A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Establishment of ingestion RNAi-based control system against western flower thrips

섭식 RNAi 를 이용한 꽃노랑총채벌레 방제 시스템 개발

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UNDER THE DIRECTION OF ADVISER SI HYEOCK LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

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SEOUL NATIONAL UNIVERSITY
August 2019

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Establishment of ingestion RNAi-based control system against western flower thrips

Major in Entomology
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Abstract

Thrips, including *Frankliniella occidentalis* and *Frankliniella intonsa*, is one of the polyphagous pest damaging flowers and leaves of horticultural and agricultural crops. Due to its rapid development of insecticide resistance, conventional insecticides are not effective to thrips control, thus requiring an alternative control method. RNA interference (RNAi)-based control strategy has been developed to control various phytophagous chewing pests. However, no successful case of RNAi-based control was reported for sucking pests including thrips. In this study, the ingested amount of nuclei and plastids following time course in the two thrips (*F. occidentalis* and *F. intonsa*) along with two reference sucking pests (*Tetranychus urticae*, a cell feeder, and *Nilaparvara*, a cap feeder) were quantified by quantitative PCR (qPCR). The ingested amount of plastids was

significantly greater than those of nuclei in the thrips and mites species. However, substantially lower and greater ratio between ingestion amount of nuclei and plastids than those in intact kidney bean leaves was identified in the two thrips and mite species, respectively, suggesting that thrips and mite has different preference for nuclei and plastids. In contrast, no plant subcellular fractions were detected in brown planthopper. These findings provide the first proof-of-concept that hairpin RNA expressed in the nucleus can be delivered to mesophyll sucking feeders such as thrips and mites. With this in mind, a total of 57 candidate genes were selected from the transcriptome data of *F. occidentalis*, and the double-stranded RNAs (dsRNA), targeting each candidate gene, were delivered to a susceptible strain of *F. occidentalis* via the leaf disc-feeding method. The dsRNA of *toll-like receptor 6* (*TLR6*), *apolipophorin* (*apoLp*), *coatomer protein subunit epsilon* (*COPE*), and *sorting and assembly machinery component 50* (*SAM50*) resulted in the highest mortality in both insecticide-susceptible strain and -resistant strain. The dsRNA-fed thrips showed 46%, 47%, 37%, and 22% reduced transcription levels of *TLR6*, *apoLp*, *COPE* and *SAM50* in insecticide-susceptible strain and 71%, 36%, 27%, and 50% in -resistant strain, respectively. This result demonstrates that the observed mortality following dsRNA ingestion was due to RNAi. The efficacy of ingestion RNAi of the lethal genes was also confirmed against an insecticide-resistant strain of *F. occidentalis*, indicating that RNAi-

based control would be effective against both susceptible and resistant populations of *F. occidentalis*. Development of transgenic tomato plants expressing dsRNA targeting these lethal genes is currently in progress as a practical tool to control thrips in the field.

Key words: Thrips, *Frankliniella occidentalis*, *Frankliniella intonsa*, Ingestion RNAi, Lethal dsRNA, Insecticide resistance, Cell feeding, Subcellular fraction, Plastid, Nucleus

Student Number: 2016-22528

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CHAPTER 1.

Ingestion amount and time course of plant subcellular fractions by two thrips species and two reference sucking pests

Ingestion amount and time course of plant subcellular fractions by two thrips species and two reference sucking pests

Abstract

Thrips are known as cell-feeding sucking pests, but little information is available on which and how much of each subcellular fraction they can selectively ingest. In this study, the ingested amounts of nuclei and plastids in two thrips species (*Frankliniella occidentalis* and *Frankliniella intonsa*) along with two reference sucking pests (*Tetranychus urticae*, a cell feeder, and *Nilaparvata lugens*, a sap feeder) were quantified by quantitative PCR using *phytoene desaturase* and *rubisco* as respective marker genes following feeding. The ingested amounts of plastids were significantly greater than those of nuclei in the thrips and mite species. In the thrips species, however, the fold differences in ingested amount between the two fractions were substantially lower than their original ratio in intact plant cells, suggesting that thrips ingest nuclei more selectively than plastids. Unlike the thrips species, the ratio between nuclei and plastids increased in *T.*

urticae. In contrast to these cell-feeding insects, no subcellular fraction was detected in *N. lugens*. Our findings further suggest that transgenic expression of foreign hairpin RNA in the nucleus would deliver a substantial amount of target molecules to cell-feeding sucking pests but not likely to sap-feeding pests when employing ingestion RNA interference-based control strategies.

1. Introduction

Thrips feed on a wide range of host plants, including vegetable crops and ornamental plants. In particular, the Western flower thrips (*Frankliniella occidentalis*) feeds on over 250 different crop plants from more than 60 families (Hunter and Ullman, 1989; Lewis, 1997; Tommasini and Maini, 1995). Thrips damage plants directly by sucking plant cell components with their piercing and sucking mouth parts (Chisholm and Lewis, 1984; Hunter and Ullman, 1992). Initial plant damage caused by thrips sucking (Shipp et al., 2000) results in malformation of fruits and horticultural products and loss of quality and yield, thereby decreasing the market price (Rosenheim et al., 1990). Viral transmission to the host plants during feeding also leads to severe loss of crops and products (Mumford et al., 1996; Ullman et al., 1997).

Unlike Homopteran insects (e.g., aphids, scale insects, whiteflies and true bugs) that suck the sap from the plant vascular system, thrips and mites feed on the mesophyll cells (Chisholmand Lewis, 1984; Kindt et al., 2003; Park and Lee, 2002). When thrips consume plant cellular components by inserting tubular stylets (Hunter and Ullman, 1989), they secrete saliva into plant tissues (Harrewijn et al., 1996; Heming, 1978). During salivation, enzymes involved in cell wall digestion

(β-glucosidase, endo-beta-glucanase and pectin lyase) and general digestion (short-chain Acyl-CoA dehydrogenase, serine protease, carboxypeptidase, chitinase, lipase andnucleotidase) are secreted from the salivary gland, thereby facilitating uptake and extra-oral digestion (Stafford-Banks et al., 2014a). Therefore, substantial amounts of plant macromolecules, including nucleic acids, are likely to be digested before they reach the insect's midgut. However, no information is available on the amounts of subcellular nucleic acid fractions ingested by thrips.

Various insecticides have been used for thrips control; however, rapid emergence of insecticide-resistant populations has occurred due to their short life cycle and high biotic potential. Therefore, chemical control of thrips by insecticides has become extremely difficult, thus requiring other control strategies that minimize insecticide resistance problems (Jensen, 2000). As an alternative control strategy, the RNA interference (RNAi), induced by double-stranded RNA (dsRNA) ingestion, has been tested against various agricultural pests (Baum et al., 2007; Huvenne and Smagghe, 2010). Recently, feeding dsRNA of selected lethal genes was able to kill the two-spotted spider mite, *Tetranychus urticae*, demonstrating the control potential of ingestion RNAi against this cell-feeding sucking pest (Kwon et al., 2016; Kwon et al., 2013). Considering that thrips are also cell-feeders, expression of lethal hairpin RNA in plant cells via transgenesis

is likely to be the most efficient way of delivering sufficient amounts of target dsRNA to thrips. Nevertheless, no information is available yet on which subcellular fractions are selectively ingested when thrips feed on plant cells and how much intact nucleic acid, including hairpin RNA (or dsRNA), once expressed in planta, can be delivered to thrips following extra-oral digestion.

In this study, the amounts of plant subcellular fractions ingested by two thrips species (the Western flower thrips, *F. occidentalis* and the garden thrips, *Frankliniella intonsa*) were determined along with those of two reference sucking pest species (the two-spotted spider mite, *T. urticae*, a cell feeder, and the brown planthopper, *Nilaparvata lugens*, a sap feeder) using *phytoene desaturase* and *rubisco* as respective marker genes of plant nuclei and plastids. In addition, the selective ingestion profiles of these two subcellular fractions were determined in these thrips and mite species.

2. Materials and methods

2.1. Insect and mite

Laboratory strains of *F. occidentalis*, *F. intonsa*, *T. urticae* and *N. lugens* were obtained from the National Institute of Agricultural Sciences (Jeonju, Korea). *F. occidentalis* and *F. intonsa* was reared on cotyledons of kidney bean, *Phaseolus vulgaris*. The thrips were fed using 150 mm of breeding dish (SPL, Pocheon, Korea), and *T. urticae* was reared on whole kidney bean and *N. lugens* was reared on whole rice seedlings, *Oriza sativa*. All insect and mite colonies were maintained 25 ± 1°C with a photoperiod of 16:8 (L:D). Relative humidity was maintained at 55 ± 5% for *F. occidentalis*, *F. intonsa* and *T. urticae* whereas at 75 ± 5% for *N. lugens*.

2.2. Host plant feeding

Female adults *of F. occidentalis, F. intonsa* and *T. urticae* (30–35, 30–35 and 200–250 individuals, respectively) were first starved in individual 5-ml glass vials and 20–25 female adults of *N. lugens* in an insect breeding dish (SPL, Korea) for 24 h. To minimize mortality during the starvation period, 1 μl of water was supplied to a filter paper disk (2 mm × 2 mm) attached onto the inside of the vial cap for each *F. occidentalis*, *F. intonsa* and *T. urticae*, and water-soaked cotton

pads (70 mm × 70 mm) were placed in the insect breeding dish (100 mm diameter × 40 mm height) for *N. lugens*. After 24 h of starvation, *F. occidentalis*, *F. intonsa* and *T. urticae* were transferred onto kidney bean leaves placed over a water-soaked cotton pad, and *N. lugens* was transferred to rice seedlings embedded in an insect breeding box (72 mm × 72 mm × 100 mm; SPL, Korea). Test insects and mites were collected at 0, 6, 12, 24, 36 and 48 h post-feeding and kept in a -80℃ deep freezer until genomic DNA (gDNA) extraction. The feeding experiment was repeated three times

2.3. Genomic DNA extraction

Various numbers of test insects or mites (20–25, 20–25, 100–200 and 15 individuals for *F. occidentalis*, *F. intonsa*, *T. urticae* and *N. lugens*, respectively) were used for each batch of gDNA extraction. *F. occidentalis*, *F. intonsa* and *T. urticae* were homogenized using a 0.1 ml glass tissue grinder (Wheaton, USA), whereas *N. lugens* were homogenized in a bullet blender with 0.9-2.0 nm stainless steel beads (Nextadvance, France) following chopping with micro-scissors (Miltex, Germany). gDNA was extracted using a DNeasy Blood & Tissue Kit according to the manufacturer's protocol (QIAGEN, Netherlands). The concentration of the extracted gDNA was measured with a Nanodrop 2000 (Thermo Fisher Scientific, USA). For gDNA extraction from host plants, leaf

disks of *P. vulgaris* and O. sativa (each 1 mg) were flash-frozen in liquid nitrogen and pulverized with a mortar and pestle. gDNA was extracted from the frozen plant powder using a DNeasy Plant Mini Kit according to the manufacturer's protocol (QIAGEN).

2.4. Quantification of ingested amounts of nuclei and plastids

To quantify the ingested amounts of host plant DNA by the four pest species, *phytoene desaturase* (CM002288.1) and *rubisco* (LT576853.1 and NC_001320.1) were selected as marker genes for nuclei and plastids (including chloroplasts) of host plants, respectively. Relative ingested amounts of the two marker genes were determined by quantitative real-time PCR (qPCR) using gDNA of the collected insect/mite specimens as the template and 18S rRNA (LOC113204774, *F. occidentalis*; KT204370.1, *F. intonsa*; AF062961.1, *T. urticae*; and JF773148.1, *N. lugens*) of each pest species as the reference gene. The qPCR mixture (10 μl) contained 2□TB Green (Takara, Japan), template gDNA (5 ng/μl), a mixture of forward and reverse primers (each 5 pM) (Table 1) and ddH2O. The qPCR was conducted with the following thermal program: one cycle of 95°C for 30 s followed by 36 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. The final mixture was incubated at 95°C for 5 s, 60°C for 60 s and then the temperature was increased to 95°C with a ramping rate of 0.2°C/s for the melting curve analysis.

3. Results

3.1. Relative levels of the plant subcellular fractions ingested by *F. occidentalis*, *F. intonsa*, *T. urticae* and *N. lugens*

The overall ingestion amount of the nuclear fraction (i.e., amount of *phytoene desaturase*) by the sucking pests was estimated to be 5-6 orders of magnitude lower than that of the 18S rRNA gene of each pest species. Even after the 24-h starvation, trace amounts of the nuclear fraction were detected in both *F. occidentalis* and *F. intonsa* (Fig. 1A and B, 0 h post-feeding). In *F. occidentalis* and *F. intonsa*, the amount of ingested nuclear fraction was slightly increased and returned to the initial level as feeding proceeded. The maximum ingestion of the nuclear fraction was observed at 24 h and 12 h post-feeding in *F. occidentalis* and *F. intonsa*, respectively, although the mean values were not significantly different ($p = 0.1533–0.8688$, ANOVA Tukey's post hoc test). In *T. urticae*, ingestion of the nuclear fraction became apparent at 6 h post-feeding (5.9×10^{-6} relative to 18S rRNA) and then remained constant during feeding.

The relative ingestion level of the plastid fraction (i.e., amount of *rubisco*) was much higher than that of the nuclear fraction in all mesophyll feeders tested over time (Fig. 1A, B and C). The ingestion levels relative to 18S rRNA reached maximum levels at 6-12 h post- feeding ($1.1\text{-}1.3 \times 10^{-4}$ in *F. occidentalis*, $2.9 \times$

10^{-4} in *F. intonsa* and 7.8 x 10^{-2} in *T. urticae*) and then stabilized. At 24 h post-feeding, undergoing stabilized feeding condition, the relative plastid ingestion amounts were 3.3-, 3.5- and 708.8-fold larger than the nuclear fractions in *F. occidentalis*, *F. intonsa* and *T. urticae*, respectively. Almost 3.3-fold higher relative ingestion level of the plastid was observed in *F. occidentalis* compared with *F. intonsa*, whereas no significant difference was found in the ingestion amount of nuclei between the two species. In contrast to the mesophyll feeders, little fractions of *phytoene desaturase* and *rubisco* were detected in *N. lugens*, demonstrating that plant cellular components were barely ingested in this sap-feeding insect (Fig. 1D).

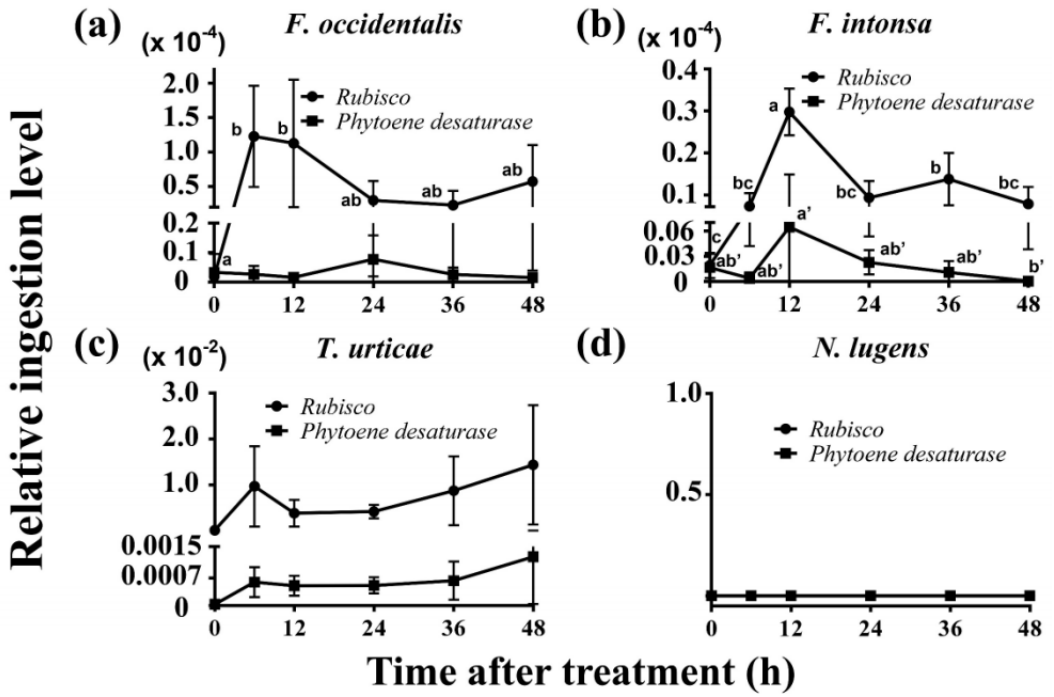

Figure 1. Relative ingestion levels of the plant nuclei and plastids from the four sucking pest species were estimated by qPCR during the time course of feeding following 24 h starvation. *Phytoene desaturase* and *rubisco* were used for marker genes for nuclei and plastids of host plants, respectively. *18s rRNA* from each sucking pest species was used as the reference gene.

3.2. Feeding ratio between nuclei and plastids ingested by *F. occidentalis*, *F. intonsa* and *T. urticae*

The ratios between the amounts of nuclei and plastids ingested by the mesophyll feeders after 6 h post-feeding were compared with those present in the kidney bean leaves to quantify the amount of selectively consumed plant subcellular components. In kidney bean leaves, nuclei and plastids exist in a ratio of 1:691 (Table 2). The ratios between nuclei and plastids ingested by the three mesophyll feeders during the observed period (6–48 h post-feeding) were decreased in both thrips (1:3.3–47.0 in *F. occidentalis*; 1:3.2–23.4 in *F. intonsa*) (Table 2 and Table 3). In *T. urticae*, however, increased ratios (1:651–1317) were generally observed (Table 2 and Table 3). The reduced ratios in *F. occidentalis* and *F. intonsa* indicate that they ingest relatively larger amounts of plant nuclei along with cytosol compared with plastids.

Table 1. Primers used in this research

Species	Target	Primer	Note
Phytoene desaturase	*P. vulgaris*	5-CGACCCACTTCATTTCGTGC-3	F
	&	5-CTTCAGGGGTTGTAGCGGAC-3	R
	O. saltiva	5-GTCGTTTGCGTCGATTATCCA-3	Nested_F
Rubisco	*P. vulgaris*	5-TCTCTACCAGGTGTTCTGCC-3	Nested_R
		5-GGGTGTCCTAAAGTTCCTTCC-3	F
	O. saltiva	5-GCAGTAGCTGCCGAATCTTC-3	R
		5-CAACGGGCTCGATGTGATAG-3	F
18s rRNA	*F. occidentalis*	5-TGGTTTCGGCCATCGTCTAC-3	R
		5-TAGGCGCAGAACCTACCATC-3	F
	F. intonsa	5-TGTCGCGGTGCTCTTCATTG-3	R
		5-TCAGGCAATATCCAGCCTGC-3	F
	T. urticae	5-GGAGCCTGCGGCTTAATTTG-3	R
		5-TAAGAACGGCCATGCACCAC-3	F
	N. lugens	5-ACTATGCCGACTAGGGATCG-3	R
		5-AATTAAGCCGCAGGCTCCAC-3	R

Table 2. Ratio between nuclei and plastids in kidney bean leaves and three mesophyll feeders

Kingdom	Species	Ratio (nucleus : platsids)
Plant	*P. vulgaris*	1 : 691
Arthropoda	*F. occidentalis*	1 : 3.3 ~ 47.0[a]
	F. intonsa	1 : 3.2 ~ 23.4[a]
	T. urticae	1 : 651 ~ 1317[a]

[a] **Average ratio between nuclei and plastids ingested by each species during the observed period (6~48 h post-feeding).**

Table 3. Ratio between plant nuclei and plastids ingested by the three mesophyll feeders during the time course of feeding

	Ratio (nucleus : plastids)					
	0 h	6 h	12 h	24 h	36 h	48 h
F. occidentalis	1 : 0.2	1 : 41.8	**1 : 47.0**	**1 : 3.3**	1 : 7.6	1 : 31.4
F. intonsa	1 : 0.8	1 : 6.1	**1 : 3.2**	1 : 3.5	1 : 6.4	**1 : 23.4**
T. urticae	1 : 269.2	**1 : 1317.3**	**1 : 650.6**	1 : 708.8	1 : 1135.6	1 : 921.7

The ratio was calculated with the relative level of *phytoene desaturase* and *rubisco* determined by using ΔΔCt method. Fudge factor '1' was added to both relative level of *phytoene desaturase* and *rubisco* when either one of them is 0 due to undetectable amount, thereby producing no ratio. The ratio written in blue and red indicates the minimum and maximum value among the results from each mesophyll feeders during the feeding period (6~48 h post-feeding).

4. Discussion

The ingested amounts of nuclei and plastids were estimated using respective marker genes. However, considering that the quantification of ingested amount of host plant DNA was performed during a period of simultaneous feeding and digestion (pre-oral and intestinal), the ingested amounts of subcellular fractions were likely to be underestimated. Because trace amounts of subcellular fractions were detected even after a 24-h starvation, complete digestion of fed material appears to require a longer time period. The ingested ratio between the nuclear and plastid fractions likely reflects more realistic values if assuming that the digestion rates of nuclei and plastids in each pest species are similar. Despite the apparent pre-oral and intestinal digestion of ingested material, substantial amounts of intact marker genes were detected, suggesting that certain portions of intact plant DNA and RNA are maintained under the steady state of feeding and digestion.

All the mesophyll feeders examined in this study ingested significantly larger fractions of plastids than nuclei (Fig. 1). This finding was expected, as plant cells contain large numbers of plastids but a single nucleus. Nevertheless, the reduced ingestion ratio between nuclei and plastids, particularly in *F. occidentalis* and *F.*

intonsa, compared with the intact ratio in the kidney bean leaf tissue suggests that thrips ingest relatively more plant nuclei along with cytosol compared with plastids. This finding further indicates that thrips ingestion of plastids, which contain most of the starch (i.e., polysaccharide energy storage), is not particularly selective.

The overall feeding pattern was similar between *F. occidentalis* and *F. intonsa* (Fig. 1). However, the ingestion amounts of plastids relative to the 18s rRNA gene of each species were different (around 3.3-fold larger in *F. occidentalis* than in *F. intonsa* at 24 h post-feeding). This difference in feeding amount is likely due to different body size of the two thrips species: *F. occidentalis* is 1.28-fold larger than *F. intonsa* by body weight (66.7 μg ± 5.5 for *F. occidentalis* vs. 52.0 μg ± 6.9 for *F. intonsa*). Nevertheless, the ingestion ratios between subcellular fractions (nucleus:plastid) were similar between *F. occidentalis* (1:3.3) and *F. intonsa* (1:3.5) at 24 h post-feeding, suggesting a common feeding physiology in these closely related thrips species. On the other hand, the ratio at 24 h post-feeding was noticeably higher (1:709) in *T. urticae*, indicating that *T. urticae* ingests plastids more selectively than thrips, which is likely due to the unique sucking physiology of mites. The differences in sucking physiology in thrips, such as the piercing mouth part, salivary enzymes, piercing position and depth and sucking power, may contribute to the observed differences

in ingested fraction ratio (Andre and Remacle, 1984; Hunter and Ullman, 1992; Jonckheere et al., 2016; Stafford-Banks et al., 2014b).

Apparent ingestion of the nuclear fraction of plant cells provides the first proof-of-concept that hairpin RNA expressed in the nucleus can be delivered to mesophyll sucking feeders such as thrips and mites. The expression of hairpin RNA in plant cells via transgenesis may be the most efficient and practical form of RNAi-based control of these sucking pests. The finding that relatively larger amounts of the nuclear fraction compared with the plastid fraction were ingested in the thrips supports the feasibility of nuclear expression of target hairpin RNA in generating transgenic plants for RNAi-based thrips control. Once in planta expressed, as in conventional transgenic plants, the amount of intact hairpin RNA required for functional RNAi is likely delivered to the target cells even after extra-oral digestion by salivary secretion. With this in mind, the lack of uptake of subcellular fractions by *N. lugens* suggests that development of transgenic plants expressing lethal hairpin RNA against phloem sap-feeding pests, including *N. lugens*, may not be feasible. Recently, the chloroplast has been considered as an ideal platform for transgene expression due to elimination of gene silencing and position effect, increased expression of transgenes and reduction of environmental dispersion of transgenes (Adem et al., 2017). As observed in this study, the thrips and mite species ingested larger amounts of plastids than nuclei due to the

abundance of plastids in cells. Therefore, chloroplast expression of lethal hairpin RNA would increase the amount of transgene, thus enhancing the efficiency of RNAi against these mesophyll feeders, if technical limitations associated with chloroplast transgenesis can be solved (Zhang et al., 2017).

CHAPTER 2.

Selection of lethal genes for ingestion RNA interference against western flower thrips, *Frankliniella occidentalis*, via leaf disc-mediated dsRNA delivery

Selection of lethal genes for ingestion RNA interference against western flower thrips, *Frankliniella occidentalis*, via leaf disc-mediated dsRNA delivery

Abstract

The western flower thrips, *Frankliniella occidentalis*, is a major pest that damages a wide variety of crops and vegetables. Following extensive use of insecticides, it has developed high levels of resistance to almost all groups of insecticides due to its high reproduction rate and short generation time. Therefore, an alternative pest control strategy, such as RNA interference (RNAi)-based control, is essential. To establish an ingestion RNAi-based control, a total of 57 genes involved in various biological processes were selected, and their double-stranded RNAs (dsRNA) were delivered to an insecticide-susceptible strain of *F. occidentalis* via the leaf disc-feeding method using a bioassay chamber optimized by 3D printing. The mortality of dsRNA-ingested thrips was examined every 24 h until 120 h post-treatment. Of the 57 genes screened, dsRNAs of the *Toll-like receptor 6*, *apolipophorin*, *coatomer protein subunit epsilon* and *sorting and assembly machinery componen*t were most lethal when ingested by thrips. The dsRNA-fed thrips showed substantially reduced transcription levels of target

genes, demonstrating that the observed mortality was likely due to RNAi. When these genes were tested for ingestion RNAi against an insecticide-resistant strain of *F. occidentalis*, bioassay results were similar. In conclusion, this study provides the first evidence that ingestion RNAi can be lethal to *F. occidentalis*, a mesophyll sucking pest, and further suggests that transgenic plants expressing hairpin RNA of these essential genes can be employed to control insecticide-resistant thrips.

1. Introduction

The western flower thrips, *Frankliniella occidentalis*, is a major agricultural pest that feeds on a variety of vegetables and ornamental plants worldwide. *Frankliniella occidentalis* ingests hosts' mesophyll cells when feeding, using its straw-shaped rasping-sucking mouth. It not only directly damages leaves and flowers, but also causes damage by transmitting plant virus diseases such as tomato-spotted wilt virus (Kirk & Terry, 2003; Yudin, Cho, & Mitchell, 1986). A variety of insecticides have been used extensively to control *F. occidentalis*, but due to its high reproduction rate and short generation time, it has developed high levels of resistance to almost all groups of insecticides (Gao, Lei, & Reitz, 2012). Currently, chemical control of *F. occidentalis* is ineffective due to resistance, thus requiring an alternative strategy for efficient management of insecticide-resistant *F. occidentalis* populations.

RNA interference (RNAi) is a post-transcriptional gene regulation system in eukaryotes, including arthropods, and silences the target gene in a sequence-specific manner. RNAi-based plant transgenesis has been attempted as a pest control strategy (Huvenne & Smagghe, 2010). Initial attempts were conducted on Coleopteran and Lepidopteran chewing pests using double-stranded RNA (dsRNA)-feeding method targeting various essential genes (Abbott, 1925; Bautista, Miyata, Miura, & Tanaka, 2009; Cao, Bao, & Wuriyanghan, 2017; Zhu,

Xu, Palli, Ferguson, & Palli, 2011). Several transgenic plants were designed to express dsRNA, and their lethality was tested against Coleopteran (Baum et al., 2007), Hemipteran (Mao et al., 2007; Zha et al., 2011), and Lepidopteran (Xiong, Zeng, Zhang, Xu, & Qiu, 2013) pest species.

Unlike chewing pests, it is difficult to deliver dsRNA to sucking pests when establishing ingestion RNAi system. To screen lethal genes for ingestion RNAi against the two spotted spider mite, *Tetranychus urticae*, a typical sucking pest, a leaf disc-mediated dsRNA delivery system was developed [15, 16]. Of 42 genes tested using this system, the *coatomer protein subunit epsilon* (*COPE*), *coatomer protein subunit beta 2* (*COPB2*) and *aquaporin 9* genes exhibited high lethality when *T. urticae* were fed with their dsRNAs. These results demonstrated that the leaf disc-mediated dsRNA delivery system is an effective method for RNAi target gene selection in sucking pests.

In case of *F. occidentalis,* a microinjection system was initially used to deliver dsRNA, and knockdown of V-ATPase-B transcripts caused a significant reduction in female survival rate and fertility (Badillo-Vargas, Rotenberg, Schneweis, & Whitfield, 2015). Later, a recombinant symbiont-mediated dsRNA delivery system was developed. When fed to *F. occidentalis* with artificial feeding mixture, the recombinant symbiont expressing dsRNA targeting tubulin gene caused a reduction of tubulin mRNA and increased thrips mortality (Whitten et al., 2016).

Neither of these methods, however, are efficient for screening multiple numbers of target genes.

In this study, the leaf disc-mediated dsRNA delivery system was optimized by 3D printing technology. A total of 57 target gene dsRNAs were evaluated for their lethality against *F. occidentalis*. Of the 57 dsRNAs tested, *toll-like receptor 6* (*TLR6*), *apolipophorin*,(*apoLp*), *COPE* and *sorting and assembly machinery component 50* (*SAM50*) dsRNAs resulted in high lethality not only against insecticide–susceptible strain, but also against insecticide-resistant strain. These target genes can be employed as candidates for developing RNAi-based thrips-resistant transgenic crops.

2. Materials and methods

2.1. Insect rearing

The insecticide-susceptible strain of *F. occidentalis* was obtained from Rural Development Administration (Jeonju, Korea) and was reared on 15~20 of fresh kidney beans (*Phaseolus vulgaris*) cotyledons in a rearing dish (150 mm diameter; SPL, Pocheon, Korea) at 25 ± 1 °C, 55±5% RH and a 16:8 (L:D) photo period. The multiple insecticide-resistant strain was initially collected from chrysanthemums in a greenhouse at Gyeonggi-do Agricultural Research and Extension Services (Hwasung, Gyeonggi-do, Korea) and further selected with emamectin benzoate (EB) three consecutive times. The EB-selected strain (EB-R) exhibited a high level of resistance to EB (1,100 fold) and moderate levels of resistance to spinosad (50 fold) and chlorfenapyr (40 fold) compared to the susceptible strain. The EB-R strain was used in the bioassay after adapting to the kidney bean rearing system.

2.2. Analysis of *F. occidentalis* transcriptome

Total RNA was separately extracted from 50 females and 50 2nd instar nymphs using 0.1 ml glass homogenizers (Wheaton, USA) and TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). The thrips homogenate in 100 μl TRI

reagent was transferred in a 1.5 ml centrifuge tube and mixed with 10 µl of 1-Bromo-3-chloropropane. The samples were centrifuged for 15 min at 13,000 g for 4℃ and the supernatant was mixed with 100 µl of isopropanol to precipitate RNA. The RNA pellet was washed with 75% ethanol for 2 times and dissolved in nuclease-free water. To remove remaining genomic DNA, 3 µg of the extracted total RNA was treated with 1.25 units of DNase I (Takara Korea Biomedical Inc., Seoul, Korea) for 30 min at 37℃. The DNase I-treated total RNA was precipitated with sodium acetate and ethanol, washed with 75% ethanol for 2 times and dissolved in nuclease-free water. . 1 µg of DNase-I treated total RNA was used for mRNA preparation, and cDNA libraries were constructed using an Illumina TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The multiple cDNA libraries from females or nymphs were paired-end sequenced (200-400 bp) using an Illumina NovaSeq 6000 Sequence System (Illumina). The resulting paired-end sequence files were trimmed using a Trimmomatic program (Illumina) to remove the adapter sequence and reads with mean quality <15 and minimum length <36 bp. The trimmed reads were mapped to the *F. occidentalis* reference genome (https://www.hgsc.bcm.edu/arthropods/western-flower-thrips-genome-project) using the Human Genome Sequencing Center (HGSC) with a HISTAT2 program (Illumina). Transcript assembly of the mapped from females or nymphs

read was proceeded separately using StringTie program (Center for computational biology, Johns Hopkins Univ.), and FPKM value (Fragment per kilobase of transcript per million mapped reads) of the assembled transcripts was calculated. After quantile normalization of the FPKM value [log2(FPKM+1)], differential gene expression (DEG) pattern between the female and nymphal stages was analyzed from the normalized FPKM value.

2.3. dsRNA synthesis

The mRNA sequences of the target genes were obtained from the transcriptome analysis (Table 1). The ~500 bp dsRNA templates containing the T7 promoter sequence were designed using the E-RNAi dsRNA designing program (https://www.dkfz.de/signaling/e-rnai 3/) (Horn & Boutros, 2010).

To prepare template DNA, cDNA was synthesized from DNase I-treated total RNA extracted from females (see section 2.2) using a Superscript IV cDNA synthesis kit (Invitrogen, Carlsbad, CA). Template DNA for dsRNA synthesis was generated by PCR using the cDNA and the primer sets (Table 1 and Table 2) Advantage II polymerase mix (Clontech, Palo Alto, CA, USA) in a C1000 Touch thermal cycler (Bio-rad, Hercules, CA, USA). PCR was conducted in 40 μl reactions with each 5 μM of primer sets, 10 mM dNTP, and 2 units of Advantage II polymerase mix using the following cycling conditions: a single denaturation

cycle at 95°C for 3 min, 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min. . Subsequently, the primary PCR product was used for nested PCR. The nested PCR was conducted in 50 µl reactions with each 5 µM of primer sets, 10 mM dNTP, and 2.5 units of Advantage II polymerase mix with following conditions: a single denaturation cycle at 95°C for 3 min, 5 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min and 35 cycles of 95°C for 30 s, 70°C for 30 s, 72°C for 1 min. The PCR product was purified with a PCR DNA cleanup kit (New England Biolabs, Ipswich, MA, USA) and then used to prepare dsRNA. The dsRNA was synthesized using *in vitro* transcription using a Megascript T7 transcription kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. dsRNA was dissolved in 200 µl of nuclease-free water, stored at -70°C and diluted to 100 ng/µl before use. The dsRNA of *Apis mellifera acetylcholinesterase 1* (*Amace1*; 551 bp) was synthesized by Genolution Inc. (Seoul, Korea) and used as negative control dsRNA after verifying that *Amace1* dsRNA does not target *F. occidentalis ace*.

Table 1. The list of primary PCR primers used for making dsRNA template of target genes.

Function	Gene		Oligonucleotide sequence (5' – 3')	Amplicon (bp)
Cell signaling	small G protein signaling modulator	F[1]	GCCAACTTGGCCAAGTTGAG	913
		R[2]	ACCAGTAGTTTCGGTCGCA	
	rho-related GTP-binding protein	F	GATGATGTGGCATGAGGCTC	856
		R	TTTACGGCCGGCAAACATTG	
	Engulfment and cell motility protein	F	CGCCTAACCTTCTCACCATC	1037
		R	TCTCCAATACGCAGCACCTC	
	Inhibitor of apoptosis	F	GCTTGAACACTCAGCGTGTG	980
		R	CCTCTGCTATGTAGTCACGC	
	Cyclin-dependent kinase 16	F	GCATTGGATCTGATGGCGAG	877
		R	GCCAAGCTGCTGATCAGATG	
	cdc42-interacting protein	F	GAGGCTGGTCAAGAACCATC	875
		R	CCTCTGGTCTGATTGAGGTG	
	Phosphatidylinositol 4-kinase	F	TGTGAACACGGCAACAACCC	870
		R	TTGCAGGCAGCTCCAAGAAC	
	Ras	F	ATGTCGAACGGTCAAGGAAG	664
		R	CAACTCGATAACCACCACAGC	
Digestion	Amylase alpha	F	CAAGCTCTGCCTTGTGTAGC	1039
		R	CGCCATCCTGCGAATAGTTG	
	Glucosidase 1	F	TTTGCTGGTGTCAGGCACTG	986
		R	TGTTCGCGTGGTATCCATCC	

Excretion	Diuretic hormone	F	CAGGAATCCGTCAGCCATGC	981
		R	AGAAGGGTGAGGAGGATGTG	
	Corticotropin releasing hormone	F	GTATACGTACGGCATACGCC	746
		R	GCTTCAGGTAGACCGTGACG	
	Diuretic hormone receptor	F	CAATCAACGACAGCCTGCTG	979
		R	ACGTAGGTCATGCCCAGCAG	
Immune response	Toll-like receptor 4	F	GCTGACCAACGTGACGTTCG	938
		R	AGGGTCGTCACCATCAAGTG	
	Toll-like receptor 6	F	TCCACCTGCACAACAACAGC	1026
		R	CCCGGATGTGGTTGTTGTTG	
Metabolism	Insulin receptor	F	GCCACGAGCTCATGATGAAC	950
		R	GTCACCAGTCAGGTCTTCTC	
	Insulin-like peptide	F	TGATAAACGCCCTGGAGCAG	1074
		R	CATCTGCCGCAAACACTTGC	
	Cytochrome c1	F	CTGCGAAGTGCTACTTTCGC	705
		R	TTCTTGCAGAACCACCCCAG	
	Trehalose-6-phosphate synthase	F	AAGCTGGAGAGGAAAGCCAG	986
		R	CGTCCATTAATACGCCCAAC	
	Vacuolar-type H^+ ATPase	F	GGAGTCCAGATTCGGCTATG	801
		R	TTACCACGCTCTCCACAACC	
	ATP synthase	F	GTGGCGAAGGCATTGCCAAA	1042
		R	CACCAAGGGTCTTGCTCATC	
metamorphosis	Chitin synthase	F	CAAGACCAAGATGATCGCGC	922
		R	CATCATGACCATGGCAT	

			AGCC	
	Ecdysone receptor	F	GAGGCTGATCGCCACAG TTCT	709
		R	GGATTGACACGTCTCAA TTCC	
Protein degradation	E3 ubiquitin-protein ligase	F	GAACTAGTCCGAGCACC ATC	910
		R	GAATAGTGTACCCAGCC GAC	
Protein expression	nucleosome-remodeling factor subunit	F	GCTGTGGCTTCAGCAAC TAC	1007
		R	GGCTTAGCCACCATGTC ATC	
	RNA-binding protein	F	AGGAGGACAAGAAGAG CTCC	874
		R	CGCTCATGTTCTCTCTCC TG	
	60S ribosomal protein L4	F	TCAGGCCTGATGTGGTG AAC	890
		R	ATTCCTCTTGAGGACAG CGG	
	40S ribosomal protein L4	F	ACCGTGTGGCCATCTAT GAG	675
		R	CAGAGCGGTTGCTCAAC ACA	
	Kruppel-like factor 13	F	GGACATCGAGATCAACC TCG	819
		R	GTTCTTCTCCCCAGTGT GAG	
Structure	dynein beta chain	F	TCATGGGCTTCCTGATG CAC	852
		R	ACATCTCCTTGACGGCC TTG	
	Tubulin alpha	F	GTATTTGTCGACCTCGA GCC	935
		R	ATGCAGACCGCTCTTGC AAC	
	Tubulin beta	F	ATCCTTGTCGACTTGGA GCC	1002
		R	CCAATGCAAGAAGGCCT TCC	
	Tubulin gamma	F	GGCTCTCTCACGAAAGT CTC	902
		R	CTATGATGCTGGAGTGG ACC	
	Golgi reassembly and stacking protein	F	TCCAGGACAAAAGGCT GGAC	979

		R	AGTAGTGGTGCCTCCAACAG	
	Integrin alpha	F	TCAACGTGGACGACTCGATC	852
		R	GTGAGGTGCCCTCTAGTATG	
	Actin	F	CCATCAGCAATTGCCATCAGG	1060
		R	CAAGCATGCTTCCACCAAAC	
Transport	Coatomer protein alpha subunit	F	TGGAGAAGTTCGACGAGCAC	930
		R	GCCGAAGGAAGCGATCTTTC	
	Coatomer protein beta subunit	F	TCCTTCCCCTGCAAGATCAC	916
		R	ACCAAGTTGTGCGTCTTGGC	
	Coatomer protein epsilon subunit	F	TCAGCAGGGGGATGTTGATG	819
		R	GCATGAGAATCTTGCAGCTG	
	Coatomer protein zeta subunit	F	CTATCTCTACCATCCGATCC	936
		R	CTGCATTAAAGTCTGCCTCC	
	Coatomer protein gamma 2 subunit	F	TTCAGGCAATCCGCGCTTTG	943
		R	GCTGGTTCAAGTGATACACGG	
	Apolipophorin	F	GCCAAGCCAAACCTTAAGCC	987
		R	GTTGCTGGAGTCCACAAGGA	
	Ferritin	F	GGCTACTTACCGAGTAAGCG	673
		R	TCTTTGAGTCCTTGGGGTCC	
	Aquaporin	F	GACTTTGAGCACCACTGGGT	928
		R	GCGAATGGAAGCTCTCCGAA	
	Exportin 7	F	CGAGGTCACCAATGCCTACA	936
		R	GCTTTCTTGGCCTCTTCAGC	
	Exportin 2	F	ACACGCCCTAAACTGCC	1048

		R	TGG TGTAGGCATTGGTGACC TCG	
Unknown	FOCC014394	F	ACACATCGCAGGATGAG GAC	901
		R	ACGCTCTCGTCGAAGTA TCC	
	Pfam12937	F	TCCTACTCGTGCTGGAG TAC	966
		R	GGCAGTCTCTTCCTGTA GTC	
	FOCC010227	F	CCGACAGTACGTATCAG GAC	941
		R	ACCTTCGTCCTCCATCA AGG	
	Pfam01433	F	CGGTGAACACAGTCAC CAAG	1001
		R	GATGTCCTTGATGCTGA CGG	
	Pfam05485	F	AGGTGTTGCGTGAAGA ACTG	960
		R	TGTCAAGAGCATTTCCG GGC	
	FOCC004156	F	CCGACAGTACCACCAA AGAG	881
		R	TCAGGAGCTGCTGCAGT ATC	

[1] Forward primer
[2] Reverse primer

Table 2. The list of nest PCR primers used for making dsRNA template of target genes.

Function	Gene		Oligonucleotide sequence (5' – 3')	Amplicon (bp)
Cell signaling	small G protein signaling modulator	F[1]	taatacgactcactatagggGGAGAC GGAGGACGTGGAT	498
		R[2]	taatacgactcactatagggGTCGAGT ATACGCCTCCCTG	
	rho-related GTP-binding protein	F	taatacgactcactatagggGAGCGC TACCGCATCTTAGT	500
		R	taatacgactcactatagggTCCTCCT CCTGTTCTCTTGC	
	Engulfment and cell motility protein	F	taatacgactcactatagggCCTAGCC TGGTTGTGCATCT	500
		R	taatacgactcactatagggTCAACAT GGTTTCTGGCAAA	
	Inhibitor of apoptosis	F	taatacgactcactatagggTGGATAC TACGACCATCGCC	400
		R	taatacgactcactatagggTCTTCCC ATCGTCCCACTTC	
	Cyclin-dependent kinase 16	F	taatacgactcactatagggAACAAA CGACTGTCCTTGCC	498
		R	taatacgactcactatagggGCGTCTT TGGTGGCAGTAAG	
	cdc42-interacting protein	F	taatacgactcactatagggTTTGGA AAGAGCCCGTAAAA	499
		R	taatacgactcactatagggAATCATC GGTTGAGCTTCCC	
	Phosphatidylinositol 4-kinase	F	taatacgactcactatagggGACCAT GGCCATCACTTACC	482
		R	taatacgactcactatagggTTTGAAT GAGACCAGTGGCA	
	Ras	F	taatacgactcactatagggCATCGCT CGTCCTTCGTTATG	450
		R	taatacgactcactatagggGTTCCAT CATACGGTGACTC	
Digestion	Amylase alpha	F	taatacgactcactatagggGCGAGAT AAACCAATGTGGC	499
		R	taatacgactcactatagggCGACAA CACACCTCTCAGGA	
	Glucosidase 1	F	taatacgactcactatagggAGGCGA ATGGTCAGCTTATG	499
		R	taatacgactcactatagggGTGAAA TGCTTCCCTTCTCG	
Excretion	Diuretic hormone	F	taatacgactcactatagggAGAGGA TAGCGCAAGAGCTG	499
		R	taatacgactcactatagggAAAAAG ATTTTTCGGCCACC	
	Corticotropin releasing hormone	F	taatacgactcactatagggACAATAC	498

		R	AGAACCTGGCGCT taatacgactcactatagggCACGAA GTCGTCCAAACCTC	
	Diuretic hormone receptor	F	taatacgactcactatagggGGAGCT GAACGGCATTAAGT	493
		R	taatacgactcactatagggGATGAG AACGTAGCAACGCA	
Immune response	Toll-like receptor 4	F	taatacgactcactatagggGCTCTGT TCGACAACAACGA	498
		R	taatacgactcactatagggGAACGC GTCGTACTGGAAGT	
	Toll-like receptor 6	F	taatacgactcactatagggCGTCAA CCGTGACACCTTC	495
		R	taatacgactcactatagggACCCGG TTGTCTATGAGGC	
	Defensin	F	taatacgactcactatagggAGCTTC ACGACTGTGGCCTTG	284
		R	taatacgactcactatagggCAGACA GCGTCCTTGCAGTA	
Metabolism	Insulin receptor	F	taatacgactcactatagggACAAAC ACAGCAACGAATGC	494
		R	taatacgactcactatagggATTGCAT TTTTCGCAGGAGA	
	Insulin-like peptide	F	taatacgactcactatagggCCTGAG GTCGATATGGCATT	497
		R	taatacgactcactatagggTTGCTCT TTGGTGGTTCCTT	
	Cytochrome c1	F	taatacgactcactatagggGCTACTT TCGCCCACATCTC	500
		R	taatacgactcactatagggATTGCAC CAGTGTCATCAGG	
	Trehalose-6-phosphate synthase	F	taatacgactcactatagggAGGTGG TGGTTACTGGGTAGG	486
		R	taatacgactcactatagggTCGTCTG ACCAAGGGAAGAG	
	Vacuolar-type H^+ ATPase	F	taatacgactcactatagggGGATGTC TGGCTCAGCTATG	457
		R	taatacgactcactatagggGTGTAAT TGCCAGGTTCTGC	
	ATP synthase	F	taatacgactcactatagggTTCAGG CTGATGAGATGGTG	499
		R	taatacgactcactatagggACAGTG GATCGCTTCTGACC	
metamorphosis	Chitin synthase	F	taatacgactcactatagggCAAGAT CCGTCACCGTAAGC	500
		R	taatacgactcactatagggAGTACTC GACCCGGTAGCCT	
	Ecdysone receptor	F	taatacgactcactatagggTGTTTCT TTCTCCAGGTCGG	467
		R	taatacgactcactatagggCAAACA	

Protein degradation	E3 ubiquitin-protein ligase	F	AAACTTGCCCTGCT taatacgactcactatagggTTTGAGC ACCACTGAGCAAC	500
		R	taatacgactcactatagggTCCACC GGAAAGTCTCAAAG	
Protein expression	polyadenylate-binding protein	F	taatacgactcactatagggGCAGAA GGAGAAGTGGGAGA	499
		R	taatacgactcactatagggTCATGCT CATGCCAGGTCTA	
	nucleosome-remodeling factor subunit	F	taatacgactcactatagggCTGACG GTAAGGGATCCAAA	499
		R	taatacgactcactatagggTCACCA GGGACAGTTGCTATC	
	RNA-binding protein	F	taatacgactcactatagggGGCCAA AGACAAGAAGAAGC	500
		R	taatacgactcactatagggCCATTTC ACGCTCCATTTCT	
	60S ribosomal protein L4	F	taatacgactcactatagggCAAGAA CCATCGTCACCAAA	499
		R	taatacgactcactatagggGAAAGC ACGAGTCACACCCT	
	RNA polymerase	F	taatacgactcactatagggTGGCGT GTGAAGAAAACTGT	499
		R	taatacgactcactatagggCTCCTGC TTGAATTCGCATC	
	40S ribosomal protein L4	F	taatacgactcactatagggACCATCG TCACCAAACCAGT	498
		R	taatacgactcactatagggTACGGA AAGCACGAGTCACA	
	Kruppel-like factor 13	F	taatacgactcactatagggAGCAGC AGCAACAACTCAAG	491
		R	taatacgactcactatagggCTCCCCA GTGTGAGTTCGAG	
Structure	dynein beta chain	F	taatacgactcactatagggGAGCAG TGGGAGAACACCA	488
		R	taatacgactcactatagggGACGTGT ACGTGTCCCAGG	
	Tubulin alpha	F	taatacgactcactatagggGGAGAT CATAGACATTGTTA	425
		R	taatacgactcactatagggCTGTCA GGTCAACATTCAAA	
	Tubulin beta	F	taatacgactcactatagggGGTACCA TGGACTCTGTCCG	489
		R	taatacgactcactatagggCATGGTG AGGGAAACGAGAT	
	Tubulin gamma	F	taatacgactcactatagggTGTGAAT CTGCTCTCTTCCA	477
		R	taatacgactcactatagggCATCCCT TGTGCTGCTGTAA	
	Golgi reassembly and stacking protein	F	taatacgactcactatagggTCAGCT	500

		R	GGTCTTCGACCTTT taatacgactcactatagggGGTGAA GGGTAAGGCTGTGA	
	Actin-related protein	F	taatacgactcactatagggGTCGTG CCGTCGTGTTTAT	413
		R	taatacgactcactatagggGATATGC TAACCCGAAGCA	
	Integrin alpha	F	taatacgactcactatagggCAGAGG GACTCCATCAGAT	498
		R	taatacgactcactatagggGCCCTCT AGTATGAGGCTC	
	Actin	F	taatacgactcactatagggCTTAAAC ACCCCCGAAAACA	497
		R	taatacgactcactatagggAAATCTC AGGGCCAAGGAAT	
Transport	Coatomer protein alpha subunit	F	taatacgactcactatagggAGACAG TCCGTGTCTGGGAT	499
		R	taatacgactcactatagggGAATCAT GACCAGCAGCAAA	
	Coatomer protein beta subunit	F	taatacgactcactatagggCTTGGTC TGCGATGCTTACA	500
		R	taatacgactcactatagggCTGCAG CTTCATAACGCACA	
	Coatomer protein epsilon subunit	F	taatacgactcactatagggCCGAGC TTACATTGCACAGA	500
		R	taatacgactcactatagggCTTGTCC ATTCAGCAGCAAA	
	Coatomer protein zeta subunit	F	taatacgactcactatagggTGTTGG GGTTGTTGATGTGT	500
		R	taatacgactcactatagggCCTCCAT GATGATTCCTCCA	
	Coatomer protein gamma 2 subunit	F	taatacgactcactatagggAGAGCA ATCCAGAAGCCAAA	488
		R	taatacgactcactatagggTACCAA AGGCTCAACAGCAG	
	Apolipophorin	F	taatacgactcactatagggCTGAGG TTTATGCCTCCGTT	500
		R	taatacgactcactatagggATTGACG ATGGACTTCAGGG	
	Monocarboxylate transporter	F	taatacgactcactatagggGAGTCC TCGCTGGAGGAAG	493
		R	taatacgactcactatagggGCTTGG GTGGACCTAACGAT	
	Ferritin	F	taatacgactcactatagggGTGTCA GCCAACAACGAGAA	410
		R	taatacgactcactatagggCAAACA GCAACAATGGAACG	
	Aquaporin	F	taatacgactcactatagggGTACACA TTCGTGTTTGCCG	497
		R	taatacgactcactatagggATTTGTG CAACTGTGCCATC	

	Exportin 7	F	taatacgactcactatagggAGAGCAGCTTTCTGTGATTGG	498
		R	taatacgactcactatagggATCTGTTCACTTCGACCCCA	
	Exportin 2	F	taatacgactcactatagggGCCCAGCTTTTCTTGACTTTT	498
		R	taatacgactcactatagggAAGGCACTGAAGCAACCATT	
Unknown	FOCC014394	F	taatacgactcactatagggACCTCAGCACACCAGAGGAC	500
		R	taatacgactcactatagggGAAGTATCCATTGTCTGCGCT	
	Pfam12937	F	taatacgactcactatagggGATGGTCTTCCACGCTGTTC	500
		R	taatacgactcactatagggCCTGTAGTCACCGTCAGGCT	
	FOCC010227	F	taatacgactcactatagggGTAGCGGTGCCTTATTCAGC	500
		R	taatacgactcactatagggATGTTCAGCCATGGCGTAGT	
	Pfam01433	F	taatacgactcactatagggCCACGTTCAAGATCAGCATC	500
		R	taatacgactcactatagggCCAGTTGTTGGTGACCAGG	
	Pfam05485	F	taatacgactcactatagggGTGCCTACGGTGTCTGTGAA	499
		R	taatacgactcactatagggCCCCTGGGAACTTAATTCGT	
	FOCC004156	F	taatacgactcactatagggTGAGTGTGTCAACTGGCAGC	500
		R	taatacgactcactatagggTTCCTGACCGGCATCTTTTA	

[1] Forward primer
[2] Reverse primer

2.4. Validation of dsRNA delivery

To confirm dsRNA delivery to the leaf disc, leaf discs were punched from kidney bean seedlings (~2 weeks old) grown in a cultivation chamber, then soaked with 100 ng/µl of Amace1 control dsRNA solution or nuclease-free water using bioassay chambers, which were assembled as described below (section 2.5), and incubated for 24 h. A leaf disc soaked in the same amount of dsRNA solution for 3 s was used as a control for detecting any residual dsRNA on the surface of the disc. Each leaf disc was washed thoroughly with ddH_2O, and the excess was blotted away with Kimwipes. The leaf discs were frozen in liquid nitrogen and homogenized using a ceramic mortar to extract total RNA using the TRI reagent. cDNA was synthesized from DNase I-treated total RNA using the same methods described above with the exception of using random hexamers instead of oligo dT. The kidney bean *actin* gene was used as a reference gene (Table 3).

To confirm dsRNA delivery to the thrips through leaf disc feeding, 10 females (2 to 3-day old after emergence) were fed leaf discs treated with five concentrations of control *Amace1* dsRNA solution (0, 50, 100, 200, and 500 ng/µl) using the bioassay chamber as described above. After 120 h, thrips were collected, and the relative amount of dsRNA detected in thrips after ingestion and the relative amount of dsRNA ingested was calculated using the same method. The *ribosomal protein small subunit 32* gene from *F. occidentalis* (*FoRPS32*) was

used as a reference gene (Table 3). The real time quantitative PCR (qPCR) was conducted using LightCycler 96 (Roche Inc., Basel, Switzerland) in 10 μl of the qPCR mixture with 10 μl reaction volume using TB green Premix Ex Taq II (Takara Korea Biomedical Inc.), 5 μM of forward and reverse primers (Table 3), 0.1 mM dNTP, and 12.5 ng of template cDNA). The following thermal conditions were as follows: pre-incubation at 95°C for 1 min, 35 cycles of 95°C for 30 s, 56°C for 15 s, 72°C for 30 s and then sequential increases of 0.2°C/s from 45 to 95°C for melting curve analysis. The relative transcription levels of the target genes were calculated using the $2^{\Delta Ct}$ method (Pfaffl, 2001). The qPCR experiment was repeated three times.

2.5. Ingestion RNAi bioassay

The dsRNA bioassay was conducted using a modified leaf disc-mediated system, which was originally established for *T. urticae* (Kwon, Park, & Lee, 2013). A cylindrical bioassay chamber with three separate units was fabricated using a 3D printer (Anycubic, China; the 3D schematic diagram was shown in Fig. 1). Each dsRNA solution (150 μl of 100 ng/μl solution, 15 μg dsRNA/chamber) was added to the bottom unit (unit III), and a kidney bean leaf disc cut by a circular punch (16 mm diameter) was placed upside down over the dsRNA solution. The middle unit (unit II) was assembled over the unit III, and 10 females

(2 to 3-day old after emergence) were infested on the leaf disc. The chamber was closed with fine mesh-covered top over the upper unit (unit I) and was kept under the conditions of 24 ± 1ºC, 60 ± 5% relative humidity (RH) and a 16:8 (L:D) photoperiod. The mortality of the thrips was examined every 24 h until 120 h post-treatment. The leaf disc and dsRNA solution were replaced with fresh ones every 24 h. When leaf disc was replaced, the bioassay chamber was inverted and gently tapped so that the thrips could move to the upper part (unit I and II) of the chamber without escaping. Then, the unit III was detached from the chamber and replaced with new one with fresh leaf disc and dsRNA solution.

For the double knockdown bioassay experiment, 10 genes that showed relatively high mortalities in the other bioassays were selected. A dsRNA solution mixture of two genes (150 µl of each 75 ng/µl dsRNA solution, 22.5 µg of dsRNA/chamber, eight combinations) was used, and the bioassay proceeded using the same method as before. Each bioassay was replicated three to five times, and the corrected mortality was calculated using Abott's method (Abbott, 1925). The toxicity index (TI) was arbitrarily calculated by dividing the corrected mortality at 120 h post-treatment (%) by LT_{50} (h) to reflect both the final lethality and overall intoxication speed (i.e., LT_{50}).

To validate RNAi gene knockdown, qPCR was conducted to quantify the transcript levels of target genes. Only surviving thrips (but with moribund signs

such as lethargy) were collected at 120 h post-treatment to ensure RNA quality. Total RNA from the survived thrips was extracted and cDNA was synthesized as described above in section 2.2. qPCR was conducted using the same protocol described in section 2.4. The *FoRPS32* gene was used as a reference gene. The qPCR conditions were the same as described above (see 2.4. Validation of dsRNA delivery). The qPCR experiment was repeated three to five times.

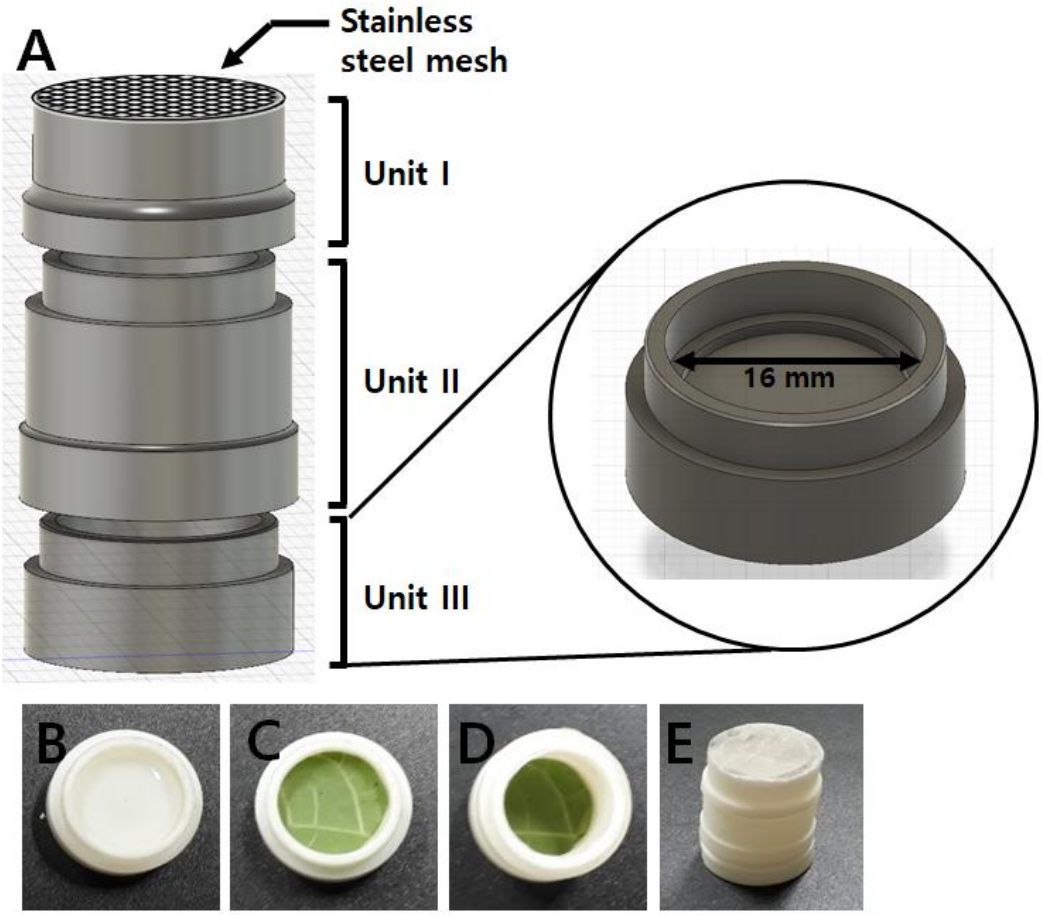

Figure 1. Schematic diagrams (A) and assembly procedures of leaf disc-mediated dsRNA bioassay chamber (B-F). The chamber is composed of three parts: unit I, upper part; unit II, middle part and unit III, bottom part. The inner diameter of unit III is 16 mm. To prepare the bioassay, dsRNA solution was added to unit III (B), a leaf disc was put over the dsRNA solution (C) and then unit II (D) and unit I (E) were assembled over the unit III.

Table 3. The list of primers used for qPCR analysis.

Gene		Oligonucleotide sequence (5' – 3')	Amplicon (bp)
Toll-like receptor 6	F	CCGCCTCACTGACATATCTG	140
	R	TGCGGTTCTGGTGCATGTCG	
Apolipophorin	F	ATCCTCTTGGTTCCAGCTGC	131
	R	AGTCAAGGGGGTTGAGAGTG	
Coatmer protein epsilon	F	AGAAGCCCAGTCTGTTCTCC	131
	R	GCTGACTAAGGTAGCGGTTG	
Sorting and assembly component 50	F	GGCAGAACATGCGACTTGCA	139
	R	CCTATTCCAAACTGCAACCC	
Insulin-like peptide	F	CCAGTAGGAGCACCAGTTCA	142
	R	CGAGGTTGCGTACTATCTCC	
Insulin receptor	F	GTCGGGCTGATCATGTTGAC	120
	R	CTTCCAGTTCCTTCACGAGG	
Aquaporin	F	GCACTGTGTTTGTGCCACAC	107
	R	CGTGAGGTAATGAAGTAGCC	
Small G protein signal modulator	F	TATGTGCACCTACGTGTGGG	180
	R	GCGAAGTGTGTATCCATGGC	
Dynein beta	F	CGAGGTCAAGAACATCGTGG	203
	R	TGGACGTGATGAGGTTCTGC	
Engulfment and cell motility protein	F	CTGGCTGGTCAGCCATTATC	122
	R	GCATGAGATTCCCACTCTTC	
Cytochrome C1	F	AGCTGATCGTTTCCCAAGCC	156
	R	GCAGGAGCTTCACAGTAACC	
Corticotropin releasing hormone	F	CTTGGGCTTTGCGACCGAAC	125
	R	TCGACACGAGGCGCACGTTG	
Exportin 7	F	TTGGAAACTCCGTGGCTGTG	171
	R	ATTCGGCCAGAGCCAACATC	

[1] Forward primer
[2] Reverse primer

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (Ver.6, GraphPad Inc., San Diego, CA, USA) software. Mean and standard deviation were calculated for each data set, and then statistical mean differences were determined by one-way ANOVA followed by Tukey's post hoc test or Student's t test.

3. Results

3.1. dsRNA delivery to leaf discs and *F. occidentalis*

The *Amace1* dsRNA detected in the leaf disc at 24 h post-treatment was 4-fold higher than the blank control, which measured residual dsRNA on the surface of leaf disc (Fig. 2A). Five different concentrations of *Amace1* dsRNA were used to feed *F. occidentalis* via the leaf disc to determine dsRNA delivery. The amount of dsRNA detected in *F. occidentalis* after feeding for 120 h increased in a dose-dependent manner significantly (Fig. 2B). Differences between treated *F. occidentalis* groups were statistically different (one-way ANOVA, $P < 0.0001$), and the relative amounts of dsRNA were significantly higher in the dsRNA-treated *F. occidentalis* than in the water-treated control *F. occidentalis* (one-way ANOVA, $P = 0.0014$). Considering that the sequence of *Amace1* dsRNA does not exist in either kidney beans or thrips, these results suggest that dsRNA was successfully delivered into the leaf discs and then to the thrips through ingestion. There was no statistical difference in mortality between water- and *Amace1* dsRNA-treated *F. occidentalis* (Fig. 3).

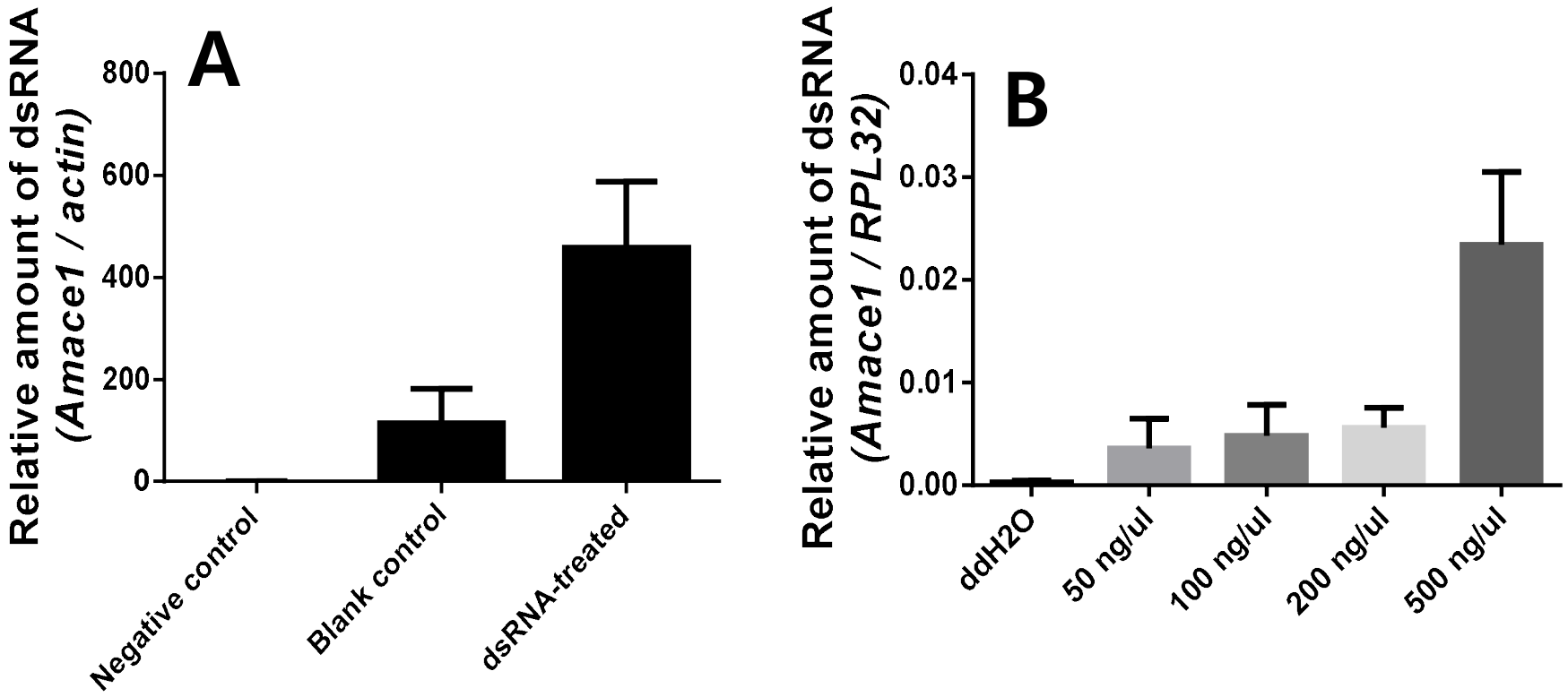

Figure. 2. Relative amount of dsRNA detected in *Phaseolus vulgaris* leaf discs at 24 h (A) and *F. occidentalis* at 120 h (B) following the control dsRNA (*Amace1*) treatment using the leaf disc-mediated bioassay system. The relative amount of dsRNA was obtained by comparison with actin for *P. vulgaris* and *RPL32* for *F. occidentalis*.

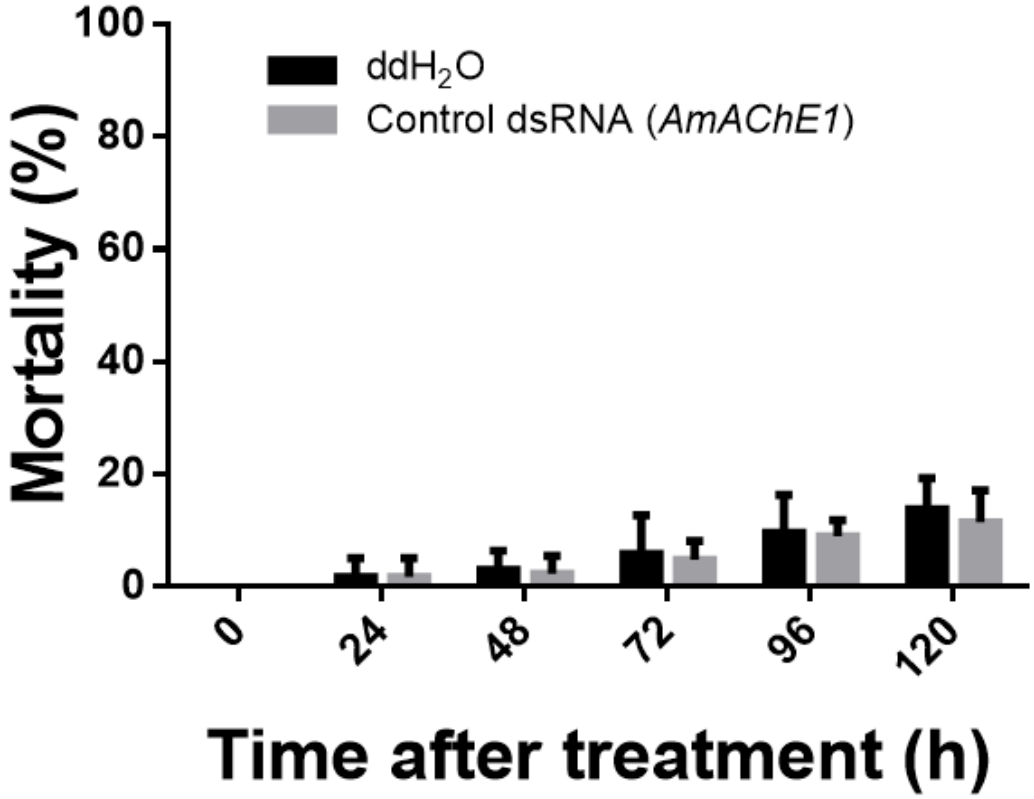

Figure 3. Comparison of *F. occidentalis* mortality after ingestion of ddH_2O or control dsRNA (*Amace1*) using the leaf disc-mediated system.

3.2. Target gene selection

Approximately 55 million and 74 million trimmed reads were generated from nymph and adult *F. occidentalis*, respectively. Among the trimmed reads, 67 million reads (90%) from the nymph sample and 51 million reads (91%) from the adult sample were mapped onto the *F. occidentalis* genome in HGSC, and a total of 6,260 transcripts were annotated. Among the housekeeping genes with similar expression levels in nymphal and adult stages (fold difference < 1.7), we selected a total of 51 target genes involved in essential biological functions, such as cell signaling, digestion, excretion, immune response, metabolism, metamorphosis, protein degradation, protein expression, structure and transport. Six *F. occidentalis*-specific genes with unknown function were also selected (Table 4).

Table 4. List of 57 target genes of *F. occidentalis* for RNAi.

Function (The number of genes)	Gene	Target insect
Cell signaling (8)	Small G protein signaling modulator	
	Rho-related GTP-binding protein	
	Engulfment and cell motility protein	
	Inhibitor of apoptosis	*L. lineolaris* (Lep.)
	Cyclin-dependent kinase 16	
	Cdc42-interacting protein	
	Phosphatidylinositol 4-kinase	
	Ras-related protein	*S. exigua* (Lep.)
Digestion (2)	Amylase alpha	
	Glucosidase 1	
Excretion (3)	Diuretic hormone	
	Corticotropin releasing hormone	
	Diuretic hormone receptor	
Immune response (3)	Toll receptor 4	
	Toll receptor 6	
	Defensin	
Metabolism (6)	Insulin receptor	*G. bimaculatus* (Orthop.)
	Insulin-like peptide	
	Cytochrome c1	
	Trehalose-3-phosphate synthase	*N. lugens* (Hemip.)
	Vacuolar ATP synthase catalytic subnit A	*B. dorsalis* (Dip.)
	ATP synthase (Mitochondrial)	
Metamorphosis (2)	Chitin synthase	*S. exigua* (Lep.)
	Ecdysone receptor	
Protein degradation (1)	E3 ubiquitin-protein ligase	
Protein expression (7)	Polyadenylate-binding protein	
	Nucleosome-remodeling factor subunit	
	RNA-binding protein	
	60S ribosomal protein L4	*B. tabaci* (Hemip.)
	RNA polymerase	
	40S ribosomal protein L4	*B. tabaci* (Hemip.)
	Kruppel-like factor 13	
Structure (8)	Dynein beta chain	
	Tubulin alpha	*B. tabaci* (Hemip.)
	Tubulin beta	
	Tubulin gamma	

	Golgi reassembly and stacking protein	
	Actin-related protein	
	Integrin alpha	
	Actin	*S. frugiperda* (Lep.)
Transport (11)	Coatomer protein subunit alpha	
	Coatomer protein subunit beta	*L. decemlineata* (Coleop.)
	Coatomer protein subunit epsilon	*L. decemlineata* (Coleop.)
	Coatomer protein subunit zeta	
	Coatomer protein subunit gamma 2	
	Apolipophorin	
	Monocarboxylate transporter	
	Ferritin	
	Aquaporin	*A. pisum* (Hemip.)
	Exportin 2	
	Exportin 7	
Unknown (6)	FOCC014394	
	Pfam 12937	
	FOCC010227	
	Pfam 01433	
	Pfam 05485	
	FOCC004156	

3.3. Target gene screening using a bioassay chamber

Ingestion RNAi experiments were conducted on the insecticide-susceptible *F. occidentalis* using bioassay chambers. TI values calculated from the bioassay are shown in Fig. 4. For this, 57 target genes were arbitrarily divided into three groups according to TI value: group A (four genes, TI>0.4), group B (seven genes, 0.4≥TI>0.25) and group C (46 genes, 0.25<TI). The genes categorized as group A were *Toll-like receptor 6* (*TLR6*), *apolipophorin* (*apoLp*), *COPE* and *sorting and assembly machinery component* 50 (*SAM50*) (Table 5). Genes categorized as Group B included *insulin receptor* (*IR*), *insulin-like peptide* (*ILP*) and *aquaporin* (*AQP*) (Table 5). Among these genes, the *TLR6* dsRNA revealed the highest lethality (58.7% corrected mortality at 120 h) and the fastest lethal time (105.1 h of LT_{50}) (Table 5) followed by *apoLp* and *COPE*. This means *TLR6* could be the best target gene for use in controlling *F. occidentalis* with RNAi. At 120 h post-treatment, the transcription level of each of the seven target genes was reduced following dsRNA feeding (46% reduction for *TLR6*, P = 0.047; 47% reduction for *apoLp*, P = 0.029; 37% reduction for *COPE*, P = 0.0009; 22% reduction for *SAM50,* P = 0.053; Fig. 5A). This finding suggests that the observed lethality was mediated by the RNAi of the target gene. However, there was no apparent correlation between TI value and target gene knockdown rate.

Table 5. Corrected mortality (%) at 120 h post-treatment, LT_{50} and Toxicity Index (TI) of the genes treated to insecticide-susceptible and resistant strains of *F. occidentalis*.

	Target gene	Susceptible strain			Resistant strain		
		Corrected Mortality (%)	LT_{50}	Toxicity Index (TI)[a]	Corrected Mortality (%)	LT_{50}	Toxicity Index (TI)[a]
Group A	TLR6	58.7	105.1	0.558	57.8	97.2	0.594
	apoLp	56.3	107.4	0.525	57.8	105.5	0.548
	COPE	55.0	118.9	0.463	47.6	131.5	0.362
	SAM50	52.0	124.9	0.416	61.0	100.2	0.609
Group B	IR	40.0	107.8	0.371			
	ILP	46.8	152.3	0.307			
	AQP	48.5	159.0	0.305			
Double knockdown	ILP + IR	60.0	130.1	0.461			

[a] Toxicity Index (TI) = Corrected mortality (%) at 120 h post-treatment / LT_{50}

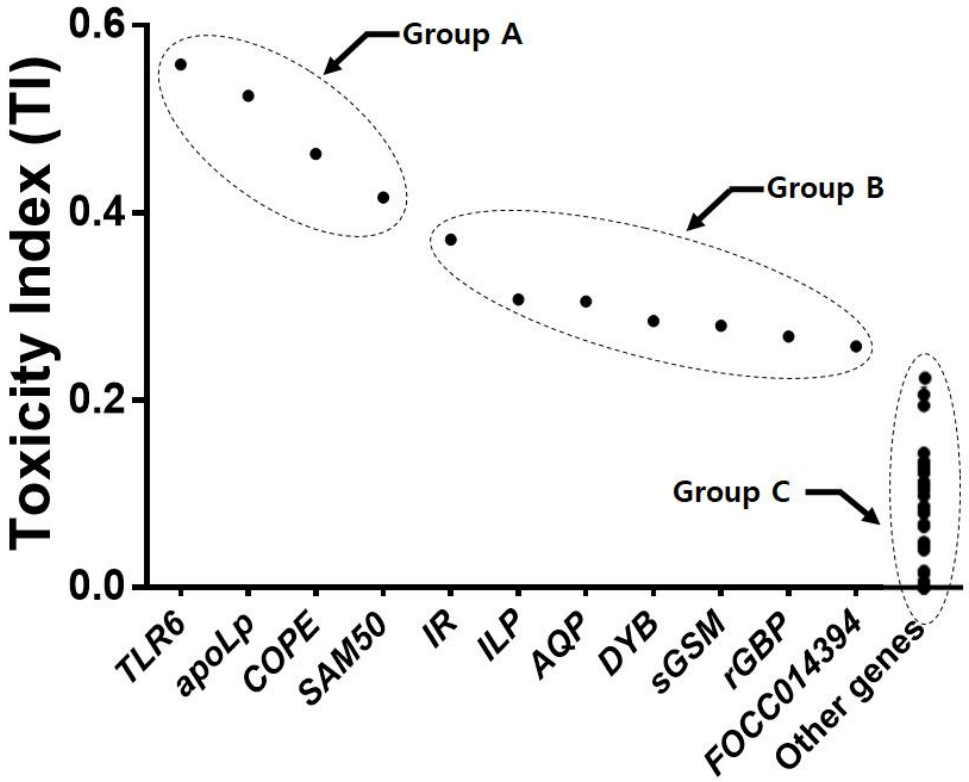

Figure. 4. Toxicity Index (TI; % mortality at 120 h post-treatment/LT50) values of 57 genes determined using a leaf disc-mediated system against *F. occidentalis*. Genes were categorized into three groups by range of TI value: group A (four genes, TI>0.4), group B (seven genes, 0.4≥TI>0.25) and group C (46 genes, TI<0.25). *TLR6*, *toll-like receptor 6*; *apoLp*, *apolipophorin*; *COPE*, *coatomer protein subunit epsilon*; *SAM50*, *sorting and assembly machinery component 50*; *IR*, *insulin receptor*; *ILP*, *insulin-like peptide*; *AQP*, *aquaporin*; *DYB*, *Dynein beta*; *sGSM*, *small G protein signaling modulator*; *rGBP*, *rho-related G protein binding protein*.

3.4. Target gene double knockdown

Among the eight combinations of two dsRNA, double knockdown of *IR* and *ILP* in group B only showed a synergistic effect by exhibiting the highest TI value (0.461), which is higher than those of the individual genes (0.371 and 0.307 for *IR* and *ILP*, respectively; Table 5). The rest of the combinations resulted in reduced lethality compared to their individual RNAi. Even simultaneous knockdown of both *TLR6* (0.558 TI) and *COPE* (0.463 TI) resulted in a lower TI value (0.113, Fig. 6).

3.5. Validation of target gene lethality against insecticide-resistant *F. occidentalis*

Based on the TI values obtained from the insecticide-susceptible *F. occidentalis*, the top four genes in group A (*TLR6*, *apoLp*, *COPE* and *SAM50*) were selected to test their ingestion RNAi efficacy against a multiple insecticide-resistant EB-R strain. The *SAM50* dsRNA exhibited the highest TI (0.609) against the EB-R strain, which was 1.46-fold higher than the insecticide-susceptible *F. occidentalis* (Table 5). The *TLR6* and *apoLp* dsRNA treatments resulted in similar TI values (0.594 and 0.548, respectively) against the EB-R strain compared to the insecticide-susceptible strain. The reduced transcription levels of each of the three target genes were statistically significant (71% reduction for *TLR6*, P = 0.003; 27%

reduction for *COPE*, P = 0.032; 50% reduction for *SAM50*, P = 0.022; Fig. 5B). As for *apoLp*, the mean transcription reduction was 36% although its P value was slightly larger than 0.05 (P = 0.089). Taken together, these results suggest that ingestion RNAi works equally well for both insecticide-susceptible and -resistant *F. occidentalis.*

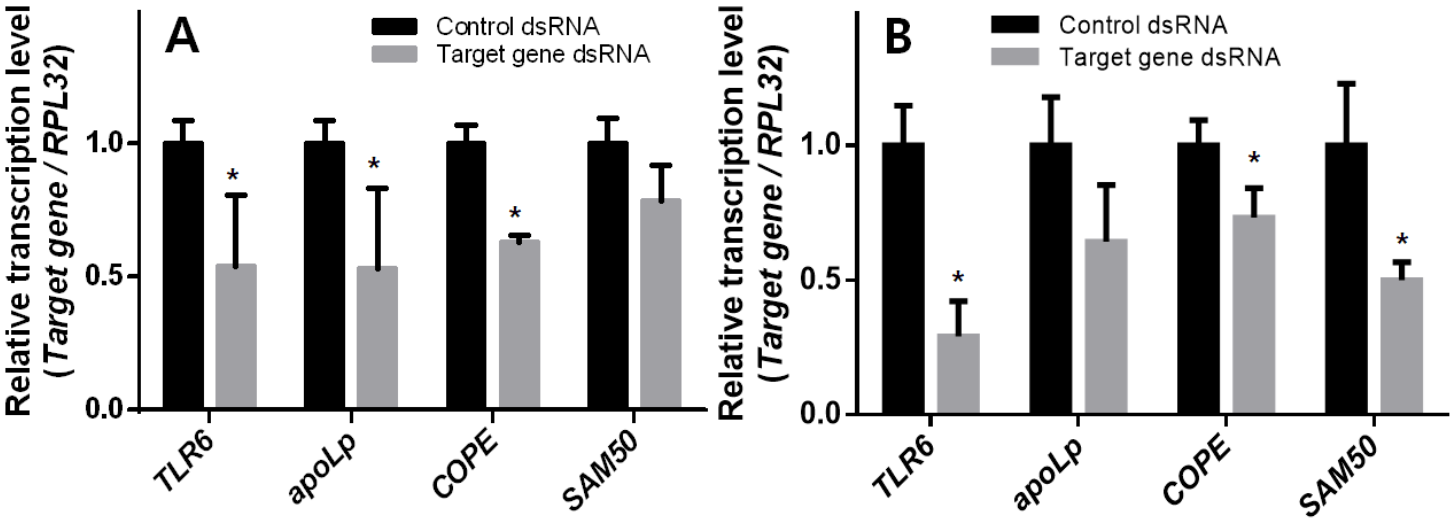

Figure. 5. Transcript reduction of insecticide-susceptible (A) and -resistant EB-R (B) strains of *F. occidentalis* following ingestion of target gene dsRNA. Relative transcription levels of target gene dsRNA-fed thrips were normalized to that of control thrips. A single asterisk (*) indicates a statistically significant decrease in transcript level using t-test ($P < 0.05$).

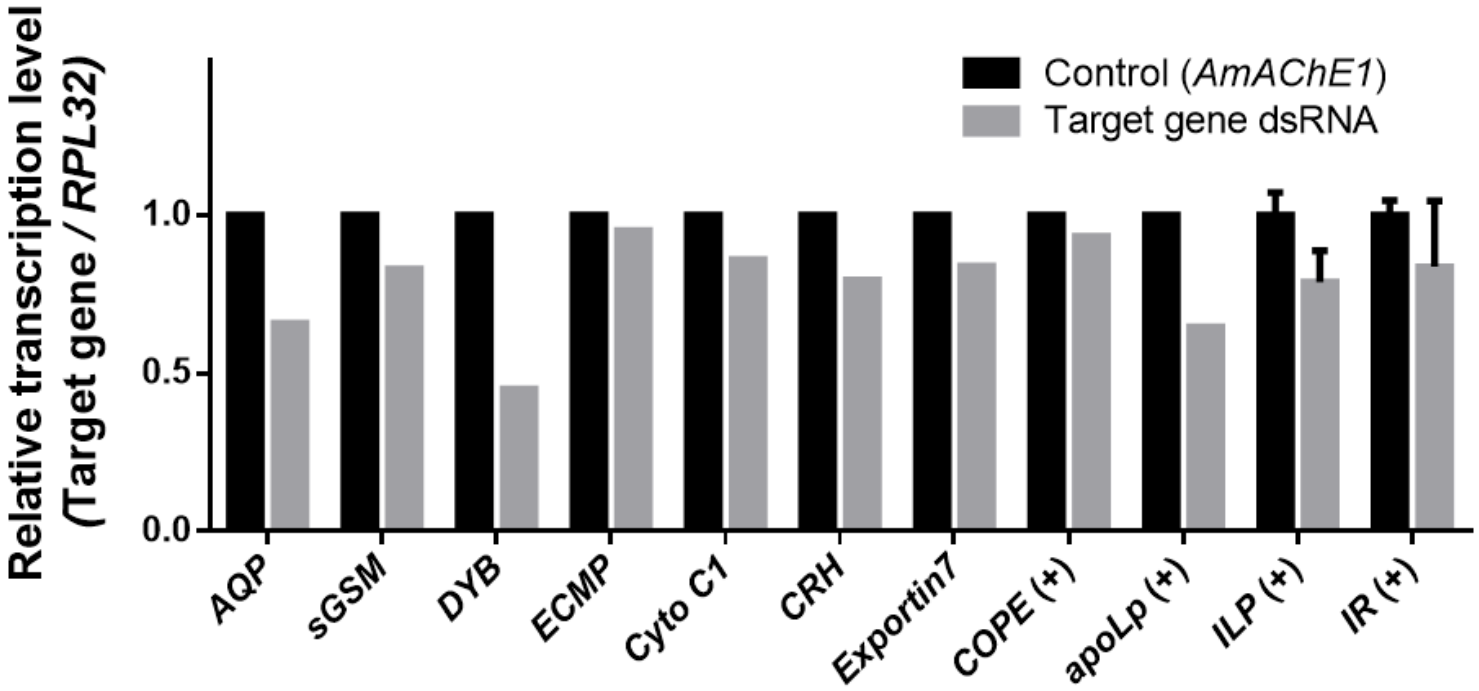

Figure 6. Transcript reduction of target genes categorized in group B and C (A) and genes treated by combination for double knockdown (*COPE-apoLp* and *ILP-IR*) following dsRNA ingestion (B).

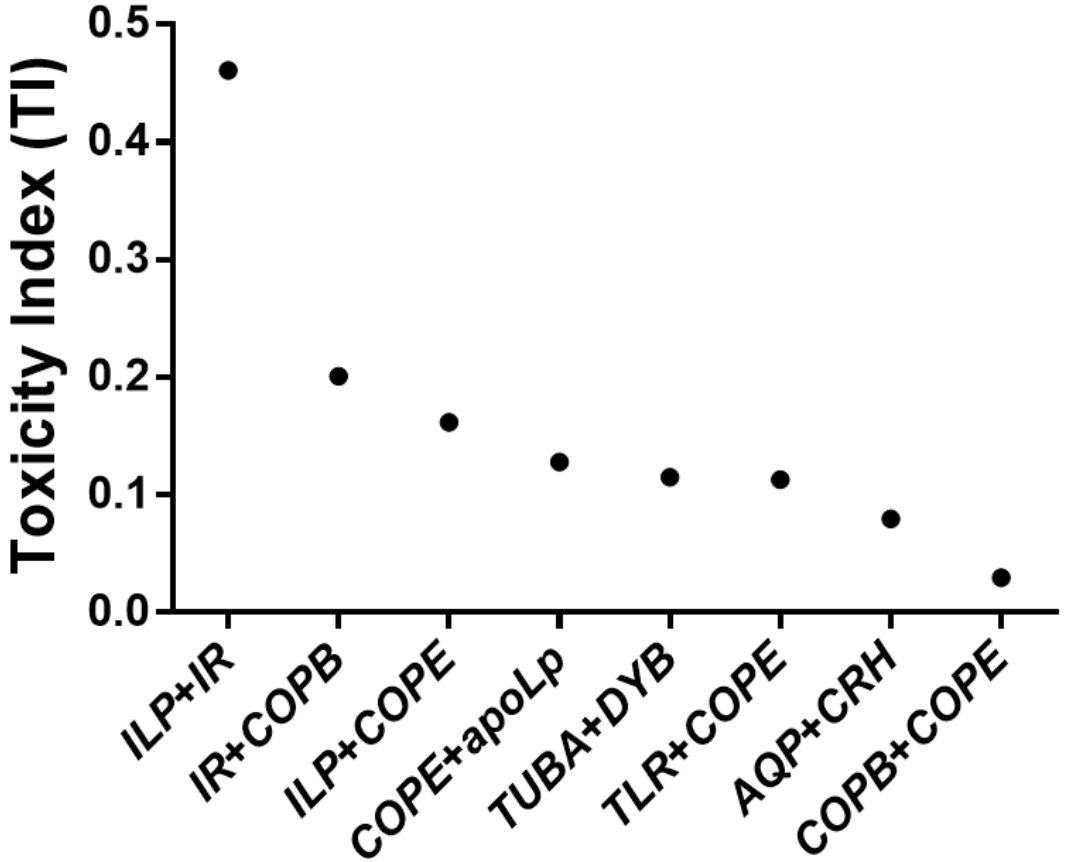

Figure 7. TI values obtained from thrips ingesting eight combinations of two targeted dsRNAs.

4. Discussion

We established an ingestion RNAi system based on the leaf discs-mediated dsRNA delivery and demonstrated its usability in evaluating lethal effects of *F. occidentalis* target genes when knocked down. Ingestion of the target dsRNA, perhaps along with leaf disc sap and cells, was confirmed without any apparent feeding avoidance as previously observed in *T. urticae* (Kwon et al., 2013). Depending on the target genes tested, ingestion of dsRNA via leaf discs was further confirmed to induce actual gene knockdown and to lead to *F. occidentalis*'s eventual death. The overall transcription reduction rates of the four target genes (*TLR6*, *apoLp*, *COPE* and *SAM50*) were not particularly high (22-71%). Considering that transcription levels of target genes were measured from the survived thrips following RNAi (dead thrips were not used to ensure RNA quality), however, the overall transcript reduction rates were likely underestimated.

Compared to the previous version used for *T. urticae* (Kwon, Park, Ashok, Lee, & Lee, 2016), the 3D-printed bioassay chamber improved overall handling procedures, including the uniform production of bioassay chambers, dsRNA dispense into the units, leaf disc alignment, introducing the test thrips onto the discs, daily mortality observation and daily dsRNA solution replacement.

Amace1 dsRNA was used as the negative control dsRNA since it did not induce any target-specific mortality to *F. occidentalis*. Alignment of *Amace1* and *F. occidentalis ace* genes (*Foace1* and *Foace2*) revealed that there were no perfect matches within any 20-bp stretches, which is important for sequence-specific RNAi in insects (Pillai, Bhattacharyya, & Filipowicz, 2007). This sequence mismatch in *ace* genes between the two species is likely the main reason for the no apparent off-target effect of *Amace1* dsRNA on *F. occidentalis*.

The ingested RNAi system enabled the screening of four lethal genes (*TLR6, apoLp, COPE* and *SAM50*) out of 57 candidate genes. These genes also worked well against an insecticide-resistant EB-R strain of *F. occidentalis*, suggesting that RNAi-based transgenesis can be employed as a feasible strategy to control insecticide-resistant *F. occidentalis* populations. To generalize this concept, however, a larger-scale experiment using various *F. occidentalis* populations with different insecticide resistance is required.

Among the 57 dsRNAs screened, *TLR6* dsRNA showed the highest level of lethality against the insecticide-susceptible strain and a similar lethality against the insecticide-resistant EB-R strain. TLRs in invertebrates are homologous to the toll receptors in mammals and play an important role in their innate immunity against pathogens (Imler & Zheng, 2004). They are a class of pattern recognition receptors that recognize the specific cellular components of pathogens, such as

lipopeptides, lipopolysaccharide and dsRNA. Thus, knockdown of *TLR6* transcription appears to suppress the innate immunity, thereby making *F. occidentalis* more vulnerable to pathogen infection or disrupting thrips's normal gut symbiont microflora, either of which may lead to death. It is also reported that knockdown of *TLR* via injection RNAi increased shrimp mortality upon bacterial challenge (Wang et al., 2010). Nevertheless, as no apparent pathogenic infection was observed during the leaf disc-mediated bioassay experiments, the high mortality induced by *TLR6* knockdown may not be directly due to immune suppression but due instead to other not-yet-known functions of the *TLR6* gene.

apoLp dsRNA induced high mortality when treated to both insecticide-susceptible and -resistant strains of *F. occidentalis*. *apoLp* belongs a family of lipid transfer protein present in the hemolymphs and functions as a lipid (diacylglycerol and triacylglycerol) transporter between lipid storage (fat body) and muscles (Shapiro, Wells, & Law, 1988). High mortality in the thrips that ingested *apoLp* dsRNA suggests that apoLp plays a pivotal role in lipid metabolism in *F. occidentalis*.

Knockdown of *COPE* also resulted in high mortality rates for both the insecticide-susceptible and -resistant strains of *F. occidentalis*. COPE is one of the components of the COPI protein complex, which consists of seven subunits (α, β, β', γ, δ, ε and ζ) (Bonifacino & Lippincott-Schwartz, 2003). In eukaryotic cells,

COPI is essential for vesicle transports of phospholipid membrane and protein between the endoplasmic reticulum to the *cis*-Golgi complex (Bonifacino & Lippincott-Schwartz, 2003; Kirchhausen, 2000). Suppression of the COPE protein likely impairs normal formation of the COPI complex and thus disturbs the traffiking of proteins and lipid, eventually leading to death. Knockdown of the *T. urticae COPE* gene via the ingestion RNAi process also resulted in a high level of mortality for *T. urticae* (Kwon et al., 2016), supporting the essential role of the COPE subunit in these pest species.

SAM50 dsRNA also showed high levels of lethality, particularly against the insecticide-resistant EB-R strain of *F. occidentalis*. SAM50 is one of the essential protein constituents of mitochondrial outer membranes. It plays a central role in integration of beta barrel proteins into the outer membrane by acting as a translocase (Becker, Voegtle, Stojanovski, & Meisinger, 2008). Knockdown of *SAM50* transcripts can cause malfunctions of the mitochondrial outer membrane, thus resulting in dysfunction in the cell's energy factory, which eventually leads to death.

In the double knockdown bioassay, combination of ILP and IR dsRNA only resulted in an apparent synergistic lethal effect when ingested by the thrips. Arthropodal ILPs are homologous to mammalian insulin. Like insulin, insect ILPs are involved in a variety of physiological pathways such as carbohydrate

metabolism (glucose, trehalose and glycogen), protein synthesis and development (Corona et al., 2007; de Azevedo & Hartfelder, 2008). Once the ILP binds to its receptor (IR), located in a fat body or other tissue cell, it initiates the signal cascade of the insulin/insulin-like growth factor signaling pathway (Wu & Brown, 2006). Down-regulation of ILP and IR transcripts can interrupt the normal energy metabolism of an organism and cause imbalance between carbohydrates in storage and in immediate need.

Simultaneous knockdown of two components (i.e., ILP and IR) within the same IIS pathway is likely more efficient in disturbing the targeted pathway, thus resulting in synergistic RNAi effects. Similar synergistic RNAi was also observed in *T. urticae*, in which simultaneous treatment of two dsRNAs (*COPE* and *COPB2*), both of which are involved in COPI complex formation, enhanced RNAi toxicity (Kwon et al., 2016). The remaining two dsRNA combinations did not induce any synergistic effects, and some combinations even showed a decrease in lethal effects. Although the reason for this phenomenon is unclear, it has been reported that double knockdown of two genes can be less effective than single knockdown perhaps due to the selective affinity of dicer enzymes to each dsRNA, resulting in RNAi only being effective for a specific type of dsRNA (Gouda, Matsunaga, Iwasaki, & Kawano, 2010). In addition, it was also reported

that when injecting multiple dsRNAs, competition happens in cellular uptake between dsRNAs, thus resulting in a poor RNAi response. (M.R. Joga et al., 2016)

Our study is the first report demonstrating the efficacy of ingestion RNA against a thrips species. The expression of target hairpin RNA in plant cells via transgenesis may be the most efficient and practical form of RNAi-based control of thrips. Kim et al. (2019) recently reported that *F. occidentalis* ingests a relatively large amount of the nuclear fraction compared to the chloroplast fraction, which supports the feasibility of nuclear expression of target hairpin RNA in generating transgenic plants for RNAi-based thrips control (Kyungmun Kim, 2019). The lethal genes screened in this study (*TLR6, apoLp, COPE* and *SAM50*) can be directly employed as candidate target genes for *in planta* expression of hairpin RNA when generating transgenic crops against *F. occidentalis*. The bioassay chambers fabricated using 3D printing would facilitate further lethal gene screening in a more efficient manner.

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KOREAN ABSTRACT

꽃노랑총채벌레 개체수 조절을 위한 섭식 RNAi 기반 방제 시스템 개발

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초록

꽃노랑총채벌레와 대만총채벌레를 포함하는 총채벌레목은 원예작물 및 농작물의 가해하는 주요 해충 중 하나이다. 살충제 저항성의 급속한 발달로 인해, 기존의 살충제는 총채벌레의 방제에 효과적이지 않기 때문에 대안적인 관리 방법이 필요한 실정이다. 현재까지 다양한 흡즙성 해충을 방제하기 위해 RNA interference (RNAi)에 기반한 방제법이 개발되었으나, 총채벌레를 포함한 흡즙성 해충에 대한 RNAi 기반 방제 연구의 성공적인 사례는 보고되지 않은 상태이다. 본 연구에서는 먼저 두 종의 흡즙성 해충 (*Tetranychus urticae*, 점박이응애, 세포섭식해충 및 *Nilaparvara lugens*, 벼멸구, 수액섭식해충)과 함께 두 종의 총채벌레 (*F. occidentalis* 및 *F. intonsa*)에 대한 식물체 섭식 후 시간 경과에 따른 핵 및 색소체 섭취량을 qPCR 을 통해 확인하였다. 그 결과 벼멸구의 체내에서는 핵과 색소체 모두 발견되지 않았으며, 총채벌레와 점박이응애에서는 핵보다 색소체가 더 많이 발견되었다.

그러나 점박이응애와는 다르게 총채벌레의 경우, 기존 강낭콩 잎에서 발견되는 색소체에 대한 핵의 비율보다 총채벌레 체내에서 발견되는 핵의 비율이 높으므로 총채벌레는 강낭콩 잎의 핵 및 색소체에 대한 선호도가 다르다는 것을 알 수 있다. 이 결과는 핵에서 발현된 hairpin RNA 가 엽육세포를 섭식하는 해충의 구기를 통해 전달될 수 있다는 것을 증명하였다. 추가적으로, 본 연구에서는 꽃노랑총채벌레의 전사체 데이터로부터 총 57 개의 RNAi 후보 유전자를 선발하고 각 후보 유전자를 표적으로 하는 double-stranded RNA (dsRNA)를 엽절편 시스템을 통해 꽃노랑총채벌레에 전달하는 실험을 진행하였다. 그 결과, *Toll-like receptor 6* (*TLR6*), *apolipophorin* (*apoLp*), *COPE* (*coatomer protein subunit epsilon*) 및 *SAM50* (*sorting and assembly machinery component 50*)의 dsRNA 는 살충제 감수성 및 살충제 저항성 계통 모두에서 높은 치사율을 보여주었다. dsRNA 를 섭식한 꽃노랑총채벌레는 감수성 계통에서 *TLR6*, *apoLp*, *COPE* 및 *SAM50* 의 유전자 발현 수준이 각각 46%, 47%, 37% 및 22% 감소했으며 저항성 계통에서는 각각 71%, 36%, 27% 및 50% 이 감소하였으므로, dsRNA 섭취 후 관찰된 치사율이 RNAi 에 의해 일어났다는 것이 증명되었다. 위 결과에서 나타나듯이 치사유전자 섭식 dsRNA 의 총채벌레 방제력은 저항성 계통에서도 효과적으로 나타나기 때문에, 앞으로 저항성 총채벌레를 방제하기 위한 새로운 대안이 될 수 있을 것이다. 또한, 이 치사유전자 dsRNA 를 발현하는 형질전환 작물체를 개발하면 이러한 RNAi 시스템이 기반 총채벌레 방제 기술을 실제 농가에서 적용 가능할 것이라고 사료된다.

검색어: 꽃노랑총채벌레, 대만총채벌레, 핵, 색소체, 세포소기관 섭식, 섭식 RNAi, 치사유전자, dsRNA, 살충제 저항성

학번: 2016-22528