A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Establishment of RCVpW-based insecticide resistance detection method and characterization of genes responding to sublethal doses of insecticides in thrips species

총채벌레류의 잔류접촉법 기반 살충제 저항성 검정체계 구축 및 살충제 아치사량 반응유전자 규명

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Min Ju Kim

Department of Agricultural Biotechnology

Seoul National University

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

By
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DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
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February, 2019

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Establishment of RCVpW based insecticide resistance detection method and characterization of genes responding to sublethal doses of insecticides in thrips species

Major in Entomology
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Abstract

Thrips (Order: Thysanoptera) are serious cosmopolitan pests that feed on the flowers, leaves and even fruits of various horticultural crops. In order to control thrips population, various types of insecticides have been heavily used; however, resistant populations have emerged due to its short life cycle and high fecundity. A residual contact vial plus water (RCVpW) bioassay method was established to monitor insecticide resistance in field populations of the melon thrips, *Thrips palmi*. Median lethal doses (LD_{50}) of six insecticides commonly used in *T. palmi* control (chlorfenapyr, cyantraniliprole, cypermethrin, dinotefuran, emamectin benzoate and spinosad), were determined at 8 h post-treatment, using a

susceptible RDA strain according to the RCVpW protocol. Diagnostic doses for on-site resistance monitoring of the six insecticides, which were set as two-fold higher doses of LD_{90} in the RDA strain, were in the range of 0.299 to 164.3 $\mu g^{-1}cm^2$. Insecticide resistance levels in five field populations of *T. palmi* were evaluated to test the applicability of RCVpW in monitoring the pest. Although the RDA strain exhibited 100% mortality in response to diagnostic doses of any of the test insecticides, field populations showed a reduced mortality in response to all test insecticides, indicating different degrees of resistance. In particular, all test field populations exhibited a significantly low mortality in response to spinosad, suggesting a wide distribution of spinosad resistance. Interestingly, an apparently reduced mortality in response to emamectin benzoate and chlofenapyr was observed in some field populations, perhaps suggesting uneven distribution of resistance to these insecticides in field populations of *T. palmi*. Our study showed that the RCVpW protocol can be employed both as an on-site resistance monitoring method for major thrip species, and in the selection of appropriate insecticides for their control.

To identify responsive genes to the treatment of insecticide, reference-based transcriptome analysis was conducted using RNA extracted from thrips treated with sublethal doses of five insecticide (chlorfenapyr, cypermethrin, dinotefuran, emamectin benzoate and spinoad) by Residual Contact Vial plus Water (RCVpW)

bioassay method. Among the annotated genes, 48, 35, 33, 429 and 18 genes were up-regulated, and 17, 142, 14, 36 and 16 transcripts were down-regulated in chlorfenapyr-, cypermethrin-, dinotefuran-, emamectin benzoate- and spinoad-treated thrips respectively. Gene ontology (GO) analysis of differentially expressed genes (DEGs) showed different GO profiles between different insecticide-treatment. The commonly responding over-transcribed genes were *ABC transporter* (G type) and two *heat shock proteins*. On the other hand, *diptericin A*, one of antimicrobial peptide, was significantly down-regulated in all treated thrips. Also, three up-regulated genes involved in detoxification (*UDP-glycosyltransferases* and *esterase*) and three muscle proteins which are able to bind with calcium ion (*myosin regulatory light chain* and *troponin C*) were additionally identified in spinosad treated thrips.

Key words: *Frankliniella occidentalis*, western flower thrips, *Thrips palmi*, melon thrips, insecticide resistance, Residual contact vial contact vial plus water bioassay method, Differentially expressed gene analysis, Gene ontology analysis

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CHAPTER 1.

Residual contact vial method for the rapid on-site detection of insecticide resistance in *Thrips palmi*

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Abstract

A residual contact vial plus water (RCVpW) bioassay method was establish to monitor insecticide resistance in field populations of the melon thrips, *Thrips palmi*. Median lethal doses (LD_{50}) of six insecticides commonly used in *T. palmi* control (chlorfenapyr, cyantraniliprole, cypermethrin, dinotefuran, emamectin benzoate and spinosad), were determined at 8 h post-treatment, using a susceptible RDA strain according to the RCVpW protocol (Kwon et al., 2015). Diagnostic doses for on-site resistance monitoring of the six insecticides, which were determined as doses two-fold higher than required to achieve LD_{90} in the RDA strain, were in the range of 0.299 to 164.3 $\mu g^{-1}cm^2$. Insecticide resistance levels in five field populations of *T. palmi* were evaluated to test the applicability of RCVpW in monitoring the pest. Although the RDA strain exhibited 100% mortality in response to diagnostic doses of any of the test insecticides, field populations showed a reduced mortality in response to all test insecticides, indicating different degrees of resistance. In particular, all test field populations

exhibited a significantly low mortality in response to spinosad, suggesting a wide distribution of spinosad resistance. Interestingly, an apparently reduced mortality in response to emamectin benzoate and chlofenapyr was observed in some field populations, perhaps suggesting uneven distribution of resistance to these insecticides in field populations of *T. palmi*. Our study showed that the RCVpW protocol can be employed both as an on-site resistance monitoring method for major thrip species, and in the selection of appropriate insecticides for their control.

1. Introduction

The melon thrips, *Thrips palmi* Karny, is a cosmopolitan pest of various ornamental and vegetable crops belonging to the Cucurbitaceae and Solanaceae. *T. palmi* directly sucks plant sap, and also causes serious damage as a primary vector of plant viruses, such as tospoviruses (Bielza et al., 2007). It is indigenous to Southeast Asia, but has spread widely over recent decades, and is now found in tropical regions of Africa, Australia, South America and Europe (Ebbels, 1993; Kawai, 1990). In Korea, *T. palmi* was first reported from peppers grown in a greenhouse on Jeju Island in 1993 (AHN, 1994), and is now distributed across the

whole Korean Peninsula.

T. palmi has mainly been controlled using various insecticide types; however, its short life cycle and high biotic potential have led to the rapid emergence of resistant populations, making it one of most hard-to-control pests. Choi et al. (2005) reported incidences of insecticide resistance in four different populations of *T. palmi* in plastic houses in the southern coastal area of Korea in 2000 to 2003. These field populations showed a 5- to 56.3-fold resistance to imidacloprid, thiamethoxam, chlorfenapyr, spinosad and fipronil. The continuous occurrence of insecticide-resistant populations of *T. palmi* have caused difficulties in controlling thrips in agricultural and horticultural crop cultivation areas, particularly in glass houses.

Kwon et al. (2015) have previously reported the use of a bioassay protocol, termed the residual contact vial plus water (RCVpW) bioassay, for the rapid and convenient detection of insecticide resistance in field populations of the western flower thrips, *Frankliniella occidentalis*. This simple bioassay method was used to predetermine diagnostic doses of test insecticides at 8 h post-treatment, and to detect resistance to the test insecticides. In our study, we applied the RCVpW bioassay protocol in the rapid detection of *T. palmi* resistance to six insecticides. We also evaluated the resistance levels of five field populations to the test insecticides using diagnostic doses in order to test the applicability of the RCVpW

bioassay in detecting *T. palmi* resistance.

2. Materials and methods

2.1. Strains and rearing

The RDA strain of *T. palmi* was reared on cucumber leaves (*Cucumis sativus*). In brief, cucumber seeds were planted in sterilized soil for six days at 28±1 °C, 55±5% relative humidity (RH) and a photoperiod of 16:8 (L:D) h. This RDA strain had originally been collected from a field and reared under laboratory conditions for over 10 years without being subject to insecticide treatment; it was therefore presumed to be relatively susceptible to insecticides and used as a reference strain for determining diagnostic doses. Five field populations of different strains of *T. palmi* were collected from the following regions: the GG_AS strain (Anseong, Gyeonggi Province; 37°02'22.7"N, 127°12'23.7"E), JG_GJ strain (Gimje, Jeonbuk Province; 35°47'30.4"N, 126°59'48.8"E), CB_CJ strain (Cheongju, Chungbuk Province; 36°38'20.8"N, 127°21'15.8"E), CN_CA strain (Cheonan, Chungnam Province; 36°46'05.2"N, 127°14'54.1"E) and GG_PT strain (Pyeongtaek, Gyeonggi Province; 37°07'40.0"N, 127°03'36.0"E).

2.2. Insecticides

Six test insecticides (chlorfenapyr, cyantraniliprole, cypermethrin, dinotefuran, emamectin benzoate and spinosad) commonly used to control *T. palmi* in Korea were selected. All the insecticides were purchased from either Sigma-Aldrich (Saint Louis, MO, USA) or Chem Service Inc. (West Chester, PA, USA). Their purities were as follows: chlorfenapyr (97.6%), cyantraniliprole (95.6%), cypermethrin (98.4%), dinotefuran (99.5%), emamectin benzoate (99.4%) and spinosad (98.0%).

2.3 Preparation of vials for *RCVpW* and determination of diagnostic doses

Insecticide-treated vials were prepared according to the method previously established (Kwon et al., 2010). In addition, a custom-made aspirator was connected to the coated test vial in order to collect thrips directly into the vial. Each insecticide-coated vial was infested with 12–15 female thrips in triplicate using the aspirator, and mortality levels were monitored at 8 h post-treatment, except for the spinosad treatment, in which mortality was confirmed at 4 h post-treatment as it showed a relatively faster intoxification response compared with the other insecticides. Thrips that were immobile for 2–3 s were regarded as dead. The LD_{50} (median lethal dose) and LD_{90} (lethal dose required to kill 90% of thrips) were determined by probit analysis using IBM SPSS Statistics software ver. 20.0

(IBM Corp., NY, USA). The diagnostic dose was set as a two-fold LD_{90} dose for each insecticide.

Table 1. Toxicity parameters of six insecticides when treated to the RDA susceptible strain of *Thrips palmi* via RCV method.

IRAC group[a]	Insecticide	Observation time	N	Slope	χ^2	df	LD_{50}[b] (95% CL)	LD_{90}[b]	DD[b,c]
13	Chlorfenapyr	8	132	3.6	33.5	7	0.1 (0.1-0.2)	0.3	0.6
28	Cyantraniliprole	8	195	2.4	191.3	13	49 (36-67.7)	164.3	328.5
3A	Cypermethrin	8	164	5.3	171.7	10	54.9 (41.8-66.1)	95.6	191.2
4A	Dinotefuran	8	272	3.1	257.4	19	3 (2.4-3.6)	7.8	15.5
6	Emamectin benzoate	8	218	2.5	81.7	13	0.3 (0.3-0.4)	1.1	2.2
5	Spinosad	4	153	3.1	89.2	10	0.9 (0.7-1.1)	2.4	4.7

[a] Group number classified by the mode of action according to the Insecticide Resistance Action Committee (IRAC).

[b] The unit of LD_{50}, LD_{90} and DD is $\mu g^{-1}cm^2$.

[c] DD represents the diagnostic dose (two fold of LD_{90})

2.4. Monitoring of field population resistance

In order to evaluate insecticide resistance levels in field populations of *T. palmi*, vials coated with the diagnostic dose of each insecticide were used. Test thrips (12–15 females) were collected from the plant host and directly transferred on-site into the precoated test vials using the aspirator; mortality was determined at 8 h post-treatment (4 h post-treatment for spinosad).

3. Results and discussion

3.1. Determination of diagnostic doses

The control efficacies of six test insecticides were determined, using the RDA strain as a susceptible reference. The LD_{90} values were estimated to be within a range of 0.299 to 164.3 $\mu g^{-1}cm^2$. Chlorfenapyr showed the lowest LD_{50}, followed by emamectin benzoate, spinosad, and dinotefuran. Cypermethrin and cyantraniliprole were least efficient against *T. palmi*; their LD_{50} values were 490- to 549-fold greater than the other insecticides tested. A two-fold amount of LD_{90} dose was arbitrarily set as the diagnostic dose to ensure 100% mortality of the RDA strain (Table 1). No mortality was observed in the control vial at 8 h post-infestation, meaning that the RCVpW bioassay can be applied safely in detecting

resistance in *T. palmi*.

To manage insecticide-resistant populations of thrips species more effectively, it is essential to establish resistance detection methods that can be easily employed, particularly in the field. In this context, use of the RCVpW bioassay protocol with a diagnostic dose for resistance detection of any fast-acting insecticide has several merits in terms of mass production of test vials, speed of decision-taking concerning insecticide resistance, a simple process for transferring insects to the test vial and less-technique-dependent procedure (Kwon et al., 2015). However, RCVpW is not suitable for resistance detection of slow-acting insecticides or growth regulators because mortality in RCVpW protocol is evaluated at <8 h post-treatment.

The glass-vial bioassay (Zhao et al., 1995; Choi et al., 2005; Shan et al., 2012) is similar to the RCVpW; however, in this method, mortality is evaluated at 24 h post-treatment and a relatively large vial (20–22 ml) is used for holding host plant as the food source, thus limiting its use for on-site resistance monitoring.

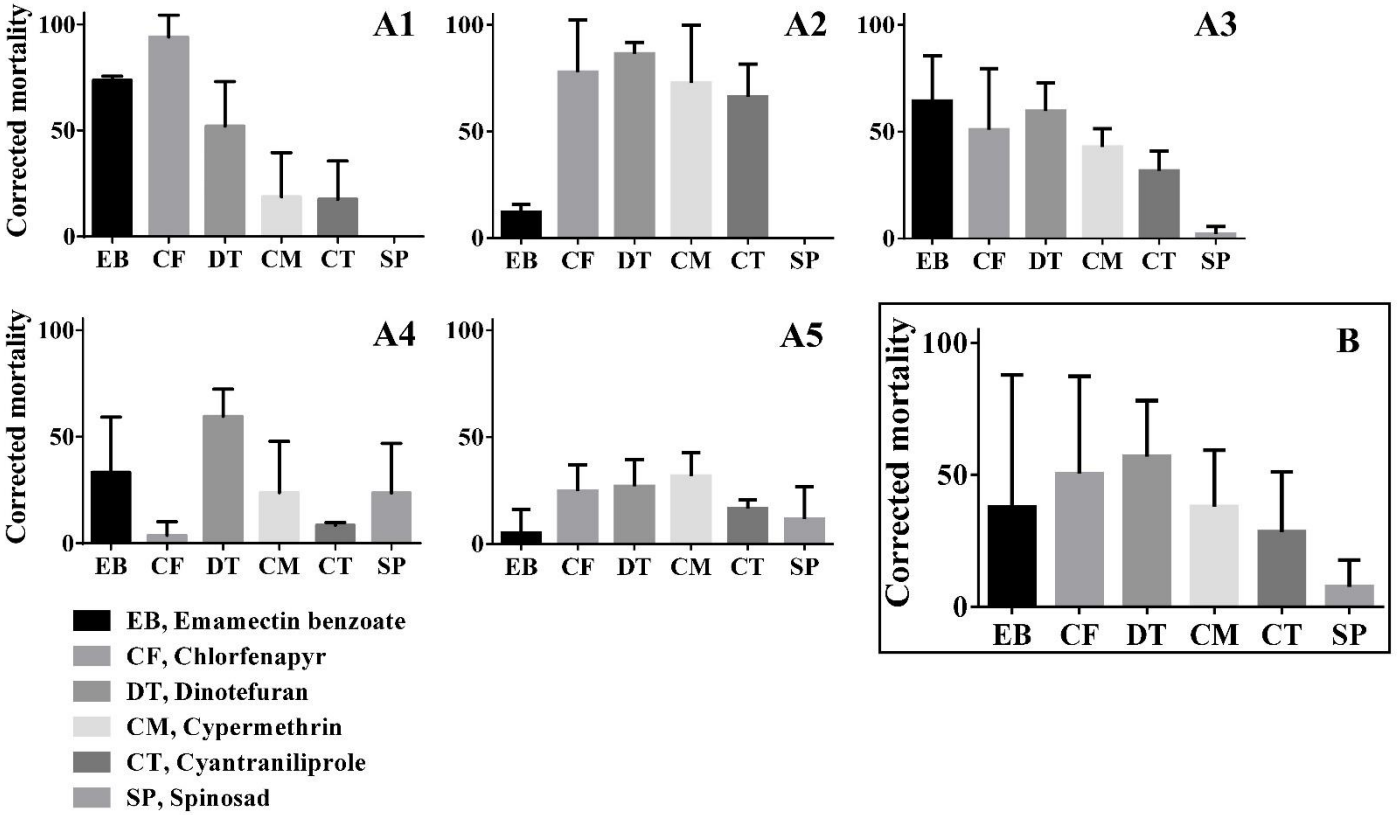
3.2. Resistance levels of field populations determined by RCVpW

In the pilot evaluation of insecticide resistance in five field populations of *T. palmi*, a reduced control efficacy was observed for all six test insecticides. All five populations (GG_AS, JB_GJ, CB_CJ, CN_CA and GG_PT populations)

collected from glass houses exhibited a reduced susceptibility (0–94.1% mortality) to all of the test insecticides (Fig. 1), suggesting the field populations had different degrees of insecticide resistance. None of the insecticides tested yielded a 100% mortality. In particular, spinosad caused the lowest mortality level (average 7.5%) in all the tested field populations, indicating the wide occurrence of spinosad resistance in the field; this is perhaps because spinosad has recently been used more extensively to control thrips in the field, compared with other insecticides. Dinotefuran exhibited the highest average mortality (56.9%) in the five field populations (Fig. 1B). Interestingly, resistance levels to chlorfenapyr had relatively more extensive variations, with the largest mortality range in the box plot (Fig. 1B) compared with the other insecticides. These results suggest that chlorfenapyr resistance is at an early stage of development, and may not yet be widespread. Although some field populations (GG_AS and JB_GJ; Fig. 1A1 and 1A2) showed 77.8–94.1% mortality to chlorfenapyr, other population exhibited greatly reduced mortality levels (3.78%; CN_CA; Fig. 1A4), implying an uneven distribution of chlorfenapyr resistance. Similarly, emamectin benzoate exhibited the second largest mortality range over the populations in the box plot (Fig. 1B), indicating that the distribution of emamectin benzoate resistance is not uniform yet either. Among the tested populations, the GG_PT population (Fig. 1A5) exhibited a relatively higher level of resistance to all the test insecticides (0–35%

mortality) compared with other populations, suggesting the development of multiple resistance in this population.

Fig 1. Comparison of the corrected mortalities of five field populations (A1,GG_AS; A2, JB_GJ, A3; CB_CJ, A4, CN_CA; and A5, GG_PT) of *Thrips palmi* in response to six insecticides. The data columns in Panel A (A1-A5) indicate the average mortality and standard deviation (SD) (n = 3). Panel B shows box plots showing the range between maximum and minimum corrected mortalities of five field populations in response to each insecticide



When susceptibilities to certain insecticides were compared between susceptible strains of *T. palmi* and *F. occidentalis* (Kwon et al., 2015), *T. palmi* was found to be generally less susceptible than *F. occidentalis*. LD_{50} values for emamectin benzoate, chlorfenapyr and spinosad against *T. palmi* were 0.34, 0.13 and 0.8 $\mu g^{-1}cm^2$, respectively, whereas 0.02, 0.011 and 0.004 $\mu g^{-1}cm^2$ against *F. occidentalis*, respectively. This indicates that the susceptibility of *T. palmi* to these insecticides is 11.8- to 200-fold lower than in *F. occidentalis* (Kwon et al., 2015). This finding may indicate endogenous differences in insecticide susceptibility between these two thrips species. Although chlorfenapyr showed a relatively higher control efficacy against *F. occidentalis* field populations compared with other insecticides tested (Kwon et al., 2015), it caused an average mortality of approximately 50% against *T. palmi* field populations with a somewhat large variation among field populations ($50.2 \pm 37.1\%$). However, it is also worth noting that the reference insecticide-susceptible RDA strains of both *T. palmi* in the present study and *F. occidentalis* used in the previous study (Kwon et al., 2015) may not be completely susceptible to the test insecticides, because these strains were established from field populations collected ~10 years ago. With this in mind, any predisposed resistance traits that were genetically stable, even in the absence of insecticide selection pressure during laboratory rearing, may have been retained within the gene pool, thereby reducing baseline susceptibility to certain

insecticides. Nevertheless, the interspecies differences in insecticide susceptibility between *T. palmi* and *F. occidentalis* observed in the present study provide useful information on the toxicological nature of these pests, which needs further consideration in the context of their control. Since the RCVpW bioassay method can be employed as an on-site resistance detection tool, it can facilitate the systematic and routine monitoring of insecticide resistance in *T. palmi*. In this study, diagnostic doses were determined for insecticides frequently used to control *T. palmi*. The diagnostic dose treatment using RCVpW bioassay provides basic information, not only on the presence or absence of resistance to the test insecticides, but also for use in selecting suitable alternative insecticides for managing insecticide-resistant populations of *T. palmi*. In order to provide more precise information on resistance profiles and their distribution in *T. palmi* populations, a large-scale resistance mapping of broader geographical regions is needed.

CHAPTER 2.

Transcriptome-based identification of responsive genes to sublethal doses of five different insecticides in the western flower thrips, *Frankliniella occidentalis*

Transcriptome-based identification of responsive genes to sublethal doses of five different insecticides in the western flower thrips, *Frankliniella occidentalis*

Abstract

The western flower thrips, *Frankliniella occidentalis* Pergande, are serious polyphagous pests that cause severe damage to horticulture and crops such as vegetables and fruits by direct sucking. Since various insecticides have been extensively used to control *F. occidentalis*, numerous studies on development of insecticide resistance were reported so far. In order to set an appropriate strategy for controlling thrips population, it is essential to understand insecticide resistance mechanism. To identify responsive genes to the treatment of insecticide, reference-based transcriptome analysis was conducted using RNA extracted from thrips treated with sublethal doses of five insecticide (chlorfenapyr, cypermethrin, dinotefuran, emamectin benzoate and spinoad) by Residual Contact Vial plus Water (RCVpW) bioassay method. Among the annotated genes, 48, 35, 33, 429 and 18 genes were up-regulated, and 17, 142, 14, 36 and 16 transcripts were down-regulated in chlorfenapyr-, cypermethrin-, dinotefuran-, emamectin

benzoate- and spinoad-treated thrips respectively. Gene ontology (GO) analysis of differentially expressed genes (DEGs) showed different GO profiles between different insecticide-treatment. The commonly responding over-transcribed genes were *ABC transporter* (G type) and two *heat shock proteins*. On the other hand, *diptericin A*, one of antimicrobial peptide, was significantly down-regulated in all treated thrips. Also, three up-regulated genes involved in detoxification (*UDP-glycosyltransferases* and *esterase*) and three muscle proteins which are able to bind with calcium ion (*myosin regulatory light chain* and *troponin C*) were additionally identified in spinosad treated thrips.

1. Introduction

The western flower thrips, *Frankliniella occidentalis* Pergande, are serious polyphagous pests that cause severe damage to horticulture and crops such as vegetables and fruits by direct sucking (Lacasa and Llorens, 1996; Lewis, 1997; Stuart et al., 2011). *F. occidentalis* causes not only direct damage like silvery scar but also indirect damage by transmitting plant viruses such as TSWV (Tomato spotted wilt virus), INSV (Impatiens necrotic spot virus), leading to economic loss (Webster et al., 2011; Zhao et al., 2014). It has been widely distributed throughout the world since 1970s as the international trade of horticultural items has

expanded from Europe to Asia (Kirk et al., 2003). In Korea, the occurrence of *F. occidentalis* was firstly reported in an orange cultivation area of Jeju Island in 1994 (Woo et al., 1994), and is now spread across the whole Korean Peninsula. Using insecticides with different mode of action types is one of important strategies for controlling *F. ccidentalis* population, but it also results in the development of insecticide resistance by its short life cycle, high fecundity (Bielza et al., 2007; Brødsgaard, 1994; Choi et al., 2005; Gholami and Sadeghi, 2016; Immaraju et al., 1992; Katayama, 1998; Wang et al., 2011; Zhao et al., 1995).

Cho et al. (1999) firstly reported insecticide resistance for *F. occidentalis* in three different field populations of Jeju island in the mid-1990s. In this resistance survey, field populations showed reduced mortalities against nine insecticides including organophosphates, carbamates, pyrethroids and neonicotinoid. In the mid-2000s, resistance against neonicotinoid insecticides, such as thiamethoxam and acetamiprid, was reported in four different field population in Korean peninsula (Choi et al., 2005).

Due to the continuous emergence of insecticide-resistant populations of *F. occidentalis* , it is necessary to understand the resistance mechanism to control. As detoxification factors including proteins responsible for efflux or hydrolyzation of xenobiotics have been suggested, characterization of these pathways were

promoted to understand how insects can resist against insecticides. The relationship between xenobiotic-inducible, over-expressed genes and insecticide-resistance traits has been introduced (Feyereisen, 2005). Insecticide-resistant fruit fly strain over-produced cytochrome P450, one of detoxification genes, accounting for detoxification genes are involved in insecticide-resistance.

Yoon et al. (2011) has claimed that metabolic process against insecticides can be progressed when insects obtain heritable traits that lead to either constitutive over-expression or alteration of protein related in detoxification metabolism. In order to identify genes working for insecticide tolerance, sublethal insecticide doses that do not cause physiological stress was applied so that genes involved in only primary detoxification process can be identified. Because the expression level of various detoxification genes can be induced by sublethal insecticide dose treatment, analysis of transcriptional profiling has been conducted to identify the major metabolic factors related in insecticide tolerance.

Gao et al. (2018) reported diamondback moth, *Plutella xylostella* detoxification genes including cytochrome P450 and cuticular proteins, which are also reported to be related in insecticide metabolism in other insects, were highly expressed by sublethal dose treatment of five different insecticides (chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinoad), meaning exposure to the insecticides resulted in tolerance.

In this study, responsive genes to sublethal doses of five different insecticides (chlorfenapyr, cypermethrin, dinotefuran, emamectin benzoate and spinosad) were identified by comparison of the transcriptome profiles between insecticide-treated and control thrips. In addition, genes that specifically responded to spinosad that showed the highest number of differentially expressed genes (DEG) were identified to check the most various detoxification-metabolism pathways among tested insecticides. Characterization of these over-expressed genes in response to sublethal dose of insecticides would help to understand detoxification (defense) elements in thrips with their mechanisms from tolerance to resistance probably

2. Materials and methods

2.1. Strains and rearing

The insecticide-susceptible RDA strain of *F. occidentalis* was obtained from Rural Development Administration, South Korea. The RDA strain of *F. occidentalis* was reared on cotyledon of kidney bean (*Phaseouls valgaris*) using previously reported method (Kwon et al., 2015). In brief, bean seeds were planted in sterilized soil for six days at 28±1°C, 55±5% relative humidity and a photoperiod of 16:8 (L:D) h. The thrips were fed on sprouted cotyledon in an insect breeding dish (91.4 ø × 40-mm height; SPL Life Sciences, Korea) with

water (5 ml)-soaked thin layered cotton. In each breeding dish, 200-300 female adults were maintained with 30-40 cotyledons. As the RDA strain was collected from a field and has been reared under laboratory conditions for more than 10 years without any treatment of insecticides, this strain was assumed to be relatively susceptible to insecticide than field strain and used as a reference.

2.2. Insecticide treatment using RCVpW method and determination of sublethal doses

Five insecticides (chlorfenapyr, cypermethrin, dinotefuran, emamectin benzoate and spinosad) which have been widely used in Korea to control *F. occidentalis* were selected. All insecticides were purchased from either Chem Service Inc. (West Chester, PA, USA) . The purities of insecticides were as follows: chlorfenapyr (97.6%), cypermethrin (98.4%), dinotefuran (99.5%), emamectin benzoate (99.4%) and spinosad (98.0%).

In order to perform bioassay, the RCV supplemented with water was designated RCVpW (abbreviated from *R*esidual *C*ontact *V*ial Bioassay *P*lus *W*ater). The insecticide-treated vials were prepared based on the method reported by Kwon et al. (2015). In brief, a 1-µl aliquot of water was dropped into a filter paper (0.5 × 0.5 mm) (Whatman, GE Healthcare, UK) attached underneath the vial screw cap for maintaining the humidity inside the vial and so that control mortality of the *F.*

occidentalis was able to be minimized. 100 µl of serially diluted insecticide solution in acetone was coated on inside wall of a 5-ml glass vial (Taeshin Bio Science, Seoul, Korea) and the vial was put on a roller mixer (Eberbach, Ann Arbor, MI, USA) for 30 min in a fume hood until acetone was completely dried. Using a custom-made aspirator, 15~20 females were transferred to each insecticide-coated vial in triplicate and the mortality was checked at 8 h post-treatment. Thrips showing immobility for 3 s were considered to be dead. The LD_{10} as a sublethal dose and LD_{50} were determined by Probit analysis using IBM SPSS Statistics software ver. 20.0 (IBM Corp., NY, USA).

Table 1. Toxicity parameters of five insecticides when treated to the RDA susceptible strain of *Frankliniella occidentalis* via RCV method.

IRAC group[a]	Insecticide	Observation time	N	LD_{10}[b]	LD_{10} 95% CL[c]	LD_{50}[b]	LD_{50} 95% CL[c]
13	Chlorfenapyr	8	240	0.48	0.04-0.89	1.41	0.66-2.59
3A	Cypermethrin	8	209	140.31	60.22-214.38	515.89	364.29-826.03
4A	Dinotefuran	8	403	5.31	2.82-7.93	29.73	21.72-44.12
6	Emamectin benzoate	8	376	2.29	0.78-4.11	12.58	4.44-7.76
5	Spinosad	8	577	1.81	0.29-0.68	2.44	1.99-2.96

[a] Group number classified by the mode of action according to the Insecticide Resistance Action Committee (IRAC).

[b] The unit of LD_{10}, LD50 and DD is $\mu g^{-1}cm^2$.

[c] CL: confident limit

2.3. Total RNA extraction for transcriptome analysis and library construction

In order to treat the sublethal dose of chlorfenapyr, cypermethrin, dinotefuran, emamectin benzoate and spinoad, RCVpW bioassay method was used as described above.

Fifty females were treated with LD_{10} doses of each insecticide for 8 h andcollected into a 1.5-ml tube. The treated were homogenized in TRIzoL (MRC, Cincinnati, OH, USA) reagent and total RNA was extracted in accordance with the manufacturer's instruction. Integrity and concentration of the RNA samples were checked by NanoDrop 8000 spectrophotometer (Thermo, Waltham, MA, USA). Only RNA samples that reach an $OD_{260/280}$ value of ≥1.8 and integrity number 7.0 were chosen for following steps. The RNA sample was treated with DNase I (Takara, Shiga, Japan) to remove extra gDNA. The RNA samples were used for mRNA preparation and cDNA library construction by Illumina TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) in accordance with manufacturer's instruction. The multiple cDNA libraries were paired-end sequenced with Illumina NovaSeq 6000 Sequence System (Illumina).

2.4. Sequence processing and annotation

Before analysing data, quality control was conducted to produce total reads quality and GC value. In order to reduce the bias of the analysis result, pre-

processing process which has low-quality or removes artifacts such as adapter sequence and contaminant was conducted. The obtained paired-end sequence files were trimmed with Trimmomatic program (Illumina), thereby eliminating adapter sequence as enough length. For the quality trimming, the read with mean quality < 15 and minimum read length of < 36 bp were trimmed. After the pre-processed reads are mapped to the reference genome using a HISAT2 program (Illumina), aligned readings were generated.

2.5. Reference-based differentially expressed gene (DEG) analysis

In order to conduct overviewing the pattern of unigenes in all samples, reads from all samples were aligned to the reference with bowtie 2 with custom parameter. After assembly, the amount of transcript abundance was estimated as the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value that is the read count and the within sample normalized value.

After filtering the transcripts that has low quality based on pre-process and quality control, quantile normalization was proceeded by $\log_2$ (FPKM+1). In addition statistical analysis was done by fold change of each sample. Gene expression differences were compared at the ratio of each different insecticide treated sample to control. For the over expressed genes showing > 2 fold changes (FC) of gene expression ratio were considered to be affected by insecticide

treatment. Likewise, for the under-expressed genes showing < -2 FC of gene expression ratio were considered to be affected by insecticide treatment as well. Among those results, genes that indicated commonly over- or under-transcribed tendency in all insecticide-treated thrips sample were selected for subsequent analysis. The over-transcribed genes in spinosad treated thrips were analysed additionally as this sample showed much larger number of over-transcribed genes compared to other samples. > 4 FC of gene expression ratio were chosen for analysis.

3. Results and discussion

3.1. Commonly over-transcribed genes following treatment of sublethal doses of insecticides

Total 30 commonly over-transcribed genes were identified in insecticide-treated thrips compared to control (Table2). Among the 30 genes, 15 genes were identified as uncharacterized proteins. First, *zinc finger protein 239-like isoform X1* was the most highly up-regulated gene and the transcription levels were an average of 1.61-fold higher compared to control. Zinc finger proteins are known to be DNA binding transcription factor. However, it is unclear that the role of this zinc finger protein is one of xenobiotic responsive nuclear factor. Also, two types

of heat shock protein (heat shock protein Hsp-16.1/Hsp-16.11-like, heat shock protein 70) were observed. The transcription levels were an average of 4.17 and 16.1-fold higher compared to control each. Heat shock proteins are known to be produced in response to exposure of stressful circumstances such as heat shock, cold shock, and UV light. This result might be due to the stimulation of sublethal doses of insecticide to thrips. In addition, *ATB-binding cassette* (*ABC*) *transporter G family member 20 isoform X3* was also commonly over-transcribed in an average of 1.63-fold. ABC transporters are involved in extracellular transport system for various substances such as lipids, metabolic products, and xenobiotics, implying the *ATB-binding cassette* (*ABC*) *transporter G family member 20 isoform X3* gene would help to excrete treated insecticide molecules. Epis et al. (2014) suggested the involvement of ABC transporters for the resistance of *Anopheles stephensi* larvae against the sublethal dose of permethrin. In case of DDT-resistant *Drosophila melanogaster*, detoxification genes including cytochrome P450s, ABC transporters were over-transcribed compared to susceptible strain (Pedra et al., 2004).

3.2. Commonly under-transcribed genes following treatment of sublethal doses of insecticides

A total 13 genes were commonly under-transcribed in insecticide-treated thrips

in comparison with control (Table 2). Among the 13 genes, 6 genes were identified as uncharacterized proteins. *Diptericin A* showed the lowest under-transcription levels (Fold change -10.1 ~ -1.5) in all treated samples. Diptericin is one of antimicrobial peptides and related in immune responses against Gram-negative bacteria in *Drosophila* (Lee et al., 2001; Lemaitre et al., 1997). According to Terhzaz et al. (2015), Cytochrome P450-4e3 knockdown flies showed approximately 10-fold elevation of diptericin gene expression, following the activation of the NF-κB pathway. The NF-κB is a protein complex that controls transcription of DNA and regulates the immune response to infection, explaining the reason why the transcription level of the NKAP (NF-κB activating protein) family protein *CG 6066*-like gene is down-regulated as well (Table2). In the point of view of trade-off concept, the genes related to immune responses were down-regulated to save the resources for detoxification so that the insect could invest limited resources more to metabolize insecticides. In addition, putative *ferric-chelate reductase 1* homolog was down-regulated in insecticide treated thrips. The ferric-chelate reductase is an enzyme that belongs to oxido-reductase family, specially oxidizing metal ion with NAD^+ as acceptor in glycolysis and citric acid cycle. It can be speculated that reduced energy generation would be beneficial to the insecticide-treated thrips with regard to acquiring tolerance; however, further study is needed to explain the relations

between energy generation and detoxification process (Gao et al., 2018).

In comparison with the commonly over-transcribed genes, another transcription factor, *zinc finger protein 32-like* gene was down-regulated, implying this gene would be involved in regulation of energy consuming processes, such as immune system, and its reduction is beneficial to metabolize insecticides as explained above.

3.3. Over-transcribed genes following spinosad treatment

Even though emamectin benzoate and spinosad have relatively complicated molecular structure among the five insecticides, only spinosad treated thrips showed the highest number of over-transcribed genes (32 genes; 104.9-fold higher number, 4 > FC) and GO terms. In comparison of metabolic process between emamectin benzoate and spinosad, the numbers of metabolites of each chemical were examined under the identical condition (in poultry-chicken). The larger number of metabolites are produced by more metabolic processes, thereby spinosad could induce more DEGs and GOs in treated thrips than the other samples.

In terms of specifically over-transcribed genes in response to sublethal dose of spinosad, a total of 32 up-regulated genes (FC > 4) were identified (Table 4). Among the 32 up-regulated genes, total 9 genes were revealed as the genes

possibly induced by spinosad. Muscle calcium-binding protein genes, *myosin regulatory light chain 2*-like and *troponin C*-like) showed the highest transcription levels (FC 6.2-105.0) among the genes. Myosin light chain and troponin C belong to the EF-hand family, enabling those proteins to bind Ca^{2+} ions. The myosin regulator light chain binds are bound with Ca^{2+} ions and phosphorylated by myosin light chain kinase, allowing muscle to modulate force transduction (Holroyde et al., 1979; Nieznanski et al., 2003). Yang et al. (2008) demonstrated that the transcription level *of myosin regulatory light chain* was 4.08-fold higher in delamethrin-resistant *Culex pipiens pallens* as well based on quantitative PCR analysis, speculating that the myosin regulatory light chain would be related in deltamethrin resistance mechanism. Troponin C is a protein actin filament of muscle and responsible for binding calcium for activating muscle contraction. According to Vontas et al. (2007), the transcription level of t*roponin C* gene in pyrethroid-resistant strain of *Anopheles gambiae* was 4.31-fold higher than susceptible strain. From a point of view with neurotoxicity, calcium ion is deeply related with excretion of acetylcholine in a synapse. Nicotinic acetylcholine receptors (nAChR) are ligand-gated, permeable ion channels to potassium, and calcium ion, being capable of switching from a closed state to an open state, when acetylcholine bind to nAChRs. Therefore, the interplay between the muscle calcium ion binding proteins (myosin regulatory light chain and troponin C) and

calcium ion would help to reduce the concentration of calcium ions in a synapse, playing a role in relieving over-stimulation of spinosad functioning as a nicotinic acetylcholine receptor allosteric modulator; however, it is still speculative and further study on relationship between muscle calcium ion binding proteins and insecticide tolerance is required.

In addition, two *UDP-glycosyltransferases* (*UGT*) were over-transcribed with FC of 4.5-6.1. UGT catalyzes the conjugation of diverse small lipophilic compounds, playing an important role in the detoxification of toxic xenobiotics. Bozzolan et al. (2014) reported the *UGT46A6* gene increased 2.2-fold in response to sublethal dose treatment of deltamethrin by topical application on aetennae in *Spodoptera littoralis*, suggesting its role in the protection of the olfactory organ towards insecticide. In the temephos-resistant strain of *Aedes albopictus*, *UGT*s were over-expressed with fold change value 4.4-16.7 along with other detoxification genes; carboyxlesterases, cytochrome P450s (Grigoraki et al., 2015). Also, one *esterase* (EST) gene was 4.6-fold overexpressed. The esterase functions by rapidly binding and slowly turning over the insecticides; sequestration or metabolism of insecticide including a common ester bond (Hemingway and Ranson, 2000; Karunaratne et al., 1995). Three types of heat shock proteins were also over-expressed (4.5-14.0 FC) in the spinosad treated thrips in addition to commonly up-regulated two.

Table 2. Commonly up-transcribed genes following the treatment of sublethal doses of five different insecticides.

Gene ID	Gene name	Fold change relative to control				
		Chlorfe-napyr	Cyperme-thrin	Dinote-furan	Emamectin-benzoate	Spinosad
FOCC000008	obscurin-like isoform X1~X5	1.46	1.0	1.26	1.09	2.12
FOCC001417	4-hydroxyphenylpyruvate dioxygenase	2.04	1.1	1.67	1.08	2.55
FOCC001994	extensin-like	1.02	2.05	1.6	1.71	1.53
FOCC003407	protein TsetseEP-like	1.18	2.04	1.42	1.49	2.96
FOCC003553	zinc finger protein 239-like isoform X1	2.11	1.19	1.32	2.21	1.2
FOCC005119	thyrotropin-releasing hormone-degrading ectoenzyme-like	2.05	1.01	2.12	1.14	4.43
FOCC005505	heat shock protein Hsp-16.1/Hsp-16.11-like	2.02	2.02	1.43	1.44	14
FOCC007529	cell growth regulator with RING finger domain protein 1-like	1.79	1.32	2.12	1.68	2.24
FOCC007777	heat shock protein 70	1.02	2.61	1.53	1.17	4.93
FOCC009415	elastin-like	1.56	1.68	1.5	2.03	2.57
FOCC012231	ABC transporter G family member 20 isoform X3	1.69	1.07	1.72	1.2	2.47
FOCC012258	immediate early response gene 5-like protein	1.46	1.05	1.39	1.1	2.84
FOCC012666	trypsin beta-like	1.5	1.32	1.34	1.28	3.1
FOCC016038	glycine-rich cell wall structural protein 1.8-like	2.28	1.85	2.03	2.18	2.95
FOCC016447	proteoglycan 4-like	1.18	2.26	1.37	1.49	3.56

Gene ID	Gene name	Fold change relative to control				
		Chlorfe-napyr	Cyperme-thrin	Dinote-furan	Emamectin-benzoate	Spinosad
FOCC001594	diptericin A	-3.35	-10.1	-5.47	-2.06	-1.5
FOCC005969	zinc finger protein 32-like	-2.03	-1.65	-2.5	-1.42	-1.86
FOCC006813	laccase-2-like	-2.36	-1.33	-1.74	-1.65	-2.01

Table 3. Commonly under-transcribed genes following the treatment of sublethal doses of five different insecticides

FOCC008498	NKAP family protein CG6066-like	-1.19	-1.41	-2.76	-1.92	-2.77
FOCC012227	putative ferric-chelate reductase 1 homolog	-1.63	-2.27	-2.54	-1.09	-1.68
FOCC016854	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1-like	-1.93	-1.0	-1.89	-1.42	-2.13
FOCC017204	cold and drought-regulated protein CORA-like	-2.43	-2.15	-1.44	-1.97	-1.84

Table 4. Specifically up-transcribed genes following the treatment of spinosad. Genes related with muscle calcium binding protein were marked with green color, the detoxification gene were marked with orange color, and stress-response gene was marked with light blue color.

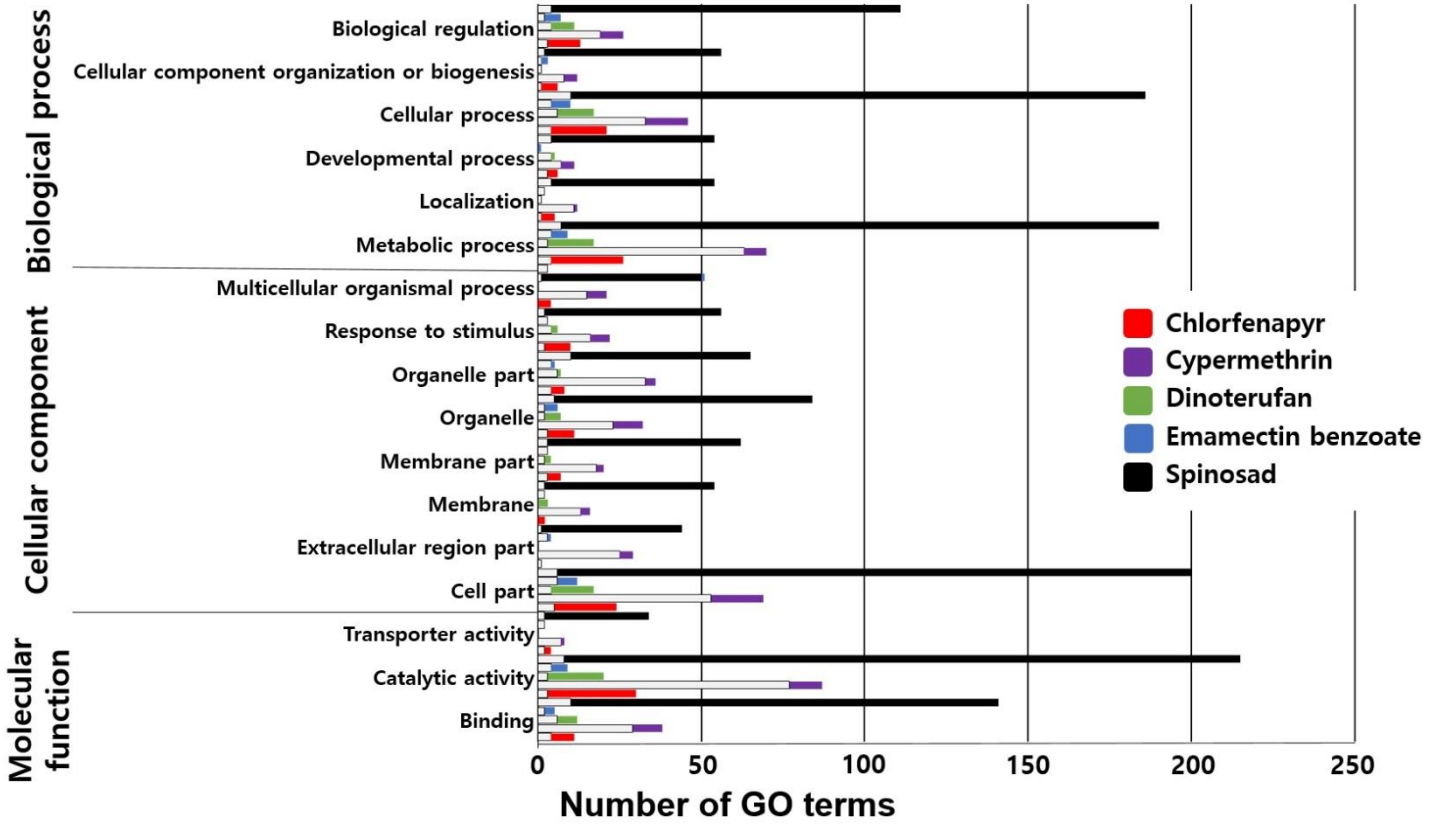
Gene ID	Gene name	Fold change relative to control
FOCC013185	myosin regulatory light chain 2-like	104.9
FOCC008072	troponin C-like	42.4
FOCC005505	heat shock protein Hsp-16.1/Hsp-16.11-like	13.9
FOCC014278	probable pectin lyase B	9.53
FOCC002259	vitellogenin-1-like	8.78
FOCC006088	alpha-glucosidase-like	8.21
FOCC011624	mucin-2-like	7.57
FOCC013211	inter-alpha-trypsin inhibitor heavy chain H4-like	7.0
FOCC015589	myrosinase 1-like	6.84
FOCC008703	troponin C-like	6.16
FOCC002665	vitellogenin-1-like	6.12
FOCC016865	UDP-glucuronosyltransferase 2C1	6.07
FOCC015862	V-type proton ATPase 116 kDa subunit a-like isoform X2	5.89
FOCC008187	actin, muscle-like isoform X2	5.34
FOCC009176	apolipophorins	5.33
FOCC014245	myrosinase 1-like	5.09
FOCC007777	heat shock protein 68-like	4.92
FOCC006731	proton-coupled amino acid transporter-like protein pathetic	4.66
FOCC001705	esterase FE4-like	4.58
FOCC012376	heat shock protein 70 B2-like	4.52
FOCC009158	proteoglycan 4-like isoform X2	4.48
FOCC003406	UDP-glucuronosyltransferase 2B2-like	4.47
FOCC004962	nucleolin 2-like	4.47

FOCC005119	thyrotropin-releasing hormone-degrading ectoenzyme-like	4.42
FOCC005903	cathepsin L1-like	4.39
FOCC003708	acyl-CoA synthetase short-chain family member 3, mitochondrial	4.30
FOCC005842	cytochrome oxidase subunit I	4.29
FOCC001545	phospholipase A1	4.19
FOCC008839	lipase 3-like isoform X2	4.19
FOCC016260	cathepsin L1-like isoform X2	4.17
FOCC004437	general odorant-binding protein 83a-like	4.12
FOCC003635	alpha-amylase 2-like	4.09

3.4. GO profiles of DEGs

Based on DEG data, GO profile analysis were performed. The overall GO profiles of the DEGs showed that the over expressed genes were categorized in the binding, catalytic activity, response to stimulus, metabolic process, cell part, cellular process, and biological regulation. As described above, relatively huge number of DEGs were observed in spinosad treated thrips, and it showed high proportion of GO terms in the categories as well. On the other hand, the genes categorized in the catalytic activity, cell part, metabolic process were under-expressed. According to the classified GO categories, these data would provide ideas to understand various detoxification-metabolism factors against tested insecticides (figure 1).

Fig 1. Gene ontology (GO) distribution of differentially expressed genes (DEGs) following the treatment of sublethal doses of insecticides. The white bar represent GO terms of down-regulated DEGs (white for all insecticides), whereas other color bars represent GO terms of up-regulated DEGs (red, chlorfenapyr; purple, cypermethrin; green, dinotefuran; blue, emamectin benzoate; black, spinosad)



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KOREAN ABSTRACT

총채벌레류의 잔류접촉법 기반 살충제 저항성 검정체계 구축 및 살충제 아치사량 반응유전자 규명

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초록

총채벌레류는 식물의 꽃, 잎, 과실부분 등 다양한 작물을 가해하는 대표적인 해충이다. 총채벌레류를 방제하기 위하여 다양한 종류의 살충제가 사용되어 왔으나, 총채벌레의 짧은 세대주기, 높은 생식력으로 인하여 살충제 저항성 개체들이 지속적으로 보고되고 있다. 본 논문에서는 오이총채벌레 야외계통의 살충제 저항성을 현장에서 간편하고 빠르게 검정하기 위한 잔류접촉법 기반 바이알법(RCVpW)을 개발하였다. 본 방법과 감수성 계통의 오이총채벌레를 이용하여 방제를 위해 많이 사용되는 여섯 가지 약제의 중간치사량(LD_{50})과 치사량(LD_{90})을 산출한 후, LD_{90} 의 2 배에 해당하는 약량을 RCVpW 를 위한 검정량으로 설정하였다. 각 약제 별

진단농도에 노출되었을 때 오이총채벌레의 감수성 계통은 100%의 사충률을 보였으나, 안성, 김제, 청주, 천안, 평택 등 5 개 지역에서 채집한 오이총채벌레 지역계통은 보다 감소된 사충률을 나타냈다. 특히, spinosad 약제에 대하여 모든 야외계통이 낮은 사충률을 보였으며, 이는 spinosad 저항성이 전국적으로 넓게 분포되어 있음을 의미한다. 반면 emamectin benzoate 와 chlorfenapyr 의 경우 지역별로 저항성 발달 정도가 크게 달랐다. 해당 연구를 통하여 RCVpW 가 주요 총채벌레류 저항성 발달 모니터링과 방제를 위한 최적의 살충제 선발에 활용될 수 있다는 것을 확인했다.

꽃노랑총채벌레 역시 저항성 계통이 지속적으로 보고되고 있으며, 살충제 저항성 계통의 적절한 방제법 선발을 위하여 살충제 저항성 발달과정에 대한 이해가 요구된다. 살충제에 노출 시 반응하는 유전자를 확인하기 위하여, 다섯 가지 약제의 아치사량(LD_{10})을 상기 잔류접촉법(RCVpW)으로 처리한 꽃노랑총채벌레의 전사체 분석을 수행하였다. 약제 간 공통으로 과발현 된 유전자로서 대표적으로 살충제 대사에 관여하는 것으로 알려진 ABC transporter (G type)와 외부자극 반응유전자인 heat shock protein 유전자가 식별되었으며, 공통 저발현 된 유전자로서 항균펩티드 종류 중 하나인 diptericin A 를 확인하였다. 특히 spinosad 를 처리한 꽃노랑총채벌레 시료에서는 다른 실험군에 비하여 더욱 많은 유전자가 과발현됨을 확인하였고, 그 중 주요한 유전자로서 해독 유전자(UDP-glycosyoltransferases and esterase)와 근육-칼슘 결합 단백질 유전자(myosin regulatory light chain, troponin C)가 spinosad 의 대사에 관여할 것으로 추정된다.

검색어: 꽃노랑총채벌레, 오이총채벌레, 살충제 저항성, 잔류접촉법, 저항성 발달, 전사체 분석

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