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Molecular Mechanism of ABC Transporter *Mdr49A* Associated with a Positive Cross-Resistance in Transgenic *Drosophila*

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형질전환 초파리를 이용한 *Mdr49A* 유전자의 살충제 교차저항성 기능 구명

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ABSTRACT: The ATP-binding cassette (ABC) transporter superfamily represents the largest transmembrane protein that transports a variety of substrates across extra- and intra-cellular membranes. In insects, the ABC transporter proteins play crucial roles in insecticide resistance. To date, no studies have investigated the involvement of ABC transporter gene for cross-resistance to insecticide chemistries. Here, we studied such possible mechanisms against six conventional insecticides using transgenic *Drosophila melanogaster* strains carrying *Mdr49* transcript variant A. For the *91-R* and *91-C* strains of *Drosophila melanogaster*, although they have a common origin, *91-R* has been intensely selected with DDT for over 60 years, while *91-C* has received no insecticide selection. Our transgenic analyses showed that overexpression of *91-R-MDR49* transcript variant A along with three amino acid variations can yield a relatively low degree of cross-resistance to carbofuran (2.0~6.7-fold) and permethrin (2.5~10.5-fold) but did not show cross-resistance to abamectin, imidacloprid, methoxychlor, and prothiofos as compared to the *Gal4-driver* control strain without transgene expression. These results indicate that the overexpression of *Mdr49A* in itself leads to a cross-resistance and three amino acid changes have additional effects on positive cross-resistance to carbofuran and permethrin.

Key words: ABC transporter, Multi-drug resistance, Cross-resistance, Transgenic fruit fly

초 록: ATP-binding cassette (ABC) transporter는 다양한 기질을 세포 밖과 세포 안으로 수송하는 대표적인 수송단백질이다. 곤충에서 ABC transporter는 살충제에 대한 저항성을 발달시키는 중요한 역할을 한다. 현재까지 모델곤충인 초파리를 대상으로 ABC transporter의 살충제 교차저항성에 관한 연구는 많이 수행되어오지 않았다. 본 연구에서는 ABC transporter에 속하는 *Mdr49A* 유전자가 여섯 종류의 살충제에 보이는 교차저항성 기작을 형질전환 초파리를 이용하여 구명하였다. 초파리 91-R과 91-C 계통은 공통된 조상으로부터 유래되었으며 91-R은 60년 이상 DDT에 노출되었지만 91-C는 어떠한 살충제에도 노출되지 않고 유지되어 왔다. 91-R 계통의 MDR49A 단백질에서 유래된 3개의 아미노산 돌연변이를 형질전환 초파리에 과발현 시켰을 때 carbofuran에 대해서 2.0~6.7배 그리고 permethrin에 대해서 2.5~10.5배의 교차저항성을 나타낸 반면 다른 약제, abamectin, imidacloprid, methoxychlor, prothiofos에 대해서는 어떠한 교차저항성도 나타내지 않았다. 이상의 결과는 *Mdr49A* 유전자의 과발현과 더불어 3개의 아미노산 돌연변이는 두 개 약제, carbofuran과 permethrin에 대해 교차저항성 기능을 한다고 제시하고 있다.

검색어: 형질전환 초파리, ABC수송체, 교차저항성

Insecticide resistance has been extensively studied as a natural phenomenon in a variety of pest species. This phenomenon is defined as a population genetic response to natural or artificial selection pressure due to the presence of toxic substances that damage pest control strategies in the field (Brown, 1959). Particular emphasis has been placed on insecticide resistance in order to discover genetic variations involved in the resistant

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phenotype in several insect species.

Insecticide detoxification processes can generally be divided into three phases. Phase I reactions include oxidation, hydrolysis, and reduction through the actions of cytochrome P450s (Feyereisen, 1999). In phase II, the byproducts of phase I can be neutralized with endogenous compounds, including sugars, amino acids, and glutathione by enzymes such as gluththione-S-transferase (GSTs; Li et al., 2007). While a number of studies have indicated that increased activity of P450s, GSTs, and esterases is a major mechanism of insecticide resistance in different insect populations (Pittendrigh et al., 1997; Feyereisen, 1999; Vontas et al., 2001), more recently, research has focused on ABC transporter genes, which play a role in phase III detoxification process (Aller et al., 2009).

Cellular membrane-spanning proteins, known as ATP-binding cassette (ABC) transporters, actively translocate a variety of substrates across cellular membranes in both prokaryotes and eukaryotes (Aller et al., 2009). ABC-B subfamily associated with multiple drug resistance (*MDR*) genes efflux a wide array of different molecules including insecticides, amino-acids, sugars, and peptides to contain intracellular concentrations of harmful toxicants below cytotoxic levels (Buss and Callaghan, 2008). Typical ABC transporters include two highly conserved nucleotide-binding domains (NBDs) that bind and hydrolyze ATP to provide energy as well as two transmembrane spanning domains (TMDs) that provide specificity and affinity on diverse substrates along with ligand biding sites (Rees et al., 2009).

The involvement of ABC transporters in insecticide-resistant phenotypes has been identified for many agricultural or medical insect species, such as *Cimex lectularius* (Zhu et al., 2013), *Pediculus humanus* (Lee et al., 2010), and *Lygus hesperus* (Hull et al., 2014). The overexpression of *Mdr* genes has also been documented in the resistant-strain *Drosophila melanogaster* (hereafter referred to as *Drosophila*; Pedra et al., 2004) and *Tribolium castaneum* species (Broehan et al., 2013). Recently, Gellatly et al. (2015) showed that several ABC transporter genes of subgroups B (*Mdr50* and *Mdr65*) and C (*Mrp1*) were overexpressed under DDT exposure in the DDT-resistant *91-R* strain of *Drosophila* as compared to the DDT-susceptible *Canton-S* strain. Moreover, work by Seong et al. (2016) suggests that amino acid changes in one protein isoform of MDR49 plays a temporal role in the DDT-resistance mechanism for the DDT-resistant *91-R* strain in comparison to the DDT-susceptible *91-C* strain.

Although ABC subgroup B proteins are involved in the multidrug resistance phenotypes in *Drosophila* and other insects, how or if the function of these *Mdr* genes also confers cross-resistance to a wider range of pesticides remains unknown. In many cases, insect populations exhibiting high levels of resistance to specific insecticides have also shown relatively high resistance to other insecticides that have never been exposured (Wilson and Cain, 1997). This phenomenon, called cross-resistance, describes how a resistance factor for one insecticide can also confer resistance to others. Miyo et al. (2000), for instance, demonstrated that genetic variations involved in DDT insensitivity across numerous *Drosophila* species also influenced resistance factors for organophosphate and pyrethroid insecticides.

While Seong et al. (2016) reported that an *Mdr49* transcript variant B plays a temporal role in DDT resistance, the involvement of *Mdr49* transcript variant A in cross-resistance to other insecticides remains unclear. The aim of this study was to investigate whether transgenic overexpression with amino acid substitutions of a *Mdr49A* transcript variant, deriving from *91-R*, play a role in cross-resistance against a set of six commonly used insecticides.

Materials and Methods

Drosophila Strains and Insecticides

DDT-resistant *91-R* and DDT-susceptible *91-C Drosophila* strains were obtained from Dr. Ranjan Ganguly (University of Tennessee-Knoxville). Both strains were maintained on a commercially available medium (Jazz-Mix *Drosophila* Food, Fischer Scientific, Cat. No. AS153) under the conditions of 25 ± 1°C, 55-70% relative humidity and a 14 h light /10 h dark cycle. *91-R* has been continually selected by rearing the flies in colony bottles with the presence of a 150 mg DDT impregnated filter paper disk, while *91-C* was reared without any exposure to insecticides. Six technical-grade insecticides were obtained from Sigma-Aldrich (St. Louis, MO, USA); abamectin, carbofuran, imidacloprid, methoxychlor, permethrin, and prothiofos for the mortality bioassay.

Transgenic Expression of *MDR49A* in *Drosophila* Strains

We used the same transgenic fly strains previously used by Seong et al. (2016). Three amino acid alterations (T374I, M388L, and E666D) were identified for *Mdr49* transcript variant A between *91-C* and *91-R* strains (Seong et al., 2016). The full-length ORFs for *Mdr49* were amplified from the cDNA of the DDT-resistant *91-R* and -susceptible *91-C* using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen, CA) and cloned into the pCR2.1 TOPO vector (Invitrogen, CA). Five positive clones from both strains were purified with a QIAprep Miniprep kit (Qiagen, Valencia, CA, USA) and sequenced to validate the correct amino acid sequences for *Mdr49A*. After sequence analysis, one clone for a *Mdr49A* gene was selected for constructing the transgenic fly strains. Each clone of *Mdr49A* from *91-C* and *91-R* strains was digested using the Kpn1 and XbaI (New England Biolabs, MA) and subcloned into the pUAST vector. Transgenic flies were generated and balanced with *CyO* for chromosome 2 and *TM3* (*sb*) for chromosome 3 by the Best-Gene Inc (Chino Hills, CA) using the w^{1118} strain.

Two transgenic fly strains contained each ORF without transgene overexpression as follows: (1) *MDR49A* from *91-R* (*UAS-91-R-MDR49A*); (2) *MDR49A* from *91-C* (*UAS-91-C-MDR49A*). Transgenic *Drosophila* flies overexpressing *Mdr49A* from *91-R* and *91-C* were generated using the *Gal4-UAS* system from the cross between the male of *UAS-MDR49A* and virgin female of ubiquitous *Gal4-driver* strain (*P{w[+mC]= GAL4-elav.L}2/CyO*) obtained from Bloomington *Drosophila* Stock Center (Bloomington, IL) as follows: *Gal4-UAS-91-R-MDR49A* and *Gal4-UAS-91-C-MDR49A*.

Validation of *Mdr49A* Expression in the Transgenic Fly Strain by Reverse Transcriptase–quantitative PCR (RT–qPCR)

To confirm the overexpression of *Mdr49A* in the transgenic fly strains as compared to the absence of expression in the control strains, total RNA was extracted from three pools of fifteen flies per replicate (three biological replicates in total) using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen), which was followed by treatment of each sample with DNase I (Qiagen) to remove contaminating genomic DNA. The first-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Subsequent RT-qPCR reactions amplifying *Mdr49A* target were performed using a StepOnePlus Real-Time PCR system (Applied Biosystems Inc., Foster City, CA), with three technical replicates across all biological replicates. The ribosomal protein 49 (*rp49*) was used as an internal control. Primer information is listed in Table 1. Relative expression levels of *Mdr49A* transgene were analyzed using the $2^{-\triangle\triangle C(t)}$ method (Livak and Schmittgen, 2001) in the F1 progeny from crossing *UAS-91-R/C-MDR49A* with *Gal4-driver* strain and their respective controls (*UAS-91-R-MDR49A* and *UAS-91-C-MDR49A* parent strains). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests to compare the results between fly strains by XLSTAT (Addinsoft, NY, USA).

Insecticide Bioassay with Topical Applications

To establish whether both the overexpression of *Mdr49A* and three amino acid alterations (T374I, M388L, and E666D) can confer cross-resistance to six insecticides, insecticide toxicity for the transgenic *Drosophila* strains was assessed by topical applications using the procedure as follows. Females (1-5 days

Table 1. Sequences of the primers used for RT-qPCR

Gene	Direction	Sequence	Tm (˚C)	GC (%)	Amplicon size (bp)
Rp49	F	CGGATCGATATGCTAAGCTGT	64	48	184
	R	GCGCTTGTTCGATCCGTA			
Mdr49A	F	CCTGGTCGTTCTGAGTTGTG	67	55	102
	R	TCGGAGTAGGACTTCAGCTC			

old, mated) from F_1 transgenically overexpressing *Mdr49A* from *91-R* and *91-C* were selected as the experimental group for the mortality bioassays. A stock solution for each insecticide was prepared and diluted in acetone to a series of concentrations suitable for bioassays. Subsequently, 0.2 µl was applied to the pronotum of female using a handheld microapplicator (Hamilton Co., Reno, NV, USA). Insecticide-treated flies were transferred to vials capped with cotton plugs moistened with a 5% sucrose solution in distilled water. For each insecticide, five doses with thirty flies per dose were biologically repeated three times on different days. An acetone-only treatment was included in each replicate as a control. Flies were considered dead when all movement and leg twitching had ceased. Mortality from the separate tests conducted with each chemical was assessed at 24 hours post-treatment. Mortality data from the triplicate experiments were pooled and analyzed to generate median lethal does (LD_{50}), confidence intervals, and slopes by probit analysis with the XLSTAT program (Addinsoft; Finney, 1964). Resistance ratio (RR) for an insecticide was calculated by dividing the LD_{50} value of each transgenic strain by the LD_{50} value of *Gal4-driver* control strain. LD_{50} values of insecticides were considered significantly different ($P < 0.05$) even after a Bonferonni correction and if their 95% confidential limits did not overlap.

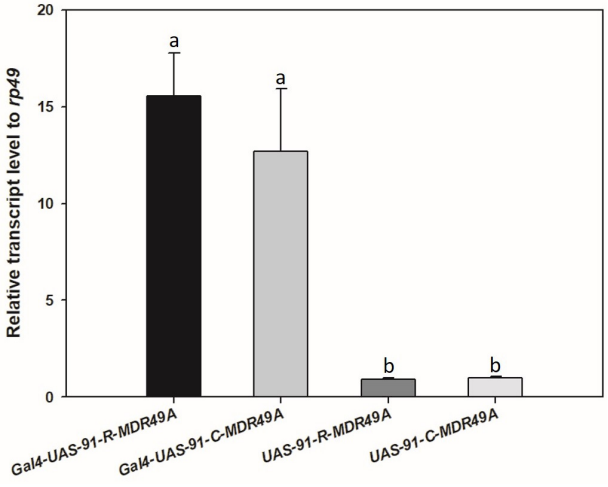

Fig. 1. The relative expression of the *Mdr49A* transgene in the transgenic *D. melanogaster* strains and the control strains with no transgene expression. The data shown are the mean ± standard error of the mean ($n = 3$).

Results

Transgenic Overexpression of *Mdr49A* in Transgenic *Drosophila* Strain

To confirm the overexpression of *Mdr49A* after crossing *Gal4-driver* strain with *UAS-MDR49A* strains, the overexpression of *Mdr49A* in adult flies was verified by RT-qPCR. The expression levels of *Gal4-UAS-91-R-MDR49A* and *Gal4-UAS-91-C-MDR49A* were significantly increased by 15.6-fold and 12.7-fold, respectively as compared to *UAS-91-R-MDR49A* and *UAS-91-C-MDR49A* parental strains ($F=149.6$, $df=3$, $P < 0.01$; Fig. 1).

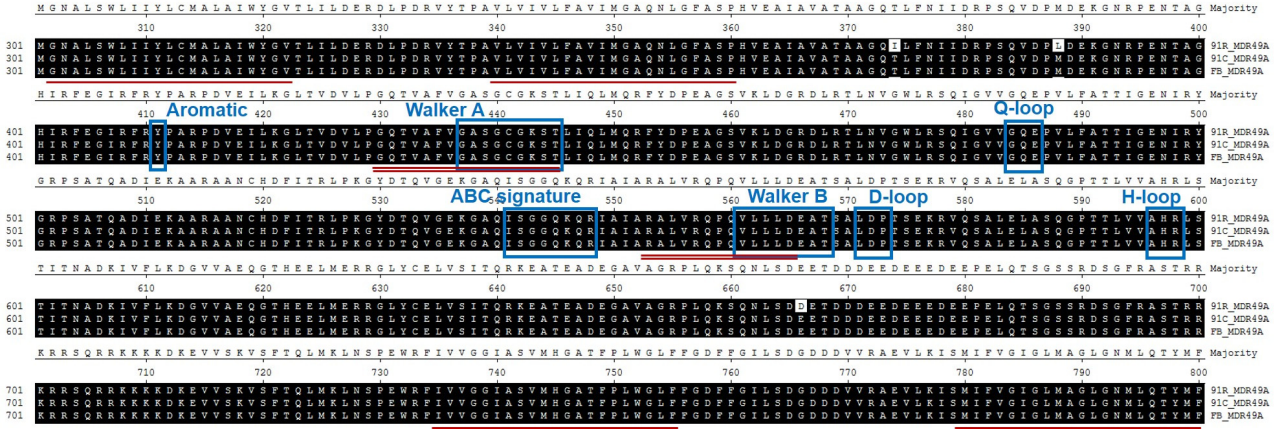

Fig. 2. Deduced amino acid sequence alignment of MDR49A from *D. melanogaster 91-R* and *91-C* strains. Amino acid changes were marked with white boxes. Walker-A/B, Q/D/H-loop, and ABC signature motifs are labeled with boxes. The predicted transmembrane and nucleotide-binding domains are marked with underlines and double underlines, respectively. FB indicates MDR49 amino acid sequence from Flybase (flybase.org).

Cross-resistance in the *MDR49A* Transgenic Strains

The structure variations (T374I, M388L, and E666D) were identified between *91-C* and *91-R* strains (Seong et al., 2016). All three mutations were observed in the intracellular loop between TMD 1 and TMD 2 (Fig. 2). Two transgenic strains (*UAS-91-R-MDR49A* and *UAS-91-C-MDR49A*) were crossed to a *Gal4-driver* strain, so that the each *Gal4-UAS-MDR49A* was ubiquitously overexpressed in the F1 progenies. Two transgenic fly strains overexpressing the MDR49A ORF of *91-C* and *91-R* with three mutations were subjected to bioassays to investigate the effect of the three amino acid variations and overexpression on susceptibility to six insecticides (abamectin, carbofuran, imidacloprid, methoxychlor, permethrin, and prothiofos).

Table 2. Resistance levels to six insecticides in the transgenic *Drosophila* strains with MDR49A from *91-R* and *91-C*

Insecticide	Strain	Slope (SE)	χ^2	LD_{50} (ng/fly; 95% CI[a])	Resistance ratio[b]
Abamectin	*Gal4-UAS-91-R-MDR49A*	1.2(0.2)	64.8	4,328.9 (3,390.1-5,885.4)	1.0
	Gal4-UAS-91-C-MDR49A	1.2(0.2)	68.7	4,665.13 (3,671.1-6,325.5)	1.1
	UAS-91-R-MDR49A	1.5(0.2)	5.3	4,379.9 (2,923.5-1,1021.2)	1.0
	UAS-91-C-MDR49A	2.1(0.2)	2.1	4,461.8 (3,825.4-5,434.4)	1.1
	Gal4-driver	1.9(0.2)	6.1	4,244.22 (2,953.3-8,675.9)	1.0
Carbofuran	*Gal4-UAS-91-R-MDR49A*	2.0(0.2)	154.9	**1.88 (1.6-2.2)***	**6.7**
	Gal4-UAS-91-C-MDR49A	1.3(0.3)	67.8	**0.57 (0.4-0.7)***	**2.0**
	UAS-91-R-MDR49A	1.6(0.2)	10.6	0.28 (0.2-0.3)	1.0
	UAS-91-C-MDR49A	1.8(0.2)	8.3	0.22 (0.1-0.3)	0.8
	Gal4-driver	1.8(0.2)	2.1	0.28 (0.2-0.3)	1.0
Imidacloprid	*Gal4-UAS-91-R-MDR49A*	1.9(0.2)	149.6	10.08 (8.6-11.8)	1.1
	Gal4-UAS-91-C-MDR49A	2.0(0.2)	157.0	12.17 (10.5-14.3)	1.3
	UAS-91-R-MDR49A	2.1(0.2)	16.6	10.34 (5.6-29.8)	1.1
	UAS-91-C-MDR49A	2.5(0.2)	18.5	8.01 (4.4-17.2)	0.9
	Gal4-driver	2.2(0.2)	3.0	9.25 (8.0-10.8)	1.0
Methoxychlor	*Gal4-UAS-91-R-MDR49A*	2.1(0.2)	167.7	45.77 (39.5-53.3)	1.0
	Gal4-UAS-91-C-MDR49A	1.8(0.2)	138.6	31.94 (26.9-37.6)	0.7
	UAS-91-R-MDR49A	1.5(0.2)	3.6	44.43 (30.6-63.6)	0.9
	UAS-91-C-MDR49A	1.9(0.2)	5.5	55.96 (38.9-82.9)	1.2
	Gal4-driver	2.0(0.2)	2.3	47.54 (40.7-55.5)	1.0
Permethrin	*Gal4-UAS-91-R-MDR49A*	1.3(0.1)	108.4	**5.8 (4.7-7.5)***	**10.5**
	Gal4-UAS-91-C-MDR49A	1.0(0.1)	71.8	**1.4 (1.0-1.8)***	**2.5**
	UAS-91-R-MDR49A	1.2(0.2)	2.6	0.78 (0.6-1.1)	1.4
	UAS-91-C-MDR49A	1.6(0.2)	3.8	0.49 (0.4-0.7)	0.9
	Gal4-driver	1.5(0.2)	4.6	0.55 (0.4-0.9)	1.0
Prothiofos	*Gal4-UAS-91-R-MDR49A*	2.5(0.2)	224.7	1.49 (1.3-1.7)	1.1
	Gal4-UAS-91-C-MDR49A	2.6(0.2)	226.9	1.31 (1.2-1.5)	1.0
	UAS-91-R-MDR49A	1.5(0.1)	2.6	1.64 (1.4-2.0)	1.3
	UAS-91-C-MDR49A	1.9(0.1)	11.0	1.21 (0.8-1.8)	0.9
	Gal4-driver	1.4(0.1)	0.2	1.30 (1.1-1.6)	1.0

[a]CI represents the confidence interval.
[b]The LD_{50} of each transgenic strain was divided by LD_{50} of *Gal4-driver* strain.
*An asterisk indicates resistance level is statistically higher as compared to *Gal4-driver* strain ($P < 0.01$).

Resistance levels were calculated as the values of the LD_{50} for each insecticide (Table 2).

While the LD_{50} values for four insecticides (abamectin, imidacloprid, methoxychlor, and prothiofos) were not significantly different among all transgenic fly strains, two transgenic strains *Gal4-UAS-91-R-MDR49A* and *Gal4-UAS-91-C-MDR49A* showed 6.7- and 2.0-fold more resistance to carbofuran as compared to *Gal4-driver* control strain: *Gal4-UAS-91-R-MDR49A* = 1.88 (1.6-2.2, 95% CI) ng per individual of LD_{50} value and *Gal4-UAS-91-C-MDR49A* = 0.57 (0.4-0.7, 95% CI) ng per individual of LD_{50} value (Table 2). For permethrin, similarly, the LD_{50} value was 5.8 (4.7-7.5, 95% CI) ng per individual for *Gal4-UAS-91-R-MDR49A*, with a resistance ratio of 10.5-fold and the LD_{50} value was 1.4 (1.0-1.8, 95% CI) ng per individual for *Gal4-UAS-91-C-MDR49A,* with a resistance ratio of 2.5 as compared to *Gal4-driver* control strain (Table 2).

Discussion

Several recent studies have shown that ABC transporters contribute to insecticide detoxification by decreasing toxic concentrations in several insect species (Buss and Callaghan, 2008). Despite extensive research into this resistance mechanism, the role of ABC transporters in cross-resistance has remained under-investigated. To the best of these authors' knowledge, the present study is the first to report specific transcript variant A of *Mdr49*, an ABC transporter, that confers positive cross-resistance to different types of insecticides and thus serves as a staging ground for future and ongoing work on positive cross-resistance mechanisms from the ABC transporter *Mdr49*.

ABC transporters in particular are also known to play an important role in cross-resistance for several classes of xenobiotics in other organisms; for instance, the cross-resistance to moxidectin, levamisole, and pyrantel was identified from the ivermectin-resistant *Caenorhabditis elegans* strains (James and Davey, 2009). This provides evidence that ABC transporters contribute to cross-resistance against several biocides. Similarly, bacterium (e.g., *Lactococcus lactis*) grown in the presence of toxicants like ethidium bromide, rhodamine, and daunomycin exhibited resistance to multiple structurally and functionally unrelated chemicals, suggesting that the cross-resistance could be due at least partly to active translocation of the toxicants (Bolhuis et al., 1994). Recently, Leprohon et al. (2011) suggested that that ABC-B4 subgroup (*MDR1*) overexpression may be involved in cross-resistance to miltefosine, an alkylphosphocholine drug able to affect daunomycin-resistant strains of the *Leishmania tropica* parasite. Consistent with previous studies, the findings in this study strongly indicate that the *Mdr49A* transcript variant contributes to cross-resistance in permethrin (a pyrethroid-class insecticide) and carbofuran (a carbamate-class insecticide) in transgenic fly strains.

For insects, differential expression patterns of P450 and GST genes typically are important metabolic systems associated with cross-resistance against several insecticides. For example, up-regulations of P450 and GST genes have been identified in *Drosophila* (Pedra et al., 2004) and *Anopheles gambiae* (Ole Sangba et al., 2017) as having a role in resistance to insecticides. Furthermore, enhanced expression of ABC transporters conferring pyrethroid and carbamate insecticide resistance have been previously documented in *Anopheles stephensi* (Epis et al., 2014a; 2014b), *Boophilus microplus* (Pohl et al., 2012), and *Myzus persicae* (Silva et al., 2012). Recently, Gellatly et al. (2015) identified differential expression patterns of the ABC-B subgroup (*Mdr50* and *Mdr65*) and the ABC-C subgroup (*Mrp*1) in the DDT-resistant *Drosophila 91-R* and DDT-susceptible *Canton-S* strains (Gellatly et al., 2015), suggesting that constitutive overexpression of these genes is a key factor in DDT resistance. Indeed, increased expression of ABC transporters involved in insecticide resistance at specific time points has been previously documented from *Anopheles stephensi* (Epis et al., 2014a; 2014b). Recent transcriptome studies also have shown that four ABC transporters were overexpressed in pyrethroid-resistant bedbug strains compared to pyrethroid-susceptible strains (Zhu et al., 2013). It is of note that the constitutive overexpression of ABC transporter genes can contribute to the development of insecticide-resistance phenotypes in insect species. Therefore, our transgenic overexpression of *Mdr49A* apparently leads to the cross-resistance to different types of insecticides in a transgenic *Drosophila* strain.

In addition to the overexpression of ABC transporter genes, it was reported that the amino acid mutations of ABC transporter genes are genetically linked to Cry toxins from *Bacillus thuringiensis* (*Bt*). Previously, Atsumi et al. (2012) demonstrated that a single amino acid mutation in an ABC transporter gene

causes resistance to the Cry1Ab in the *Bombyx mori* (Atsumi et al. 2012). Our results showed that the overexpression of *Mdr49A* with three amino acid changes in *Gal4-UAS-91-R-MDR49A* leads to more resistance to carbofuran and permethrin insecticides as compare to *Gal4-driver* strain with no transgene expression. These results indicate that the overexpression of *Mdr49A* in itself leads to a cross-resistance and three amino acid changes have additional effects on positive cross-resistance to carbofuran and permethrin.

Previous work by Seong et al. (2016) characterized that the *91-R* strain carries three unique amino acid mutations (T374I, M388L, and E666D) in the intracellular loop between two transmembranes for MDR49A protein variant as compared to *91-C* strain (Seong et al., 2016). Thus, one or a combination of the three amino acid mutations from MDR49A in *91-R* could be directly lead to enhancing the translocation of xenobiotics like permethrin and carbofuran across cellular membranes. Additionally, Seong et al. (2016) characterized different structural features in the transmembrane domains of MDR49A and MDR49B and demonstrated that only the MDR49B variant from *91-R* played a role in DDT resistance. Since transmembrane domains form ligand binding sites and provides the substrate specificities, this observed variation may afford unique specificity to structurally unrelated insecticides like DDT, permethrin, and carbofuran with respect to extracellular translocation. Also, the transport cycle for each *Mdr49* transcript variant may differentially bind specific substrates to a high-affinity pocket formed by the transmembrane domains and thus translocate its substrates across cell membranes as well. Additional experiments are needed to clarify this.

In conclusion, there are few studies that show the involvement of ABC transporter in cross-resistance to a wide range of insecticides in insects. From the evidence of the cross-resistance mediated by *Mdr49A* noted above, this suggests that transgenic overexpression of *Mdr49A* can directly result in a cross-resistance to carbofuran and permethrin. Furthermore, since the transgenic *Drosophila* strain carrying these three mutations leads to cross-resistance mechanism against these two insecticides, an additional effect of the three amino acid changes on cross-resistance has been suggested from our transgenic fly strains. To the authors' knowledge this represents the first documented case of transcript variants in an ABC transporter being the basis for positive cross-resistance to different classes of insecticides.

Finally, we make no claims as to the role of these mutations in cross-resistance in *91-R*.The *91-R* strain shows very little cross-resistance to pesticides (beyond the fact that it is DDT resistant), and *Mdr49* is not over-expressed in *91-R*. However, our results here show the potential to understand, beyond this study's scope, the basic functioning of MDR49, and amino acid changes in the protein, concerning different classes of pesticides through the use of transgenic *Drosophila*.

Statements for Authorship Position & contribution

Seong, K.M.: Kyungpook National University, Professor, Designed the research, conducted the experiments, and wrote the manuscript.

Pittendrigh, B.R.: Michigan State University, Professor, Conceived and designed the experiments, Contributed to the writing and editing of the manuscript.

All authors read and approved the manuscript.

Literature Cited

Aller, S.G., Yu, J., Ward, A., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I., Chang, G., 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323, 1718-1722.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M.R., Noda, H., 2012. Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin *Cry1Ab* in the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. U. S. A. 109, E1591-1598.

Bolhuis, H., Molenaar, D., Poelarends, G., van Veen, H.W., Poolman, B., Driessen, A.J., Konings, W.N., 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J. Bacteriol. 176, 6957-6964.

Broehan, G., Kroeger, T., Lorenzen, M., Merzendorfer, H., 2013. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. BMC Genomics 14, 6.

Brown, A.W., 1959. Insecticide resistance as a world problem. Can.

J. Biochem. Physiol. 37, 1091-1097.

Buss, D.S., Callaghan, A., 2008. Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. Pestic. Biochem. Physiol. 90, 141-153.

Epis, S., Porretta, D., Mastrantonio, V., Urbanelli, S., Sassera, D., De Marco, L., Mereghetti, V., Montagna, M., Ricci, I., Favia, G., Bandi, C., 2014a. Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector *Anopheles stephensi*. Sci. Rep. 4, 7435.

Epis, S., Porretta, D., Mastrantonio, V., Comandatore, F., Sassera, D., Rossi, P., Cafarchia, C., Otranto, D., Favia, G., Genchi, C., Bandi, C., Urbanelli, S., 2014b. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi*. Parasit. Vectors. 7, 349.

Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507-533.

Finney, D.J., 1964. Probit analysis: Statistical treatment of the sigmoid curve. Cambridge University Press, London.

Gellatly, K.J., Yoon, K.S., Doherty, J.J., Sun, W., Pittendrigh, B.R., Clark, J.M., 2015. RNAi validation of resistance genes and their interactions in the highly DDT-resistant 91-R strain of *Drosophila melanogaster*. Pestic. Biochem. Physiol. 121, 107-115.

Hull, J.J., Chaney, K., Geib, S.M., Fabrick, J.A., Brent, C.S., Walsh, D., Lavine, L.C., 2014. Transcriptome-based identification of ABC transporters in the western tarnished plant bug *Lygus hesperus*. PLoS One 9, e113046.

James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. Int. J. Parasitol. 39, 213-220.

Lee, S.H., Kang, J.S., Min, J.S., Yoon, K.S., Strycharz, J.P., Johnson, R., Mittapalli, O., Margam, V.M., Sun, W., Li, H.M., Xie, J., Wu, J., Kirkness, E.F., Berenbaum, M.R., Pittendrigh, B. R., Clark, J.M., 2010. Decreased detoxification genes and genome size make the human body louse an efficient model to study xenobiotic metabolism. Insect Mol. Biol. 19, 599-615.

Leprohon, P., Legare, D., Ouellette, M., 2011. ABC transporters involved in drug resistance in human parasites. Essays Biochem. 50, 121-144.

Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol. 52, 231-253.

Livak, K.J., Schmittgen, T.D., 2011 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle C(t)}$ method. Methods 25, 402-408.

Miyo, T., Akai, S., Oguma, Y., 2000. Seasonal fluctuation in susceptibility to insecticides within natural populations of *Drosophila melanogaster*: Empirical observations of fitness costs of insecticide resistance. Genes Genet. Syst. 75, 97-104.

Ole Sangba, M.L., Sidick, A., Govoetchan, R., Dide-Agossou, C., Osse, R.A., Akogbeto, M., Ndiath, M.O., 2017. Evidence of multiple insecticide resistance mechanisms in *Anopheles gambiae* populations in Bangui, Central African Republic. Parasit. Vectors 10, 23.

Pedra, J.H., McIntyre, L.M., Scharf, M.E., Pittendrigh, B.R., 2004. Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 101, 7034-7039.

Pittendrigh, B.R., Aronstein, K., Zinkovsky, E., Andreev, O., Campbell, B., Daly, J., Trowell, S., Ffrench-Constant, R.H., 1997. Cytochrome P450 genes from *Helicoverpa armigera* : Expression in a pyrethroid-susceptible and -resistant strain. Insect Biochem. Mol. Biol. 27, 507-512.

Pohl, P.C., Klafke, G.M., Junior, J.R., Martins, J.R., da Silva Vaz Jr, I., Masuda, A., 2012. ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus (Boophilus) microplus*. Parasitol. Res. 111, 2345-2351.

Rees, D.C., Johnson, E., Lewinson, O., 2009. ABC transporters: the power to change. Nat. Rev. Mol. Cell. Biol. 10, 218-227.

Seong, K.M., Sun, W., Clark, J.M., Pittendrigh, B.R., 2016. Splice form variant and amino acid changes in MDR49 confers DDT resistance in transgenic *Drosophila*. Sci. Rep. 6, 23355.

Silva, A.X., Jander, G., Samaniego, H., Ramsey, J.S., Figueroa, C.C., 2012. Insecticide resistance mechanisms in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) I: A transcriptomic survey. PLoS One 7, e36366.

Vontas, J.G., Small, G.J., Hemingway, J., 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochem. J. 357, 65-72.

Wilson, T.G., Cain, J.W., 1997. Resistance to the insecticides lufenuron and propoxur in natural populations of *Drosophila melanogaster* (Diptera: Drosophilidae). J. Econ. Entomol. 90, 1131-1136.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456.