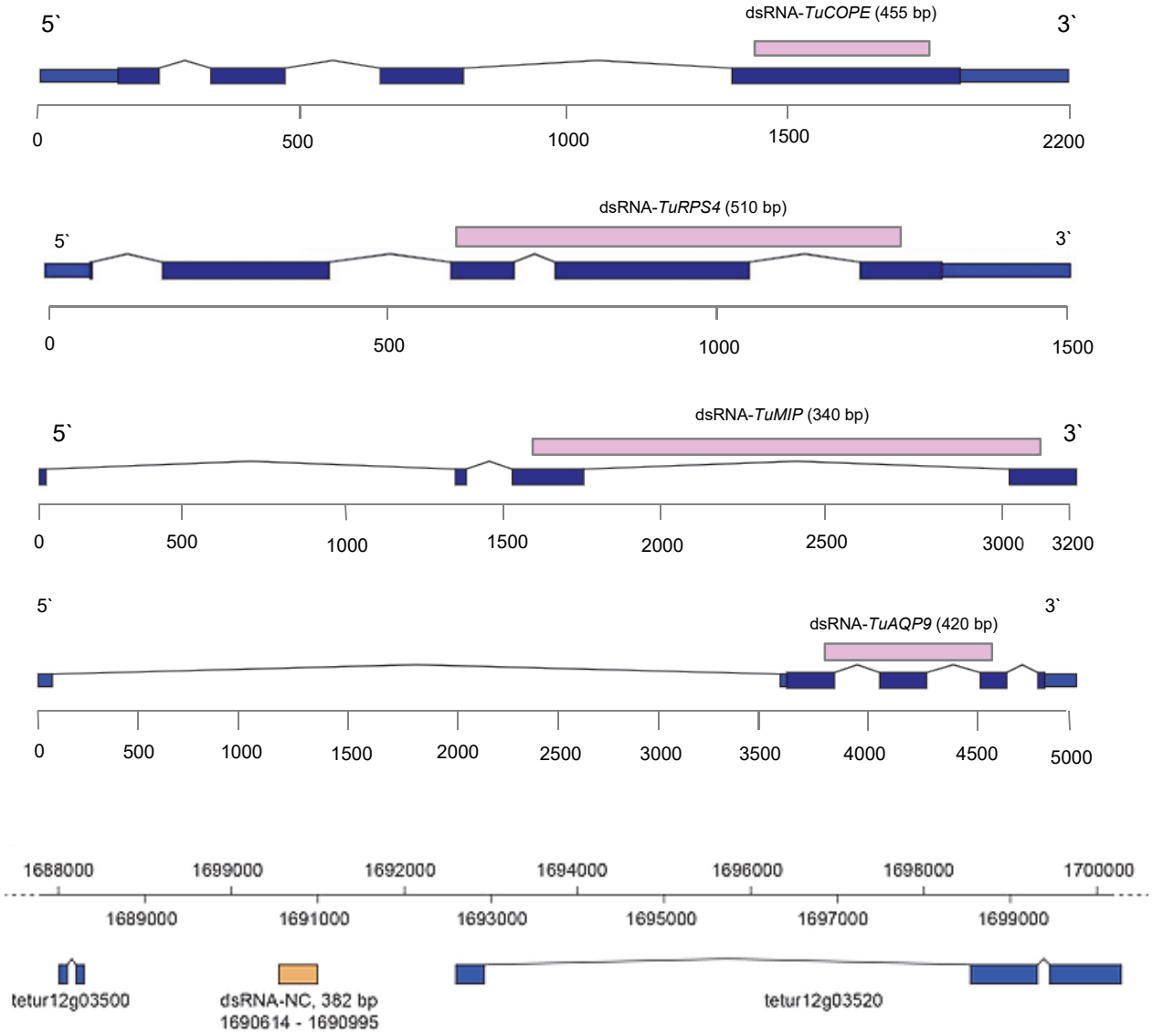

Fig. 15. DNA fragments used for the synthesis of dsRNA-*TuCOPE* (tetur08g03990), dsRNA-*TuRPS4* (tetur24g01600), dsRNA-*TuMIP* (tetur05g03630), dsRNA-*TuAQP9* (tetur35g00530) and dsRNA-NC. The intergenic region was used for synthesizing the negative control dsRNA (dsRNA-NC).

4.3.3. dsRNA preparation

From 100 adult females frozen in liquid N_2, total RNA were extracted with a spin column kit (NucleoSpin® RNA Plus; Macherey–Nagel, Düren, Germany) and quantified using a spectrophotometer (NanoPhotometer N60; Implen, Munich, Germany). cDNA (3 µg) was synthesized from 3 µg of the extracted total RNA with a reverse transcriptase (SuperScript II Reverse Transcriptase; Thermo Fisher Scientific, Waltham, MA) and an oligo $(dT)_{12–18}$ primer (Thermo Fisher Scientific) according to the manufacturer's protocol, and stored at −30°C until farther use. Genomic DNA (gDNA) was extracted with a spin column kit (NucleoSpin Tissue; Macherey–Nagel) and stored at −30°C. cDNA was used as a template to PCR amplify the fragment of *TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9*. gDNA was used as a template to amplify the control sequence (NC). All primer sequences were shown in Table 3. The DNA fragments were PCR amplified with a DNA polymerase (Phusion High-Fidelity DNA Polymerase; New England Biolabs, Hitchin, UK) and a PCR thermal cycler (TaKaRa PCR Thermal Cycler Dice® Touch; Takara Bio). The amplified DNA fragments were then purified with a spin column kit (NucleoSpin® Gel and PCR Clean-up; Macherey–Nagel). After purification, DNA fragments were confirmed with 2% (w/v) agarose gel electrophoresis and quantified with the spectrophotometer, and stored at −30°C.

The RNA fragment for *TuCOPE*, *TuRPS4*, *TuMIP*, *TuAQP9*, or NC was synthesized from 100 ng of each DNA template with a transcription kit (*in vitro* Transcription T7 Kit; Takara Bio, Shiga, Japan) by incubating the reaction at 42°C for 2 h, then adding 2 µL of RNase free DNase I and incubated for 30 min at 37°C, then denaturated at 95°C, followed by slow cool-down to room temperature for ~5 hours to facilitate the formation of dsRNA. dsRNA for *TuCOPE*, *TuRPS4*, *TuMIP*, *TuAQP9*, or NC (dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-

TuMIP, dsRNA-*TuAQP9*, or dsRNA-NC) was purified by phenol-chloroform extraction followed by ethanol precipitation as described in Kirby (1965). After purification, dsRNA dissolved in RNase free water was confirmed with 2% (w/v) agarose gel electrophoresis and quantified using the spectrophotometer. dsRNA was stored at −80°C until used.

4.3.4. RNAi test

The sandwich method (Abouelmaaty et al. 2019) was used to conduct the following experiments as mentioned in as described in the Chapter 2. Newly-emerged adult females were inoculated onto leaf discs (ϕ10 mm; 1 adult female mite/disc) coated with 6 µL (80 ng μL^{-1}) of dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, dsRNA-*TuAQP9*, or dsRNA-NC. The leaf discs where replaced with newly-coated ones every 2 days. The survival, fecundity, and body phenotype were observed up to 6 days. All experiments were repeated three times.

4.3.5. RT-qPCR analysis

Thirty adult females were allowed to feed on leaf discs coated with dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, dsRNA-*TuAQP9*, or dsRNA-NC for 4 days. The leaf discs were replaced with newly-coated ones every 2 days. Mites were incubated at 25±1°C and 50 to 60% RH. In the fourth day after the onset of the experiment, mites were transferred from leaf discs and into a 1.5-mL tube, frozen in liquid N_2, and stored at −80°C until use. Total RNA was extracted from the frozen mites and cDNA were synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RT-qPCR was performed with three technical replicates using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and a real-time PCR system (StepOnePlus™; Applied Biosystems). The ribosomal protein gene

RP49 (tetur18g03590) was used as a reference gene (Demaeght et al. 2013). Primers sequences and efficiency (*E*) are shown in Table 3. Gene expression values were plotted based on the average of cycle threshold (*Ct*) values (Livak and Schmittgen 2001). The normalized relative quantity (*NRQ*) values were calculated by following formula:

$$NRQ = \frac{(1 + E_{\mathrm{R}})^{Ct_{\mathrm{R}}}}{(1 + E_{\mathrm{T}})^{Ct_{\mathrm{T}}}}$$

where, T and R indicate target and reference genes, respectively.

4.3.6. Data analysis

Survival curves were calculated using the Kaplan–Maier method (R function ‘survfit’, package ‘survival’) and compered using the log-rank test (R function ‘survdiff’, package ‘survival’). Significant differences in the fecundity of mites among treatments were analyzed by using the Wilcoxon–Mann–Whitney test with Bonferroni correction (R function ‘pairwise.wilcox.test’). Normalized NRQ values between the control and treatments were analyzed with the Dunnett’s test. Analyses and graphics were performed with R 3.3.2 (R Core Team 2016).

4.4. Results

4.4.1. Gene expression

In TSSMs fed on leaf discs coated with 80 ng μL^{-1} of dsRNA-*TuCOPE*, no significant downregulations were observed for neither *TuCOPE* nor other three genes (Fig. 16A). In TSSMs fed on 80 ng μL^{-1} of dsRNA-*TuRPS4*, significant downregulations were observed in *TuRPS4* ($p < 0.05$), *TuMIP* ($p < 0.001$), and *TuAQP9* ($p < 0.01$) (Fig. 16B). In TSSMs fed on 80 ng μL^{-1} of dsRNA-*TuMIP*, significant downregulations were observed in *TuMIP* ($p < 0.001$), *TuRPS4* ($p < 0.001$), and *TuAQP9* ($p < 0.01$) (Fig. 16C). In TSSMs fed on 80 ng μL^{-1} of dsRNA-*TuAQP9*, downregulations were observed in *TuAQP9* ($p < 0.01$), *TuRPS4* ($p < 0.01$), and *TuMIP* ($p < 0.01$) (Fig. 16D).

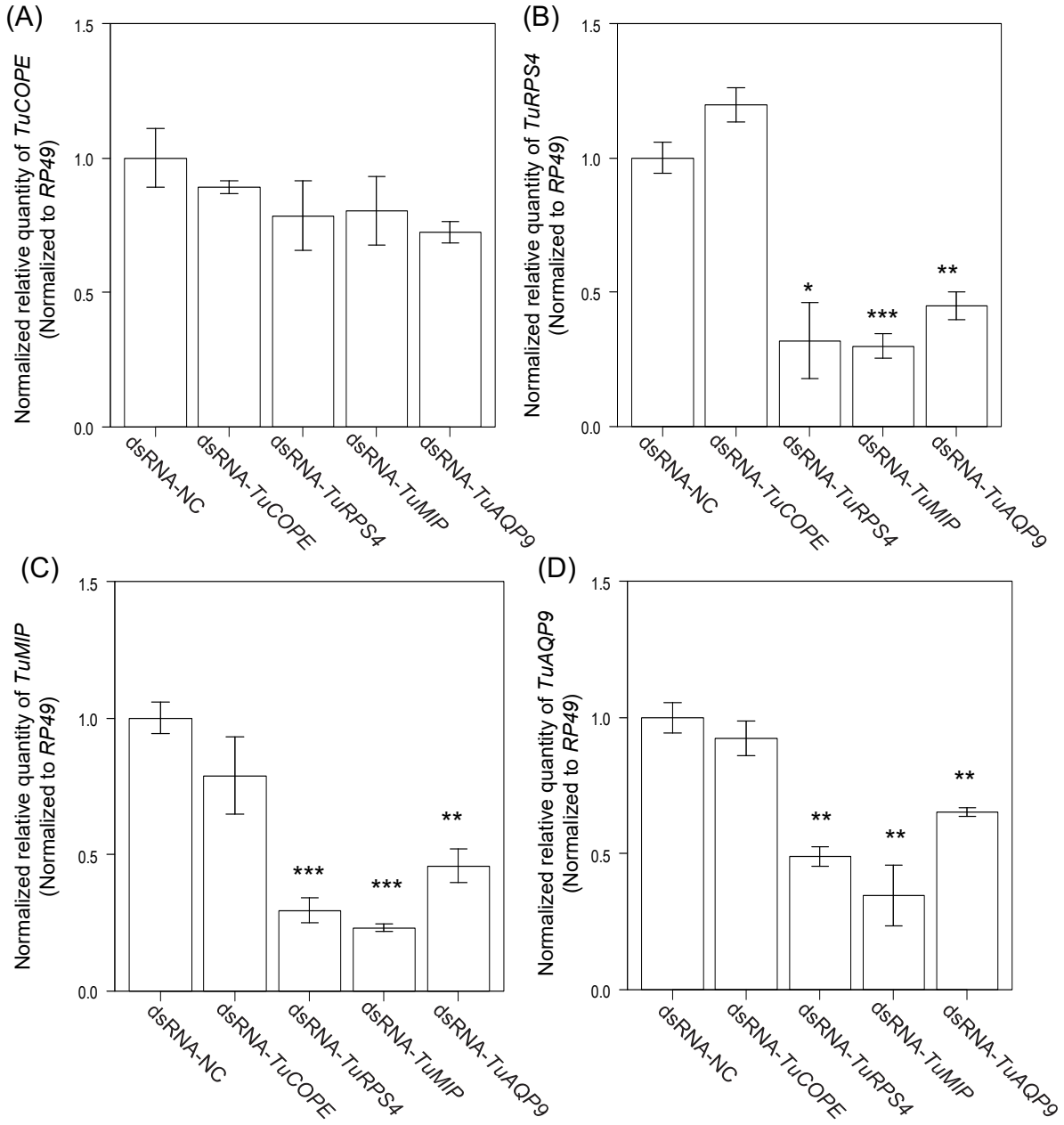

Fig. 16. Gene expressions of (A) *TuCOPE*, (B) *TuRPS4*, (C) *TuMIP*, and (D) *TuAQP9* relative to the expression of *RP49* reference gene in adult female mites after 4-day feeding on bean leaf discs coated with the solution of 80 ng μL^{-1} of dsRNA-NC, dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, or dsRNA-*TuAQP9*. All leaf discs used for dsRNA delivery were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs (n = 10 to 25, each) and were compared using the Dunnett's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data are represented as mean ± SE.

4.4.1. Survival

No significant reduction in survival was observed when TSSMs fed on leaf discs coated with 80 ng μL^{-1} of dsRNA-*TuCOPE* compared with those of treated with dsRNA-NC ($p = 0.412$) (Fig. 17). In contrast, a significant mortality ($p < 0.001$) was observed in TSSMs fed on leaf discs coated with 80 ng μL^{-1} of dsRNA-*TuRPS4*, dsRNA-*TuMIP*, or dsRNA-*TuAQP9* compared with those of treated with dsRNA-NC.

TSSMs fed on leaf discs coated with dsRNA-*TuRPS4* or dsRNA-*TuCOPE* showed normal body size while the body color was yellowish in mites treated with dsRNA-*TuCOPE* (Fig. 18) and slightly dark in those treated with dsRNA-*TuRPS4* (Fig. 19). After feeding on leaf discs coated with dsRNA-*TuMIP* or dsRNA-*TuAQP9*, mites showed small body phenotypes, and most of them died (Figs. 20 and 21).

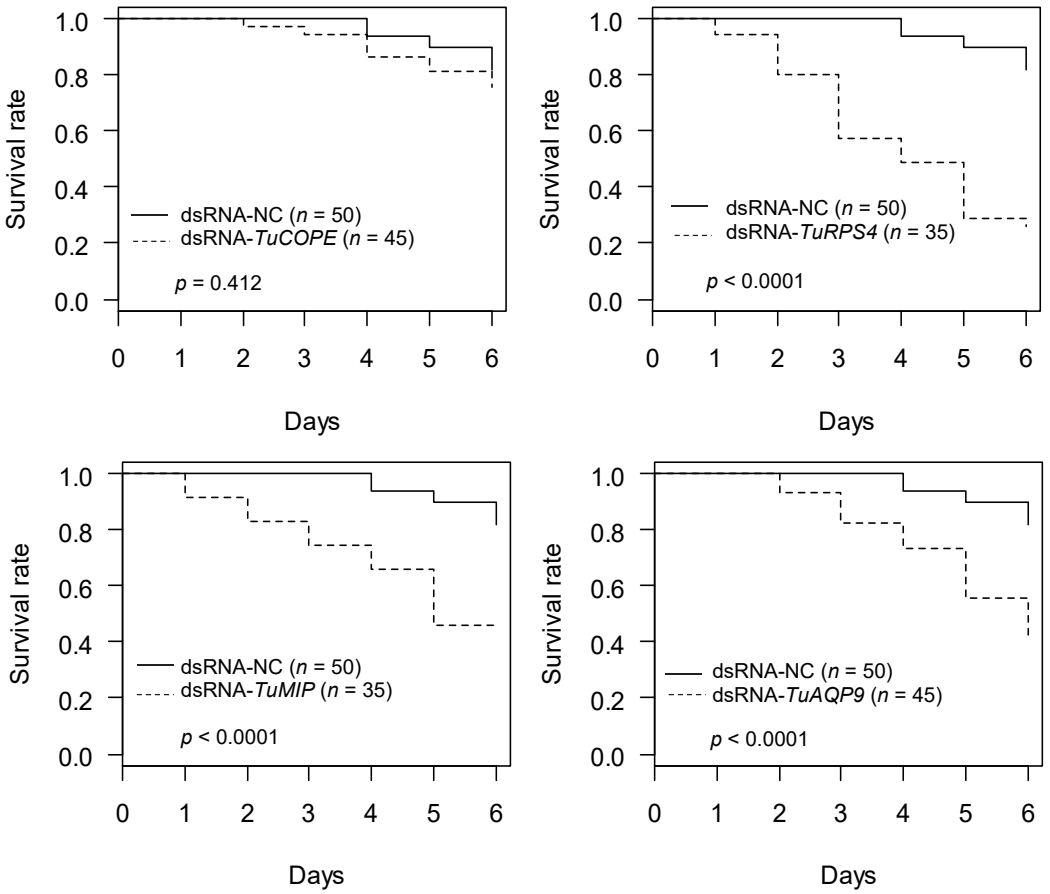

Fig. 17. Survival of adult female mites fed on bean leaf discs treated with the solution (80 ng μL^{-1}) of dsRNA-NC, dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, or dsRNA-*TuAQP9*. All leaf discs used for dsRNA delivery were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs. Survival curves were plotted using the Kaplan–Meier method and compared using the log rank test.

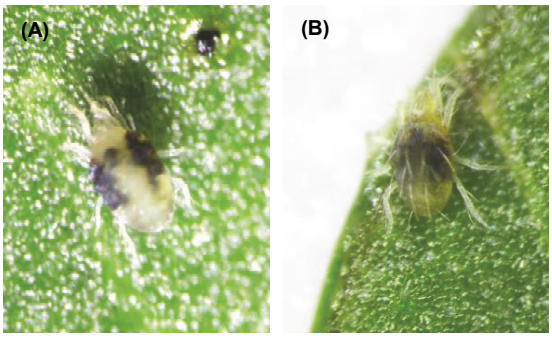

Fig. 18. The effect of dsRNA-*TuCOPE* delivered by the sandwich method (Abouelmaaty et al. 2019). Adult female mites fed on (A) dsRNA-NC or (B) dsRNA-*TuCOPE*. The mite in (B) is dead mite.

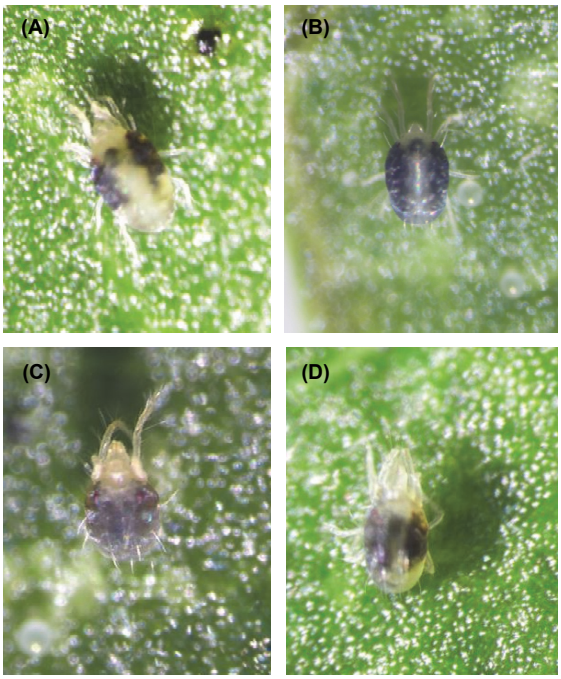

Fig. 19. The effect of dsRNA-*TuRPS4* delivered by the sandwich method (Abouelmaaty et al. 2019). Adult female mites fed on bean leaf discs coated with the solution (80 ng μL^{-1}) of (A) dsRNA-NC or (B–D) dsRNA-*TuRPS4*. (B) Dark-body phenotype in alive mites. (C and D) dead mites after feeding on dsRNA-*TuRPS4*.

Fig. 20. The effect of dsRNA-*TuMIP* delivered by the sandwich method (Abouelmaaty et al. 2019). Adult female mites fed on bean leaf discs coated with the solution of 80 ng μL^{-1} of (A) dsRNA-NC or (B–F) dsRNA-*TuMIP*. (B, C, D, E) dead mites after feeding on dsRNA-*TuMIP*. (F) Small-body surviving mites

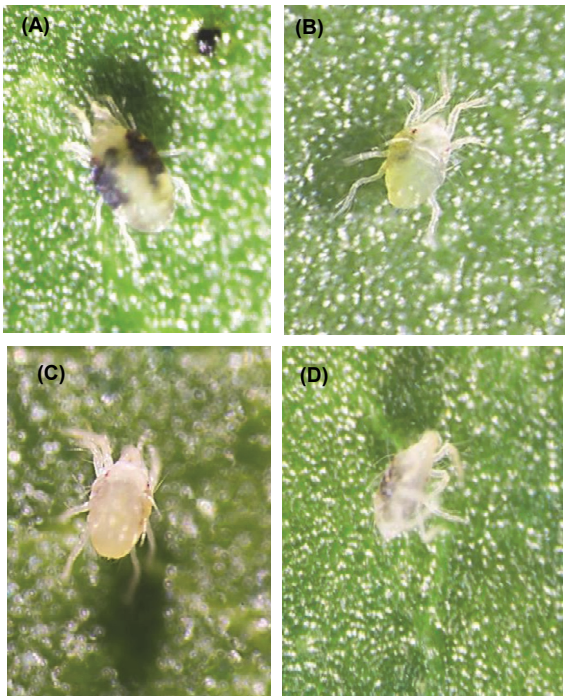

Fig. 21. The effect of dsRNA-*TuAQP9* delivered by the sandwich method (Abouelmaaty et al. 2019). Adult female mites fed on bean leaf discs coated with the solution of 80 ng μL^{-1} of (A) dsRNA-NC or (B–D) dsRNA-*TuAQP9*. (B) Small-body surviving mites and (C, D) dead mites after feeding on dsRNA-*TuAQP9*.

4.4.2. Fecundity

In TSSMs fed on leaf discs coated with 80 ng μL^{-1} of dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, or dsRNA-*TuAQP9* for 6 days, significant reduction in fecundity ($p < 0.001$) was observed when compared with dsRNA-NC treatment (Fig. 22).

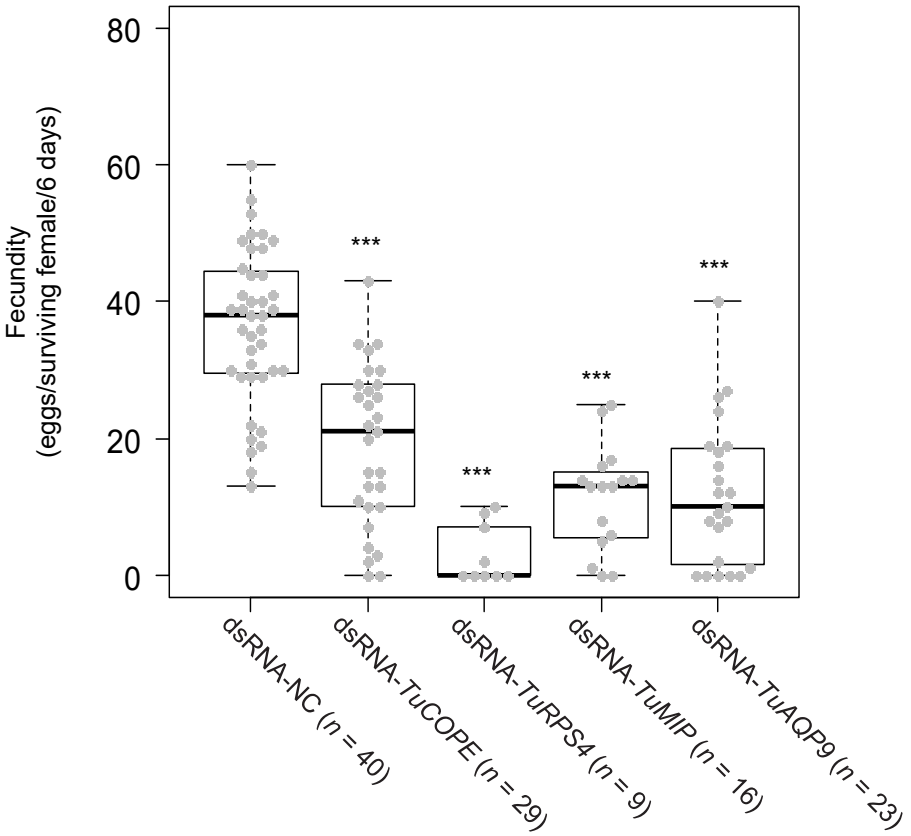

Fig. 22. Fecundity of adult female mites fed on bean leaf discs coated with the solution of 80 ng μL^{-1} of dsRNA-NC, dsRNA-*TuCOPE*, dsRNA-*TuRPS4*, dsRNA-*TuMIP*, or dsRNA-*TuAQP9*. All leaf discs used for dsRNA delivery were prepared with the sandwich method (Abouelmaaty et al. 2019). Data were collected from three independent experimental runs and were compared using the Wilcoxon–Mann–Whitney test with Bonferroni's correction (***, corrected $p < 0.001$).

4.5. Discussion

Although RNAi has a potential for application in pest control field as a novel pesticide technology, it encounters several difficulties particularly in delivering dsRNA into target pest species. The methodology of dsRNA delivery is considered as a cornerstone in the process of RNAi screen of target genes for pest control. An ideal method for dsRNA delivery should be effective, simple, reproducible, time-saving, and resource (i.e. dsRNA)-saving. The sandwich method (Abouelmaaty et al. 2019), a leaf disc-mediated oral delivery method, may suit the abovementioned requirements. The method was proved to be effective in inducing RNAi, because when dsRNA-*TuVATPase* were orally delivered into TSSMs, the *TuVATPase* knockdown-related phenotypes that were black body color, mortality, and reduction of fecundity (Suzuki et al. 2017b) were observed (Chapter 3). To further confirm the efficacy of the sandwich method, the dsRNA of four TSSM genes that showed the RNAi-mediated mortality in TSSMs (Kwon et al. 2016) were investigated in this Chapter.

Unlike Kwon et al. (2016), no significant differences in survival and gene expression level were observed between mites fed on leaf discs coated with dsRNA-*TuCOPE* and dsRNA-NC (Figs. 16 and 17) but a significant reduction was observed only in their fecundity (Fig. 22). Although the dsRNA concentration used in the present study (80 ng μL^{-1}) was same with Kwon et al. (2016), the reason of disagreement in the mortality results is unknown. It might be due to the difference in the strains where the rearing population of TSSMs collected in London (Ontario, Canada) was used in this study whereas the “PyriF strain” collected in Korea was used in Kwon et al. (2016). Previous studies have showed that the sensitivity to RNAi may vary among strains even in a same species, which depends on the ability to degrade alien dsRNA (Asikainen et al 2005; Bellés 2010).

In addition to the reductions of survival and fecundity (Figs. 17 and 22), downregulations of *TuRPS4*, *TuMIP*, and *TuAQP9* were observed mites fed on dsRNA-*TuRPS4* when compared with the control treatment (Fig. 16). The functions of ribosomes are translation and post-translational modifications, including phosphorylation, acetylation, methylation, *O*-linked β-*N*-acetyl-glucosaminylation, and ubiquitylation (Xue and Barna 2012). Therefore, some transcriptional factors for *TuRPS4*, *TuMIP*, and *TuAQP9* might be downregulated by RNAi of *TuRPS4*. As another hypothesis on the off-target effects of dsRNA-*TuRPS4*, identity of the sequence with a gene other than *TuRPS4* might be affected. In the primers for amplified the DNA fragment of *TuRPS4* which were designed by Kwon et al. (2016) and also used in the present study, the sequence of 20 consecutive nucleotides in the fragment was identical with that of the gene encoding Histone H2B (tetur13g00510) which is one of the core members of nucleosome. In *Drosophila*, 21- to 23-bp small interfering RNA (siRNA) is generated from the dsRNA processing by Dicer-2, whereas microRNA (miRNA) which is a single-stranded RNA (19 to 22 nt) is generated from the pre-miRNA processing by Dicer-2 (Lee et al. 2004). In the TSSM genome, there are two orthologs each for the gene encoding Dcer-2 (tetur07g00990) and Dicer-1 (tetur19g00520). Therefore, the gene encoding Histone H2B might be downregulated by siRNA or miRNA generated from dsRNA-*TuRPS4*; thus other target genes might also be downregulated because the formation of nucleosome could be suppressed. In this hypothesis, the exception of *TuCOPE* cannot, however, be explained. RPs play a crucial role in the organism's biological regulation of growth and development (Ferreira-Cerca and Hurt 2009). In the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), RNAi of the gene encoding L5 which is one of RPs inhibited the ovarian development and fecundity (Zhu et al. 2017). In the adult female of *M. domestica* injected with 5μg dsRNA of the gene encoding RPS6, the ovarian

developments was inhibited which resulted in low or no fecundity (Sanscrainte et al. 2018). This further indicates the broad function and regulation mechanism of ribosomal proteins.

Mites fed on leaf discs coated with dsRNA-*TuMIP* and dsRNA-*TuAQP9* also showed significantly lower survival and fecundity than in the control treatment (Figs. 17 and 22). Both *TuMIP* and *TuAQP9* belong to the MIP family that is a membranous channel group. The family members play fundamental rules in the reabsorption, secretion, and homeostasis behavior by regulating the water and small solutes across cell membranes (Heymann and Engel 2000). The available data on RNAi of *TuMIP* and *TuAQP9* are reported only by Kwon et al. (2016) in which oral delivery of dsRNA-*TuMIP* and dsRNA-*TuAQP9* resulted in LT_{50} of 110 and 98 h, respectively. Using the same sequence of the fragments and dsRNA concentration (80 ng μL^{-1}) as described in Kwon et al. (2016), the present study also demonstrated the high toxicity of dsRNA-*TuMIP* and dsRNA-*TuAQP9* to the London population of TSSM. In addition, downregulations of *TuMIP* and *TuAQP9* were observed in this population (Fig. 16). Interestingly, silencing of *TuMIP* or *TuAQP9* resulted in the significantly low expression level each other and that of *TuRPS4* as well. The former result may be due to the high homology between the fragments *TuMIP* and *TuAQP9* up to 73% whereas the mechanism of the latter result (downregulation of *TuRPS4*) was unclear. Further investigations are needed to figure out the relationships among *TuMIP*, *TuAQP9*, and *TuRPS4*.

4.6. Conclusion

This Chapter was devoted to the efficiency of the sandwich method (Chapter 2; Abouelmaaty et al. 2019) on RNAi of four genes (*TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9*) that are a potential target for TSSM control. Survival of TSSMs was significantly deceased compared to control after oral delivery of dsRNA of these target genes except *TuCOPE*. All dsRNA tested in this Chapter inhibited the fecundity of TSSMs. These results may contribute developing the high-throughput RNAi screen system using the sandwich method to aim the goal of RNAi-based control of TSSMs.

Chapter 5. General discussion

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae) is a worldwide-spread agricultural pest. TSSM is distributed in more than 120 countries and has been found in association with more than 1100 host plants (Migeon and Dorkeld 2006-2017). Chemical pesticides are commonly used to suppress TSSM population. However, due to its short developmental time and high reproductive rate, TSSM is able to evolve resistance to most known chemical pesticides and in a manner faster than beneficial organisms (Stumpf and Nauen 2001; Van Leeuwen et al. 2010; Schmidt-Jeffris and Beers 2015). In such circumstances, repeated pesticide applications are needed which exert further risk on human health, agroecosystems and non-target species (Devine and Furlong 2007). In addition to that, the excessive uses of chemical pesticides promote the selection of pesticide-resistant individuals among pest population that leads to control failure (Stumpf and Nauen 2001). Therefore, efforts need to be directed toward developing a new non-chemical pest control measures.

RNA interference (RNAi)-mediated gene silencing technology is being exploited as a potential alternative to chemical-based pest control against insects and mites (Tian et al. 2009; Kwon et al. 2016; Cao et al. 2017; Suzuki et al. 2017). RNAi machinery is initiated through the introduction of sequence-specific double-stranded RNA (dsRNA) that degrades cognate mRNA and disrupts gene expression. The delivery of dsRNA to a target organism is an essential first step (Yu et al. 2013). Injection of dsRNA into a test organism is widely applied in insects but limited in mites due to their small body sizes (Khila and Grbić 2007). The oral administration of dsRNA with diet (i.e. artificial diet or leaf-mediated delivery systems) are less invasive delivery tactic and represents a likely pathway for an RNAi pesticide. The success in RNAi induction via

leaf-meditated oral delivery has increased the feasibility of this technology (e.g. Kwon et al. 2013, 2016; Suzuki et al. 2017).

The leaf disc floating on dsRNA solution (Kwon et al. 2013, 2016) and leaf disc coated with dsRNA solution (Suzuki et al. 2017b) were recently developed as a mean to deliver liquid molecules to TSSM. However, because a large solution volume is used in the leaf disc floating method, it is considered labor-intensive and less applicable for high-throughput RNAi screen. On the other hand, the leaf disc coating method uses a smaller solution volume; however, the method requires surfactant such as Silwet L-77 for enhancing distribution of a solution over the surface of leaf discs. Such surfactants often induce mite mortality or interrupt normal fecundity and may affect the overall outcome of the RNAi assay. Therefore, the major goal of this study was to first develop a method for leaf disc-mediated oral delivery of small molecules, which should be surfactant-independent and labor-saving by reducing the solution volume of test compounds and minimizing the handling time for the preparation of experimental materials. Then, the developed method was evaluated for its utility in the high-throughput RNAi screen to find a potential target gene for the foliar application of dsRNA as a next-generation pesticide.

In the Chapter 2, I developed a new method named “sandwich method” for oral delivery of small molecules to TSSMs. In the Chapter 3, the sandwich method was then used for RNAi by oral delivery of dsRNA targeted on the *Vacuolar-type H^+-ATPase* (*TuVATPase*) in which an RNAi-induced phenotype on mite body color was known to be useful for evaluating the RNAi effect (Suzuki et al. 2017b). In the Chapter 4, promising target genes (*TuCOPE*, *TuRPS4*, *TuMIP* and *TuAQP9*) screened from the mortality induced by RNAi in TSSMs (Kwon et al. 2016) were tested with the sandwich method.

5.1. Oral delivery of small molecules

The present study first focused on developing a new method for oral delivery of small molecules to TSSMs. In the Chapter 2, I developed the sandwich method in which no surfactant is required for coating the aqueous solution of test compounds over an entire leaf surface. In this method, a droplet of solution (6 μL) is sandwiched between the surface of leaf discs (ϕ10 mm) and a polypropylene sheet (Fig. 2). The polypropylene sheet is then placed gently and kept for 8 min at 25°C. After that, the polypropylene sheet is removed and the test compound can be distributed over the leaf disc surface. The efficiency of the sandwich method for oral delivery of a tracer dye was compared to the leaf-coating method that requires the surfactant Silwet L-77 (Suzuki et al. 2017a). A fluorescent tracer was delivered into the midgut of TSSMs fed on leaf discs prepared with the sandwich method, which the function was equivalent to the coating method. Although both sandwich and coating methods did not affect mite survival for 6 days, mite feeding on leaf discs and fecundity on leaf discs prepared with latter method were significantly lower than with the former method. As Silwet L-77 used in the coating method has known to be harmful to TSSMs (e.g. Cowles et al. 2000), it should be avoided to provide more precise bioassays. Therefore, the sandwich method that allows to orally deliver a test compound of interest into TSSMs in the absent of surfactant will be useful for a wide range of bioassays.

5.2. Dose-response effect of dsRNA-*TuVATPase*

The vacuolar H^+-ATPase (V-ATPase) present in all eukaryotic organisms is an essential protein which functions as H^+ pump and maintains the acidic condition in the lumen using energy released from ATP hydrolysis (Nelson et al. 2000; Nishi and Forgac 2002; Forgac 2007). In the previous studies using insects, oral delivery or direct injection of dsRNA targeted on the *V-ATPase* gene induced high mortality, which suggest that the gene is one of the promising targets for RNAi-based pest control (e.g. Zhu et al. 2010; Upadhyay et al. 2011; Basnet and Kamble 2018). Kwon et al. (2013) reported that leaf disc-mediated oral delivery of 160 ng μL^{-1} of dsRNA targeted on *TuVATPase* for TSSMs (dsRNA-*TuVATPase*) induced 21% mortality at 120 h after treatment. In addition, a distinctive dark body color phenotype was observed after oral delivery of dsRNA-*TuVATPase*, which is associated with a significant reduction in the fecundity (Suzuki et al. 2017b). Therefore, the *TuVATPase* gene can be a suitable target gene to evaluate the sandwich method for feeding RNAi. For this evaluation, in the Chapter 3, I investigated the dose-response effect of dsRNA-*TuVATPase* using different concentrations (0.01, 0.1, and 1 μg μL^{-1}) and the role of Tween 20 (with/ without 0.1% Tween 20), which potentially increases the permeability of dsRNA through cell membranes, for enhancing the RNAi effect.

A significant reduction of survival was observed in mites fed on dsRNA-*TuVATPase* at 1 μg μL^{-1} with and without Tween 20 for 6 days post-treatment. In addition, a significant reduction in fecundity was observed in mites fed on dsRNA-*TuVATPase* at all concentrations without Tween 20 and at 0.1 and 1 μg μL^{-1} with Tween 20. Nearly 40% of mites fed on dsRNA-*TuVATPase* exhibited a dark body color, which was comparable with the previous study (Suzuki et al. 2017b). The dark body color could be due to disturbance in marinating the acidic condition inside vesicles upon the loss of V-ATPase function (Mauvezin et al. 2015). In TSSMs, digestive

cells contains vesicles that become dark and large with taking up foods for intracellular digestion are distributed in the midgut lumen located in almost entire volume of the dorsal part of their body (Bensoussan et al. 2018). Digestive cells are originated from the midgut epithelial cells and developed in the lumen, and disruption of the acidity inside vesicles might cause the reduction of intracellular digestion. Therefore, the dark color phenotype might be related with the reduced function of digestive cells distributed in the midgut lumen. These findings proved the effectiveness of the sandwich method for RNAi with oral delivery of dsRNA and the potential of *TuVATPase* as a target for RNAi-based TSSM control, which can be achieved with the foliar application of dsRNA-*TuVATPase* at >0.1 μg μL^{-1}.

5.3. RNAi effects on four potential target genes

In the Chapter 4, I carried out feeding RNAi with the sandwich method targeted on four TSSM genes encoding i) coatomer protein (COP) subunit epsilon, COPE; ii) ribosomal protein (RP) S4, RPS4; iii) major intrinsic protein (MIP); iv) aquaporin (AQP) 9, AQP9. COPs function in vesicle transport between the endoplasmic reticulum and Golgi apparatus, RPs function in post-translational modifications, and MIPs and AQPs function in the transportation of water and small solutes across membranes.

In TSSMs, LT_{50} of 81 to 110 h resulted from oral administration of dsRNA (80 ng μL^{-1}) targeted on *TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9* genes to TSSMs with the floating leaf disc system (Kwon et al. 2016). For these promising targets for RNAi-based TSSM control, I investigated the RNAi effects in detail with the sandwich method using same dsRNA fragments and concentration with the previous study (Kwon et al. 2016). I conducted the experiments using the sandwich method and examined the survival, fecundity and gene expression levels. ,. The results in general are in agreement with Kwon et al. (2016). In the result, oral administration of dsRNA-*TuRPS4*, dsRNA-*TuMIP*, and dsRNA-*TuAQP9* induced mite mortality and reduced fecundity and the expression level of these genes. Interestingly, positive correlation relationships were found among the downregulations of *TuRPS4*, *TuMIP*, and *TuAQP9*. Mechanism of the interaction of these genes is unclear, but the high degree of homology in the sequence between *TuMIP* and *TuAQP9* might explain their simultaneous downregulation by off-target effects each other. Only dsRNA-*TuCOPE* did not follow these trends; no significant effects on mortality and gene expression level were observed. This could be due to different sensitivity to dsRNA-*TuCOPE* between the TSSM populations used in the previous and present study. Sensitivity to RNAi differs depend on the ability to degrade alien dsRNA (Asikainen et al 2005; Bellés 2010).

5.4. Conclusion

TSSMs have rapidly developed the resistance to virtually all synthetic acaricides due to their great capacities for reproduction and metabolic detoxification. Thus, the major focus in the present study was to examine the potential of RNA interference (RNAi)-mediated gene silencing technology for being utilized as a novel measure alternative to synthetic acaricides to control TSSMs. In this strategy, one of the most important elements is oral delivery of small molecules such as dsRNA, which triggers RNAi, for its foliar application for TSSM control. Although several methods were previously developed for oral delivery of dsRNA into TSSMs, mite feeding on leaf discs treated with dsRNA has been the most promising approach to assess the RNAi effect aiming to foliar application of dsRNA. However, the conventional methods require a harmful surfactant for TSSMs or manual painting to spread dsRNA over the surface of leaf discs (Suzuki et al. 2017a, b), a huge volume of dsRNA solution (Kwon et al. 2013, 2016, 2017), or time (~5 h) for the leaf disc preparation (Shi et al. 2015, 2016; Liao et al. 2016; Xia et al. 2016; Li et al. 2017; Xu et al. 2017a, b). Therefore, I developed the sandwich method (Abouelmaaty et al. 2019) in which the small volume (6 μL) of dsRNA solution was distributed by using a polypropylene sheet within 8 min and the abovementioned problems were solved. The sandwich method was then used to orally deliver dsRNA targeting on *TuVATPase* into female TSSM adults and it successfully induced downregulation of the expression of *TuVATPase*, the dark body color related indigestion, and significant reductions in survival and fecundity. In addition, the sandwich method proved that *TuRPS4*, *TuMIP*, and *TuAQP9* out of genes previously screened (Kwon et al. 2016) would be promising candidates for RNAi-based TSSM control because oral delivery of these dsRNAs induced high mortality. Taken together, the

sandwich method developed in the present study will be useful for high-throughput RNAi screens aiming for foliar application of dsRNA to control TSSMs.

Summary

1- The two-spotted spider mite (TSSM), *Tetranychus urticae* (Acari: Tetranychidae), is a common pest feed on over 1100 plant species worldwide and causes a severe damage in the agricultural crops. In addition to the polyphagy, TSSMs have a short life cycle and a variety of genes capable of detoxifying xenobiotics; thus these functions help them to rapidly develop the resistance to almost all acaricides. To overcome the difficulty in controlling this pest species, I examined the reverse genetic approach based on RNA interference (RNAi), which has the potential to be alternative to conventional acaricides. In this strategy, double-stranded RNA (dsRNA), the initiator of RNAi, was orally delivered into TSSMs through leaf discs, which mimics the foliar application of dsRNA as a sprayable acaricide. Since organosilicone surfactants such as Silwet L-77 often increase the efficacy of pesticides because of their highly wetting function on plants, great attentions have been paid to them for practical use as a dsRNA spreader in the foliar application. However, I found that Silwet L-77 had negative effects on feeding and fecundity of TSSMs. In the Chapter 2, I therefore developed the sandwich method to coat test compounds onto the leaf disc in absence of surfactant (Abouelmaaty et al. 2019), which helps to conduct accurate bioassays that eliminate any surfactant effects on TSSMs.

2- In the sandwich method, I used a polypropylene sheet instead of Silwet L-77 to spread the test solution on the entire surface of leaf discs. The function of oral delivery of a tracer dye in the sandwich method was comparable to that in the leaf disc coating method with Silwet L-77. However, orally-delivered Silwet L-77 was toxic to TSSMs and induced less feeding followed by the reduction of fecundity was observed when comparing with those fed on the leaf discs

prepared with the sandwich method, the latter showed comparable biology to those fed on untreated leaf discs. Thus, my novel method may be useful for the delivery of small molecules such as dsRNA to the digestive system of leaf cell feeders such as TSSMs without inducing negative effects on their biology.

3- With the sandwich method (Abouelmaaty et al. 2019), I examined RNAi targeting on the Vacuolar-type H^+-ATPase (V-ATPase) encoding a protein that functions as proton pump using energy releasing from ATP hydrolysis to maintain the acidity inside vesicles (Chapter 3). TSSMs were allowed to feed on the leaf discs coated with an aqueous solution of dsRNA targeting on *V-ATPase* (dsRNA-*TuVATPase*) or an intergenic region as a control (dsRNA-NC) at three different concentrations (0.01, 0.1, or 1 μg μL^{-1}) with or without Tween 20 that has been known to be no toxic to arthropods and expected for improving drug passage across the cell membrane. In the results, downregulation of *TuVATPase* was observed in TSSMs at 4 days after feeding on the leaf discs coated with dsRNA-*TuVATPase*. In addition, the fecundity of TSSMs fed on leaf discs coated with dsRNA-*TuVATPase* was significantly reduced even when the concentration was as low as 0.01 μg μL^{-1}. Reduction of survival was more robust than fecundity; it was significantly reduced only when the concentration was as high as 1 μg μL^{-1}. Furthermore, almost 40% of TSSMs showed a black body color that was a typical indigestion-related phenotype when the concentration of dsRNA-*TuVATPase* was higher than 0.1 μg μL^{-1}. However, no significant improvement was observed in the RNAi efficacy with the application of Tween 20. The results suggest that dsRNA-*TuVATPase* at >0.1 μg μL^{-1} can be potentially used as a foliar-applied acaricide for RNAi-based TSSM control.

4- To find genes that can be used as a promising target for RNAi-based TSSM control, TSSMs were allowed to feed on the leaf discs coated with 80-μg μL^{-1} of dsRNA of four potential target genes (*TuCOPE*, *TuRPS4*, *TuMIP*, or *TuAQP9*) which were previously screened by Kwon et al. (2016) (Chapter 4). All leaf discs were prepared with the sandwich method (Abouelmaaty et al. 2019). A significant mortality was observed in TSSMs fed on leaf discs coated with dsRNA targeting on *TuRPS4*, *TuMIP*, or *TuAQP9*. A significant reduction in the fecundity was observed in the RNAi treatments targeting on all four candidate genes. Interestingly, downregulations of *TuRPS4*, *TuMIP*, and *TuAQP9* were interacted with each other. Among the tested genes, *TuRPS4* was the most promising target for the RNAi-based TSSM control.

5- In this study, I examined RNAi in TSSMs at three steps: 1) Development of the leaf disc-mediated method for oral delivery of test compounds to mites without inducing any negative effects of surfactants. 2) Evaluation of the dose-response effect of dsRNA targeting on *TuVATPase* to determine the concentration that will be useful for the future application of dsRNA as a sprayable acaricide. 3) Investigation of RNAi effects on the highly potential target genes *TuCOPE*, *TuRPS4*, *TuMIP*, and *TuAQP9*. Taken together, this study revealed that the sandwich method can be useful for testing dsRNA as a foliar-applied acaricide in the laboratory-scaled RNAi screen and that *TuRPS4* as well as *TuVATPase* was the most promising target for the RNAi-based TSSM control, which will paves the way for next-generation IPM.

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