A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Identification of Point Mutation on Acetylcolinesterase1 and Candidate Genes Related to Carbofuran Resistance in *Laodelphax striatellus* (Hemiptera: Delphacidae)

애멸구에서 나타나는 아세틸콜린에스터레이즈 1 의 점돌연변이와 카보퓨란 저항성 관련 후보 유전자의 동정

By

Hyung Bum Kim

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

By
Hyung Bum Kim

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
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Identification of Point Mutation on Acetylcolinesterase1 and Candidate Genes Related to Carbofuran Resistance in *Laodelphax striatellus* (Hemiptera: Delphacidae)

Major in Entomology
Department of Agricultural Biotechnology, Seoul National University

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Abstract

Laodelphax striatellus is an important pest of rice due to not only sucking rice seedlings, but also transmitting serious plant viruses. Among various kinds of insecticide groups (carbamates, organophosphorus, neonicotinoids, etc.), carbofuran, a systemic carbamate insecticide, has been most extensively used to control rice pests including *L. striatellus*, resulting in widespread carbamate resistance in Korea and other Northeast Asia countries. To establish high-throughput screening systems for insecticide resistance management, molecular diagnostic markers are required. Here, we have used the carbofuran selected strains (SEL0 to SEL9) to find a single amino acid point mutation associated with carbamate resistance in the type-1 acetylcholinesterase (Lsace1). When the

phenotype resistance of the SEL9 strain, which was selected by carbofuran for 9 generations, was compared with that of the susceptible strain using topical application method, the resistance was determined to be 14-fold higher. In the biochemical enzyme assay, the esterase activity was not significantly different but the median inhibitory concentration (I_{50}) value against acetylcholinesterase (AChE) was 4.3-fold higher. This suggests that insensitive AChE is likely involved as a resistance factor. Comparison of the *type-1 acetylcholinesterase* (*Lsace1*) gene sequences of five strains (SUS, SEL0, SEL3, SEL6, SEL9) revealed two types of amino acid substitutions (F330Y and F331H). Correlation analysis between the genotype and phenotype suggested that resistance allele frequency of F331H was strongly correlated with the I_{50} value. Interestingly, the F331H mutation was negatively associated with the transcript level of *Lsace1*. This suggests that the selection pressure might result in a reduction of the target gene. We also conducted transcriptome analysis and compared the results from the resistant SEL9 and SEL0 (susceptible control) strains to reveal molecular biological factors involved in carbofuran resistance. A total of 96,185,150 reads were analyzed, of which 62,860,430 reads were mapped. From these reads, 28,332 transcripts were annotated. A total of 24 up-regulated and 15 down-regulated genes were identified in the resistant SEL9 strain compared to SEL0 strain by DEG analysis in statistical condition ($p < 0.05$, $q < 0.15$) and Log_2FC value (> 1, < -1). As a result of gene

ontology (GO) analysis, we determined the composition of GO terms in biological process group, cellular component group and molecular function group. Overall, there was no significant difference between up-regulated and down-regulated genes in the composition of GO terms. But it was found that GO terms in catalytic activity and binding group occupied a high portion were, suggesting that many genes belonging to these groups can be important factors associated with carbofuran resistance.

Key words: Acetylcholinesterase, Carbofuran resistance, *Laodelphax striatellus*, Point mutation, Transcriptome analysis, Cytochrome P450, Larval cuticular protein, Carboxylesterase, Differentially expressed gene (DEG), Gene ontology (GO)

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Carbofuran resistance mechanism mediated by target site insensitivity in *L. striatellus*

CHAPTER 2.
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LITERATURE REVIEW

1. What is *Laodelphax striatellus*

Small brown planthopper, *Laodelphax striatellus* is widely distributed worldwide from Philippines, Siberia to Europe (Kisimoto, 1989). It is an important pest of rice due to not only sucking rice seedlings, but also transmitting serious plant viruses such as the rice strip virus (RSV) and the rice black-streaked dwarf virus (RBSDV) during early seedling periods (Chung and Lee, 1971; Falk and Tsai, 1998; Park, 1973). Especially, damage by RSV has been reported continuously in Korea. In 2009, it was reported that RSV outbroke in 21,541 ha area of the whole west coast area, which caused destructive damage in rice production field (Kim et al., 2011) Although *L. striatellus* used to be considered as domestic indigenous pests, migration of *L. striatellus* to Korea was also suggested by the unusually large number of *L. striatellus* and high infection rate of RSV in the Western seaside region from 2008 to 2010 (Kim et al., 2011).

2. Insecticide resistance problem in *Laodelphax striatellus*

Among various control methods, insecticide use is the most common and efficient way to control insects. However, development of insecticide resistance is a major factor that makes chemical control of pests difficult. For this reason, regular

resistance monitoring is required for proper insecticide resistance management of *L. striatellus*.

Previously, resistance to chlorpyrifos (Ban et al., 2012) and fipronil (Mu et al., 2016) was reported in China. In Japan, resistance to imidacloprid was found and the resistance ratio was higher in local populations (Mu et al., 2016). Also, thiamethoxam resistance was reported in various regions of Korea (Jeong et al., 2016b).

In recent study, both indigenous and migratory *L. striatellus* populations were found to exist in Korea, where migratory populations arrive irregularly from China along the western coastal area during late May to early June (Kwon et al., 2018). In a comparative toxicity study of eight insecticides, migratory populations exhibited higher resistance than indigenous populations (Jeong et al., 2016a). Therefore, proactive resistance monitoring and management programs are required for the migratory resistance population to prevent a fatal decrease to rice yields.

3. Previous Studies on Mechanisms of Insecticide Resistance

The insecticide resistance mechanisms are composed of three major factors: target site insensitivity, increased expression of genes involved in detoxification and cuticular penetration factors. Many studies have been reported on target site insensitivity using inhibition assay for organophosphorus (chlorpyrifos) (Wang et

al., 2010a), neonicotinoid (imidacloprid) and pyrethroid (deltamethrin) (Gao et al., 2008) insecticide resistance in *L. striatellus*. The I_{50} values of target enzyme in resistant strain was found to be higher than that of susceptible strain, suggesting that the increased insensitivity of enzyme is associated with insecticide resistance. In addition, many studies have revealed that overexpression of the detoxifying enzyme induces insecticide resistance. It was confirmed that Cyp450 genes were overexpressed in resistant populations of *L. striatellus* strains that are resistant to various insecticides, such as chlorpyrifos (Wang et al., 2010a), deltamethrin (Gao et al., 2008), buprofezin (Zhang et al., 2012), imidacloprid (Elzaki et al., 2016) and ethiprole (Elzaki et al., 2015). Knock-down of detoxifying enzyme genes via RNAi increased the mortality by insecticide treatment, suggesting the involvement of detoxification enzymes in resistance (Elzaki et al., 2015; Elzaki et al., 2016; Xu et al., 2014).

Transcriptome analysis has been extensively used as a powerful tool for identifying genes related to insecticide resistance. Any comparative transcriptome analysis with the focus on identifying resistance-associated genes in *L. striatellus* has not been performed yet. Instead, studies on various detoxifying enzymes have been carried out based on the transcript analysis of susceptible strain (Xu et al., 2013, 2014). Also, transcriptome analysis was only performed by *de novo* assembly because of absence of genome data for *L. striatellus* (Zhang et al., 2010).

Recently, a genome analysis of *L. striatellus* was performed, allowing reference-based transcriptome analysis (Zhu et al., 2017).

CHAPTER 1.

Carbofuran resistance mechanism mediated by target site insensitivity in *L. striatellus*

Carbofuran resistance mechanism mediated by target site insensitivity in *L. striatellus*

Abstract

Molecular diagnostic markers are necessary for establishing high-throughput screening systems to support insecticide resistant population management. Here, we identified single amino acid substitution mutations related to carbamate resistance in *Laodelphax striatellus* Fallén *type-1 acetylcholinesterase* (*Lsace1*) using carbofuran-selected strains. The phenotypic resistance profiles of the final selection strain (SEL9) compared to the susceptible strain revealed a 14-fold higher resistance ratio based on topical application, 1.2-fold higher general esterase activity, and 4.3-fold higher acetylcholinesterase insensitivity based on the 50% inhibitory concentration (I_{50}), suggesting that insensitivity of the target site could occur as a resistance factor. Comparison of the nucleotide sequences of *Lsace1* of five strains (SUS, SEL0, SEL3, SEL6, and SEL9) revealed two amino acid substitutions (F330Y and F331H). To understand the roles of these mutations, we determined the allele frequency of both point mutations in the selected strains using quantitative sequencing methods. In addition, several quantitative genotypic traits (e.g., gene copy numbers and transcript levels of *Lsace1*, *Lsace2*, and *LsCarE1*) were assessed. A correlation analysis of genotypic and phenotypic traits revealed

strong correlations between resistance level and I_{50} with F331H allele frequency. Interestingly, the F331H mutation was negatively correlated with transcript levels of *Lsace1*, suggesting that selection pressure might result in a reduction of the target gene. Overall, the F331H mutation and reduced mRNA are important factors in the development of carbamate resistance. Furthermore, the point mutation can be used to monitor rapid carbofuran resistance in conjunction with molecular diagnostic methods such as quantitative sequencing.

1. Introduction

The small brown planthopper (*Laodelphax striatellus* Fallén) is an important pest in rice cultivation areas, which sucks directly on seedlings and transmits plant viral viruses, such as the rice strip virus, during early seedling periods (Chung and Lee, 1971; Falk and Tsai, 1998; Park, 1973). Both indigenous and migratory *L. striatellus* populations exist in Korea, where migratory populations arrive irregularly from China along the western coastal area during late May to early June (Kwon et al., 2018). In a comparative toxicity study of eight insecticides, migratory populations exhibited higher resistance than indigenous populations (Jeong et al., 2016a). Thus, it is necessary to improve the assessment of resistance levels to various insecticides, such as carbofuran, by establishing rapid detection methods to enhance the efficiency of control measures. Pesticide application has been used as a major insect control agent since the 1960s. In Korea, there are 183 registered commercial products composed of 24 active ingredients for the control of *L. striatellus* (Korea Crop Protection Association, 2018b). Among them, carbofuran is the top-selling pesticide, with sales equivalent to approximately 1.1 million dollars, used to control rice pests, including *L. striatellus* (Korea Crop Protection Association, 2018a).

The emergence of carbamate-resistant populations in rice fields has been reported in Korea (Lee et al., 1987) and Japan since the 1970s (Ozaki and Kassai,

1971). The resistance mechanisms are related to target site insensitivity and enhanced detoxification enzymes based on a biochemical study conducted in Japan (Endo and Tsurumachi, 2000). For example, the multiple-resistance Kumamoto strain exhibited 18~39-fold higher resistances to four types of carbamate insecticides (fenobucarb, carbaryl, carbofuran, and carbosulfan). Meanwhile, an enzymatic analysis revealed a 4.8~6.5-fold reduced sensitivity to acetylcholinesterase (AChE) and 4.3-fold higher general esterase (EST) activity. Moreover, it exhibited partial cross-resistance to organophosphate insecticides, suggesting that both types of insecticides might share AChE as a common target site. The putative target gene of carbamate insecticides in *L. striatellus*, type-1 AChE (*Lsace1*), was first cloned from an organophosphate insecticide-resistant strain (Zhang et al., 2013a). An F331(439)H substitution (where the amino acid numbers outside and inside the parentheses are those of AChE from *Torpedo californica* and *L. striatellus*, respectively) located in an acyl pocket near the active site gorge of AChE was identified from a chlorpyrifos-resistant strain. The strain also possessed a 5-fold reduced insensitivity toward AChE and 3.9-fold higher EST activity due to overexpression (Zhang et al., 2013a).

In this study, the carbamate resistance mechanism was primarily assessed with molecular biological approaches in carbofuran-selected strains due to an absence of reports providing direct evidence of target site insensitivity mediated by point

mutation in the carbamate-resistant *L. striatellus* population. We identified point mutations associated with target site insensitivity to carbofuran and established quantitative sequencing (QS) methods that were successfully applied in head lice (Kwon et al., 2008) and two-spotted spider mite (Kwon et al., 2015) studies for the rapid detection of resistance-associated gene allele frequencies. We also validated the resistance factors with a correlation analysis of phenotypic resistance parameters (i.e., resistance ratio, enzyme activity, and AChE inhibition rate by carbofuran) vs. qualitative traits (i.e., point mutation allele frequency) or quantitative traits (i.e., gene copy number and target gene transcript level) in consecutively selected strains.(Bao et al., 2013)

2. Materials and methods

2.1. Strains and Insect rearing

The susceptible strain (SUS) was obtained from the National Academy of Agricultural Science (Jeonju, South Korea), which has been maintained since 2003 without exposure to any insecticides. The field populations were collected from Gimpo city, Haenam county, and Taean county, South Korea, by sweeping rice fields with a collection net during August and September 2015. These three strains

were mixed into a single cage and designated as the SEL0 strain to obtain a carbofuran-resistant strain via consecutive selection. All strains were reared on rice seedlings (var. Chucheong; 15~20 days after germination) in acrylic cages (26 × 30 × 20 cm) at 26 ± 1°C with a long-day photoperiod (16:8 light:dark).

2.2. Bioassay

Carbofuran (Chem Service, Inc., West Chester, PA, USA) was applied topically to determine the median lethal dose (LD_{50}) of several *L. striatellus* strains. Carbofuran was dissolved in acetone and serially diluted in the range of 25~1,000 ppm. Each concentration was tested on 15 individual 2~4-day-old females by applying 0.1 μL of carbofuran with a droplet to parts of the thorax of individuals that had been anesthetized with carbon dioxide for 30 s using a PB600-1 repeating dispenser (Hamilton Company, Reno, NV, USA). Carbofuran-treated *L. striatellus* were placed in glass tubes (6.3 diameter × 15 cm) with rice seedlings. Mortality was assessed after 24 h and the LD_{50} was calculated using the PoloPlus program (LeOra Software LLC, Parma, MO, USA). The resistance ratio was obtained by dividing the LD_{50} of the resistant strain by that of the SUS strain.

2.3. Carbofuran selection

Carbofuran selection was performed nine times following the seedling soaking

and spray methods. The selected strains were designated consecutively as SEL1, SEL2, … SEL9 following the carbofuran selection frequencies. The seedling soaking method was performed using a commercial carbofuran product (Furadan, Kyung Nong Inc., Seoul, South Korea) up to SEL3 strain. The selection pressure was empirically chosen by brief mortality screening using commercial product. Briefly, 0.2 g of carbofuran granules was dissolved in 1 mL of 100% ethanol for 5 min and the solution was subsequently diluted with 400 mL of water (final concentration: 500 ppm). The solution was carefully poured into square containers (21.5 × 17.5 × 2.5 cm) to inoculate 14~16 days rice seedlings containing 400~500 *L. striatellus* individuals. The carbofuran solution was replaced with tap water after 48 h to prohibit excessive *L. striatellus* mortality. The approximate mortality of adults was 20~50% in each selection after 48 h.

We changed the selection method from SEL4~SEL9 strain by spray method by using the selection pressures. The selection pressure were determined by the bioassay methods using the technical-grade carbofuran. The spray method was applied for the consecutive selection of strains SEL4~SEL9 using technical-grade carbofuran dissolved in acetone. First, a 100-ppm carbofuran solution dissolved in 0.1% Triton X-100 was sprayed on rice seedlings and dried for 30 min. Then, carbofuran-treated rice seedlings were inoculated with approximately 300 third-instar nymphs for 24 h, after which live nymphs were transferred to untreated rice

seedlings.

2.4. Protein extraction

Females (10~14 mg) from the SUS, SEL0, and SEL9 strains were homogenized in 350 μL extraction buffer (0.1 M Tris-HCl, 0.02 M NaOH; pH 7.8) using a Bullet Blender (Next Advance, Inc., Averill Park, NY, USA) and the homogenate was centrifuged at 12,000 × g for 15 min. The supernatant was used as the enzyme source for the EST and AChE activity measurements. Protein concentrations were determined using a BCA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

2.5. Biochemical activity assay and enzyme activity

EST activity was measured following the method of Van Aspern (Van Asperen, 1962) using two substrates, 1-naphthyl acetate (1-NA) and 4-nitrophenyl acetate (4-NPA), with slight modifications. For the activity measurement with 1-NA, 7.5 μg of enzyme was incubated with 0.4 mM of 1-NA in a total volume of 100 μL for 5 min. The reaction was stopped by adding 50 μL of stop solution (0.2% fast blue RR salt and 0.2% sodium dodecyl sulfate) and its activity was measured at 562 nm using a VersaMax microplate reader (Molecular Devices LLC, San Jose, CA, USA). For the activity measurement using 4-NPA, 7.5 μg of enzyme was incubated with

0.125 mM of 4-NPA for 5 min and measured at 405 nm.

AChE activity was measured following the method of Elman (Ellman et al., 1961) with slight modifications. In a total reaction volume of 100 μL, 15 μg of AChE was incubated with 0.4 mM DTNB and 1 mM of acetylthiocholine iodide (ATChI) for 30 min, and the solution was measured at 415 nm. The inhibition assay was conducted by preincubation of the enzyme source within a carbofuran concentration range of 6 × 10-3 to 2.0 μM for 30 min. The median inhibitory concentration (I_{50}) was calculated by preincubating the enzyme source (15 μg protein) with various concentrations of carbofuran (0.005 nM to 2.0 μM) for 30 min before adding 1 mM ATChI and 0.4 mM DTNB. The remaining AChE activity was measured as described above. The I_{50} was determined by Probit analysis using SPSS software (IBM Corp., Armonk, NY, USA) with three replicates. All experiments included three biological replicates.

2.6. Total RNA and genomic DNA extraction

Genomic DNA (gDNA) and total RNA were extracted from the SUS, SEL0, and SEL3~SEL9 strains. Each sample was collected independently with three replicates. Approximately 20 female adults (2~4 days old) were homogenized with 200 μL of Tri reagent (Sigma-Aldrich) using a Bullet Blender according to the manufacturer's instructions. The extracted total RNA (10 μg) was treated with 4

units of DNase I (Takara Korea Biomedical Inc., Seoul, Korea) to avoid contamination of gDNA, and then used for the synthesis of single-stranded complementary DNA (cDNA) using a Superscript IV cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was stored in a deep freezer at -80°C until experimentation. The remaining intermediate phase from the total RNA extraction was used for gDNA extraction according to the manufacturer's protocol with slight modifications. Briefly, the intermediate phase was precipitated with the same volume of 100% ethanol and centrifuged for 15 min at 12,000 × g. The gDNA in the precipitant was extracted with a gDNA extraction kit (GeneAll Biotechnology, Seoul, South Korea), where the precipitant was completely dissolved in 200 μL of Buffer A (GeneAll Biotechnology) and 100 μL of ethanol according to the manufacturer's instructions. gDNA was isolated using a binding column and eluted with elution buffer.

2.7. Mutation screening of type-1 ace cloned from *L. striatellus* and haplotypes

A partial fragment (~1,658 bp) of the *Lsace1* clone was obtained using the homology probing method (Kwon et al., 2012). Briefly, degenerate primers showing high homology of amino acid sequences with hemipteran insects were used to obtain two partial fragments (200 bp and 500 bp) (Table 1). The 3′ ends of the cDNA were amplified with rapid amplification of the cDNA ends (RACE) using

a SMART RACE kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The sequences of 5′-end RACE is referred to the GeneBank under the accession no. KC470080.1. PCR amplification was conducted in 20-μL reactions with 250 μM dNTPs, 10~20 μM primers, 50~200 ng cDNA template, and 1 unit of Advantage® 2 DNA polymerase mix (Clontech) with 35 cycles under the following conditions: 95°C for 30 s, 55~62°C for 30 s, and 68°C for 70~180 s. Partial sequence fragments (860 bp) were compared to screen for mutations in *L. striatellus* and *Nilaparvata lugens* by PCR amplification of the partial sequences under the aforementioned conditions using the 5_LSAChE1_Scr and 3_LSAChE1_Scr primers (Table 1) in three strains (SUS, SEL0, and SEL9). Homogenous PCR products were purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA) and directly sequenced using an ABI Prism 3730 DNA sequence analyzer (PE Applied Biosystems, Foster City, CA, USA). Heterogeneous PCR products obtained from the 3′ RACE were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and individual positive clones were sequenced. Haplotype analysis of two mutations was conducted by obtaining PCR fragments of the partial fragment for QS from two individual SEL3 specimens (Individual A and B) exhibiting high mutation frequencies and possessing the F330Y and F331H mutations in simultaneously on the chromatograms (signal ratio of individual A: 0.6 and 0.3 for F330Y and F331H, respectively; signal ratio of individual B: 0.3 and 0.2 for F330Y and F331H, respectively). Cloning was performed as described above.

Table 1. Primers used in this study.

Purpose	Primer name	Oligonucleotides	Size (bp)	Remarks
Lsace1 cloning and mutation screening	5'PLAce1[N]	GARAAYGARTGGGGNACNYT	cDNA & gDNA: 306, nd	
	3'PLAce1[N]	ARCCARTCNGTRTAYTCRAA		
	5'AChE1(for)	ATHCCNTAYGCNCARAARCC	cDNA & gDNA: 993, nd	Fragment 'a', 'b' and 'd' by Kwon et al., 2011
	3'LSAChE1(N)	ATTCGTCCCTGTTCACGTAGA		
	5'LSAChE1-3'RACE	TGTCGACGGCACCTTCCTCGACGACA	cDNA & gDNA: 1102, nd	
	5'LSAChE1-3'RACE(N)	AATGGGTAGTAACACGGAGGA		
	5_LSAChE1_Scr	TCGGAGGACTGTCTCTACATA	cDNA & gDNA: 860, nd	Major mutation screening
	3_LSAChE1_Scr	CCAATCAGTGTACTCGAACACA		
	5_LS_Ace1_G119	TCGGAGGACTGTCTCTACA	cDNA: 398; gDNA: 1316, 1738	Intron Variation check
	3_LS_Ace1_G119	GCATGATAGCCTGACTGAACA		
QS	5_LSAChE1_F331_2	CCAACATCCTAATGGGTAGTA	cDNA & gDNA: 284	QS standard template amplification
	3_LS_AChE1_F331_R0	CCTATGCGCCAGCTCATTGACAT		
	3_F331_R0_QS_seq	CGTGAACTGATAATCTCCCATCA	nd	For QS mutation sequencing
qPCR	betatublin3_5_F	GACAATGAGGCACTCTACGAC	cDNA & gDNA: 93	1st qPCR reference
	betatublin3_5_R	CATGGTGAGAGACACAAGG		
	5_SBPH_RPS3a	TTGAGGGCCAACTCGTCTTC	cDNA & gDNA: 81	2nd qPCR reference
	3_SBPH_RPS3a	GCGCTTCGTCATGGACAATC		
	5_LSAChE1_FYqPCR	GTAACACGGAGGAAGGCTA	cDNA & gDNA: 93	Lsace1 transcript and gene copy number
	3_LSAChE1_FYqPCR	GGAATTCGTCCCTGTTCAC		
	5_LSAChE2_qPCR	ATGCATCACAGTGACCCGTT	cDNA & gDNA: 96	Lsace2 transcript and gene copy number
	3_LSAChE2_qPCR	CGGTGAACACGTGTACCTCA		
	5_LSCar1_qPCR_1	CTTTTCCGCTTCGCACAACA	cDNA & gDNA: 99	LsCarE1 transcript and gene copy number
	3_LSCar1_qPCR_1	CTCAATTGTGCGGTTGGGTG		

2.8. Establishment of quantitative sequencing

The QS methods generally followed the methods of Kwon (Kwon et al., 2008). Briefly, 284-bp intron-free partial PCR products yielding three point mutations (F330Y, F330S, and F331H) were amplified, and susceptible and resistant templates were prepared via cloning following the methods described above. PCR amplification was conducted in 20-μL reaction mixtures containing 250 μM dNTPs, 5 μM of each primer, 25~50 ng gDNA template, and 1 unit AmpONETM-α-Taq DNA polymerase (GeneAll Biotechnology) for 35 thermal cycles under the following conditions: 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The amplified PCR product was directly TA-cloned into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. Plasmids were purified from positive clones, and nucleotide sequencing was determined via cycle sequencing (NICEM, Seoul, South Korea). Three representative amplicons were prepared, and the signal ratios of each mutation were prepared in proportions of 0, 0.17, 0.33, 0.50, 0.67, 0.83, and 1.0 by mixing of the three templates. The signal ratio of each mutation was read and calculated using chromatogram software (Chromas, Technelysium Pty. Ltd., Austrailia). The observed versus expected signal ratios were plotted against each other and the best fitting linear or non-linear equation was calculated using SigmaPlot software (Systat Software, Inc., San Jose, CA, USA).

2.9. Determination of transcription level, gene copy number, and mutation allele frequency

The transcription levels of *Lsace1*, *Lsace2*, and *LsCarE1* and gene copy number of *Lsace1* were determined by quantitative real-time PCR (LightCycler® 96; Roche, Basel, Switzerland) in 10-μL reaction mixtures containing 3~15 ng cDNA or gDNA template, 2 μM primers (Table 1), and 5 μL SYBR reaction solution (Takara Korea Biomedical Inc.). The PCR conditions were as follows: one cycle at 95°C for 30 s; 30 cycles with three steps at 95°C for 5 s, 58°C for 10 s, and 72°C for 10 s; and a melting step from 60~90°C at an increment of 1°C/s. The amplification efficiencies of both the references and target genes were 1.8~2.0, and the relative transcript levels were determined by the 2-ΔΔCT method (Schmittgen and Livak, 2008) using *β-tubulin* and *RPS3* as references.

2.10. Statistical analysis

Analysis of variance with Tukey's test was performed to identify statistical differences in the average transcript level and gene copy number among strains using SPSS software (IBM corporation).

3. Results

3.1. Carbofuran resistance development

The LD_{50} values of the five strains ranged from 5.5 to 77.7 ng per insect (Table 2), where the LD_{50} of the field population (SEL0) was 1.2-fold higher than that of the SUS population. By contrast, after the ninth selection, the SEL9 strain exhibited a 14-fold greater resistance than the SUS strain and 11.7-fold greater resistance than the SEL0 strain. These results suggested that the SEL0 strain showed consecutive selection from carbofuran insecticide treatment

Table 2. Carbofuran insecticide toxicity profiles determined using topical application in five *L. striatellus* strains.

Strain	Selection pressure(ppm)	Selection method	N	LD_{50} (ng/insect) (95% CL)	Slope±SE	RR** by SUS	RR by SEL0
SUS	0	nd*	385	5.5 (4.5-6.9)	1.7±0.2	1	nd
SEL0	0	nd	243	6.7 (4.5-11)	2.7±0.3	1.2	1.0
SEL3	50	Root dipping	176	7.8 (3.4-27.6)	1.2±0.2	1.4	1.2
SEL6	100	Spray	150	42.9	1.4±0.3	6.8	5.7
SEL9	125	Spray	193	77.7 (32.3-116.8)	2.1±0.5	14	11.7

* Not determined

** RR represent the resistance ratio which was determined by dividing with LD_{50} of SUS strain.

3.2. Comparison of enzymatic activity

Next, the EST and AChE activities of five strains were assessed. The SEL9 strain had the highest EST activity against 1-NA and 4-NPA as substrates, which were approximately 1.2- and 1.4-fold those of the SUS strain, respectively, although the differences were not significant (Table 3). The SEL9 strain exhibited higher AChE activity as 1.36-fold than SUS strain but not significant (Table 3). However, it revealed the significantly higher activity (1.7-fold) comparing those of SEL0 strain.

Table 3. General esterase and acetylcholinesterase activity against two substrates in three *L. striatellus* strains.

Enzyme (Substrate)	Enzyme activity of strain (OD/mg/sec)		
	SUS	SEL0	SEL9
Esterase (1-NA)	0.274 ± 0.017 [a *] (1.0)[**]	0.330 ± 0.015 [a] (1.2)	0.336 ± 0.038 [a] (1.2)
Esterase (4-NPA)	0.109 ± 0.013 [a] (1.0)	0.143 ± 0.014 [a] (1.3)	0.157 ± 0.033 [a] (1.4)
AChE (AChI)	0.012 ± 0.003 [ab] (1.0)	0.010 ± 0.0006 [a] (0.8)	0.017 ± 0.002 [b] (1.36)

[*] Statistical difference by Tukey's test ($P < 0.05$)
[**] The value in parenthesis represent the relative ratio which was divided by the value of SUS strain.

3.3. Carbofuran inhibition against *AChE*

We compared the enzyme inhibition rates of three strains. The SEL9 strain showed a 4.3-fold greater insensitivity than the SUS strain (Table 4) and 3.6-fold greater insensitivity than the SEL0 strain, suggesting the existence of target site insensitivity against AChE mediated by a point mutation in AChE.

Table 4. Median acetylcholinesterase inhibition concentration (I_{50}) of carbofuran in *L. striatellus*.

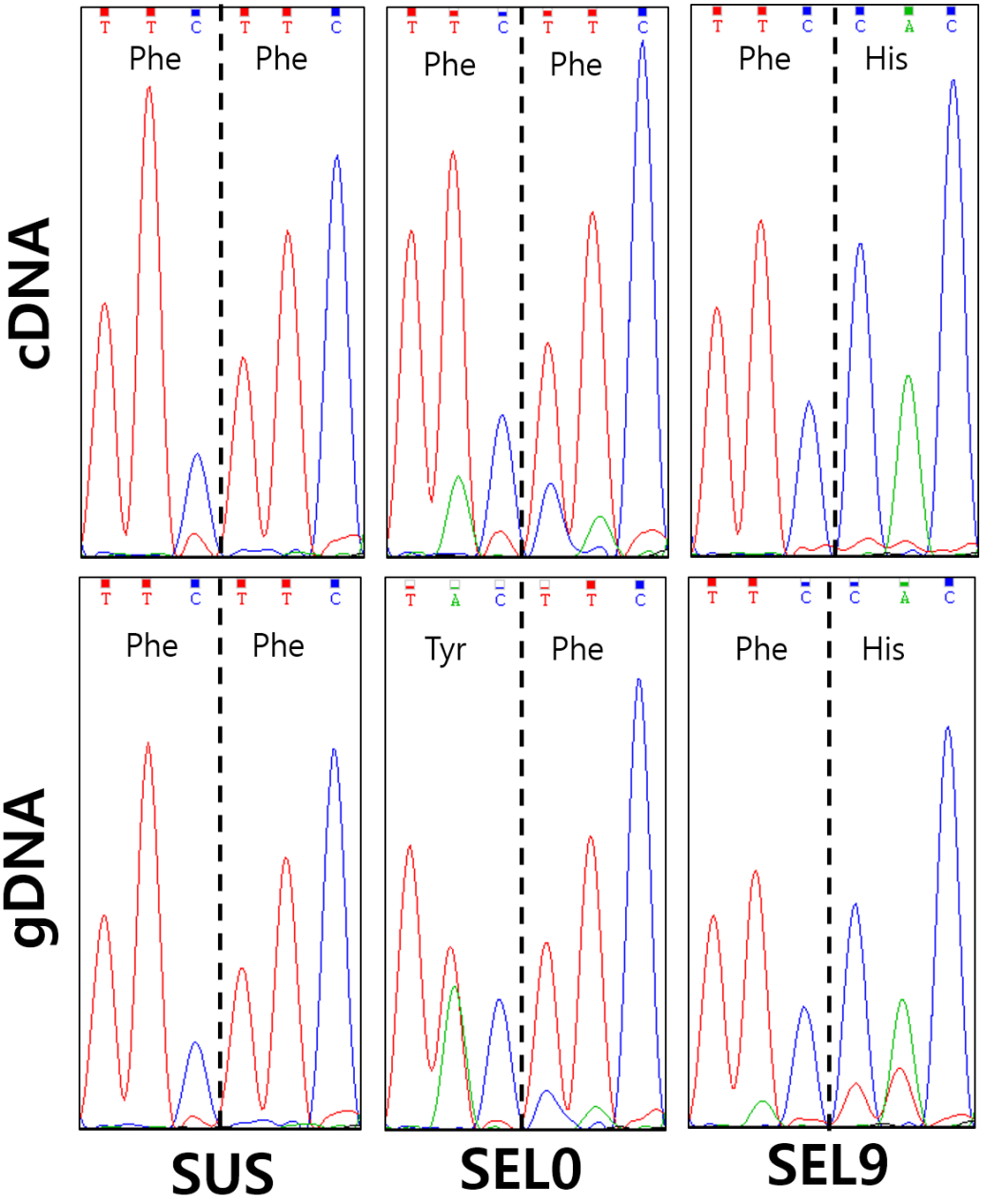
Insecticide	I_{50} (μM)		
	SUS	SEL0	SEL9
Carbofuran	0.077 ± 0.019 [a*] (1.0) [**]	0.091± 0.017 [a] (1.2)	0.327 ± 0.02 [b] (4.3)

[*] Statistical difference by Tukey's test ($p < 0.05$)
[**] The value in parenthesis represent the relative ratio which was divided by the value of SUS.

3.4. Point mutation screening and haplotype analysis of *Lsace1*

We compared point mutations in the type-1 AChE gene of three strains. Two mutations, F330Y (T**T**C-to-T**A**C substitution) and F331H (**TT**C-to-**CA**C substitution), were observed in *Lsace1* from SEL0 (Fig. 1). F331H has been previously reported in a chlorpyrifos-selected strain (Zhang et al., 2013a). To understand the haplotype composition between two different amino acid

Fig 1. Comparison of point mutation region in *Lsace1* of 3 strains (SUS, SEL0, and SEL9). It shows F330 and F331 region of *Lsace1* in each strain.



substitutions, we conducted the haplotype analysis using 15 clones from two representative individuals possessing two point mutations from the SEL3 strain. The expected haplotype compositions were designated as LS-A (F330 + F331), LS-B (F330Y + F331), LS-C (F330 + F331H) and LS-D (F330Y + F331H) haplotypes (Table 5). In the haplotype analysis, the LS-B and LS-C haplotypes were each observed in proportions of approximately 46.7%. One clone of individual A possessed the LS-E (F330Y + F331S) haplotype, with a phenylalanine (T**T**C)-to-serine (T**C**C) substitution at position 331. No haplotype of LS-D was observed that simultaneously possessed the F330Y and F331H mutations (Table 5). Interestingly, individual A possessed three different haplotypes, LS-B, LS-C, and LS-E, suggesting the existence of gene duplication of *Lsace1* in *L. striatellus*.

Table 5. Haplotype composition from 15 clones of *Lsace1* from two SEL3 strain individuals. The underlining bold alphabet represent the nucleotide substitution corresponding to F330Y, F330S and F331H.

Haplotype (Amino acid substitution and combination)	Individual A (N=5)	Individual B(N=10)	Total (= 15)
LS-A {F(TTC)330+ F(TTC)331}	0	0	0
LS-B {F(T**T**C)330Y(T**A**C) + F(TTC)331}	2	5	7
LS-C {F(TTC)330 + F(**TT**C)331H(**CA**C)}	2	5	7
LS-D* {F(T**T**C)330Y(T**A**C) + F(**TT**C)331H(**CA**C)}	0	0	0
LS-E {F(T**T**C)330Y(T**A**C) + F(T**T**C)331S(T**C**C)}	1	0	1

* Not observed but expected haplotype.

3.5. QS regression and allele frequency dynamics of the two mutations in the selection strains

We determined the QS regression equations of F330Y and F331H for the rapid determination of mutation allele frequencies. Both equations had high correlation coefficients of 0.971 and 0.985, respectively (Fig. 2), indicating their reliability. In the previous haplotype analysis, the F331 (TTC) position was substituted with two alternative mutations, histidine (CAC) and serine (TCC) (Table 5). Therefore, we used the second nucleotide, which could distinguish three amino acids for the accurate determination of the allele frequency, for further analysis.

Based on the established QS equation, we determined the allele frequency of both mutations from cDNA and gDNA templates in the nine strains (SUS, SEL0, and SEL3~SEL9). With carbofuran selection, the F330Y substitution was not significantly increased, whereas the F331H substitution increased (Fig. 3). Moreover, the saturation of F331H in mRNA occurred more rapidly than in gDNA. No saturation of F331H in the SEL9 strain was observed in gDNA. These results suggest that F331H could be a major factor corresponding to target site insensitivity in *Lsace1*, while F330Y might not be directly associated with carbofuran resistance.

Fig 2. Quantitative sequencing regression of F330Y and F331H in *Lsace1*.

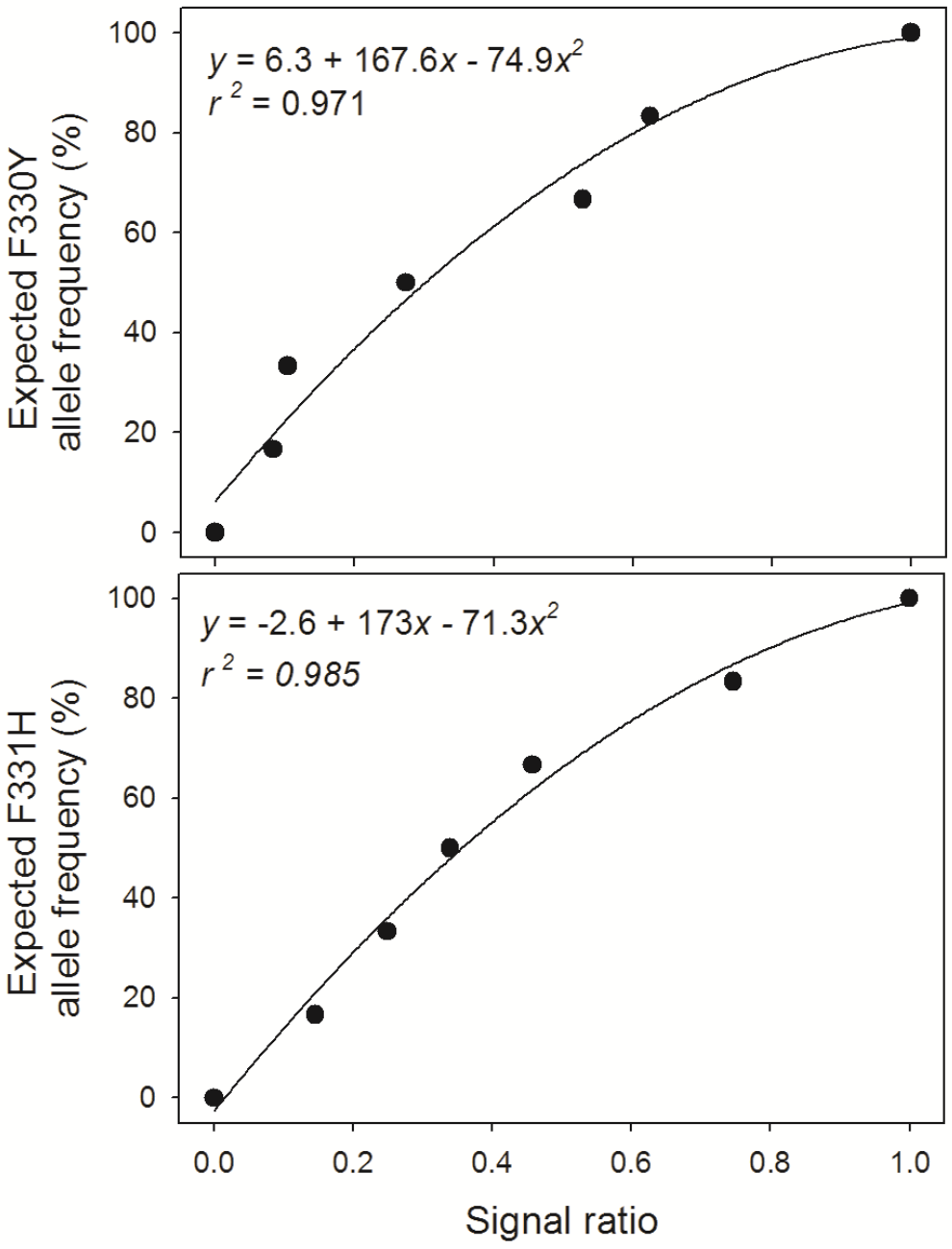
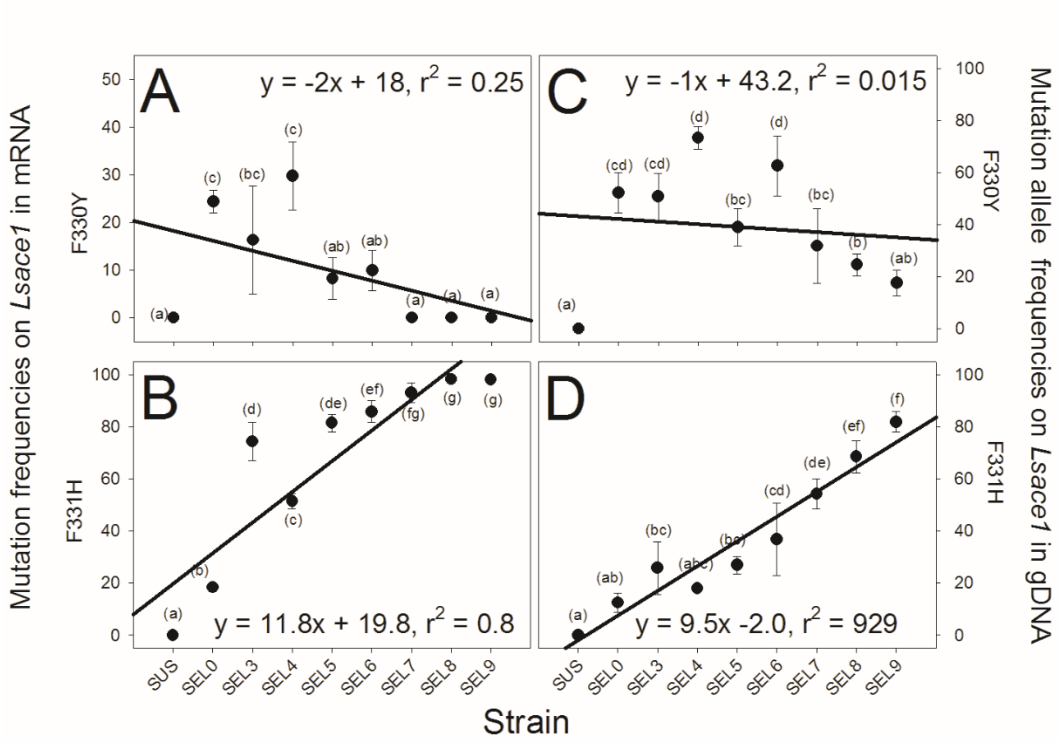


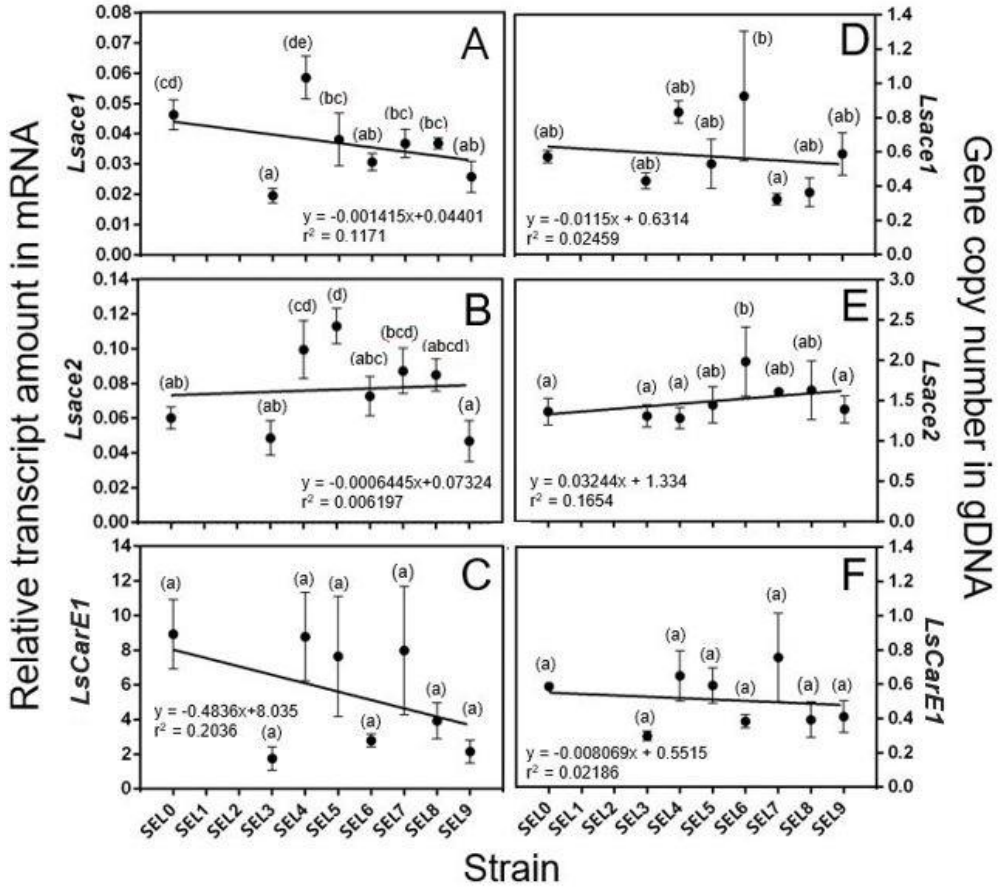
Fig 3. Mutation frequencies of two point mutations (F330Y and F331H) on *Lsace1* in cDNA and gDNA.



3.6. Dynamics of *Lsace1*, *Lsace2*, and *LsCarE1* based on mRNA levels and gene copy number

To understand the dynamics of the qualitative traits in terms of gene expression and effects on carbofuran resistance, we measured the gene copy number and transcript levels of three genes (*Lsace1*, *Lsace2*, and *LsCarE1*) (Fig. 4). The mRNA levels of both the *Lsace1* and *Lsace2* genes were not significantly changed in

Fig 4. Comparison of relative transcript amount on cDNA and gene copy number on gDNA in 9 strains (SUS, SEL0, …, SEL9).



the SEL9 strain compared with the other strains (Fig. 4A and 4B). Also, there was no significant reduction in the gene copy numbers of gDNA (Fig. 4D and 4E). The mRNA levels and gene copy numbers of *LsCarE1*, a known chlorpyrifos resistance factor, showed changes of 0.7~2.9-fold and 0.5~1.7-fold, respectively; however,

there were no significant differences among the nine strains (Fig. 4C and 4F). Thus, quantitative genetic traits of *Lsace1*, *Lsace2* and *LsCarE1* are unlikely to be associated with the development of carbofuran resistance.

3.7. Correlation analysis of phenotypic resistance and the genotypic strains

We conducted a correlation analysis to identify the resistance factors conferring carbofuran resistance in *L. striatellus* (Fig. 5). A significant positive correlation was observed between the LD_{50} with EST activity, I_{50}, and F331H allele frequency based on mRNA and gDNA levels (Fig. 5). In addition, the I_{50} was correlated with F331H allele frequency. Interestingly, a significant negative correlation was observed between F330Y and F331H allele frequencies based on mRNA and gDNA levels.

These results indicate that carbofuran resistance can be developed based on target site insensitivity mediated by F331H on *Lsace1*.

4. Discussion

We investigated the carbamate resistance mechanism in *L. striatellus* via correlation analysis of several phenotypic and genotypic traits in consecutively selected strains. The F331H substitution in *Lsace1* exhibited strong correlations

with several phenotypic traits (see the LD_{50}-based resistance ratio and I_{50}-based insensitivity rate). The same point mutation was reported in a chlorpyrifos-resistant strain showing 214-fold resistance and 5-fold insensitivity against AChE (Wang et al., 2010a; Zhang et al., 2013a). The F331H substitution is located in an acyl pocket near the active site gorge of AChE, which confers reduced hydrophobicity to the substrate, resulting in the disruption of the regular role of catalytic histidine. The AChE activity of SEL9 revealed significantly higher activity than those of SEL0 about 1.7-fold (Table 2). It is necessary to confirm the F331H role by using the purified AChE which was prepared in vitro expression system for further analysis.

The observed frequency of the other point mutation, F330Y, was 24.4% (mRNA basis) in the SEL0 strain, which disappeared completely in the SEL9 strain. Considering its strong negative correlation with the F331H mutation, it might not be directly associated with carbofuran resistance. Interestingly, its gDNA allele frequency was 17.7% in the SEL9 strain (Fig. 3C), suggestive of post-transcriptional regulation of *Lsace1* mRNA expression by the existence of alternative exons or multiple copy of *Lsace1*. Further analysis is necessary to understand its basal mechanism.

Gene duplication of AChE has been proposed as a resistance mechanism in many insect species (Lee and Kwon, 2011). In *Tetranychus urticae*, the extensive gene duplication of *ace1* was correlated with mRNA expression related to

monocrotophos resistance (Kwon et al., 2010). In *L. striatellus*, the existence of gene duplication is supported by the three haplotypes in individual A (Table 5).

The copy number variations (CNVs) were observed on the *Lsace1* and *LsCarE1* showing low gene copy number in a few strains (SEL7 and SEL8 in *Lsace1*; SEL3 in *LsCarE1*) by qPCR (Fig. 4). We checked the gene copy number and its structure of two genes from the genome database of *L. striatellus* (Zhu et al., 2017). The *Lsace1* and *LsCarE1* were composed with two {evm.model.Contig214.6 (528 a.a.) and evm.model.Contig2913.1 (92 a.a.) showing 94% and 97% identities with AGN31549.1, respectively} and three {evm.model.Contig855.1 (1449 a.a.), evm.model.Contig1004.5 (230 a.a.), evm.model.Contig229.18 (222 a.a.) showing 99%, 85% and 73% identities with ADR73023.1, respectively} partial fragments, respectively, suggesting that the nucleotide sequence for two genes might not be completed (data not shown). However, the two partial fragments of *LsCarE1*, evm.model.Contig1004.5 and evm.model.Contig229.18, were obtaining identical partial nucleotide sequences (about 87 nucleotide) with the *LsCarE1* which was locating different contig on genome, suggesting the existence of multiple gene of the *LsCarE1*. It is necessary to understand the CNVs role after confirmation of each gene structure and their intrinsic gene copy number by employing long-read sequencing technology for further analysis.

The increase in the F331H allele frequency was negatively correlated with mRNA levels of *Lsace1* based on selection frequency (Fig. 5). Considering the negative correlation between the F330Y and F331H allele frequencies from cDNA and gDNA templates, some individuals possessing the LS-B haplotype might be preferentially chosen during carbofuran selection. But, it is difficult to clarify the specific haplotype reduction of LS-B with respect to carbofuran resistance, and the functional role of each mutation and amount should be clarified based on their *in vitro* expression.

In addition to target site insensitivity conferred by point mutation and gene duplication, EST is considered as a minor factor of carbamate resistance. The activity of EST based on two substrates was increased 1.2-fold in the SEL9 strain compared to the SUS strain (Table 3). Similar results have been reported for carboxylesterase in organophosphate- and carbamate-resistant strains (Endo and Tsurumachi, 2000). Zhang (Zhang et al., 2013a) suggested that overexpression of *LsCarE1* was also related to organophosphate resistance. In this study, we assessed the transcript levels and gene copy numbers of *LsCarE1* in the nine strains to identify its relationship with carbofuran resistance. We observed 1~3-fold variations; however, there were no significant correlations based on selection pressure (Fig. 5). Thus, *LsCarE1* is unlikely to be associated with carbamate resistance, suggesting that other genes are associated with resistance in these strains.

As such, it is necessary to identify other resistance factors via transcriptome analysis.

Carbamate is a top-selling commercial insecticide used widely to control *L. striatellus*; therefore, it is necessary to periodically monitor for carbamate resistance. In this study, we established a QS method for the rapid detection of resistance associated with the point mutations F330Y and F331H in several selected strains. This method can be used to determine carbamate resistance in various field specimens.

In summary, we investigated the carbamate resistance mechanism using molecular and biochemical approaches. F331H was the major point mutation associated with target site insensitivity, showing a strong correlation with resistance levels and the I_{50}. Therefore, it is an appropriate genetic marker for the determination of carbofuran resistance in conjunction with molecular diagnostic techniques such as QS in *L. striatellus*. Moreover, it can be applied for the rapid detection of carbamate and organophosphate resistance in both indigenous and migratory insect populations.

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CHAPTER 2.

Identification of putative genes associated with carbofuran resistance in *L. striatellus*

Identification of putative genes associated with carbofuran resistance in *L. striatellus*

Abstract

Laodelphax striatellus is an important pest of rice due to not only sucking rice seedlings, but also transmitting serious plant viruses. Among various kinds of insecticide groups (carbamates, organophosphorus, neonicotinoids, etc.), carbofuran, a systemic carbamate insecticide, has been most extensively used to control rice pests including *L. striatellus*, resulting in widespread carbamate resistance in Korea and other Northeast Asia countries. To identify the genes associated with carbofuran resistance, we obtained a 14-fold higher resistant strain (SEL9) from the mixed-field population (SEL0) by consecutive selection. A transcriptome-based analysis was conducted and differentially expressed genes (DEG) were compared between the SEL9 and SEL0 strains. A total of 93,531,356 clean reads were analyzed, of which 75,567,939 reads were mapped. From these reads, 28,332 transcripts were annotated. DEG analysis between SEL0 and SEL9 strain showed total 327 up-regulated genes and total 275 down-regulated genes and among these genes, gene ontology (GO) analysis was performed and identified the function of 365 genes (more 2-fold changes and $p < 0.2$). Also, we got the list of total 24 up-regulated and 15 down-regulated genes, which were in statistical condition ($p < 0.05$, $q < 0.15$) and Log_2FC value (> 1, < -1).

1. Introduction

Data on transcripts and expression levels are important for understanding the biochemical characteristics of pests. Transcriptome analysis has been an effective tool for understanding the mechanisms of resistance development at the molecular level. To this end, transcriptome analyses have been carried out in various pest species, including *Nilaparvata lugens* (Bao et al., 2012; Xue et al., 2010), *Plutella xylostella* (Gao et al., 2018; He et al., 2012; Lin et al., 2013), *Bemisia tabaci* (Karatolos et al., 2011; Wang et al., 2010b; Xie et al., 2012), *Frankliniella occidentalis* (Zhang et al., 2013b) and *Anopheles sinensis* (Zhu et al., 2014) to several kinds of insecticides such as pyrethroid (deltamethrin and cypermethrin), organophosphate (chlorpyrifos), phenylpyrazole (fipronil), neonicotinoid (imidacloprid, dinotefuran and thiamethoxam), diamide (chlorantraniliprole), spinosyn (spinosad) and oxadiazine (indoxacarb).

In the case of *Laodelphax striatellus*, transcriptome analysis has been used to find molecular factors involved in various insecticide resistance such as deltamethrin, buprofezin, chlorpyrifos, ethiprole and imidacloprid, through comparison of gene expression levels between susceptible and resistance strains. Analysis of the gene expression level of the selected resistant strains by continuous insecticide exposure revealed that CYP5AY3v2, CYP6FU1 and LSCE12 were involved in deltamethrin resistance (Xu et al., 2013). Overexpression of the

CYP6CW1 and *carboxylesterase-1* gene was found to be involved in the buprofezin (Zhang et al., 2012) and chlorpyrifos resistance (Zhang et al., 2013a), respectively. In the case of ethiprole resistance, overexpression of the *CYP4DE1* and *CYP6CW3v2* genes was also found to be responsible (Elzaki et al., 2015). In addition, *CYP6AYv2*, *CYP306A2v2*, *CYP353D1v2* and *LSCE36* genes were also involved in chlorpyrifos resistance (Xu et al., 2014). Especially overexpression of *CYP353D1v2* gene was also found to be related in imidacloprid resistance (Elzaki et al., 2017).

In this study, we obtained carbofuran resistance strain by selection process, and the transcriptome data was compared between resistance and susceptible strains using DEG and GO analysis. Identified genes that are possibly involved in carbofuran resistance would be helpful to understand mechanisms of resistance development and establish resistance management strategy.

2. Materials and methods

2.1. Strains and insect rearing

The field strain of *L. striatellus* used for transcriptome analysis was obtained by combining insects collected from three domestic fields (Gimpo city, Haenam

country and Taean country). In addition, the carbofuran-selected strain (SEL9) along with the progenitor strain (SEL0) were also used for transcriptome analysis. All insects were reared at 24 ° C, 60% humidity and a 16-h light: 8-h dark cycle.

2.2. Transcriptome samples preparation

To synchronize the age of *L. striatellus* for transcriptome analysis, approximately 100 fifth instar larvae were transferred into a plastic cage (28 cm × 20 cm × 18 cm) containing rice seedling pad (15 × 18 cm). After 24 hours, newly emerged adults were transferred into a new rearing cage and reared for additional 4 days. Total RNA was extracted from10-15 short-wing females with 200 μl of Trizol (Sigma-Aldrich). Following extraction, DNase I (Takara Korea Biomedical Inc., Seoul, Korea) was treated to remove contaminated gDNA according to the manufacturer's protocol.

2.3. Total RNA quality check, library construction and sequencing

Total RNA quality was assessed by analyzing rRNA band integrity using an Agilent RNA 6000 Nano kit (Agilent Technologies, CA). Before making the cDNA library, poly(A)RNA was enriched using total RNA and magnetic beads with Oligo dT. Thereafter, the purified mRNA was size-fractionated and double-stranded cDNA was synthesized. cDNA was subjected to end-repair for poly A addition and

ligated with sequencing adapters using the TruSeq Stranded mRNA sample prep kit (Illumina, CA). The fragments with desired size, which were automatically purified by BluePippin 2% agarose gel cassette (Sage Science, MA), were used as template for PCR amplification. The size and quality of the final library were assessed by electrophoresis using the Agilent High Sensitivity DNA kit (Agilent Technologies, CA), and the fragment was found to be 350 to 450 bp. The generated library was sequenced using an Illumina HiSeq2500 sequencer (Illumina, CA).

2.4. Transcriptome data filtering and sequence alignment

Reads containing more than 10% of skipped bases (labeled as 'N'), reads containing more than 40% bases with less than 20 quality score and reads containing less than 20 for average quality score of each read were filtered out as low-quality reads. The filtered reads were then mapped to the *L. striatellus* genome (Zhu et al., 2017) using the aligner STAR v.2.3.0e (Dobin et al., 2013).

2.5 Gene expression estimation and differentially expressed gene (DEG) analysis

Gene expression levels were measured by Cufflinks v2.1.1 (Trapnell et al., 2009) using the gene annotation database of Ensembl release 72. Non-coding gene regions were removed using the -mask option. To improve the accuracy of the

measurements, multi-read-correction and fragbias-correct options were applied. Other options were set by default values.

For differential expression analysis, the gene level count data were generated using the HTSeq-count v0.5.4p3 (Anders et al., 2015) tool with the option "-m intersection-nonempty" and -r option considering paired-end sequence. Based on the calculated read count data, DEGs were confirmed using the R package called TCC (Sun et al., 2013). Tag count data was compared by the TCC package using robust normalization strategies. Normalization factors were calculated using the iterative DEGES / edgeR method. The Q-value was calculated based on the p-value using the p.adjust function of R package with default parameter settings. Differentially expressed genes were identified based on the q value threshold of less than 0.05.

3. Results

3.1. Assembly of transcriptome data by reference-based mapping

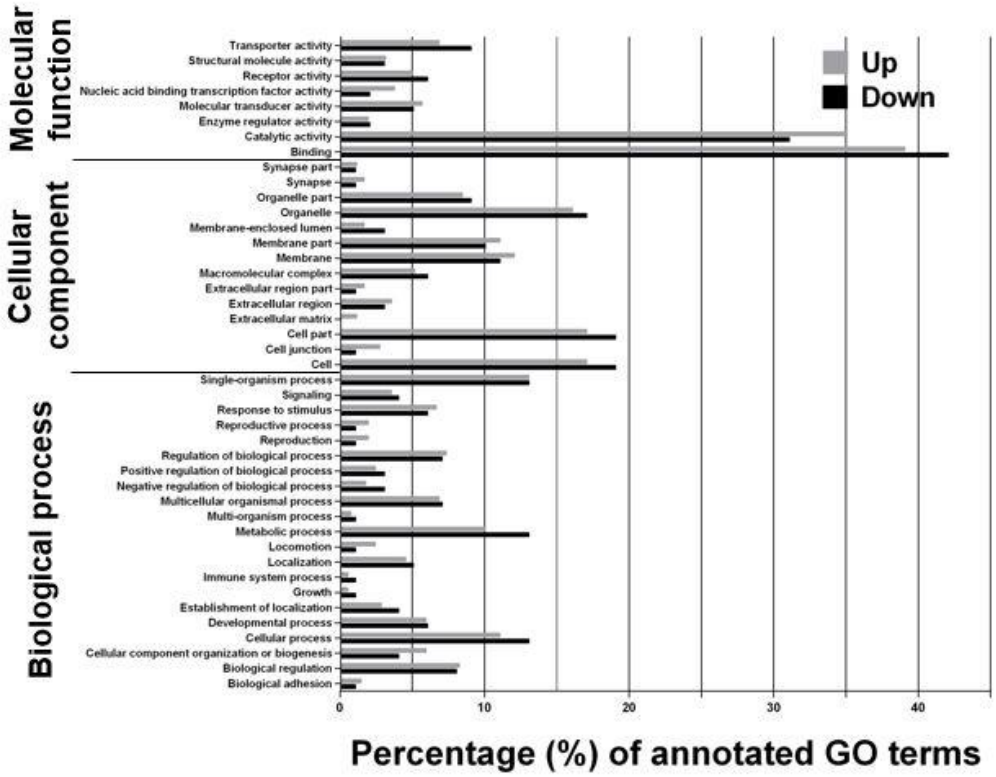
Transcriptome analysis was performed to compare SEL0 and SEL9 strains with the same genetic background. As a result, 46,449,648 and 49,735,502 raw reads

were obtained from the SEL0 and SEL9 strain samples, respectively. Of these, 45,159,328 (97.2%) and 48,372,028 (97.3%) of clean reads were obtained from the SEL0 and SEL9 strain samples, of which 36,643,874 (81.1%) and 38,924,065 (80.5%) reads were mapped to genes, respectively. Properly paired reads were 35,320,756 (78.2%) and 37,479,500 (77.5%), respectively. With these reads, 14,257 (92.8%) and 14,075 (91.7%) genes were annotated by the reference of 15,356 transcripts. Among the 602 differentially expressed genes (DEGs), 327 genes were up-regulated whereas 275 genes were down-regulated in SEL9 compared to SEL0.

3.2. GO profiles of DEGs

GO analysis was performed to investigate the role of 365 genes broadly selected from DEG analysis. The genes showing no detectable expression and with p value lower than 0.2 were not included for GO profiles. Genes related cellular component, molecular function, and biological process, were analyzed up to the level 2 GO term, and the GO term of up-regulated and down-regulated genes in the resistant strain were confirmed. Differences in the percentage of GO term of up-regulated and down-regulated genes were compared; however, there was no significant difference between the two groups. The categories of catalytic activity and binding have the largest number of both up- (35 and 39 %) and down- (31 and 42 %) regulated genes.

Fig 1. Gene ontology (GO) distribution of differentially expressed genes (DEGs) in carbofuran resistance strain. X axis mean the percentage of annotated GO terms. The gray bars mean GO terms for up-regulated genes, and the black bars mean GO terms for down-regulated genes.



3.3. DEGs in carbofuran resistance strain

Through the DEG analysis of SEL0 and SEL9 strains, a total of 24 up-regulated genes ($Log_2FC > 1$, $p < 0.05$, $q < 0.15$) (Table 1) and 15 down-regulated genes ($Log_2FC < -1$, $p < 0.05$, $q < 0.15$) (Table 2) were identified. The *60S ribosomal protein L32* (*RpL32*) showed the highest transcription level, followed by the fused toxin protein gene, *IVBI_SISCA* and the *Bardet-Biedl syndrome 5 protein homolog*, *bbs5*. However, it is unclear whether these genes are directly involved in insecticide resistance. Two cytochrome P450 (Cyp) genes (*Cyp6ER2* and *CYP4g15*) were up-regulated in the resistant strain and Log_2FC values were 2.3 and 1.04, respectively. In addition, the expression level of *Carboxylesterase* (*CarE*) gene was 1.2-fold higher in SEL9 strain. The *Uro* and *mixo* encoding proteins possessing oxidoreductase activity were also overexpressed and their Log_2FC values were 1.44 and 1.15 respectively. In contrast, *defensin* gene, *DEFI_PYRAP,* showed the lowest transcription level, followed by *atrial natriuretic peptide receptor 1* (*Npr1*) and *larval cuticle protein 14* (*LCP-14*). Among these, the transcription level of two *CarE* (*CarE12* and *CarE35*) were 2.2- and 2.3- fold lower in SEL9 strain than SEL0 strain.

Table 1. Up-regulated genes in carbofuran resistance strain ($p < 0.05$, $q < 0.15$, $Log_2FC > 1$). The cyp450 and carboxylesterase genes were marked with red and orange colors, respectively.

Gene ID	Gene name	Log2FC relative to control
		Up-regulated
TBIG008342	RpL32	4.56
TBIG007823	IVBI_SISCA	4.07
TBIG012586	bbs5	3.77
TBIG005681	ppk28	3.61
TBIG010124	Ssr4	3.23
TBIG006719	GARNL3	2.41
TBIG014838	Cyp6ER2	2.32
TBIG000810	Bai3	2.3
TBIG007329	BTBD6	2.08
TBIG014066	ENDOU_DROME	1.83
TBIG000686	RpS12	1.68
TBIG012787	Tpk1	1.61
TBIG003483	PRR3_JUNVI	1.57
TBIG014992	Uro	1.44
TBIG007557	Agmo	1.34
TBIG010992	BTBD3	1.33
TBIG002678	chac1	1.22
TBIG017167	RpS6	1.22
TBIG011485	CarE18	1.2
TBIG000013	miox	1.15
TBIG012806	SFRP5	1.13
TBIG010046	pug	1.08
TBIG007067	Cyp4g15	1.04
TBIG012254	awd	1.03

Table 2. Down-regulated genes in carbofuran resistance strain ($p < 0.05$, $q < 0.15$, $Log_2FC < -1$). The carboxylesterase and larval cuticular protein genes were marked with orange and green colors, respectively.

Gene ID	Gene name	Log_2FC relative to control
		Down-regulated
TBIG013537	DEFI_PYRAP	-3.04
TBIG016909	Npr1	-2.78
TBIG013390	LCP-14	-2.33
TBIG011483	CarE35	-2.3
TBIG005866	CarE12	-2.2
TBIG005803	psr-1	-1.89
TBIG009596	RpL8	-1.83
TBIG007578	KIAA0195	-1.38
TBIG004443	ACT_SPOLI	-1.36
TBIG000692	ND42	-1.28
TBIG017325	TTPAL	-1.2
TBIG009898	ATP5B	-1.17
TBIG008183	Gld	-1.05
TBIG013170	ZNF813	-1.04
TBIG003501	PebIII	-1.01

4. Discussion

As the result of GO analysis, we identified GO categories of up- and down-regulated genes. There was no difference in the percentage of the GO term between the two groups. However, the categories of catalytic activity and binding showed the highest proportion in both up-regulated and down-regulated genes, suggesting that the expression of genes possessing catalytic activity and binding function were changed during development of carbofuran resistance. Overexpression of genes

related to detoxification, which is the typical metabolic resistance factor, can be speculated to suppress the expression of other gens due to fitness cost.

Two cyp450 genes (*Cyp6ER2* and *Cyp4g15*) were overexpressed 4-fold and 2-fold in the resistant strain, respectively. *Cyp6ER*2 is known as a Cyp gene that responds to high temperature stress in *Sogatella furcifera* (Huang et al., 2017). Insect Cyp6 family genes are well known to be involved in insecticide metabolism (Feyereisen, 2006). In a study of transcriptome analysis in *Sogatella furcifera*, it was presumed that *CYP6ER2* was overexpressed in the high temperature condition as an antioxidant defense system activated by increasing oxidative stress (Huang et al., 2017). *Drosophila* Cyp4g1, a gene in the same Cyp4g family as *Cyp4g15*, in is known as a key enzyme in hydrocarbon biosynthesis and also is involved in acquiring physical resistance that inhibits the penetration of insecticide in DDT resistant strain (Kim et al., 2018). Similarly, *Cyp4g16* and *Cyp4g17* were overexpressed in a pyrethroid-resistant strain of *Anopheles gambiae* (Balabanidou et al., 2016). These genes were involved in alteration of cuticular haydrocarbon composition, thereby increasing cuticle thickness, which eventually results in resistance. In *Aphis mellifera*, Cyp4g11, which is an abundant gene in antennas, is associated with the production of cuticular hydrocarbons and the release of pheromones or physiochemicals. Therefore, Cyp4g15 of *L. strialtellus* would be

possibly involved in cuticular hydrocarbon biosynthesis and likely function as a penetration factor increasing resistance.

On the other hand, it is noteworthy that a larval cuticular protein, *LCP-14* was down-regulated. Considering that insects protect themselves against xenobiotics using limited resources, it is natural that they can down-regulate some non-essential genes to save their resources. With this in mind, the *LCP-14* could be down-regulated in the resistant strain as it is a relatively less important in adult stage. The two *CarE*s (*CarE12* and *CarE35*) also down-regulated in SEL9 are commonly known to show reduced expression in the resistant strain against chlorpyrifos, one of the organophosphate insecticide (Elzaki et al., 2016; Xu et al., 2013, 2014).

However, there was no difference in expression levels of the two *CarE*s between susceptible and other insecticides (deltamethrin and imidacloprid) resistant strains (Elzaki et al., 2016; Xu et al., 2013). The relationship between uricase and insecticide resistance has not been clarified yet. However, in case of *Nilaparvata lugens*, uric acid is used as a nitrogen source and the uricase of symbiotic bacteria is essential for growth. Without uricase, the metabolism of uric acid becomes problematic and the insects are not able to grow into the adult stage (Hongoh and Ishikawa, 1997; Hongoh et al., 2000; Sasaki et al., 1996). In addition, *uricase* is known to be changed commonly in three species of planthoppers following temperature treatment (Huang et al., 2017). Also, overexpression of inositol

oxygenase was confirmed in the resistant strain. However, the relationship between inositol oxygenase and insecticide resistance has not yet been clarified.

Further studies would be necessary to understand the exact functions of identified candidate genes in the development of carbofuran resistance.

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KOREAN ABSTRACT

애멸구에서 나타나는 아세틸콜린에스터레이즈 1 의 점 돌연변이와 카보퓨란 저항성 관련 후보 유전자의 동정

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초록

애멸구(*Laodelphax striatellus*)는 벼의 주요 해충으로서 어린 유묘를 흡즙하여 고사시킬 뿐만 아니라 벼에 치명적인 식물 바이러스를 매개하는 것으로 알려져 있다. 다양한 계통의 살충제 중, 침투이행성 카바메이트계 살충제인 카보퓨란은 애멸구를 비롯한 벼과 해충 방제에 광범위하게 사용되어 왔으며, 이에 따라 한국 및 다른 동북 아시아 국가에서 광범위하게 그 저항성이 보고되어 왔다. 그러므로 분자 진단 마커의 개발은 살충제 저항 관리를 위한 고효율 스크리닝 시스템을 확립하기 위하여 분자 진단 마커의 개발이 요구되고 있는 실정이다.

본 논문에서는, 카보퓨란으로 도태시킨 저항성 계통의 애멸구에서 카바메이트 저항성과 관련된 type-1 acetylcholinesterase(Lsace1) 내 점 돌연변이를 발견하였다. 미량 국소처리법을 이용하여 9 세대 도태시킨 SEL9 저항성 계통과 감수성 계통의 표현형 저항성을 비교한 결과, 저항성 비는 14 배, estarase 활성은 1.02 배, 중간 저해 농도(I_{50}) 값

기반 acetylcholinesterase 불감응성은 4.3 배 더 높음을 확인하였다. 이것은 표적 부위의 불감응성이 저항성 인자로서 발생할 수 있음을 시사한다.

또한 5 개의 애멸구 계통(SUS, SEL0, SEL3, SEL6, SEL9)의 Lsace1 유전자 서열을 비교하여 2 가지 유형의 아미노산 치환체(F330Y 와 F331H)를 발견하였다. 또한 정량적 시퀀싱 방법을 사용하여 도태 저항성 계통에서 해당 두 가지 점 돌연변이의 대립 유전자 빈도를 확인하고, *Lsace1* 과 *Lsace2, LsCarE1* 에 대한 유전자 카피 수 및 발현량을 조사하였다. 그 결과, F331H 유전자 변이와 저항성 수준은 I_{50} 와 밀접한 관련이 있음을 확인하였다. 따라서, F331H 돌연변이와 발현량의 감소는 카바메이트 저항성 발달에 중요한 요인으로 보인다. 또한 해당 점 돌연변이는 양적 시퀀싱과 같은 분자 진단 방법과 함께 신속한 카보퓨란 저항성의 모니터링에 사용될 수 있을 것이다.

한편, 카보퓨란 저항성과 관련된 분자생물학적 요인을 확인하기 위하여 야외 계통(SEL0)과 연속적인 도태를 통하여 획득한 저항성 계통(SEL9)을 사용하여 전사체 분석을 실시하였다. 총 96,185,150 개의 read 중 62,860,430 개의 read 가 mapping 되었고, 28,332 개의 염기서열을 annotation 하였다. 차등발현 된 유전자(DEG)를 통계적 조건($p < 0.05$, $q < 0.15$) 및 Log_2FC 값(> 1, <-1) 하에 조사한 결과, SEL0 에 비해 SEL9 에서 발현량이 높은 24 종의 유전자와 발현량이 낮은 15 종의 유전자를 확인하였다. 또한 GO 분석을 수행하여, 차등 발현된 유전자들이 높은 비율로 촉매 활성 혹은 다른 분자들과 결합할 수 있는 능력을 지니고 있음을 밝혔다.

검색어: 아세틸콜린에스터레이즈, 카보퓨란 저항성, *Laodelphax striatellus*, 점 돌연변이, 전사체 분석, 사이토크롬 P450, 유충 큐티클 단백질, 카복실에스터레이즈, 차별적 발현 유전자(DEG), 유전자 온톨로지(GO)

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