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Overexpression of cytochrome P450 genes in *Aphis gossypii* (Glover) in the cotton fields of the Çukurova region, Turkey

Çukurova bölgesi pamuk alanlarında *Aphis gossypii* (Glover)'da aşırı düzeyde sitokrom P450 genleri ekspresyonu

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ABSTRACT

Aphis gossypii Glover, 1877 (Hemiptera: Aphididae), which is a polyphagous species, is among the main pests of the cotton plants in the whole world. The Çukurova region is an area where polyculture agriculture activities are carried out. While insecticides in the neonicotinoid group are prevalently used in the cotton fields in this region for controlling *A. gossypii*, the problem of resistance is frequently encountered. This study determined the relative expression levels of the CYP6CY22 and CYP6CY13 genes, cytochrome P450, acetylcholine esterase (AChE) and glutathione S-transferase (GST) enzyme activities in six different populations of *A. gossypii* collected in 2018 from cotton fields in the province of Adana in Southern Turkey. In comparison to the reference culture, there was an increase in gene regulation by 81.9 fold in the CYP6CY22 gene in the Hamitbey population and by 6 fold in the CYP6CY13 gene in the Gazipaşa population. Additionally, the Hamitbey population showed 4.3 times higher (0.74 U/ml) cytochrome P450 enzyme activity. The Çiftlikler population showed 5.9 fold higher AChE (0.82 U/ml) and 4.3 fold higher GST (2.68 U/ml) activities. The analyses revealed that the *A. gossypii* populations were exposed to high amounts of insecticides. Consequently, overexpressed genes, high levels of enzyme activities and metabolic resistance in based on cytochrome P450 activity were observed in the study.

INTRODUCTION

Cotton aphid, *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae), is one of the significant pests among aphids that reaches the economic loss threshold. *A. gossypii* is a

polyphagous species that has a very broad spectrum of hosts. As in the entire world, they create harm on especially cotton and Cucurbitaceae species in Turkey (Ozgun and Sekeroglu

1986, Tomizawa and Casida 2005). Insecticide application has become prominent today due to its economical and easy to implement nature in controlling *A. gossypii* which leads to reductions in yield and quality in cotton farming. While there are several natural deterrents that are effective on *A. gossypii* in cotton, chemical control is the most frequently preferred method among producers (Godfrey et al. 1997). In addition to this, increased resistances to insecticides (organophosphates, carbamates, pyrethroids and neonicotinoids) in different ratios were reported in several countries (Ahmad et al. 2003, Sparks and Nauen 2015, Wang et al. 2007). Neonicotinoids are among insecticides with high market share that are effectively used in recent times for controlling especially aphids and whiteflies in the Coleoptera, Diptera and Lepidoptera families. However, varying ratios of development of resistance against this group of insecticides were reported in both the entire world and the Mediterranean Region due to its widespread and irresponsible usage (Jeschke et al. 2011, Nauen et al. 2008, Ulusoy et al. 2018). Resistance mechanisms are caused by strengthened metabolic enzyme activities in organisms and reduction in the levels of sensitivity against xenobiotics as a result of mutations in target proteins (Nauen 2007). In the process of detoxifying xenobiotics in living organisms, there are activities of several enzymes in the multigene group such as esterase, glutathione S-transferase (GSTs) and cytochrome P450. This activity is closely related to the transcription levels of these genes on the cellular level (Bass and Field 2011, Field et al. 1999). In *A. gossypii*, target-site insensitivity against neonicotinoids and point mutations in the nAChR beta 1 loop D field (R81T) are significant factors in the development of resistance (Bass et al. 2011, Hirata et al. 2015, Ihara et al. 2008). Likewise, detoxification caused by cytochrome P450 in insects is one of the main factors in the development of resistance against insecticides (Nauen and Elbert 2003, Scott 1999, Zhao et al. 2014). Cytochrome P450 monooxygenases are a broad family of enzymes that have functions of metabolic activities such as hormone synthesis in the insect's body and making xenobiotics less toxic or water-soluble in their detoxification (Rauch and Nauen 2003, Scott and Wen 2001). High P450 enzyme activity levels were reported in neonicotinoid-resistant *A. gossypii* populations (Seyedbrahimi et al. 2015, Shang et al. 2012, Ulusoy et al. 2018). It was stated that this situation is dependent on the changes in the expression levels of P450 genes, changes in catalytic activity or changes in substrate-specificity (Bergé et al. 1998, Hirata et al. 2017, Nikou et al. 2003, Wu et al. 2018). Various next-generation sequencing (NGS) studies have been carried out in relation to P450 expression levels (Hirata et al. 2017, Wu et al. 2018). Hirata et al. (2017) reported 45 times

higher activity levels in comparison to reference values in the CYP6CY13 and CYP6CY22 genes in 10 different genes in the cytochrome P450 gene family in thiamethoxam-resistant *A. gossypii* populations. Widespread and irresponsible usage of insecticides takes place in Çukurova, which is one of the most important regions involved in polyculture agriculture in the Mediterranean Region in Turkey. Neonicotinoid resistance was reported in *A. gossypii* in cotton fields in this region (Ulusoy et al. 2018).

This study determined the relative expression levels of the CYP6CY22 and CYP6CY13 genes, cytochrome P450, acetylcholine esterase (AChE) and glutathione S-transferase (GST) enzyme activities in six different populations of *A. gossypii* collected in 2018 from cotton fields in the province of Adana in Southern Turkey.

MATERIALS AND METHODS

A. gossypii individuals were collected from six different locations (Gazipaşa, Hamitbey, Solaklı, Körkuyu, Akyuva, Çiftlikler) in the province of Adana where widespread cotton production took place in June 2018. The *A. gossypii* population that had been cultured for the five previous years under greenhouse conditions at 22 °C, 65±5% RH and 16:8 h L:D photoperiod at the Directorate of the Biological Control Research Institute was taken as the susceptible culture. PCR analysis was utilized at thermalcycler (Eppendorf Mastercycler Nexus Thermal Cycler, USA) and real-time Light cycler (Roche LightCycler 96, Switzerland). PCR products were run at agarose gel (ThermoFisher Scientific Mini Gel Electrophoresis Systems, USA) and viewed in gel visulation (Quantum Vilber gel visulation, Germany). Enzyme analyses were read via microplate reader spectrophotometer (Multiscan Go microplate reader, ThermoFisher Scientific, USA). It was worked with individual *A. gossypii* apterous in all analyses.

Determining acetylcholine esterase activity

Acetylcholine esterase activity was determined by modifying and using the method reported by Ulusoy et al. (2018). 50 *A. gossypii* individuals were homogenized with a homogenizer in an Eppendorf tube containing a 500 µl phosphate buffer (0.1 M pH: 7.5) 0.1% Triton X-100. The homogenate was centrifuged at 10 000 g and 4 °C at 5 min, and the supernatant was used as an enzyme source after 10 times of dilution. For measuring AChE activity, 100 µl (0.5 mM) acetylcholine iodide (ATChI), 100 µl 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 100 µl enzyme solution were added to the microplate cells. AChE activity was measured for 10 min at 412 nm in a kinetic microplate reader with 3 replications at 25 °C. The control cells were read without

adding the homogenate. The enzyme readings were made with 3 replications. The activity levels were determined by comparison to the controls. The specific enzyme activity was determined using the extinction coefficient of chromophore (Kranthi 2005).

Determining glutathione S-transferase activity

The method by Ulusoy et al. (2018) was modified and used to determine activity. 50 aphid individuals were homogenized in a 500 µl Tris HCL buffer (0.05 M, pH: 7.5) by a homogenizer, the homogenate was centrifuged at 11 000 g at 4 °C for 7 min, and the supernatant was used as an enzyme source. 100 µl 0.4 mM 1-chloro-2,4-dinitrobenzene (CDNB), 100 µl 4 mM reduced glutathione (GSH) and 100 µl enzyme were added to the microplate cells and the reaction was started. Reading was carried out in a microplate reader at 340 nm and 25 °C for 10 min. The control cells were read without adding the homogenate. The enzyme readings were made with 3 replications and the activity levels were determined by comparison to the controls. Changes in absorbance per minute were converted into nM CDNB conjugated/min/mg protein using the extinction coefficient of the resulting 2,4-dinitrophenyl-glutathione (Habig et al. 1974).

Determining cytochrome p450 monooxygenase activity

Cytochrome p450 activity was measured based on the method describe by Ulusoy et al. (2018). 50 aphid individuals were homogenized in a Na - phosphate buffer (0.1 M pH: 7.5), the homogenate was centrifuged as 10 000 g at 4 °C for 10 min, and the supernatant was used as an enzyme source. 90 µl of enzyme and 100 µl 2 mM of p-nitroanisole (substrate) were mixed and left for 3-4 min at 34 °C. Then, 10 µl 9.6 mM of nicotinamide adenine dinucleotide phosphate (NADPH) was added to this mixture, the resulting mixture was put into the microplate cells, and measurements were made at 40 s intervals for 20 min at 405 nm and 25 °C by using microplate reader. The control cells were read without adding the homogenate. The enzyme readings were made with 3 replications and the activity levels were determined by comparison to the controls. The specific enzyme activity was determined using the extinction coefficient of p-nitrophenol (Kranthi 2005).

A. gossypii total RNA extraction and real-time polymerase chain reaction (RT-PCR)

Aphid individuals were frozen at -80 °C, 50 of the individuals were homogenized, and their total RNAs were extracted by using a RNA purification kit (ThermoFisher Scientific RNA purification kit, USA). After the extraction, the total RNA amounts were measured by spectrophotometer (ThermoFisher Scientific NanoDrop Spectrophotometer,

USA), and equal concentrations of 60 ng/µl each were diluted by a TE buffer.

In real-time PCR analysis, the relative activity of the cytochrome P450s enzyme gene was determined by examining the expression levels of the CYP6CY22 and CYP6CY13 genes (Hirata et al. 2017). Using the NCBI primer blast software for this genes, primers that recognized the CYP6CY22 (NCBI Accession No: XP_001948421) and CYP6CY13 (NCBI Accession No: XP_008184269) gene parts were designed. As the genuine primer for the CYP6CY22 gene, forward: 5'-TTCCGCCATTACGCCAAGAT-3', reverse: 5'-ACGGTTTCTGAACCAGCAGT-3' and for the CYP6CY13 gene, forward: 5'-AGGAAAGCAAACGTGCCTCA-3', reverse: 5'-GAATCCGTGGTCCGTGAAGT-3' primers were designed. As the housekeeping gene, this study used the β-actin gene, forward 5'-AGCTCTATTCCAACCTTCCTTCT-3', β-actin reverse 5'- TGTATGTAGTCTCGTGGATACCG-3'. In the classical PCR experiment, the primers were tested by using a One-Step RT-PCR kit (ThermoFisher Scientific One-Step RT-PCR Kit, USA), and band widths were determined. In the classical PCR process, there were one cycle of 15 min at 50 °C, one cycle of 15 min at 95 °C, 40 cycles of 15 s at 95 °C, waiting time of 30 s at 59 °C and another 30 s at 72 °C. The real-time PCR process was carried out with 3 replications for each gene in each population by using One-Step qRT-PCR kit (ThermoFisher Scientific, One-Step qRT-PCR kit, USA) the Ct values were determined, and these values are taken into analysis by taking the average of 3 replications. In order to confirm the specificity of the real-time reactions, a melting curve cycle was applied. The same temperature table was used in the RT-PCR process. The melting curve program included a cycle of 30 s at 95 °C, a cycle of 10 s at 60 °C and a waiting time of 10 s at 60 °C. The relative activity levels of the CYP6CY22 and CYP6CY13 genes were determined by making $\Delta\Delta C_t$ calculations based on the reference population by taking the average of the obtained Ct values (Livak and Schmittgen 2001).

RESULTS AND DISCUSSION

Enzyme assays and gene expression analyses were carried out on *A. gossypii* populations that were collected from different locations in the province of Adana. As a result of the analyses, higher levels of AChE, GSTs and cytochrome P450 activities were observed in all the collected populations in comparison not the reference population. The highest AChE activity was seen in the Çiftlikler population (0.82 U/ml), while this was followed by the Hamitbey population (0.62 U/ml). The lowest activity was observed in the Akyuva population (0.34 U/ml). 5.9 times more AChE activity was observed in comparison to the reference population (Table

1). In parallel with these results, another study that was carried out in this region found 6.5 times more AChE activity in comparison to the reference population for *A. gossypii* populations (Ulusoy et al. 2018, Velioglu et al. 2008). As in the cases of AChE enzyme activity levels, the highest GST enzyme activities were found in the Çiftlikler population (2.68 U/ml) and Hamitbey population (2.33 U/ml). 4.3 times higher GST enzyme activity was observed in comparison to the reference population. The weakest GST activity was found in the Akyuva population (1.05 U/ml) (Table 1). Likewise, it was reported that, 3.3 times more enzyme activity was found in the neonicotinoid-resistant *A. gossypii* populations in the province of Adana in 2016 (Ulusoy et al. 2018). These results provide evidence that these populations have metabolic resistance still in this region. Previous studies reported that the general levels of esterase and GST activities may be higher in populations that are resistant against the insecticides in the groups of organophosphates, carbamates and pyrethroids (Devonshire and Moores 1982, Hemingway and Georghiou 1984, Rauch and Nauen 2003). Especially the Çukurova region is an agricultural production area where there is a widespread usage of insecticides from different groups in the agricultural fields where polyculture agriculture activities including vegetables, fruits and cotton are carried out. Although different agricultural products are grown, these agricultural fields are almost intertwined. Therefore, considering that *A. gossypii* is a polyphagous species and is exposed to substantial amounts of insecticides with different effect mechanisms such as organophosphates, carbamates, pyrethroids and neonicotinoids, it is inevitable to consider a metabolic resistance. The observation of cytochrome P450 enzyme activities revealed the highest enzyme activity in the Hamitbey population (0.74 U/ml) followed by the Solaklı population (0.64 U/ml), while the lowest activity was found in the Körkuyu population (0.29 U/ml). The Hamitbey population showed 4.3 times higher enzyme activity in comparison to the reference population (Table 1). A similar study that was carried out in this region also reported higher cytochrome P450 enzyme activity (Ulusoy et al. 2018).

In the study, RT-PCR analysis was carried out to observe *A. gossypii*'s cytochrome P450 CYP6CY13, CYP6CY22 and β -actin genes in agarose-gel (Figure 1). When the gene expression of cytochrome P450 CYP6CY13 and CYP6CY22 were analyzed by RT-PCR, higher relative activities were recorded in comparison to the reference population (Figure 2). The relative activity levels of the CYP6CY22 gene were 81.9 higher in the Hamitbey population and 46.3 times higher in the Solaklı population. The highest relative activity level of the CYP6CY13 gene was found in the Gazipaşa population (6 times) (Figure 2). The relative activity levels of both genes

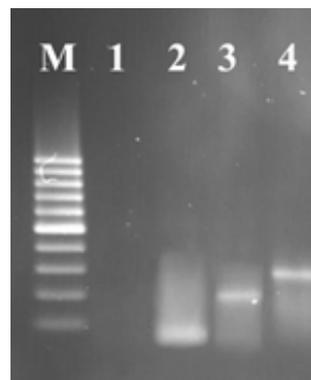


Figure 1. *Aphis gossypii* CYP6CY13, CYP6CY22 and β -actin genes RT PCR agarose gel electrophoresis photograph. M: 100 bp ladder, 1. Negative control, 2. β -actin: 60 bp, 3. CYP6CY13: 234 bp 4. CYP6CY22: 326 bp

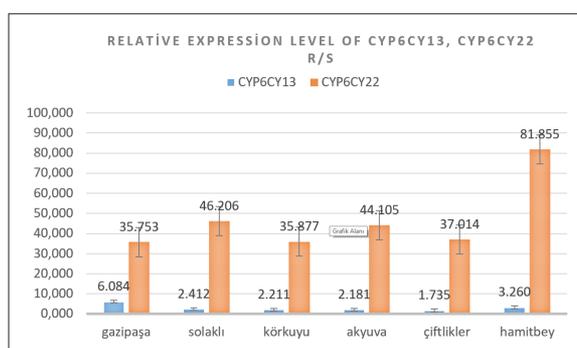


Figure 2. Cytochrome P450 CYP6CY13 and CYP6CY22 genes relative expression levels of *Aphis gossypii* populations (R/S: resistant population/susceptible population)

were found to be higher in comparison to the reference. Hirata et al. (2017) reported 45 times higher relative activity in the CYP6CY13 and CYP6CY22 genes of thiamethoxam-resistant *A. gossypii* individuals in comparison to the reference individuals. The finding of a study that was carried out in 2016 in this region on the presence of thiamethoxam-resistant *A. gossypii* populations supports the finding of high relative activity levels (Ulusoy et al. 2018). Previous studies conducted in the Eastern Mediterranean Region reported resistance against neonicotinoids in *A. gossypii* in the cotton production fields especially in the year 2016 (Ulusoy et al. 2018). Previous studies stated that especially the activity of the P450 enzyme group was effective in the development of such resistance (Rauch and Nauen 2003, Scott 1999, Zhao et al. 2014). Additionally, studies have demonstrated that, as well as the presence of mutations in resistance against neonicotinoid insecticides, the metabolic activity and gene

Table 1. Acetylcholin esterase (AChE), glutathione S-transferase (GTS), cytochrome P450 monooxygenase (P450) enzyme activities of resistant and susceptible *A. gossypii* populations collected from Cukurova region

Population	AChE U/ml	GSTs U/ml	Cytochrome P450 U/ml
Gazipaşa	0.52 ± 0.04c	1.63±0.73bc	0.45±0.12bcd
Solaklı	0.57 ± 0.08cd	1.72±0.38bc	0.64±0.14de
Körkuyu	0.45± 0.12bc	1.40±0.7b	0.29±0.12ab
Akyuva	0.34±0.06b	1.05±0.06b	0.52±0.07cd
Çiftlikler	0.82±0.05e	2.68±0.28d	0.39±0.06bc
Hamitbey	0.67±0.02 d	2.33±0.33cd	0.74±0.14e
Susceptible	0.14±0.05 a	0.63±0.06a	0.15±0.12a

a, b, c, d, e – Duncan's multiple range test ($p < 0.05$)

expressions of the P450 group were also higher (Hirata et al. 2017, Nauen et al. 2008, Wang et al. 2009, Wu et al. 2018). While the CYP4, -6, -9, and -12 gene family in insects plays a role in the biological detoxification process, it was reported that the CYP4 and CYP6 subfamilies were the most effective members in the xenobiotic mechanism and observation of resistance (Berenbaum 2002, Li et al. 2007). Consequently, this study showed that the samples that were collected from locations of the cotton production fields in the province of Adana in 2018 were highly exposed to insecticides, and there was an increased metabolic resistance. The high levels of the AChE, GST and cytochrome P450 enzyme activities that were observed in the collected *A. gossypii* populations supported the development of resistance. Additionally, as shown by the total RNA and RT-PCR analyses, in parallel with cytochrome P450 activity, the expression levels of the CYP6CY13 and CYP6CY22 genes were also higher. The presence of increased metabolic resistance was observed in an accelerating manner considering the results of previous studies on resistance against insecticides in the neonicotinoid group in the Çukurova region. It was also revealed that there might be a development of resistance caused by cytochrome P450.

These days, the discovery and development of new insecticides is very difficult and costly task. Therefore, strategies that slow down the rapid development of resistance to new and existing insecticides need to be implemented in all agricultural environments (Sparks and Nauen 2015). If insecticide resistance management (IRM) is not seriously taken into account in pest control, the development of resistance will be inevitable. The basis of an IRM strategy is

reduce selection pressure and avoiding selection of resistance mechanisms (Bielza 2008). The repeated use of the same insecticide compounds of the same modes of action over many generations is cause insecticide resistance development. An Integrated Pest Management (IPM) strategies, cultural control (proper watering and fertilization, sanitation, weed removal, crop rotation), biological control (use of predators, parasitoids and pathogens), and genetic control (host plant resistance) should not be ignored (Biondi et al. 2018, Guedes and Picanço 2012). The use of one or more of these alternative strategies may reduce the need for insecticides, thus decreasing the selection pressure on pest population. In the light of these results, it was shown that the insecticide-based control against *A. gossypii* should be reevaluated in the cotton field in the Çukurova region. Consequently, in the management of *A. gossypii*, it is necessary to take a more conscious and serious approach by considering the IRM and IPM strategies again.

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ÖZET

Polifag bir tür olan *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae) tüm dünyada pamuk bitkisinin ana zararlılarından. Çukurova bölgesi polikültür tarım yapıldığı bir bölgedir. Bu bölgede pamuk ekim alanlarında *A. gossypii* mücadelesinde neonicotinoid grubu insektisitler yoğun bir şekilde kullanılmakta olup, direnç problemi ile

karşılaşmaktadır. Çalışmada 2018 yılı, Adana ili pamuk alanlarından toplanmış altı farklı *A. gossypii* popülasyonu sitokrom P450 (CYP)'ye ait CYP6CY22 ile CYP6CY13 genleri relatif ekspresyon düzeyleri, sitokrom P450, asetilkolin esterase (AChE) ve glutathion S-transferaz (GST) enzim etkinlikleri belirlenmiştir. Hassasa oranla Hamitbey popülasyonunda CYP6CY22 geni 81.9 kat ve Gazipaşa popülasyonunda CYP6CY13 geni 6 kat daha fazla gen regülasyonu belirlenmiştir. Ayrıca Hamitbey popülasyonunda 4.3 (0.74 U/ml) kat daha fazla sitokrom P450 enzim aktivitesi saptanmıştır. Çiftlikler popülasyonunda 5.9 kat AChE (0.82 U/ml) ve 4.3 kat daha fazla GST (2.68 U/ml) aktivitesi gözlenmiştir. Yapılan analizlerde *A. gossypii* popülasyonlarının yoğun şekilde insektisitlere maruz kaldığı saptanmıştır. Sonuç olarak, aşırı gen ekspresyonu ve yüksek düzeydeki enzim etkinlikleri, sitokrom P450 aktivitesine dayanan metabolik direncin varlığını ortaya koymuştur.

Anahtar kelimeler: *Aphis gossypii*, pamuk, sitokrom P450, aşırı ekspresyon

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