IDENTIFICATION OF MUTATION IN THE *BEMISIA TABACI* (GENN.) *PARA* SODIUM CHANNEL GENE ASSOCIATED WITH RESISTANCE TO PYRETHROIDS

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Abstract

Mechanism of resistance to lambda-cyhalothrin in highly resistant *Bemisia tabaci* strain can be explained by the voltage-gated sodium channel which is the primary target site of pyrethroid insecticides. The super knockdown resistance (super-kdr) to pyrethroids is caused by changes at specific sites on *the para*-type sodium channel protein domain II (IIS4-6). The *B. tabaci para*-type sodium channel gene was RT-PCR amplified from lab-strain (reference), Parent (first generation after treatment with the insecticide) and generation thirteen after treatment was considered. The mechanisms of resistance to Lambda-cyhalothrin (Karat 20% EC) in a Q biotype, highly resistant *Bemisia tabaci* strain. Analysis of the sequence of the IIS4-IIS6 region of *the para* sodium channel gene of lab-strain, Parent and resistant strain (G13) revealed two amino acid replacements compared to that of the SUD-S susceptible strain. One is the leucine to isoleucine substitution at position 925 (L925I) and allele r1-Q1 (GenBank accession no. DQ205206) has identical intron sequences with samples of parent and lab-strain, other is a novel kdr resistant mutation for *B. tabaci*, a threonine to valine substitution at position (T929V) and alleles r2-Q1 (GenBank accession no DQ205207) has identical intron sequence with G_{13}..

Keywords: *Bemisia tabaci*, sodium channel gene, insecticide resistance, pyrethroids

INTRODUCTION

The whitefly, *Bemisia tabaci* is one of the most serious and key against pests of numerous crops worldwide. The ability of *B. tabaci* to develop resistance to insecticides after only a few applications makes its control problematic in the long term (Denholm,I. et al 1998)

For the past thirty years, *B.tabaci* control in Egypt has been based almost exclusively on conventional insecticides such as organophosphates, pyrethroids and carbamates. Little work quantifying resistance in *B.tabaci* in Egypt has been published, but it is clear that, by the late 1980s, at least some of these broad-spectrum insecticides were failing in some situations, the pyrethroid cypermethrin, lost its efficacy during 1989 and 1990, severe resistance to organophosphate and pyrethroids in *B.tabaci* from Egyptian cotton fields were established (Abdallah 1991). More recent

data on insecticide efficacy from field trials on cotton shows that many traditional insecticides are no longer effective (Sobiha *et al.*,1997) and 10 to 12 insecticide applications per season are now common in the cotton crop.

Insecticide resistance selection is even more marked on vegetable crops, where the risk of whitefly mediated virus transmission (particularly in tomatoes) has led farmers to apply organophosphate and/or pyrethroid insecticides two or three times per day (Hafez El-Kady & Gregor J.D. 2003). Monitoring high resistance levels to several insecticide classes in field *B. tabaci* populations from different governorates in seasons 2000 and 2001, particularly pyrethroids and organophosphates, resistance factors to the pyrethroid lambda-cyhalothrin was up to 1161.54 fold (Farghaly 2005).

Resistance to pyrethroids in *B.tabaci* has been associated with enhanced detoxification by oxidative and hydrolytic pathways. In addition, target site insensitivity was recently implicated in *B. tabaci* pyrethroid resistance, (Morin *et al.*, 2002). Although the kdr mutation L1014F found in many important agricultural pests, (Soderlund and Knipple 2003) has not been detected in the sweet potato whitefly, two mutations in the IIS4-5 linker of *B.tabaci* sodium channel gene, a methionine to valine substitution at position 918 (M918V) and a leucine to isoleucine alteration at position 925 (L925I) conferred high levels of fenpropathrin resistance in *B. tabaci* strains isolated from Arizona, (Morin *et al.*, 2002). A third sodium channel resistance mutation, a threonine to valine substitution at position 929 (T929V), was implicated with pyrethroid resistance, in a highly resistant *B. tabaci* strain (GRMAL-RP) isolated from Crete, Carboxylesterase (COE) - and cytochrome P450 dependent monoxygenase-based detoxification was also implicated in pyrethroid resistance in GRMAL-RP, (Bass *et al.*, 2004)

In the present study, the level of resistance to lambda-cyhalothrin was estimated in population of *B.tabaci*. Subsequent insecticide selection was carried out to build up resistance, and identification of mutations within the voltage-gated sodium channel gene. This information is fundamental to an understanding of insecticide resistance and for the development strategies to reduce the risk for lambda-cyhalothrin.

MATERIALS AND METHODS

1.1-Insecticide used

Lambda-cyhalothrin (Karat 20% EC)
(S)   (Z)-(1 R)-*c is* -

+

(R)   (Z)-(1 S)-*c is* -
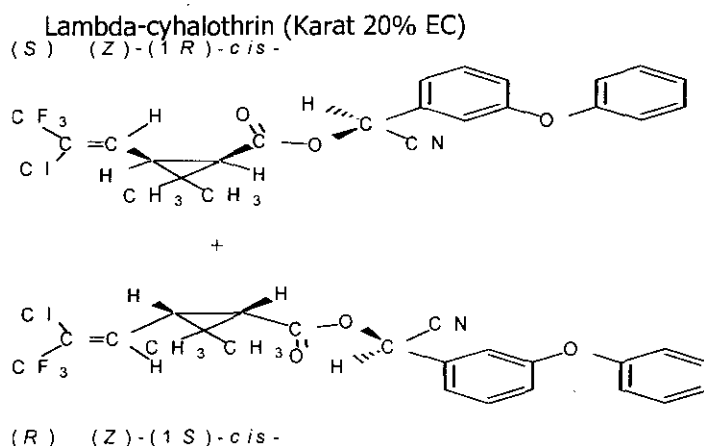
1.2-*Bemsia tabaci* strain

Field strain of *B.tabaci* was collected from Behera governorate (2000) and maintained without insecticide selection pressure for more than 8 years in the lab of Central Agricultural Pesticides Laboratory, department of rearing standard insects. Resistant-strain derived from the Lab-strain was selected with lambda-cyhalothrin for 13 generations (127.08 fold) as shown in table (2).

1.3-Bioassay

The bioassay method for obtaining concentration-response regressions lines was described by (Prabhaker *et al.*, 1985) with some modification, attached cotton leaves were dipped for 5 sec. in 100 ml of the desired concentration of each insecticide and allowed to dry, the treated leaves were laid on a thin layer of 2% agar in small cage and then twenty adults were transferred into the cage by an aspirator. Mortality was recorded 24 and 48 hr after treatment. Six concentrations At least were tested for each insecticide, each test was replicated on five different. Results were expressed as percentage mortalities, corrected using (Abbott formula, 1925).

1.4-Selection pressure

The population of the resistant-strain which consisted of many thousands was subjected to laboratory selection pressure with insecticide, at a level producing 30% mortality to the adult stage. The level of developing resistance was determined at generations 3, 6, 9 and 13, Resistance ratio (RR) was determined by dividing the LC_{50} of the R-strain by the LC_{50} of lab-strain.

2.1-RT-PCR analysis of *B. tabaci* sodium channel gene sequences

The IIS4-5 region of the *B.tabaci para*-type sodium channel gene was initially amplified by reverse transcriptase-mediated PCR (RT-PCR). Total RNA was extracted from susceptible strain, parent types and generation thirteen (S, P and G13). In each sample, 100 adults were homogenized according to the manufacturer's instructions. One RNA sample was produced for each sample. First strand cDNA was synthesized from total RNA (3μg) by reverse transcriptase (superscript III, RNase H⁻, ready togo RT-PCR kit Pharmacia, Aniasham) with oligodT (20 μM) as a primer, according to the supplier recommended protocol. The cDNA fragments served as templates for subsequent PCR amplification using the forward primer kdr-1 (5′ GCCAAATCCTGGCCAACT 3′) and reverse Kdr-4 (5′ GAAATTACTCAGCAACAACGC 3′). The thermal conditions were : denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min.

2.2-Sequence analysis

The presence or absence of the L925I and T929V mutation in individual *B.tabaci* adult was analyzed using the manufacturer recommendations (Ahmadian *et al.*, 2000). The gene fragments of interest were amplified using the primers *Bemisia* Na-channel pyro f biotin (5′ TGGCCAACTTTGAATCTGTT 3′) and *Bemisia* Pyro r (5′ GCATTCCCATCACAGCAAAA 3′) that amplify a 112 bp fragment comprising the L925I and the T929V mutations.

2.3-Phylogenetic analysis

To determine the phylogenetic relationship between resistant and susceptible allel sequences, the sequences were aligned using the (Bioeditver 201) for maximum likelihood analysis. Maximum likelihood analysis were performed by the branch and bound search, with the tree bisection and reconnection option (TBR).

RESULTS

3.1- RT-PCR amplification of the ΠS4-6 region of the *B. tabaci* para-type sodium channel gene.

The ΠS4-5 region of the *B. tabaci* para-type sodium channel gene was RT-PCR amplified from lab-strain (reference), Parerɪ (first generation after treatment with the insecticide) and generation thirteen. Alignment of the different cDNA clones revealed the presence of four mismatches and five different sequences (Table 1 and Fig.1).

Compare with susceptible laboratory strain (Sudan) at position 61, cDNA clones from both lab and parent had A nucleotide instead of T nucleotide the second substitutions, T nucleotide instead of A nucleotide at position 63, caused a L925I

substitution (sodium channel numbering follows the housefly *para*-type sodium channel protein sequence EMBL X96668), and were designated as r1-Q1 (GenBank accession no. DQ205206). The L925I mutation was previously identified in a B biotype strain from Arizona and found the linked to synergized pyrethroid resistance (Morin *et al.*, 2002). They identified in a Q biotype strain (Alon *et al.*, 2006). The third and fourth substitution, A to C at position 73 and C to T at position 74, occurred only in cDNA clones from lab-strain and parent type. These substitutions cause a threonine to leucine substitution at position 929 (T929L). But 13th generation had G instead of A at position 73 and T instead of C at position 74. These substitutions cause a threonine to valin (T929V). An identical mutation was recently associated with pyrethroid resistance in cat flea (Bass *et al.*, 2004, Alon *et al.*, 2006) which agrees with our results. The cDNA sequence harbouring the T929V mutation was designated as r2-Q1 (GenBank accession no. DQ205207). The two wild-type sequences lacking the L925I and T929V mutations, but differing in the codon usage of V981, were designated as s-B and s-Q (GenBank accession nos. AJ440727 and DQ205204, respectively). The five identified cDNA sequence had the kdr mutation L1014Q and L1014K [lab-strain, parent and generation 13th respectively in ΠS4-6 region].

Fig. 1. Nucleotide sequence of the IIS4-6 region of *B. tabaci para*-type sodium channel gene, amplified from the (susceptible strain, parent type and generation thirteen). The identified cDNA sequences differed by nucleotide substitutions at positions 61 (L925I) and 73 (T929V).The position of the kdr (L1014) and super kdr (M918) mutation sites, found in other insects but not in *B.tabaci* .

Table 1. Nucleotide variation in resistant generation parent and susceptible lab-strain of *B.tabaci para*-type sodium channel alleles. The sequences span last 145 bp of the first exon and the complete sequence of the first intron in the ΠS4-6 region

Generation	61	63	73-74	91	115	116	185	186	244	245	247	248	250
DQ205207.1	t	a	gt	a	g	g	t	t	a	'a	a	t	g
DQ205201.1	a	-	ac	-	-	-	-	-	-	-	-	a	-
DQ205206.1	a	-	ac	-	-	-	-	-	-	-	-	-	-
Sample 1	a	t	ct	g	t	t	c	c	c	t	t	g	a
Sample 2	a	t	ct	g	t	t	c	c	c	t	t	g	a
Sample 3	t	a	gt	a	g	g	t	t	a	a	a	t	g

	252	253	254	255	259	260	317	318	327	328
DQ205207.1	a	t	g	a	t	g	t	t	a	a
DQ205201.1	-	a	t	g	-	t	-	-	-	-
DQ205206.1	-	-	-	-	-	-	-	-	-	-
Sample 1	t	g	a	t	g	a	a	c	t	c
Sample 2	t	g	a	t	g	a	a	c	t	c
Sample 3	a	t	g	a	t	g	t	t	a	a

3.2-The role of L925I and T929V in resistance

To examine the association between the L925I and T929V mutations and resistance to pyrethroids, bioassay of different generations of resistant strain: G_3, G_6, G_9 and G_{13} was carried out (Table 2).

Table 2. Rate of resistance development for Lambda-cyhalothrin in whitefly *B.tabaci* during selection for 13 generations.

strain	Karate selected 13 generations strain		
	Slope ± EC	LC$_{50}$ppm(95%FL)	RR*
Lab.strain	1.39 ± 0.43	6.86 (3.43-11.39)	-----
Parent	1.12 ± 0.44	5.36 (1.77-13.53	0.78
G_3	2.86 ± 0.82	21.10 (--------)	3.10
G_6	1.63 ± 0.24	30.63 (23.78-41.28)	4.46
G_9	1.12 ± 0.37	98.94 (27.02-215.42)	14.42
G_{13}	1.45 ± 0.19	127.08 (97.02-175.51	18.52

RR* (Resistance ratio) = LC$_{50}$ of the resistant strain / LC$_{50}$ of Lab-strain

Table 3. Mutation found in the transmembrane segment IIS4 of the para sodium channel gene in different strains of B. tabaci

Strain	Description	Sequence at position 925[a]	Sequence at position 929[a]
SUD-S[b]	Lab susceptible (Sudan)	TTA Leucine	ACT threonine
Sample1 Parent[c]	Pyrethroid resistant lab strain 1st generation	ATT isoleucine	CTT leucine
Sample2 susceptible[c]	Susceptible lab strain	ATT isoleucine	CTT leucine
Sample3 Generation13[c]	Pyrethroid resistant lab strain 13th generation	TTA Leucine	GTT[d] Valin[e]
GRMAL-RP[d]	Pyrethroid resistant from Crete	ATA[e] Isoleucine[e]	GTT[d] Valin[e]

a Numbering of amino acid residues follows translation of *Musca domestica* sodium channel gene (X96668).
b Sequences reported by Morin et al.,(2002)
c This study
d Sequences reported by Emmanouil et al.,(2006)
e Replacements occurred in single haplotypes

which has the r2-Q1 carrying the T929V mutations), parent and lab-strain which has both the r1-Q1 sequences (carrying L925I mutations) (Table 3) and extracted RNA from lab-strain (s), parent (p) and three generations of resistant strain(G_4, G_9 and G_{13}) which appeared bands with M.W. 399, 417, 416, 418 and 420 KDa respectively. (Table 4 and fig. 2)

The role of L925I has been well documented in *B. tabaci* pyrethroid resistance (Morin *et al.*, 2002), the second mutation, threonine (ACT)-valine (GIT) replacement at amino acid 929 (T929V) identified in the G_{13} pyrethroid resistant strain (Table 3), is the same kdr resistance mutation previously reported in the cat flea *Centocephalids felis* (Bass *et al.*, 2004) and pyrethroid resistance from Crete in the *B. tabaci* (Roditakis *et al.*, 2006) the role of the T929V in pyrethroid resistance has been established by site directed mutagenesis and functional expression studies in *Drosophila* (Atkinson, 2002).
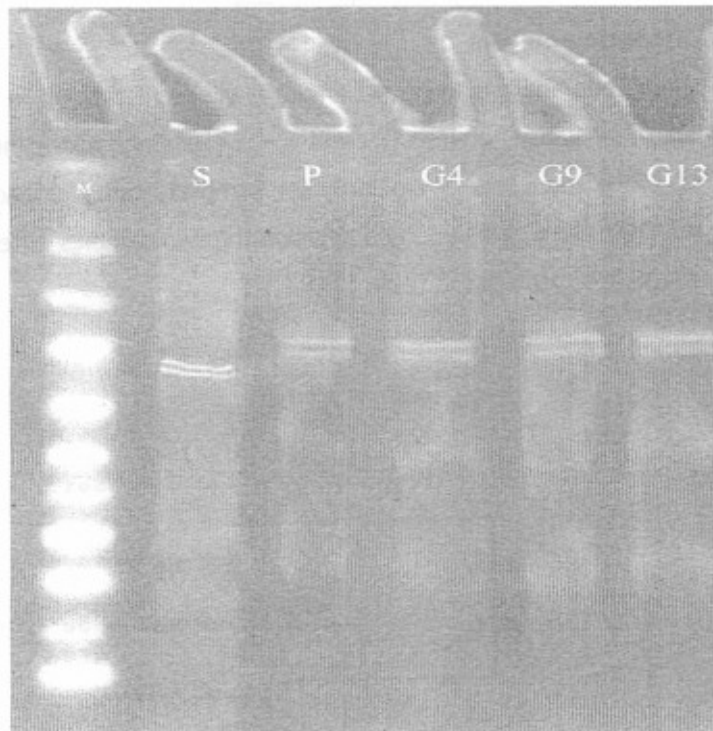
Fig. 2. Gel analysis of RT-PCR amplification using Kdr-1 forward and Kdr-4 reverse. The wild-type allele was amplified from three generations. DNA was extracted from 100 whitefly laboratory-strain (s), three generations of resistant strain (G_4, G_9, G_{13}). The position of the amplified PCR fragment was (370 bp)

Table 4 . Molecular weight of RNA bands for different samples of resistant strain

Generation	Molecular weight
Lab. strain	399
Parent	417
Generation 4	416
Generation 9	418
Generation 13	420

3.4- Nucleotide variation in the first axon and first intron of domain ΠS4-6

In the Q-biotype, it is found that two resistant alleles r2-Q1 (GenBank accession nos. DQ205207) has identical intron sequence with G_{13}. The overall nucleotide variation was 0.000. and allele r1-Q1 (GenBank accession no. DQ205206) has identical intron sequences with samples of parent and lab-strain. The overall nucleotide variation for samples was 0.000 but the samples differs from r1-Q1 by 22 nucleotide substitutions (table 1). The overall nucleotide variation for samples with r1-Q1 (GenBank accession no. DQ205206) was 0.066.

3.5- Phylogenetic analysis

Phylogenetic analysis using maximum likelihood is shown in (Table 5 and Fig 3). The value of similarity between G_{13} and r2-Q1 (GenBank accession no. DQ205207) was 100.00. While the value of similarity for sample1, sample2 and r1-Q (GenBank accession no. DQ205206) was 93.38% because the two samples differs from allele r1-Q by 22 nucleotide substitution .(Table 1).
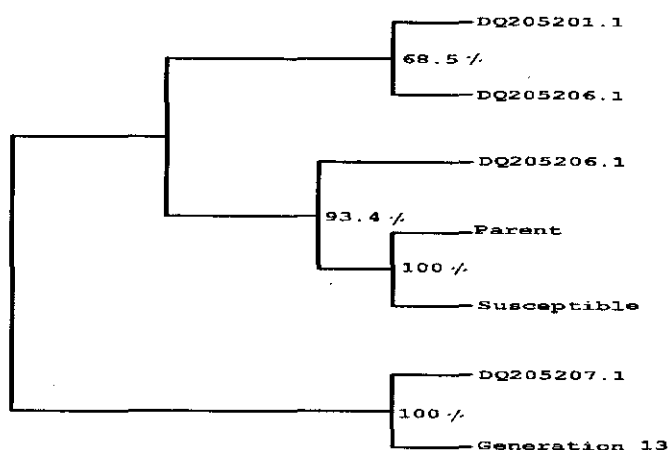

Fig 3. Maximum likelihood analysis of resistant and susceptible allele Sequences produced from the first exon (145bp) and first intron in (~730bp) the IIS4-6 domain of the *B.tabaci para*-sodium channel gene. Analyses were performed by the branch and bound search, with the tree bisection and reconnection option (TBR).

Table 5. Estimated similarity for allele sequences produced from the first exon (145 bp) and first intron (~730 bp) in the IIS4-6 domain of the *B. tabaci para*-type sodium channel gene.

	DQ205207	DQ205201	DQ205206	Sample1	Sample2	Sample3
DQ205207	100.00	-------	--------	-------	-------	------
DQ205201	67.10	100.00	--------	-------	-------	------
DQ205206	99.18	68.45	100.00	-------	-------	------
Sample1	93.38	59.42	93.38	100.00	-------	------
Sample2	93.38	59.42	93.38	100.00	100.00	------
Sample3	100.00	67.10	99.18	93.38	93.38	100.00

DISCUSSION

Point mutations in the insect para sodium channel gene that reduce the sensitivity to pyrethroids have been identified in several insect species. Most studies have focused on the IIS4-5 region, using degenerate primers designed from conserved sequences in these membrane-spanning segments (Martinez-Torres *et al.,* 1997). Using this strategy, we amplified from *B. tabaci* (a resistant lab strain from Egypt) the cDNA fragment spanning the II4-6 region. Through cloning and sequencing we identified two mutations, (L925I and T929V). The two mutations have been previously reported in the GRMAL-RP (a resistant lab strain from Crete) of *B. tabaci* (Roditakis *et al.,* 2006). The L925I mutation was reported by (Morin *et al.,* 2002), while functional expression studies in oocysts showed that it is able to reduce channel sensitivity to deltamethrin by over 2000-fold (Atkinson 2002). In contrast, the mutation at position 918 (methionine), which was associated with pyrethroid resistance in a laboratory selected whitefly strain [but not in pyrethroid-resistant field-collections from Arizona (Morin *et al.,* 2002)] was not also found in GRMAL-RP (a resistant lab- strain from Crete) (Emmanouil *et al.,* 2006), which identical with this investigation in pyrethroid resistant-lab strain from Egypt.

The level of resistance conferred by each of them is difficult to infer. We are currently undertaking experiments to separate the two mutations, as well as to obtain susceptible whiteflies of similar genetic background, to evaluate the exact level of the resistance conferred by each mutation and their impact (if any) on fitness. As in many agricultural pests, showing that whiteflies which carry the V929 (or I959) alone are viable, in line with previous studies (Morin *et al.,* 2002 and Bass *et al.,* 2004).

Horowitz *et al.,*(2003) suggested that although B-type may survive better than Q-type *B. tabaci* under untreated conditions, the faster occurrence and development of resistance in Q biotype favours its predominance in areas with heavy insecticide pressure history. The classification of the highly resistant strains (field strain and lab resistant) whiteflies isolated from Behera Governorate as Q biotype, indicates that the use of insecticides may have increased the occurrence of these biotype in Egypt. However further studies are needed to explain the B and Q biotypes genetically isolation. Nevertheless, our data show little or no gene flow between B and Q biotypes of *B. tabaci* since the introduction of the widespread use of synthetic insecticides that target *the para-*type voltage gated sodium channel such as pyrethroids.

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تحديد الطفرات فى قناة الصوديوم المرتبطة بمقاومة الذبابة البيضاء لمركبات البيرثرويدز

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تعتبر قناة الصوديوم هى الموقع الأساسى لتأثير مبيدات البيرثرويدز، ففى بعـض الحـشرات يكون ظهور جين المقاومة الصاعق ناتجا عن التغير الطفرى المحصور بين الجزء الرابع والـسادس (6 - 4 S II) فى الشريط الوراثى الخاص بقناة الصوديوم .

تم استخدام جهاز البلمرة (RT-PCR) لمعرفة جين المقاومة فى الموقع بارا لقناة الـصوديوم وذلك لكلا من السلالة المعملية (الحساسة) ، وسلالة الأباء بعد جيل واحد مـن المعاملـة بمركـب لمبداسيهالوثرين وأيضا بعدالإنتخاب لمدة ١٣ جيل متعاقبة بنفس المركب. وفى هذا العمل تم دراسة ميكانيكية المقاومة لمركب لمبدا سيها لوثرين   فـى سـلالة الذبابـة البيـضاء المنتخبـة بمركـب لمبداسيهالوثرين لمدة ١٣ جيل متعاقبة .

أوضحت نتائج التحليل الجينى للشريط الوراثى فى عينات كلا من السلالة المعملية وسـلالة الاباء والسلالة المقاومة وجود نوعين من الأحماض الأمينية ظهرت فيهما إستبدالات مقارنة بسلالة السودان الحساسة ، وكان أحد هذين الحامضين هو الحامض الأمينى ليوسـين الـذى تحـول الـى الحامض الأمينى أيزوليوسين ، و قد تم هذا الأستبدال فى الموقع 925 (L925 I) ولوحظ وجود الأليل r1-Q1 الموجود فى بنك الجينات برقم DQ205206 وذلك فى عينات كلا من السلالة المعملية وسلالة الآباء ، وكان الحامض الأمينى الثانى الذى حدثت لة إستبدالات فى جين المقاومـة للذبابـة البيضاء هو الحامض الأمينى الثيورين الذى تحول الى الحامض الأمينى الفالين وقد تم هذا الإستبدال فى الموقع   929 (V 929 T) فى الشريط الوراثى ولوحظ ايضا وجود الأليل r2-Q1 الموجود فى بنك الجينات برقم DQ205207 وذلك فى عينة السلالة المقاومة.