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Molecular Detection of Toxicodynamic and Metabolic Factors Associated with the Pyrethroid and Carbamate Resistance in *Myzus persicae* (Sulzer)

복숭아혹진딧물의 피레스로이드와 카바메이트계 살충제 저항성요인의 분자적 진단

By

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

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ABSTRACT

The green peach aphid (*Myzus persicae*) is an economically serious pest of agricultural and horticultural crops all over the world and developed resistance almost all kinds of insecticides. In this study, target site mutations were investigated by sequencing the voltage-gated sodium channel (*vgsc*) and acetylcholinesterase (*ace*) genes in 7 and 10 local strains collected in 2010 and 2011, respectively. In addition, leaf dip bioassay was conducted to determine LC_{50} values of bifenthrin and methomyl. Based on the mutation survey, quantitative sequencing (QS) was employed for investigation of resistance allele frequencies in local strains. Mutation survey of the *vgsc* revealed that M918L and L1014F mutations were present in local strains. Especially, M918L mutation showed a higher correlation than L1014F

between the resistance allele frequency and actual resistance level. Thus, QS using newly identified M918L mutation should facilitate the detection and monitoring of pyrethroid resistance levels in the field.

As putative mutations for target site insensitivity to carbamates, frequencies of A301S and S431F mutations in *ace* were investigated by sequencing. However, only S431F mutation was revealed in local strains. QS was employed to detect S431F mutation frequency. Correlation analysis of mutation allele frequency versus actual resistance level revealed that S431F mutation itself does not appear to play a significant role.

To identify whether higher expression of *M. persicae* carboxylesterase (CbE) E4 is due to gene duplication, gene copy number was determined by quantitative real-time PCR. To determine CbE E4 quantity, Western blotting in conjunction with activity staining were conducted. Correlation analysis was carried out to investigate the possible connection between CbE expression level and its gene copy number, activity and protein quantity. Qualitative changing from point mutation was considered and mutation survey was conducted by PCR. However, new mutation G134C was revealed in oxyanion hole. To predict the functions of this mutation, altered CbE E4 3D structure was predicted and correlation analysis was conducted using sequence chromatogram of G134C mutation. Finally, we expect that altered CbE E4 might play a significant role to hydrolyze and/or sequestrate

the carbamate insecticide in *M. persicae*.

Key words: *Myzus persicae*, *M. persicae*, insecticide resistance, Voltage-gated sodium channel, acetylcholinesterase, quantitative sequencing, carboxylesterase

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LITERATURE REVIEW

1. *Myzus persicae*

The green peach aphid is one of the most serious aphid pests of crops. The life cycle varies depending on the presence of winter season and could develop from nymph to adult within 12 days (van Emden et al. 1969). As in the case of aphid, *M. persicae* can transmit more than 100 plant viruses including potato leafroll virus (PLRV) and cucumovirus (Chen and Francki 1990; Eigenbrode et al. 2002). Thus, *M. persicae* causes not only direct damage but also indirect damage by transmitting plant viruses (Robert et al. 2000).

Resistance development to various insecticides in *M. persicae* has been reported in many countries, including Greece, Italy, Scotland, UK, and Japan (Kwon et al. 2009). Imidacloprid resistance of *M. persicae* has been reported by several studies (Puinean et al. 2010; Srigiriraju et al. 2010). In South Korea, organophosphate and carbamate resistance in *M. persicae* has been reported since 1985 (Choi 1985; Choi and Kim 1986; Kwon et al. 2009). In spite of increasing level of pyrethroid resistance in Korea, detailed information on *M. persicae* pyrethroid resistance mechanisms and resistance distribution in Korea has not been reported. Therefore, it is still important to investigate resistance mechanisms, particularly ones mediated by target site insensitivity, to major insecticides used to control *M. persicae* and

characterization of target site mutations associated with resistance in *M. persicae*.

2. Insecticide resistance monitoring

Bioassay is the most basic method to monitor resistance level in the field. As alternatives to bioassay, various methods based on molecular twchniques have been established such as PCR amplification of specific allele (PASA) and restriction fragment length polymorphism analysis of PCR product (RFLP) (Sommer et al. 1989; Cassanelli et al. 2005). More recently, population genotyping methods based on DNA samples such as real-time PCR amplication of specific allele (rtPASA)(Germer et al. 2000; Kwon et al. 2004; Baek et al. 2006), and quantitative sequencing (QS) (Amos et al. 2000; Kwon et al. 2008; Seong et al. 2010) have been developed and these method have been introduced to determine the resistance allele frequencies at the population. Nowadays, these methods were combined with microarray technologies to detect insecticide resistance more rapid and accurate (Le Goff et al. 2003; David et al, 2005; Vontas et al. 2007).

3. Carboxylesterase (CbE) as co-resistance factor

CbE is an enzyme with α,β-hydrolase fold protein that catalyzes the hydrolysis reaction, specifically acting on carboxylic ester bonds (Satoh et al 2006). CbE is known to be involved in the resistance of insecticides containing ester bond (Herath et al.1987; Hemingway et al.1995). Point mutation of CbE E3 transformed the substrate specificities in *Lucilia cuprina* (Campbell et al. 1998). Additionally, overexpression of CbE caused by gene amplification was reported to be a major factor of sequestration (Field et al. 1988). Increased expression of CbE E4 in *M. persicae* was associated with a wide range of insecticide resistance by hydrolysis and sequestration (Devonshire and Moores 1982).

CHAPTER 1.

Pyrethroid resistance monitoring in local strains of *Myzus persicae* and characterization of the voltage-gated sodium channel gene

Pyrethroid resistance monitoring in local strains of *Myzus persicae* and characterization of the voltage-gated sodium channel gene

Abstract

Two mutations (M918L and L1014F) were found in local strains collected in 2010 and 2011. A quantitative sequencing (QS) protocol was developed to detect a resistance allele frequency of *M. persicae* population. The nucleotide signal ratio at each mutation site was generated from sequencing chromatograms and plotted against the resistance allele frequency. To investigate the correlation between *vgsc* mutation frequency and resistance level to bifenthrin, leaf dip bioassay was conducted. As determined by correlation, M918L mutation showed a higher correlation ($r^2 > 0.942$) than L1014F ($r^2 < 0.121$) between the resistance allele frequency and resistance ratio in local strains of *M. persicae*. Nevertheless, no saturated level of M918L mutation frequency was observed in any of local strains showing high levels of bifenthrin resistance, suggesting the possibility of duplication of the entire *vgsc* or an exon containing the mutation site. However, *vgsc* gene copy number determination and genome structure analysis revealed no clear evidence of either gene duplication or alternative exon splicing, requiring further in-depth investigation. Nonetheless, the newly identified mutation M918L should be an effective marker for detecting pyrethroid resistance levels in the field.

1. Introduction

The green peach aphid, *Myzus persicae* (Sulzer), represents the greatest threat for a variety of agricultural crops (Cocu et al. 2005; Ramsey et al. 2007; Puineal et al. 2010). In addition, more than 100 plant viruses could be transmitted by *M. persicae* (Kennedy et al. 1962; Blackman et al. 2000). Various insecticides have been used to control this pest but resistance to these insecticides developed rapidly due to the high selection pressure (Foster et al. 2000). Among the insecticides used to control *M. persicae*, pyrethroids act on the voltage-gated sodium channel (*vgsc*) in the nervous system (Soderlund and Bloomquist 1989) and pyrethroid resistance is known to be mainly conferred by reduced sensitivity of *vgsc* (Martinez et al. 1999). This resistance mechanism is responsible for the cross-resistance between pyrethroids and DDT. Besides, it could be increased pyrethroid resistance more easily in the most insect species (Soderlund and Bloomquist 1989). Two mutations (M918T and L1014F) on the *vgsc* were revealed and these confer an extremely insensitive form of pyrethroid resistance known as the *supe-kdr* trait (Eleftherianos et al. 2008). Carboxylesterase (CbE) E4 is also known to be involved in pyrethroid resistance as a metabolic factor in M. persicae. This CbE E4 is also confers resistance to organophosphate and carbarmate insecticides (Devonshire and Moores 1982). In addition, this metabolic resistance mechanism mediated by CbE E4 enhanced deltamethrin resistance when *kdr* mutation was present (Martinez-Torres et

al.1999). Detection of resistance at an early stage is important for efficient resistance management. Various molecular tools to detect the resistance have been developed in *M. persicae*,

In this study, a quantitative sequencing (QS) protocol for the detection of point mutations on *vgsc* was established to predict the resistance allele frequencies on a population basis. In addition, correlation analysis was conducted to confirm the role of *vgsc* mutation in resistance.

2. Materials and methods

2.1 *M. persicae* strain

Five local strains were collected from cultivation area in 2010 and eight local strains were additionally collected from Chinese cabbage cultivation area in 2011. Insecticide-susceptible and -multiple resistant *M. persicae* strains were obtained from RDA. *M. persicae* was maintained in the laboratory condition on Chinese cabbage at 25 °C with a 16:8 h photoperiod (light:dark).

2.2 Pyrethroid resistance monitoring

To investigate of pyrethroid resistance level of *M. persicae*, Target site mutations were investigated by sequencing in voltage-gated sodium channel (*vgsc*) and leaf dip bioassay method was performed to determine the LC_{50} of bifenthrin. The methods will be discussed in greater length in 2.2.1 and

2.2.2.

2.2.1 Local strain genotyping for the detection of resistance mutation

The local strains were collected from the field and immediately used for mutation survey. Genomic DNA (gDNA) was extracted using gDNA extraction kit (Bioneer, Seoul, Korea) according to the manufacturer's protocol. Briefly 10 *M. persicae* were soaked in 200 μL TL buffer and homogenized using plastic pestle (Bel-art product, NJ). The homogenate was treated with protease K for 60 min at 60°C and filtrated through a DNA-binding column. gDNA was finally collected using 20 μL of elution buffer. A partial gDNA fragment containing two mutation sites (M918L and L1014F) was PCR-amplified from gDNA templates from all local strains using sequence-specific primer set (Table 1). The PCR was performed for 35 cycles of 95°C for 5 min, 95 °C for 20 s, 62 °C for 20 s, 72 °C for 1 min, 72 °C for 5 min. The PCR products were directly sequenced to determine the presence or absence of mutation with an ABI prism 3730 DNA sequence analyzer (Nicem Sequencing Facility, Seoul National University, Seoul. Korea).

Table1. PCR and sequencing primer used in this study

Primer name	Sequence	Remark
5 *vgsc*_M918L	AAGCCGCTCTGAAACTGATGGCG	For sequencing
3' *vgsc*_M918L	TCTCCGACGTGTAAACAGTCCCAC	
5' *vgsc* _L1014F	AGACCACGAGCTTCCCCGGTG	
3' *vgsc* _L1014F	GATGGTTGTAGGTTCTGGATAGC	
5' *vgsc*_AS	GAATTACGTACACATCGCACG	For the cloning of partial *vgsc* fragment
3' *vgsc*_AS	CTCCACTGGTTCTTTAGTGTC	
5'AS_Rest_F_ApaI	AATGGGCCCCTTCCAAATGGGCTGGAAC	
3'AS_Rest_R_NsiI	GCCATGCATGGCGCTATAACGTCTACAA	
5′MpActin	CAAATCATGTTTGAAACCTTCA	For qrtPCR to determine the gene duplication
3′MpActin	AATGCATAACCTTCATAGATG	
5'qrt_ *vgsc*_exon2	GAGTATTTAAGTTGGCAAAATC	
3'qrt_ *vgsc*_exon2	GTAGTTTTTTCCAAATAACTGC	

2.2.2 Bioassay of local strains by bifenthrin

The local strains were collected from the field and immediately used for bioassay. In case of aphids collected in 2010, bioassay was conducted using the recommend concentration from the pesticide manual and the mortality at the concentration was determined. However, LC_{50} was calculated from all strains collected in 2011. Commercial grade bifenthrin (Tasta, WP, 2%, Bayer CropScience, Seoul, Korea) was dissolved in water to appropriate concentration. Chinese cabbage leaf discs were dipped into an appropriate concentration of solution for 1 min and dried in a darkened hood for 30 min. 10-15 wingless aphids (adult) were placed into a 50-mm petri dish with a

cabbage disc and filter paper. The mortality were calculated at 48 h post-treatment and LC_{50} values were determined by Probit analysis using SPSS 12K software (IBM corporation, Armonk, NY). All tests were performed at 25±2 °C and repeated at least three times.

2.2.3 Establishment of QS protocol for the detection of resistance allele frequency

A QS protocol was developed according to the method of Kwon et al. (2008) and Seong et al. (2010) with some modification. The gDNA fragments of *vgsc* containing either the M918L and L1014F mutations site were PCR-amplified from all strains as described above. The PCR product was subcloned into pGEMTeasy vector (Promega, Madison, WI) and transformed into One Shot TOP10® *Escherichia coli* competent cells (Invitrogen, Carlsbad, CA). A total of 10 positive clones were sequenced to identify the mutations. The template plasmid DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and used as standard templates. The standard DNA templates were mixed in the following ratios: 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, 10:0 (susceptible allele:resistance allele at each mutation site). Total 10ng of standard templates were sequenced using a sequencing primer (Table 1). The nucleotide signal intensities of susceptible and resistance allele at each mutations were measured from the sequence chromatogram using Chromas

2.31 software (Technelysium Pty Ltd, Tewantin, Australia) and signal ratios were calculated according to the signal ratio equation [resistance signal/(resistance signal + susceptible signal)]. Standard regression equations were generated using the SigmaPlot10 software (Systat Software Inc., San Jose, CA). The lower and upper prediction equation was generated to determine the prediction intervals at the 95% confidence level.

2.2.4 Correlation analysis between the *vgsc* mutation frequency and bifenthrin resistance ratios

To investigate the correlation between *vgsc* mutation frequency and resistance levels of aphid to bifenthrin, the M918L and L1014F allele frequencies were evaluated by QS. The LC_{50} values were calculated following bioassay for all strains. The M918L and L1014F allele frequencies were plotted against LC_{50} values and correlation equation and regression coefficients were predicted using SigmaPlot10 software (Systat Software Inc., San Jose, CA).

2.2.5 Analysis of exon-intron structure in partial genomic DNA fragment of *vgsc*

To determine the alternative splicing possibility, individual gDNA was extracted as described above. A gDNA fragment flanking exon regions containing the two mutation sites (M918L and L1014F) was PCR-amplified from gDNA template using sequence-specific primer set (Table.1). The PCR

was performed for 35 cycles of 95 °C for 3 min, 95 °C for 30 s, 56 °C for 30 s, 72 °C for 3 min, 72 °C for 5 min. Nested PCR was performed using the initial PCR product as template to incorporate restriction enzyme sites (ApaI and NsiI, Table 1). Apa I (Koschem, Sungnam, Korea)- and Nsi I (Koschem, Sungnam, Korea)-digested PCR product and pGEMTeasy vector (Promega, Madison, WI) were ligated and transformed into One Shot TOP10® *E. coli* competent cells (Invitrogen, Carlsbad, CA). A total of 10 positive clones were sequenced to determine the exonal organiztion.

2.2.6 Analysis of *vgsc* gene copy number ratio by quantitative real-time PCR(qPCR)

To determine the *vgsc* gene copy number, individual gDNA was extracted as described above. Primers specific to *vgsc* exon 2 (Table 1) was used for amplification. The actin gene was used as a reference gene to normalize the relative copy number of target gene. qPCR was conducted using Chromo4 real-time thermal cycler (MJ research analysis, Waltham, MA) and DyNAmo HS SYBR Green master mix (Finnzymes, Vantaa, Finland) containing SYBR Green I fluorescent dye, $MgCl_2$, dNTP, and *Tbr* DNA polymerase. The qPCR reaction mixture was composed of 20 ng of 2 ng of gDNA, 10 μL of 2X DyNAmo HS SYBR Green master mix, and 5 μM of primers. The qPCR was performed with the following step-wise thermal program: 1 cycle at 95 °C for 15 min for DNA polymerase activation followed by 45 cycles at 95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec. Melting

curve analysis was performed to confirm the homogeneity of the PCR products. Gene copy number was determined by following equation (ratio=$2^{-[Ct\ target-Ct\ actin]}$).

3. Result

3.1 Bifenthrin resistance level in local strains

In the result of the leaf dip bioassay, MR, Chungju, Bonghwa, and Sachun strain had a >130-fold resistance to bifenthrin (Table 2). Especially, MR strain, which is known to have cross resistance to carbamates, showed a 540-fold resistance to bifenthrin. Comparing between 2010 and 2011 strains, bifenthrin resistance was maintained in MR and Bonghwa strains. The results of bioassay in 2010 and 2011 are shown in Fig. 1 and Table 2.

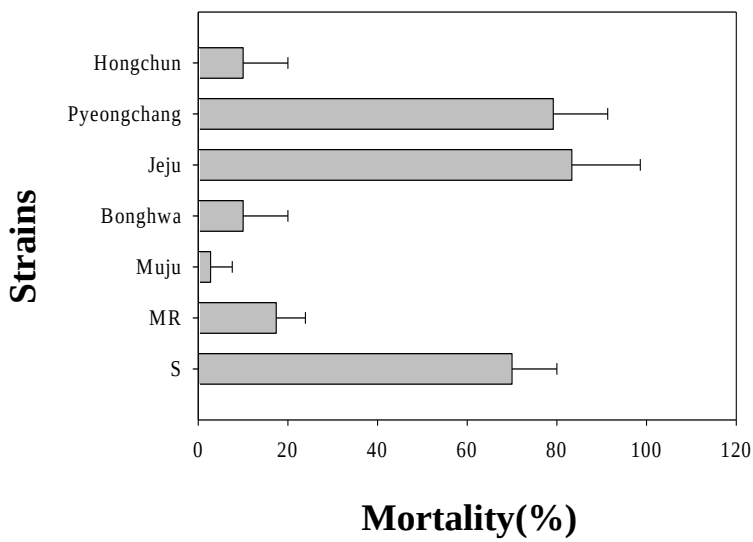

Figure 1. Mortalities of the 2010 local strains of GPA in the pyrethroid (bifenthrin) leaf dipping bioassay

Table 2. Evaluation of LC_{50} of bifenthrin for the 2011 local strains of GPA as determined by leaf dipping bioassay

Strain	N	Slope	SE	LC_{50}	χ^2	Lower	upper	DF	RR[a]
Sus	143	1.08	0.19	0.1	3.55	0.1	0.2	10	1
MR	144	0.72	0.23	540	5.12	248.1	4260.2	10	5183
Hongchun	151	1.7	0.29	68	7.08	43.6	98.1	10	654
Anbanduk	143	1.35	0.27	86	9.59	48.5	133.9	10	826
Pyeongchang	150	1.85	0.45	24	8.41	8.3	37.8	10	225
Chungju	132	4.82	0.97	188	1.54	146.1	237.3	10	1803
Bonghwa	138	2.73	0.43	167	10.01	123.8	222.6	10	1598
Muju	156	1.68	0.28	75	13.88	48.9	107.7	10	720
Sachun	149	1.4	0.23	130	10.94	82.6	202.1	10	1249
Jeju	148	2	0.36	56	3.39	35.8	78.5	10	536

[a] Resistance ratio = LC_{50} of Local strain / LC_{50} of Susceptible strain

3.2 Genotypes of the *vgsc* mutation sites

A total of 10 local strains were analyzed. In the mutations of M918L and L1014F, sequence chromatograms revealed than the frequencies of all mutation was shown in Figs. 2 and 3. Significantly, two different genotypes (homozygous susceptible and heterozygous resistance) were existed in all local strains. In 2010, both two mutations were distributed separately and equally except Hongchun strain (Fig. 2). In contrast, distribution of M918L mutation was identified by genotyping and it was absolute majority in all strains (Fig. 3).

3.3 Establishment of the QS protocol

In the result of sequencing, nucleotide signal intensity increase according to the allele frequency in template plasmid DNA mixture (Fig. 4). The regression plots and equations of each mutation were generated from resistance allele frequencies (Fig. 5). Both of the two regression lines revealed high correlation coefficients (r^2 = 0.999 and 0.993). The prediction equations were generated from the antisense directional sequencing for both mutations. 95% prediction intervals (Fig. 5) were similar between both mutations.

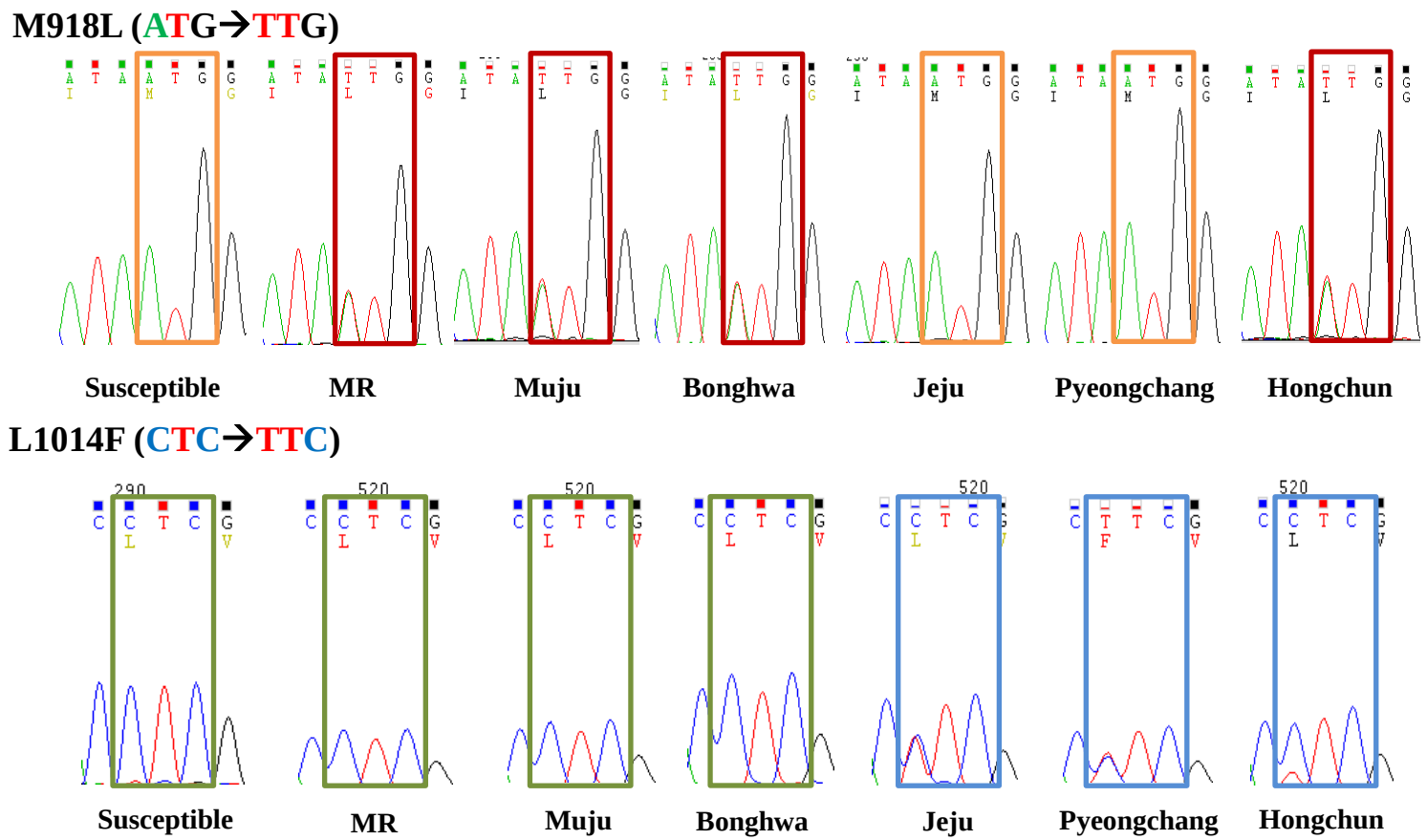

Figure 2. *vgsc* gene mutation survey in the 2010 local strains of GPA

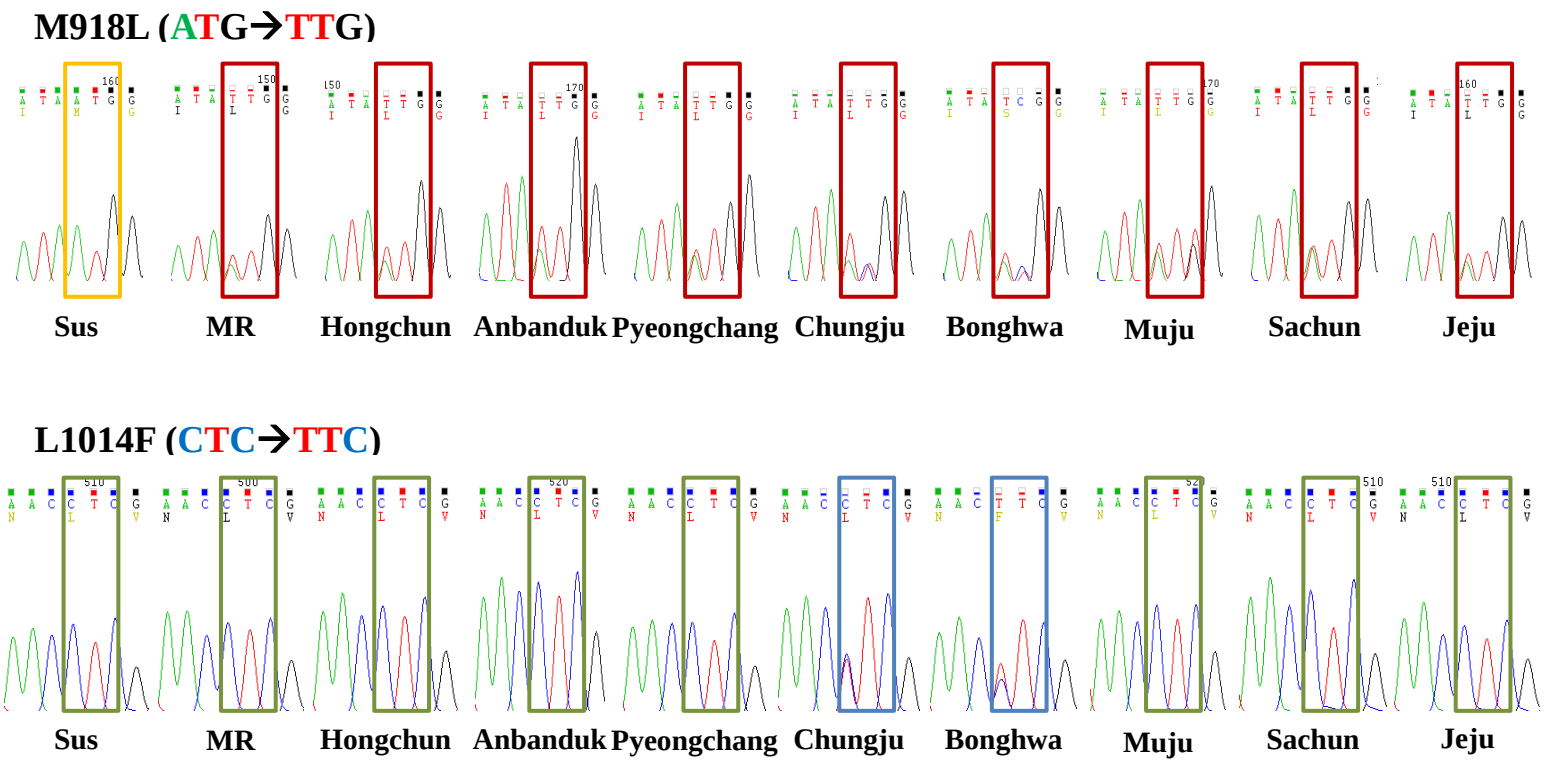

Figure 3. *vgsc* gene mutation survey in the 2011 local strains of GPA

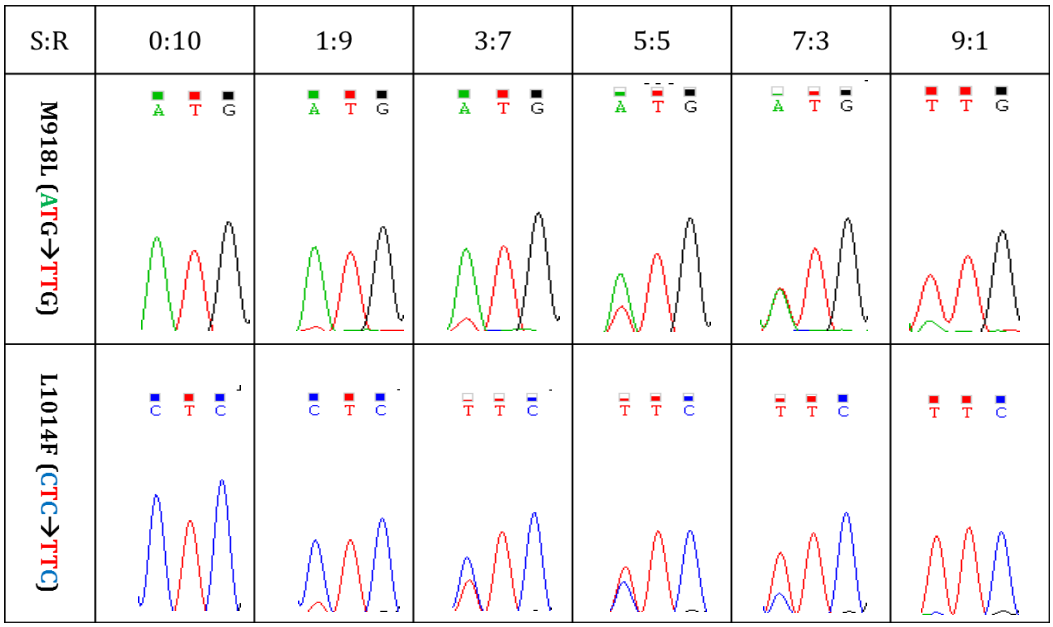

Figure 4. Sequencing chromatograms of the standard template plasmid DNA mixtures with difference ratios of resistance and susceptible allele at the M918L and L1014F mutation sites.

3.4 Correlation between the two *vgsc* mutations and bifenthrin resistance levels

To determine the correlation between two *vgsc* mutation and resistance levels in local strains of *M. persicae*, linear regression was conducted between the mutation frequencies and LC_{50} values. In case of the local strains collected in 2010, linear regression was conducted between the mortality and resistance allele. The high correlation coefficient was revealed in M918L mutation (r^2 = 0.986),

whereas considerably low correlation coefficient was obtained for the L1014F mutations in the local strains collected in 2011 (r^2 = 0.457). In case of the 2011 local strains, the M918L mutation was associated with a high level of pyrethroid resistance (r^2 = 0.942), whereas the L1014 mutation result showed lower coefficient (r^2 = 0.121). The M918L mutation, which was newly identified in the local strains of Korea, appears to confer a high level of pyrethroid resistance as judged by the high correlation between its frequency and actual level of resistance.

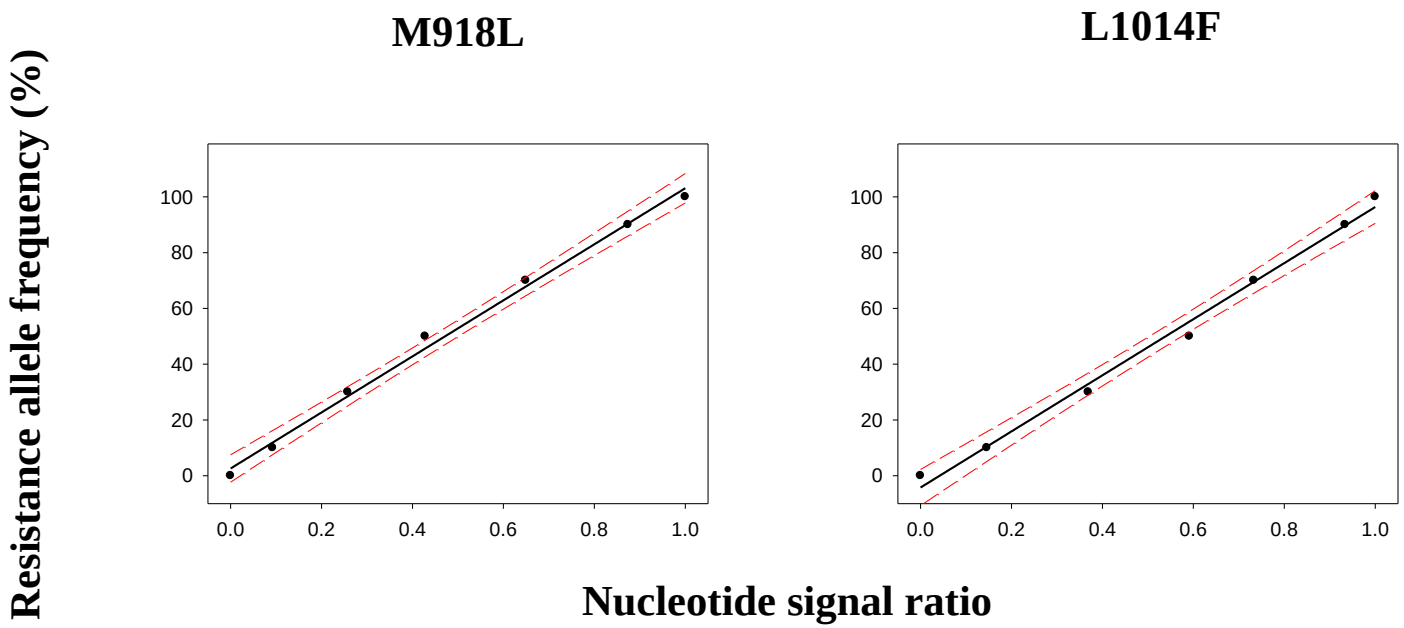

Figure 5. Resistance sequence signal ratios obtained from the antisense sequencing were plotted with corresponding resistance allele frequencies at the (A) M918L and (B) L1014F mutation sites. Linear regression lines are indicated by solid black lines with upper and lower 95% prediction lines indicated by dotted red lines. Nucleotide signal ratio (x-axis) was calculated as [resistant nucleotide signal / (resistant nucleotide signal + susceptible nucleotide signal)].

Table 3. Linear regression and prediction equation of the resistance nucleotide signal ratio versus corresponding resistance allele frequency at M918L and L1014 mutation sites.

Mutation	Sequencing direction	Regression equation [1]	Regression coefficient (r^2)	95% prediction equation	
				Lower	Upper
M(ATG)301L(TTG)	Antisense	y =-1.2+100.4x	0.999	y =-6.0+100.4x	y =6.0+100.4x
L(CTC)1014F(TTC)	Antisense	y =-4.2+100.6x	0.993	Y =-15.2+100.8x	y =6.8+100.3x

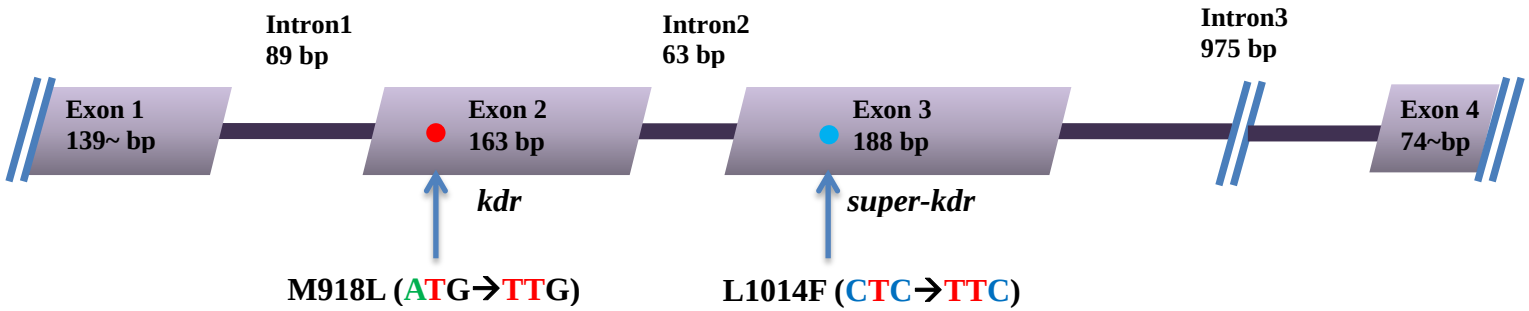

Figure 6. Exon-Intron structure of partial genomic fragment of *vgsc*, encompassing the M918L and L1014F mutation sites

3.5 Exon-intron structure of *vgsc* genomic DNA fragments

The individual gDNA fragment of *vgsc* (1661 bp containing the M918L and L1014F mutation sites) was PCR-amplified and their sequences determined. The exon–intron structure is illustrated in Fig.6. The 163-bp exon (designated as exon 2) and the 188-bp exon (designated as exon 3) contain the M918L and L1014F mutation sites, respectively. No apparent tandem array of alternative exons was identified within the gDNA fragment (Fig. 6).

3.6 *vgsc* gene copy number ratio

qPCR analysis was conducted to determine the gene duplication of *vgsc*. The gene copy number of *vgsc* of the suceptible strain was determined to be 1.6-fold higher than that of the MR strain although the difference was insignificant (Fig. 7).

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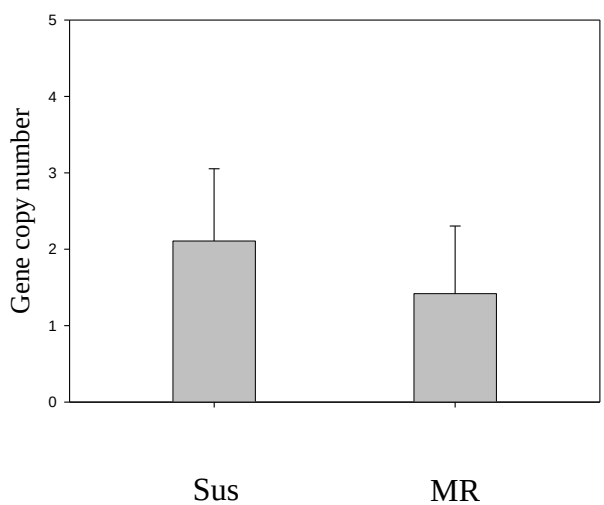

Figure 7. The relative gene copy number to determine the gene duplication in *vgsc*

Table 4. Correlation of two mutations between mortality and allele frequency to pyrethroid resistance in the 2010 and 2011 local strains

Mortality (%) *vs*. Allele frequency (%)				
Mutation	M918L		L1014F	
Year	2010	2011	2010	2011
Correlation coefficient (r^2)	0.986	0.942	0.457	0.121

4. Discussion

Two mutations (M918L and L1014F) were identified from the local strains of *M. persicae* in Korea from this research. QS protocol was developed and used for the prediction of *vgsc* mutation frequencies in field population. In addition, bioassay was conducted to determine which mutation is more responsible for resistance. Although Chungju and Bonghwa strains have both mutations, increasing tendency of insecticide resistance was not apparent. In contrast, MR strain, which has a single mutation of M918L, showed a high level of resistance to bifenthrin. Taken together with correlation analysis ($r^2 = 0.942$), this newly identified M918L mutation may have a significant role in bifenthrin resistance.

Although L1014F mutation is well known to be responsible for the *kdr* trait in a number of insects (Soderlund and Knipple 2003), the correlation between L1014F mutation frequency and resistance level was unclear. It remains to uncover whether other resistance factors or conter-acting

mutations are involved in the strains showing relatively low correlation between L1014F mutation and actual resistance level. With these considerations in mind, additional experiments are needed to identify additional resistance factors.

Even in the genotyping of pathenogenetic individual aphids, no apparent homozygous mutation allele was found at either M918L or L1014F mutation site. Therefore, it was assumed that the duplication of entire *vgsc* gene or partial exonal fragment, resulting in duplicate fragments possessing the same mutation site, may be responsible for this phenomenon. However, there was not clear evidence on gene duplication or the presence of alternative exons.

Chapter 2.

Carbamate resistance monitoring in local strains of *M. persicae* and characterization of the acetylcholinesterase gene

Carbamate resistance monitoring in local strains of *M. persicae* and characterization of the acetylcholinesterase gene

Abstract

Two mutations (A301S and S431F) on the acetylcholinesterase gene that are known to be associated with organophosphate and carbamate resistance were investigated by genotyping. Only one mutation (S431F) was observed in local strains of *M. persicae* in Korea. A quantitative sequencing (QS) protocol was developed to detect the resistance allele frequency in field strains of *M. persicae*. The nucleotide signal ratio at each mutation site was generated from sequencing chromatograms and plotted against the resistance allele frequency. To investigate the correlation between AChE mutation frequency and resistance level of aphid to methomyl, leaf dip bioassay was conducted. As determined by correlation, S431F mutation showed a negative correlation (r^2 <0.255) between the resistance allele frequency and resistance ratio in local strains. Many *M. persicae* strains with relatively high levels of resistance also possessed high frequencies of susceptible allele, suggesting that other resistance factors likely play major roles in methomyl resistance. In other words, the S431 mutation itself does not appear to play a significant role in methomyl resistance, thereby not being able to be used as a resistance marker. Nevertheless, the QS protocol established in this study could be useful in tracing the dynamics of the

S431F mutation in field strains of *M. persicae*.

1. Introduction

Resistance development to several insecticides that have been used to control *M. persicae* is a common phenomenon in many countries (Vucetic et al. 2008; Bass et al. 2011). Various target site insensitivity mechanisms that are resulted from point mutations have already been described in this species for a variety of insecticide targets, including AChE (MACE) (Moores et al. 1994; Nabeshima et al. 2003), *vgsc*, GABA-*Rdl* receptor, and nAChR (Bass et al. 2011). AChE is a member of the serine hydrolase family of enzymes and hydrolyzes the neurotransmitter acetylcholine in the cholinergic synapses. AChE is inhibited by organophosphate (OP) and carbamate (CB) insecticides acting on the post synaptic nerve (Javed et al. 2003). Mutations on *ace*, such as S431F mutation of the *ace*1 gene (called as MACE phenotype) have been reported to be associated with pirimicarb resistance in M. persicae. The A302S mutation on *ace*1 has been reported in *Aphis gossypii* as a causative mutation for OP resistance (Benting and Nauen 2004).

In spite of relatively high toxicity, consumption of CB insecticides has not been decreased dramatically in the field thanks to their wider range of

application and lower cost. Therefore, increased selection pressure by CB insecticides has driven the development of CB resistance in *M. persicae*. As an attempt for early resistance detection, QS protocol was established to predict the resistance allele of carbamate and correlation analysis was conducted to confirm the role of *ace* mutation in resistance.

2. Materials and methods

2.1 *M. persicae* strain

The same local strains as described above were used in this study. The methods was had discussed in Chapter 1.

2.2 Carbamate resistance monitoring

To investigate of carbamate resistance level of *M. persicae*, target site mutations were investigated by sequencing type 1 *ace* (*ace1*) and leaf dip bioassay method was performed to determine the LC_{50} values for methomyl. The methods will be discussed in greater length in 2.2.1 and 2.2.2.

2.2.1 Local strain genotyping for the detection of acetylcholinesterase mutation

Local strains were collected in 2010 and 2011 and immediately used for mutation survey. Genomic DNA was extracted using gDNA extraction kit

(Bioneer, Seoul, Korea) according to the manufacturer's protocol. Briefly, 10 *M. persicae* were soaked in 200 μL TL buffer and homogenized using plastic pestle (Bel-art product, NJ). The homogenate was treated with protease K for 60 min at 60 °C and filtrated through a DNA-binding column. gDNA was finally collected using 20 μL of elution buffer. Two mutation sites (A301 and S431F) were PCR-amplified from the gDNA templates from all local strains using sequence-specific primer set (Table 1). The PCP was performed by 35 cycles of 95 °C for 5 min, 95 °C for 20 s, 60 °C for 20 s, 72 °C for 1 min, 72 °C for 5 min. The PCR products were directly sequenced to determine the mutation with an ABI prism 3730 DNA sequence analyzer (Nicem Sequencing Facility, Seoul National University, Seoul. Korea).

Table 1. PCR and sequencing primer used in this study

Primer name	Sequence	Remark
5'AChE_S431F	ACTTAGGCTGGCAAGGGCAATGG	For amplification and sequencing
3'AChE_S431F	TCACCCCTGTCCATTTCGGCCA	

2.2.2 Bioassay of local strains by methomyl

As described in the previous chapter, leaf dip bioassay was conducted. Local strains were collected in 2010 and 2011 and used immediately for bioassay. In case of 2010, bioassay was conducted using a recommend concentration

from the pesticide manual and only mortality was estimated from the result. However, LC_{50} was calculated for all strains collected in 2011. Commercial grade methomyl (methomyl, WP, 45%,Young Il chemical, Sungnam, Korea) was dissolved in water to appropriate concentrations. Chinese cabbage leaf discs were dipped into an appropriate concentration of solution for 1 min and dried in a darkened hood for 30 min. 10-15 Wingless aphids (adult) were placed onto a cabbage disc placed in a 50-mm petri dish. Mortality was evaluated at 48 h post-treatment and LC_{50} value were determined by Probit analysis using SPSS 12K software (IBM corporation, Armonk, NY). All tests were performed at 25±2 °C and repeated at least three times.

2.2.3 Establishment of the QS protocol to predict resistance allele frequency

A QS protocol was developed as described in the previous section. The gDNA fragments of *ace* containing either the A301S and S431F mutations site were PCR-amplified from all strains as described above. To separate the resistance allele and susceptible allele, the PCR product was subcloned into pGEMTeasy vector (Promega, Madison, WI) and transformed into One Shot TOP10® *Escherichia coli* competent cells (Invitrogen, Carlsbad, CA). A total of 10 positive clones were sequenced to identify the mutations. The template plasmid DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and used as standard templates. The standard DNA templates were mixed in the

following ratios: 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, 10:0 (susceptible allele:resistance allele at each mutation site). Total 10ng of standard templates were sequenced using a sequencing primer (Table 1). The nucleotide signal intensities of susceptible and resistance allele at each mutations were measured from the sequence chromatogram using Chromas 2.31 software (Technelysium Pty Ltd, Tewantin, Australia) and signal ratios were calculated according to the signal ratio equation [resistance signal/(resistance signal + susceptible signal)]. Standard regression equations were generated using the SigmaPlot10 software (Systat Software Inc., San Jose, CA). The lower and upper prediction equation was generated to determine the prediction intervals at the 95% confidence level.

2.2.4 Correlation analysis between the *ace* mutation frequency and methomyl resistance ratios.

To investigate the correlation between *ace* mutation frequency and resistance levels of aphid to methomyl, the S431F allele frequencies was evaluated by QS. The LC_{50} values were calculated by bioassay for all strains. The S431F allele frequencies were plotted against LC_{50} values and correlation equation and regression coefficients were established using SigmaPlot10 software (Systat Software Inc., San Jose, CA).

3. Results

3.1 Methomyl resistance level in local strains

According to the leaf dip bioassay, MR, Hongchun strains showed >12-fold resistance to methomyl (Table 2). Especially, Hongchun strain exhibited a 46-fold resistance to methomyl. In the cross-comparison between 2010 and 2011, Hongchun strain collected in 2011 did not have methomyl resistance. In contrast, this strain showed the highest methomyl resistance when collected in 2010. The results of bioassay in 2010 and 2011 are shown in Fig. 1 and Table 2.

3.2 Genotypes of the *ace* mutation sites

A total of 10 local strains were analyzed. In the S431F mutation, sequence chromatograms revealed than the frequencies of all mutation was shown in Fig. 2. However, A301S mutation was not found in the local strains either in 2010 or 2011. In 2010, there was no 100% S431F mutation allele in any of local strains. However, three different genotypes were observed in 2011 local strains. Homozygous resistance allele was found in Muju strain in 2011 and homozygous susceptible allele was only found in the susceptible strain. Although there was no A301S mutation in *ace* gene from all local strains, we identified the S431F mutation which shown as heterozygous allele.

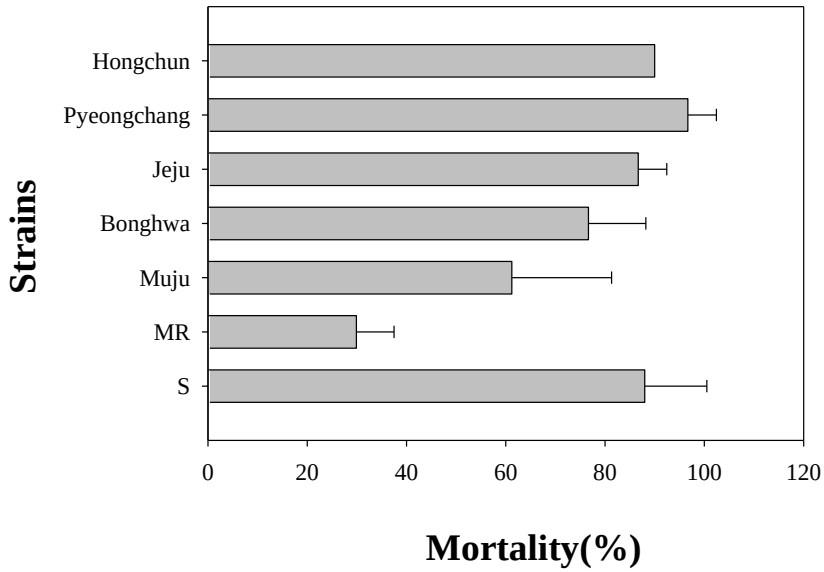

Figure 1. Mortalities of the 2010 local strains of GPA in the carbamate (methomyl) leaf dipping bioassay

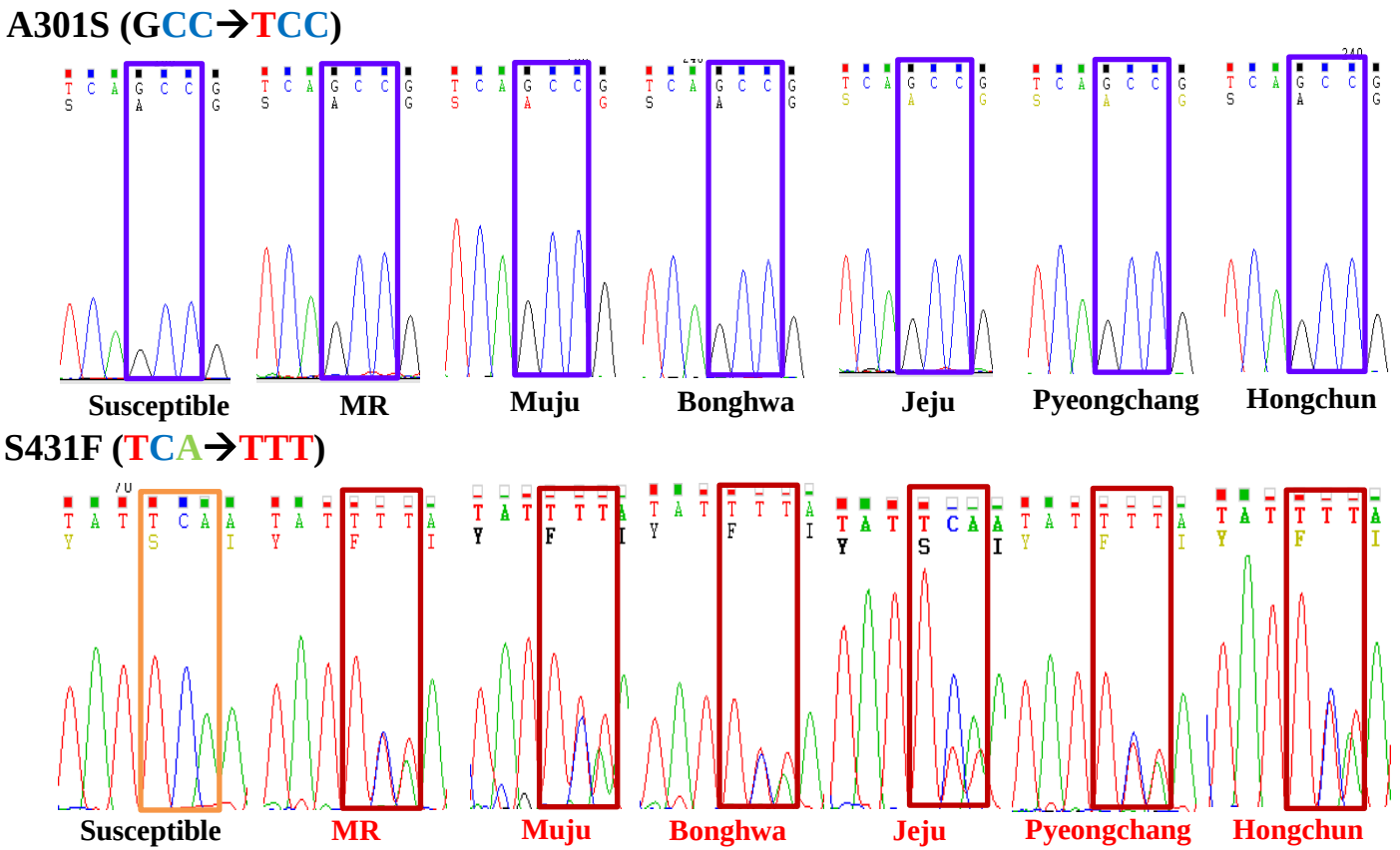

Figure 2. AChE gene mutation survey in the 2010 local strains of GPA

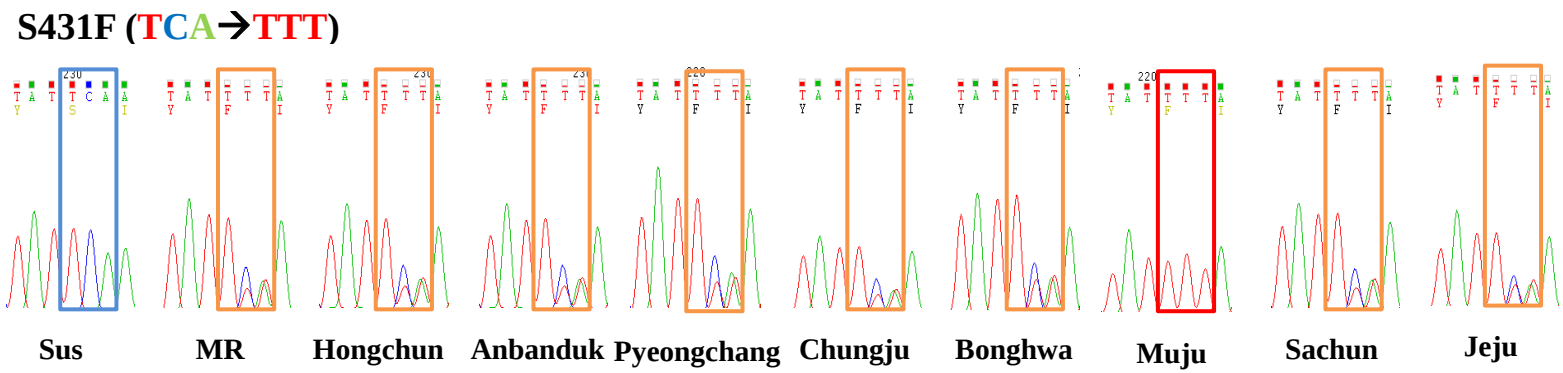

Figure 3. AChE gene mutation survey in the 2011 local strains of GPA

Table 2. Evaluation of LC_{50} of methomyl for the 2011 local strains of GPA as determined by leaf dipping bioassay

Strain	N	Slope	SE	LC_{50}	χ^2	Lower	upper	DF	RR[a]
Sus	150	2.17	0.39	39	21.36	22.16	85.9	10	1
MR	140	1.72	0.33	474	15.8	273.37	1202.92	10	12
Hongchun	121	1.01	0.31	1806	9.49	700.9	43701.07	9	46
Anbanduk	140	2.27	0.41	52	12.83	34.68	72	10	1.3
Pyeongchang	144	3.09	0.49	124	17.93	80.96	192.56	10	3.2
Chungju	145	1.85	0.33	60	13.74	36.58	86.34	10	1.5
Bonghwa	150	2.76	0.41	112	20.43	66.55	177.05	10	2.9
Muju	156	2.21	0.33	171	7.54	126.95	229.49	10	4.4
Sachun	142	1.66	0.37	35	18.13	5.19	68.03	10	0.9
Jeju	147	2.11	0.32	189	13.94	136.52	258.9	10	4.8

[a] Resistance ratio = LC_{50} of Local strain / LC_{50} of Susceptible strain

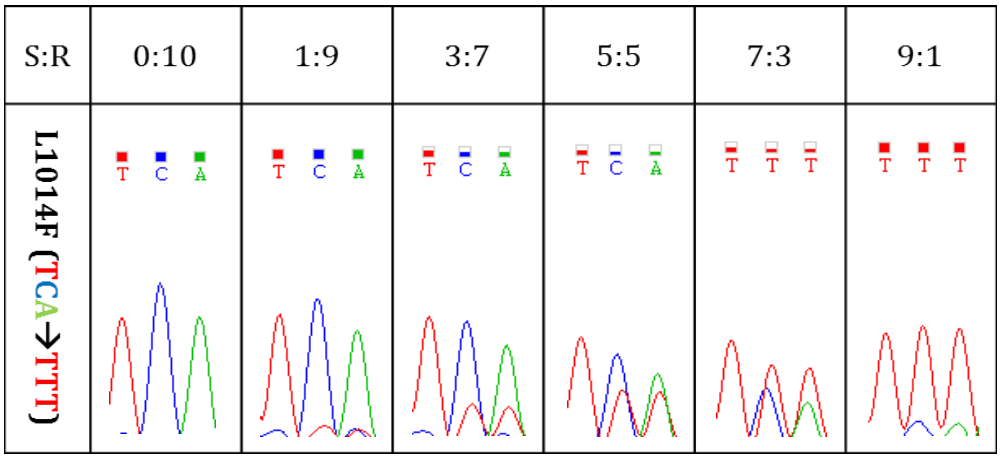

Figure 4. Sequencing chromatograms of the standard template plasmid DNA mixtures with difference ratios of resistance and susceptible allele at the S431F mutation site

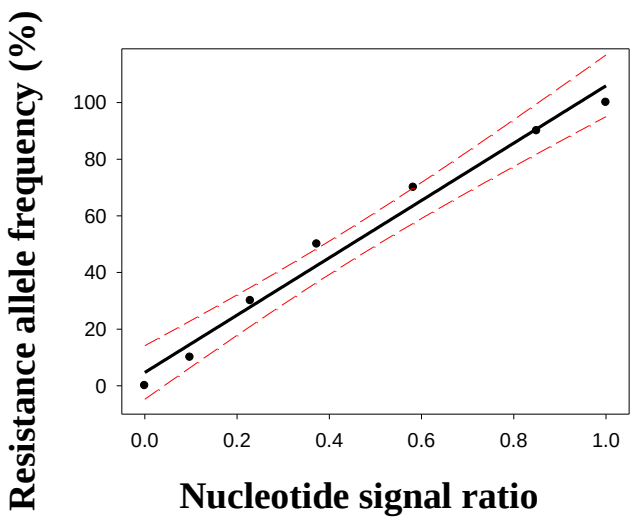

Figure 5. Resistance sequence signal ratios obtained from the antisense sequencing were plotted with corresponding resistance allele frequency at the S431F mutation site. Linear regression lines are indicated by solid black lines with upper and lower 95% prediction lines indicated by dotted red lines. Nucleotide signal ratio (x-axis) was calculated as [resistant nucleotide signal / (resistant nucleotide signal + susceptible nucleotide signal)].

3.3 Establishment of QS protocol

Cause of absence of A301S mutation, nucleotide signal intensity increase according to the allele frequency in template plasmid DNA mixture in S431F mutation (Fig. 4). prediction equations for mutation frequency were generated from the regression plots of resistance allele frequencies versus actual resistance level (Fig. 5). The regression line revealed high correlation coefficient (r^2 = 0.980). The prediction equations were generated from antisense directional sequencing of mutation.

3.4 Correlation between the *ace* mutation and methomyl resistance levels

To determine the correlation between *ace* mutation and resistance level in local strains, linear regression was conducted between the LC_{50} values and resistance allele frequencies. In the result of correlation analysis, low correlation coefficient suggested that the S431F mutation itself does not appear to play a significant role in methomyl resistance. Thus, other factors are likely more important in methomyl resistance in *M. persicae*.

Table 3. Linear regression and prediction equation of the resistance nucleotide signal ratio versus corresponding resistance allele frequency at S431F mutation site.

Mutation	Sequencing direction	Regression equation [1]	Regression coefficient (r^2)	95% prediction equation	
				Lower	Upper
S(TCA)431F(TTT)	Antisense	y =-0.025+102.3x	0.996	y =-8.1+102.2x	y =8.2+102.5x

Table 4. Correlation of mutation between mortality and allele frequency to carbamate resistance in the 2010 and 2011 local strains

Mortality (%) *vs*. Allele frequency (%)		
Mutation	S431F	
Year	2010	2011
Correlation coefficient ($\boldsymbol{r^2}$)	0.255	0.018

4. Discussion

AChE is well known as the target site of OPs and CBs (Javed et al. 2003). In this research, mutation survey was conducted using genotyping and QS protocol and used for the prediction of *ace* mutation frequencies in field strains of *M. persicae*. S431F mutation was found in all strains except susceptible strain, whereas there was no A301S mutation in any of the strains. In the bioassay, MR, Hongchun strains exhibited a >12-fold resistance to methomyl and a >654-fold resistance to bifenthrin. Cross-

resistance among OPs, CBs, and pyrethroids in *M. persicae* was reported to be due to nonspecific metabolic factor, such as carboxylesterase (CbE) E4 (Devonshire et al. 1982; Field et al. 1993; Field et al. 1997; Field and Blackman 1998; Field 2000; Field and Blackman 2003; Kwon et al. 2009). Based on this research, cross-resistance between the CBs and pyrethroid existed in many field populations. Taken together, the moderate levels of methomyl resistance (i.e., ~12 fold resistance) may not be caused by insensitive AChE and S431F mutation not be the causative mutation for insensitive AChE. We expect that other nonspecific metabolic factor such as CbE E4 and cytochrome p450 may be involved in methomyl resistance in local strains of *M. persicae* in Korea.

Although the S431F mutation does not appear to play a significant role in methomyl resistance, it is likely responsible for other OP or CB resistance as the same mutation has been reported to be associated with OP and CB resistance in *Aphis gossypii* (Benting and Nauen 2004). Therefore, the QS protocol established in this study could be useful in tracing the dynamics of the S431F mutation, which may have a potential for resistance development to OP and CB, in field strains of *M. persicae*.

Chapter 3

Investigation and Identification of Carboxylesterase (E4) of local strain comparing with two insecticides

Investigation and Identification of Carboxylesterase (E4) of local strain comparing with two insecticides

Abstract

To identify whether higher expression of carboxylesterase (CbE) E4 in *Myzus persicae* is due to gene duplication, gene copy number was determined by quantitative real-time PCR. In addition, to determine the actual protein concentration of CbE E4 and it activity, Western blotting and activity staining were conducted. CbE gene copy number was highly correlated with carbamate resistance ratio (r^2 = 0.934). However, CbE E4 expression level was little correlated with insecticide resistance ratio (r^2 < 0.046) and no apparent correlation was observed among the gene copy number, protein quantity and total activity of CbE E4. Therefore, it was assumed that not only quantitative changing but also qualitative alteration of CbE E4 occurred in *M. persicae*. To investigate any potential alteration of CbE E4, mutation survey was conducted by sequencing of CbE E4 from various local strains of *M. persicae*. G137D and W251L mutations have been known as the main mutations associated with structural change leading to resistance. Interstingly, a new G134C mutation, which is in proximity of G137D mutation, was identified in the oxyanion hole of CbE E4. To predict the functional role of this mutation in resistance, 3-dimensional structure modeling was conducted. In summary, CbE E4 appears to be involved in

resistance to both pyrethroids and carbamates as a nonspecific hydrolase or sequestration protein in *M. persicae*.

1. Introduction

Carboxylesterase (CbE) belongs to the esterase superfamily with several isozymes and represents a metabolic detoxification factor in insecticide resistance (Satoh et al 2006). As described above, *M. persicae* has involved in resistance to organophosphate (OP), carbamate (CB) and pyrethroid insecticides (Devonshire et al. 1986). In the resistance mechanism, not only toxicodynamic factors but also metabolic factors are important in developing insecticide resistance. As a well known metabolic resistance factor, CbE E4 was overexpressed in *M. persicae* and conferred nonspecific resistance to various insecticides including OPs and pyrethroids (Devonshire et al. 1982; Field et al. 1993; Field et al. 1997; Field and Blackman 1998; Field 2000; Field and Blackman 2003). Similarly, overproduced cytochrome p450 was responsible for enhanced detoxification of insecticides, resulting in metabolic resistance (Philippiu et al. 2009; Puinean et al. 2010; Bass et al. 2011). Besides, structural change of CbE has been reported to be associated with OP resistance in *L. cuprina*. The G137D and W251L mutations were expressed using baculovirus and shown to be responsible for resistance to OPs (Newcomb et al. 1997).

In this study, we generated polyclonal antibody to semi-quantify the

overexpressed CbE E4 and activity staining using 1-naphthyl acetate was conducted to measure its total enzymatic activity. In addition, gene copy number was estimated by quantitative real-time PCR (qPCR). Presence of CbE E4 mutation was investigated by extensive sequencing and a new G134C mutation was identified in oxyanion hole. Fanally, correlation analysis was conducted among these multi factors. Although, correlation coefficient was not positive, we found the possibility that a new mutation from CbE E4 could be regulate the insecticide resistance.

2. Materials and methods

2.1 qPCR of CbE E4 gene

qPCR protocol of CbE E4 was developed according to the method of Kwon et al. (2009) with some modification. To determine the CbE E4 gene copy number, gDNA was extracted from 50 wingless aphids as described above. Primers specific to CbE E4 (Table 1) was used. Actin gene was PCR-amplified as a reference gene to normalize the relative copy number of target gene. qPCR was conducted using Chromo4 real-time thermal cycler (MJ research analysis, Waltham, MA) and DyNAmo HS SYBR Green master mix (Finnzymes, Vantaa, Finland) containing SYBR Green I fluorescent dye, $MgCl_2$, dNTP, and *Tbr* DNA polymerase. The qPCR reaction mixture was composed of 20 ng of 2 ng of gDNA, 10 μL of 2X DyNAmo HS SYBR Green master mix, and 5 μM of primers. The qPCR was performed with the following step-wise thermal program: 1 cycle at 95 °C for 15

min for DNA polymerase activation followed by 45 cycles at 95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec. Melting curve analysis was performed to confirm the homogeneity of the PCR products. Gene copy number was determined by following equation (ratio=$2^{-[Ct\ target-Ct\ actin]}$).

Table 1. PCR and sequencing primer used in this study

Primer name	Sequence	Remark
5′MpE4FE4	AAACTTTCCTTTTACACCGTTT	For qrtPCR to determine the gene duplication
3′MpE4FE4	TCTAAGCCAAGAAATGTTGAAA	
5′MpActin	CAAATCATGTTTGAAACCTTCA	
3′MpActin	AATGCATAACCTTCATAGATG	
5′CbE_G151D	TCCTGGAAGTGCCTGTTTAG	For amplification and sequencing
3′CbE_G151D	ATCGCCGGTGGAAGCAAATC	
5′CbE_W271L	CATCGTTGCATTCGGTGGTG	
3′CbE_W271L	CTATGGCCTTTGCTGGTCTG	

2.2 Generation of anti-CbE E4 polyclonal antibody

Rabbit anti-MpCbE_E4 ploYclonal antibody was generated by Ab Frontier (Seoul, Korea). Considering the hydrophobicity and antigenicity analysis, two peptide fragment [KPISKETKSNLSKMISDRS-C (position 395-413) and VYDNEEDRKMIKTMVN-C (position 478-493)] were synthesized. The synthesized immunogenic peptides were injected to rabbits intravenously three times. Final serum was affinity-purified using columns immobilized with antigen peptides.

2.3 Activity staining and Western blotting

50 wingless aphids presoaked in 300 μL of 0.1M Tris-HCl (pH 7.8) were homogenized using 1.0 mm zirconium oxide beads and a Bullet blender (Next advance Inc. Averill Park, NY). The homogenates were centrifuged for 10 min at 12,000 g at 4 °C. The supernatant was filtered through glass wool filter to remove excess lipids. The filtrate was used as a template of electrophoresis. To determine CbE activity, templates (12 μg protein) were separated by native polyacrylamide gele electrophoresis (PAGE) (7.5%) in triplicate at 120 V for 90 min in a cold chamber using a Tris-glycine buffer. Following native page, gel was incubated with 1 mM 1-naphthyl acetate (Sigma-Aldrich, St. Louis, MO) for 5 min at 25 °C. The reaction was terminated by 0.1% Fast blue RR salt (Sigma-Aldrich, St. Louis, MO) solution. To investigate the anchor properties, another gel was transferred onto a Hybond-N nitrocellulose membrane (GE Healthcare, Pittsburgh, PA) by electroblotting. Blocking solution (5% skim milk in PBS buffer) with primary antibody (anti-MPCbE_E4) was treated for overnight at 4 °C. The membrane was incubated with anti-rabbit IgG secondary antibody (Pierce Bio-Technology, Rockford, IL) for 1 h at 25 °C. The antigen-antibody complex was visualized using chemiluminescence kit according to manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4 Mutation survey of CbE E4 gene of local strains

To investigate the possibility of structural alteration of CbE E4, mutation survey was conducted. gDNA fragment flanking the G151D and W251L mutation sites was PCR-amplified from gDNA template from all local strains using sequence-specific primer set (Table 1). The PCR was performed for 35 cycles of 95 °C for 3 min, 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, 72 °C for 3 min. The PCR products were directly sequenced to determine the mutation with an ABI prism 3730 DNA sequence analyzer (Nicem Sequencing Facility, Seoul National University, Seoul. Korea).

2.5 3D structure modeling

The 3D structure prediction of CbE E4 with or without the mutation was conducted using the modeling server of SWISS-MODEL (http://swissmodel.expasy.org/). Structure comparison was performed using UCSF Chimera MatchMaker ver1.4 (University of California, CA). The modified model for improving visualization was generated by Swiss PDB viewer 4.0.1 (Swiss Institute of Bioinformatics, Lausanne, Switzerland).

2.6 Correlation between insecticides resistance ratio, gene copy number and expression levels

To determine the correlation factor, many factors, including gene copy

number, expression levels and total enzymatic sctivity, were compared with resistance level to each insecticide. Mortally was plotted against different factors, and regression coefficients were established using SigmaPlot10 software (Systat Software Inc., San Jose, CA).

3. Results

3.1 Gene copy number of CbE E4 in local strains

Hongchun strain showed a significantly higher gene copy number than other strains (Fig 1). With the exception of Hongchun strain, the gene copy number of all strains was 2.0- to 4.39-fold higher than susceptible strain. In general, gene copy number showed a high correlation with bioassay result of carbamate (methomyl).

3.2 CbE E4 expression levels in local strains

To determine the expression levels of CbE E4, activity staining and Western blotting were conducted. Following Western blotting and activity staining, band intensity was estimated by Kodak Imaging software4.0 (Kodak, Rochester, NY). All CbE E4 bands with activity were confirmed by being recognized by anti-MpCbE E4 polyclonal antibody. Bands of activity staining and Western blotting were detected at same position on the native PAGE gels (Fig 2). Based on the intensity of Western blotting, the amounts of CbE E4 in different local strains were 0.22 to 2.48 fold greater compared

to the one in the susceptible strain (Table 2). However, no apparent correlation between CbE E4 protein quantity and its activity was observed.

Table 2. Signal ratio from Western blotting and activity staining

	Local strains in 2011									
	S	MR	Hong chun	An ban duk	Pyeong chang	Chung ju	Bong hwa	Muju	Sa chun	Jeju
Signal ratio from Western blotting	1	2.07	2.32	2.84	2.48	1.81	2.36	0.22	1.92	0.7
Signal ratio from activity staining	1	1.76	1.76	1.57	1.67	1.57	1.21	1.08	2.28	1.71

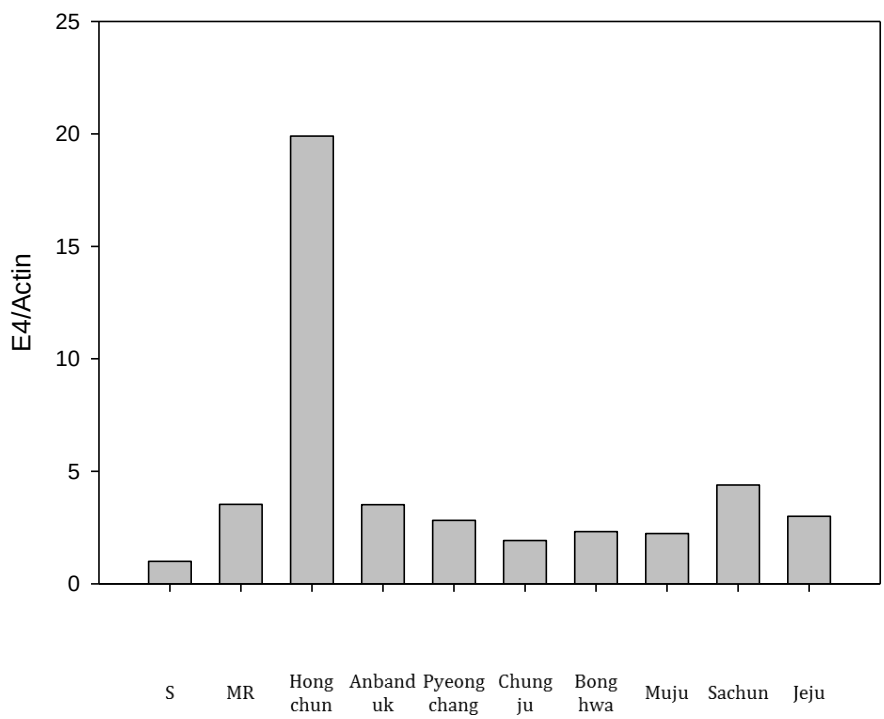

Figure 1. The relative gene copy number of CbE E4 in local strains of GPA.

The actin gene was used as an internal reference for comparison.

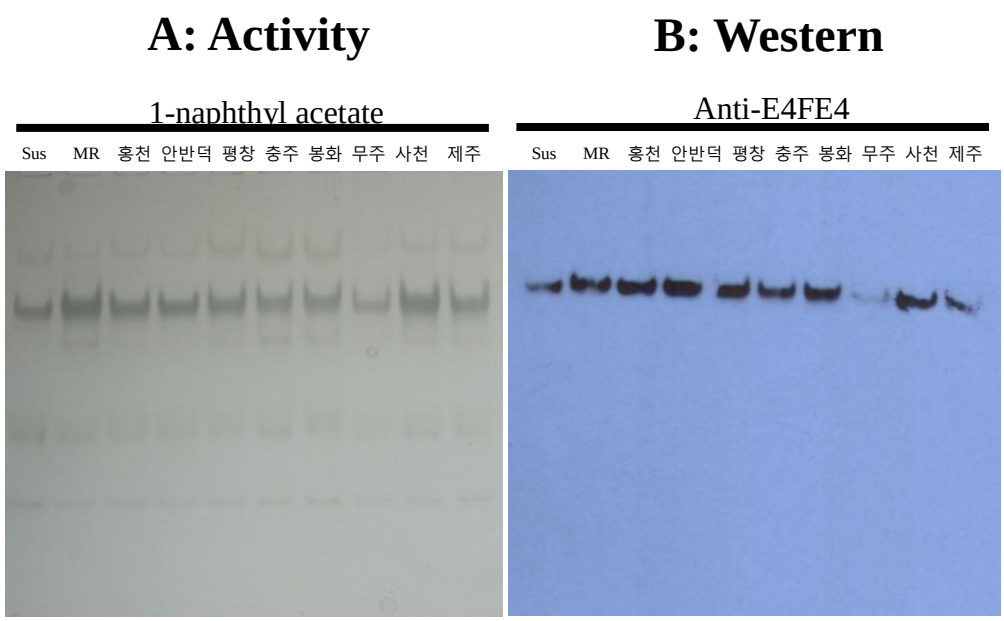

Figure 2. Native PAGE in conjunction with activity staining and western blotting for detection of carboxylesterase in local strains. After native PAGE, one gel was stained fom activity using 1---naphthyl acetate as a substrate (A) whereas another gel was analyzed by western blotting using anti-MPCbE_E4 polyclonal antibody (B), respectively.

3.3 Comparison of CbE E4 sequences among local strains

To investigate the possibility of qualitative change, presence or absence of the well characterized CbE mutations (G137D and W271L) in CbE E4 was investigated (Fig. 3 and 4). No similar mutations at the corresponding locations were found but a new G134C mutation was identified in the oxyanion hole (Fig. 3 and 4). Susceptible and Sachun strains revealed

susceptible sequence signal. In contrast, mixed signals of both susceptible and putative resistant allele sequences were observed in all other strains.

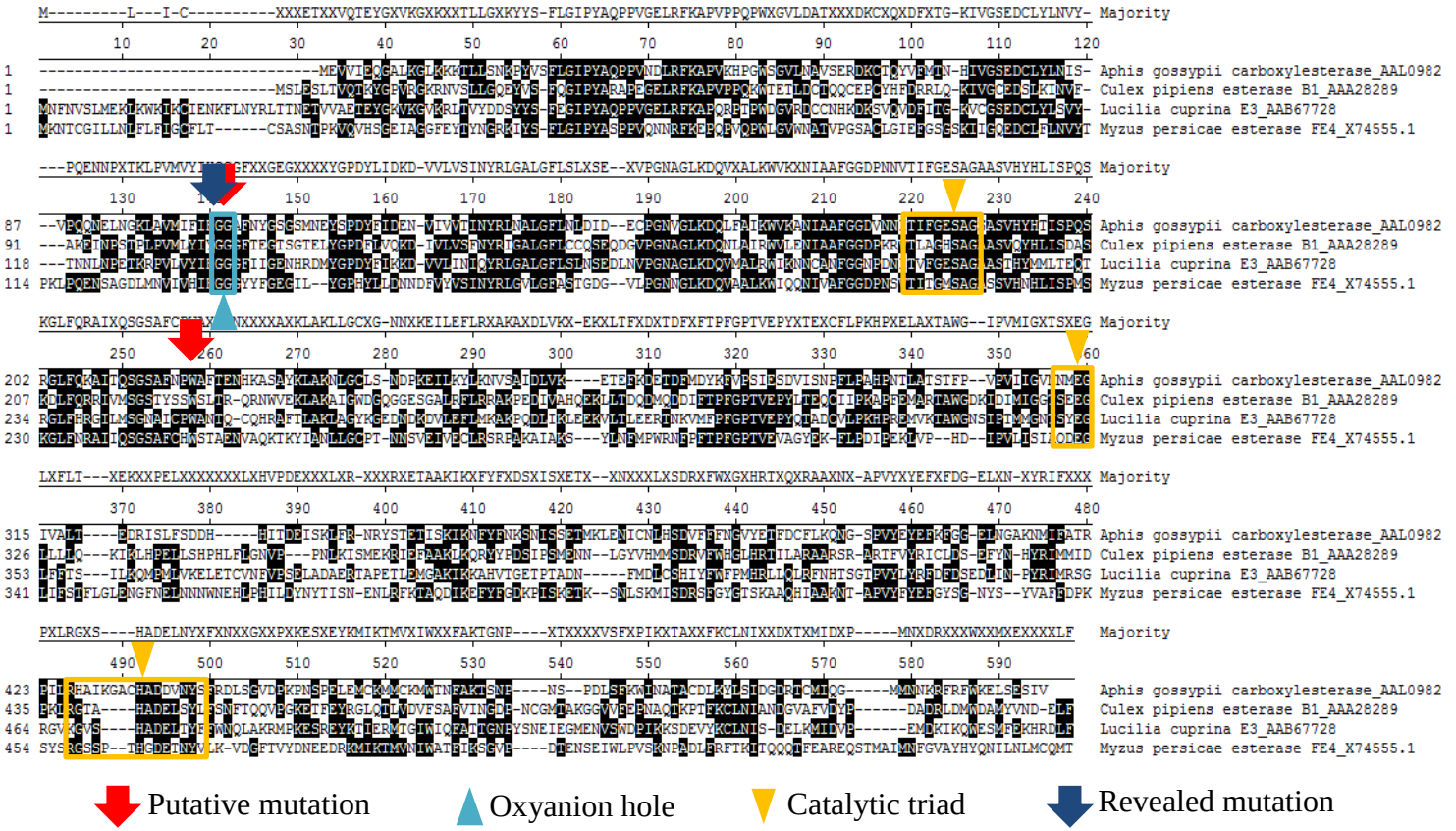
Putative mutation
Oxyanion hole
Catalytic triad
Revealed mutation

Figure 3. Alignment of carboxylesterase amino acid sequences by the Clustal V Method using DNA STAR. The catalytic triads and oxyanion hole were indicated. *Aphis gossypii* (AAL0982); *Culex pipiens* (AAA28289); *Lucilia cuprina* (AAB67728) ; *Myzus persicae* (X74555.1)

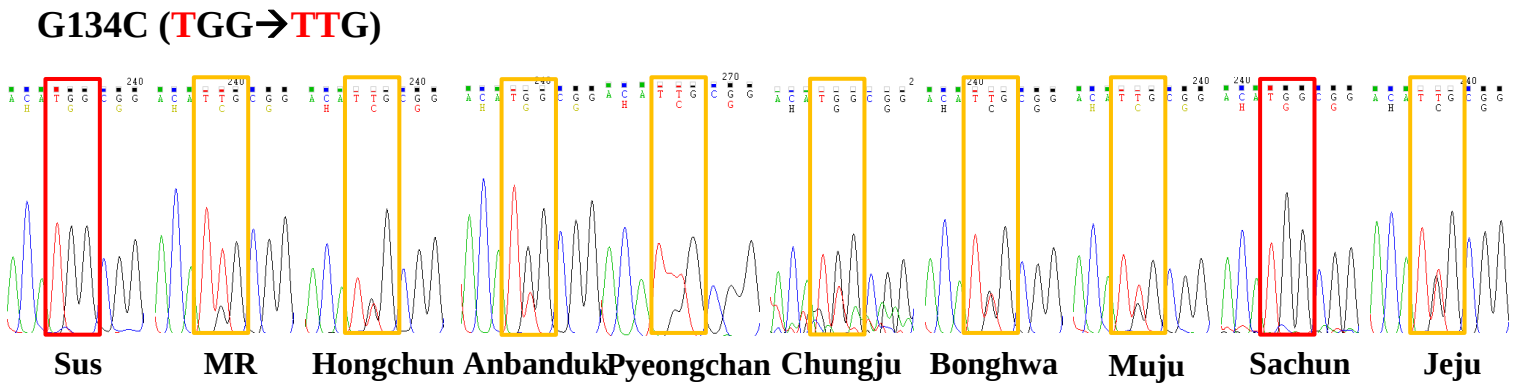

Figure 4. CbE E4 gene mutation survey in the 2011 local strains of GPA

3.4 Three-dimensional (3D) modeling of CbE E4

To investigate the functions of G134C mutation, the 3D structure of CbE E4 was predicted. The overall 3D structures of wildtype and mutant type CbE E4s were similarly folded (Fig. 5). The amino acid residues forming the oxyanion hole (blue) and catalytic triad (black) and the side chain of mutated residue (red) were indicated in Fig. 3 and 5.

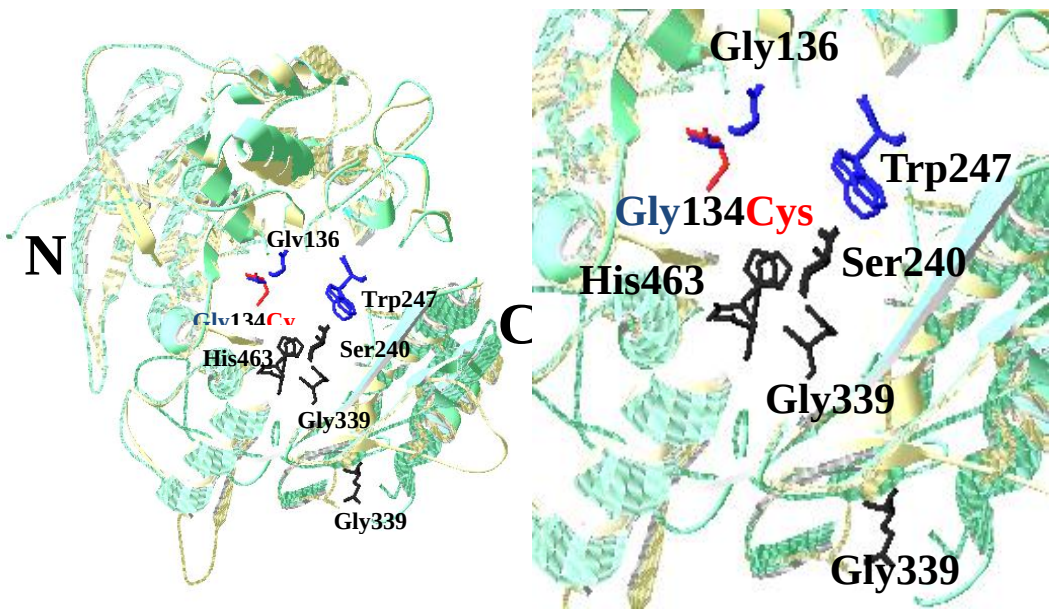

Figure 5. Predicted three-dimensional (3D) structure of CbE E4 as ribbon diagrams. The three residues comprising the catalytic triad (S240, G339, and H463)

3.5 Determination of correlation factor related with insecticide resistance

As a result of correlation between bioassay result and many factors, gene copy number significantly correlated with carbamate resistance level as indicated by high correlation coefficient (r^2=0.934)(Table 4). In contrast, correlation coefficient of pyrethroid resistance level shown negative correlation (r^2=0.008) (Table 3). To investigate the quantitative change of CbE E4, expression level such as activity staining and western blotting correlated with each insecticide but it indicated a negative correlation (r^2 < 0.046) (Table 3 and 4). In case of CbE mutation, G137C shown negative correlated with many factors (r^2 = 0.038), however correlation coefficient was substantially enhanced (r^2 = 0.491) when Hongchun strain was ignored (Table 5). On the basis of this finding, we assumed that quantitative and qualitative changing of CbE E4 might be occurred in aphid.

Table 3. Correlation analysis between the pyrethroid resistance ratio and CbE E4 resistance factors in the 2011 local strains

Pyrethroid (bifenthrin)	Correlation coefficient (r^2)
Resistance ratio *vs*. Allele frequency(M918L) (%)	0.942
Resistance ratio *vs*. Gene copy no.	0.008
Resistance ratio *vs*. Western activity (ratio)	0.046
Resistance ratio *vs*. Esterase staining activity (ratio)	0.063

Table 4. Correlation analysis between the carbamate resistance ratio and CbE E4 resistance factors in the 2011 local strains

Carbamate (methomyl)	Correlation coefficient (r^2)
Resistance ratio *vs*. Allele frequency (%)	0.018
Resistance ratio *vs*. Gene copy no.	0.934
Resistance ratio *vs*. Western activity (ratio)	0.041
Resistance ratio *vs*. Esterase staining activity (ratio)	0.008

Table 5. Correlation analysis between the carbamate resistance and allele frequency (G134C) except Hongchun strain (Concerning the possibility of over-expression of E4/FE4 protein by gene duplication)

Carbamate (methomyl)		Correlation coefficient (r^2)
Before exception	Resistance ratio *vs*. Allele frequency(G134C) (%)	0.038
After exception	Resistance ratio *vs*. Allele frequency(G134C) (%)	0.491

4 Discussion

In this work, we examined the role of CbE E4 in bifentrhin and methomyl resistance as the metabolic resistance factor in *M. persicae*. CbE E4 is well known for hydrolysis and/or sequestration of various insecticides (Herath et al.1987; Hemingway et al.1995). The molecular basis for overexpressed CbE E4 was confirmed by determining gene copy number, total activity and protein quantity. In case of CbE E4 gene copy number, Hongchun strain showed a remarkably higher copy number than other strains. Based on this

result, highly overexpressed CbE was expected in Hongchun strain. Therefore, polyclonal antibody (MPCbE_E4) was generated to determine the amount of CbE E4 in aphids by Western blotting. Based on these results, correlation analysis was performed to determine the main factor of resistance. Bifenthrin resistance was not little correlated with the quantity of CbE E4, suggesting that the role of CbE E4 in bifenthrin resistance is likely negligible. In contrast, CbE E4 quantity showed better correlation with the level of methomy resistance.

In addition to the overexpression of CbE, structural changes induced by point mutation in CbE was reported in *Lucilia cuprina* (Campbell et al. 1998). To investigate the possibility of structural alteration of CbE E4, mutation survey was conducted, particularly for the G137D and W251 mutation regions. However, a new G134C mutation was found in the oxyanion hole. Since the location of G134C mutation is only two amino acids away from the well characterized G137D mutation in the oxyanion hole, G134C mutation likely affects the catalytic properties of CbE E4, perhaps enhances hydrolysis and/or sequestration of CB insecticides, thereby resulting in resistance. To determine the role of this mutation in resistance, however, functional expression of CbE E4 would be mandatory.

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KOREAN ABSTRACT

복숭아혹진딧물의 피레스로이드와 카바메이트계 살충제 저항성요인의 분자적 진단

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초록

100가지 이상의 식물바이러스를 매개하며 작물의 심각한 피해로 수확량을 감소시키는 복숭아혹진딧물의 지역계통을 채집하고 실제 포장에서 많이 사용되는 두 가지 계통 약제 (pyrethroid, carbamate) 에 대한 저항성 수준 판단을 위해 생물 검정을 실시하였다. 또한 저항성 관련 요인 규명 및 저항성 형질빈도분포조사를 위한 분자생물학적 진단 기법을 개발하였다. 총 10지역의 계통

이 본 연구에서 사용되었으며 엽침지법으로 반수치사량을 구하였다. Bifenthrin (pyrethroid) 약제의 경우 voltage-gated sodium channel 유전자 내에서 M918L와 L1014F 돌연변이를 발견하였으며 Quantitative sequencing을 실시하여 군집 내 저항성 형질빈도를 조사 하였다. 이 중 새롭게 발견된 M918L 돌연변이는 생물검정결과의 상관도검정시 높은 상관관계 (r^2=0.942)를 보였으며 이는 효과적인 Bifenthrin저항성 진단 마커로 사용될 것으로 예상된다.

Methomyl (carbamate) 약제도 생물검정을 실시하였으며 acetylcholinesterase 유전자 내에서 S431F mutation이 발견되었으나 상관도 검정 시 낮은 상관도(r^2 <0.02)를 보여 여러 요인들이 복합적으로 저항성에 관여할 것으로 예상된다.

마지막으로 두 종류 살충제 저항성에 복합관여 할 것으로 예상되는 carboxylesterase E4의 정량적, 정성적 변화에 대해서 조사하였다. 정량적인 변화를 측정하기 위해 qPCR을 통한 gene copy number를 측정하고 polyclonal antibody를 제작 후 western blot 및 esterase staining을 통해 비교 검정하였다. Gene copy number의 경우 methomyl (carbamate) 생물검정 결과와 높은 상관도(r^2 =0.934)를 나타내었으나 western 및 esterase

staining의 경우 두 종류의 살충제 모두 낮은 상관도(r^2 <0.07)를 보였다. 이에 carboxylesterase 유전자 내의 돌연변이로 인한 정성적 변화를 예측하고 조사하였으며 몇몇의 지역계통에서 G151C 돌연변이가 발견되었다. 이 돌연변이의 기능을 예측하기 위한 3D prediction결과 작용점 근처에서 기질에 반응성을 높이거나 가수분해 효율을 높일 것으로 예상되며 이를 통한 종합적 살충제 진단에 있어서 효과적인 마커로 사용될 것으로 기대되고 있다.

검색어: 복숭아혹진딧물, 피레스로이드 저항성, 카바메이트 저항성, 나트륨 이온채널 돌연변이, 아세틸콜린에스터라아제 돌연번이, 정량염기서열분석법, 카복실에스터라아제.

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