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Molecular cloning and sequence analysis of vira-like chitinase gene from the cotton leaf worm, *Spodoptera littoralis* Bosid (Lepidoptera: Noctuidae), treated with two insect growth regulators

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Abstract: *The vira-like chitinase gene, one of the genes involved in the formation of chitin and concerned with development of insect. PCR- RFLP technique was used in the present study to compare the effect of sub-lethal doses of two IGRs, flufenoxuron (cascade) as a chitin synthesis inhibitor and tebufenozide (mimic) as a moulting hormone agonist, on this gen in Spodoptera littoralis pest. As the 2nd instar larvae were treated with sublethal doses LC_{25}, LC_{50} and LC_{90} of the two tested IGRs. The endonucleases SpeI, EcoRI and BbsI were used to detect the molecular changes in the gene at different time intervals 6, 12, 24 and 48 hours post treatment. Bioinformatical obtained results showed significant changes in the gene sequences (mutations) of the treated groups which were dose and time dependants. For each studied group, the RNA secondary structure stems nucleotide sequences; positions and free energy were recorded.*

Keywords: *IGRs- vira-like chitinase gene- PCR- RFLP- Bio informatics- Spodoptera littoralis*

I. Introduction

Spodoptera littoralis is one of the major pests of cotton and other crops in Egypt and the Near East. This insect acquired resistance to several chemical insecticides (Ibrahim et al., 2008). The recent control intensive research is concerned mainly with avoiding the serious problems resulted from using harmful insecticides that cause harmful residues in the food chain, hazards of the surrounding natural enemies and pest resistance (Abo-Arab and Salem, 2005).

The use of insect growth regulators IGRs compounds in insect control is known as insect developmental inhibition, which inhibits or prevents normal metamorphosis of immature stages to the adult. Tebufenozide (mimic) belongs to a class of IGRs, bisacyl hydrazine ecdysteroid agonists, mimicking the natural insect moulting hormone 20-hydroxyecdysone (20E) (Dhadialla et al., 1998). While, the flufenoxuron (cascade) **N-{4-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenylaminocarbonyl}-2,6-difluoro-benzamide** is one of IGR pesticides, highly used to control pests by inhibiting of chitin synthesis (Cutler et al., 2007). Most laboratory and field experiments with IGRs so far have focused on their effect on S. liitoralis larvae (Wang and Tian, 2009).

Chitin, an insoluble structural polysaccharide that occurs in the exoskeletal and gut linings of many insects, is a metabolic target of selective insect pest control agents. One potential control agent is the insect molting enzyme, chitinase, which degrades chitin to lower molecular weight soluble and insoluble oligosaccharides (Kramer and Muthukrishnan, 1997). Chitinases (-1,4-poly-N-acetylglucosaminidase; EC 3.2.1.14) are members of the O-glycoside hydrolase superfamily and are found in many species, including microbes, plants, insects, and mammals (Kramer and Muthukrishnan, 2005). Insect chitinases, which belong to family 18 glycosylhydrolases, have been detected in molting fluid and gut tissues and are predicted to mediate the digestion of chitin present in the exoskeleton and peritrophic membrane in the gut (Kramer and Muthukrishnan, 1997; Kramer and Koga, 1986). Genes and cDNAs encoding insect chitinases have been identified and characterized from several lepidopteran, dipteran, and coleopteran insects (Kramer et al., 1993; de la Vega et al., 1993; Royer et al., 2002). A viral-like chitinase was found to be up-regulated. The degradation of cuticular chitin by chitinases is a vital step prior to ecdysis and metamorphosis (Kim et al., 1998).

In this work, we used *S. littoralis* as a model target to compare the effect of sub-lethal doses of two IGRs (tebufenozide and flufenoxuron) with different mode of action on the vira-like chitinase gene and using bioinformatics to detect the induced mutations.

II. Material And Methods

Test insect

The culture of the cotton leaf worm, *S. littoralis* Bosid was initiated from freshly collected egg masses supplied from the division of cotton leaf worm, of Plant Protection Research Institute, Dokki, Egypt. All rearing steps of the colony and experiments were kept under laboratory conditions of 27±2 Cº and R.H. 70±5 %.

Tested Compounds

One moulting hormone agonist, Ecdyson agonist, mimic10 % (tebufenozide) and one chitin synthesis inhibitors cascade10% (flufenoxuron) were used in this study.

Susceptibility of *S. littoralis* to IGRs:

Newly moulted 2nd larval instars were segregated from the stock colony in clean glass Petri dishes and starved for 24 hrs. Pieces of castor been leaves, were treated by the leaf-dipping technique in the different concentrations of tested compounds (tebufenozide and flufenoxuron) according to Bakr et al. (2010). The data of susceptibility test were subjected to Probit analysis (Finney, 1972) to evaluate the values of LC_{25}, LC_{50} and LC_{90}.

Isolation of DNA:

Isolation of DNA:

Total nucleic acid (nuclear DNA and mtDNA) was extracted from 2nd instar larvae treated with sublethal doses (LC_{25}, LC_{50} and LC_{90}) of the two tested IGRs at different time intervals post treatment (6, 12, 24 and 48hrs). Whole larvae were homogenized in 0.5 ml STM (0.32M sucrose, 50 mMTris pH 7.25, 10 mM MgCl2, 0.5 % NP detergent) and 0.1 ml of 0.5 M EDTA. The suspension was centrifuged at low speed for 4 min and the supernatant was removed. The pellet was resuspended in 0.5 ml STE (75mM NaCl, 25 mM EDTA, 10 mMTris pH 7.8) and 1% SDS. The homogenate was digested with 500 µg proteinase K for 1h and extracted with phenol and chloroform. The DNA was ethanol precipitated, resuspended in 60 µl 10 mMTris pH 8.0 and stored at -20ºC (Levy et al., 2002).

PCR-primers:

Nucleic acid sequence for *S. littoralis* vira-like chitinase gene was obtained from Gen Bank of the National Center for Biotechnology Information (Tyne & Possee 2005). The genetic computer programs Bioface, DNA counter and DNA Baser were used to detect the primers used,
GTAAAGGAGATTGAAGGCAGTTTC 5-prime candidate
and TAAACTTTTTTTTCATATTAACTTACA 3-prime candidate.

PCR-RFLP analysis:

For PCR amplification, the PCR mixture for each reaction contain a total volume 50 µl of (6 µl of 250ng/µl of the total DNA extracted from *S. littoralis* larvae used as template, 5 µl of 2m M dNTPs, 2 µl of 50Mm Mgcl2, 5 µl of PCR buffer, 4 µl of 10 µM primer, 1 µl of 5U/µl Taq. Polymerase and 27 µl of distal water). Temperature cycling was carried out by Perkin Elmer thermal cycler 2400 with 15 min at 96Cº and then 30 cycles of the following profile: 40 sec at 96Cº, 40 sec at 36Cº, and 2min at 72 C º. After the 30 cycles the reaction were held at 72 Cº for 7 min. The PCR amplified gene products were digested with the endonucleases SpeI, EcoRI and BbsI (New England Biolabs) to cut down the gene to detect the mutations induced by the different sub-lethal doses of the used IGRs. Samples were incubated overnight at 37 Cº. The restriction enzyme profiles were visualized with ethidium bromide on 1.5% agarose gel and photographed under UV light.

Gene and Bioinformatical analysis

All the details of the normal *S. littoralis* vira-like chitinase gene such as origin, translation, percentage of bases, codons and amino acids …etc are explored by the (Bioface and DNA counter programs). The alignments of the vira-like chitinase gene sequences, the encoded amino acids for the studied groups were obtained by (CLC Main Workbench program version 5.5). RNA secondary structure, stems nucleotide sequences, positions and free energy were recorded.

III. Results

Sublethal doses of the tested IGRs against 2nd instar larvae are summarized in Table (1). Toxicity bioassays showed that flufenoxuron was more toxic against 2nd larval instars at sub-lethal concentrations LC_{25}, LC_{50} and LC_{50} than tebufenozide.

Table (1) Toxicological evaluation of IGRs against 2nd larval instars of *S. littoralis.*

IGRs	Toxicity of IGRs ppm			Slop function
	LC_{25}	LC_{50}	LC_{90}	
Tebufenozide	1.1	1.5	2.5	5.6
Flufenoxuron	0.1	0.2	1.3	1.8

The bands of vira-like chitinase gene for the different groups of treated *S. littoralis* larvae with sublethal doses (LC_{25}, LC_{50} and LC_{90}) of the two IGRs at different time intervals (6, 12, 24 and 48hrs) and untreated larvae have the same length 137pb and

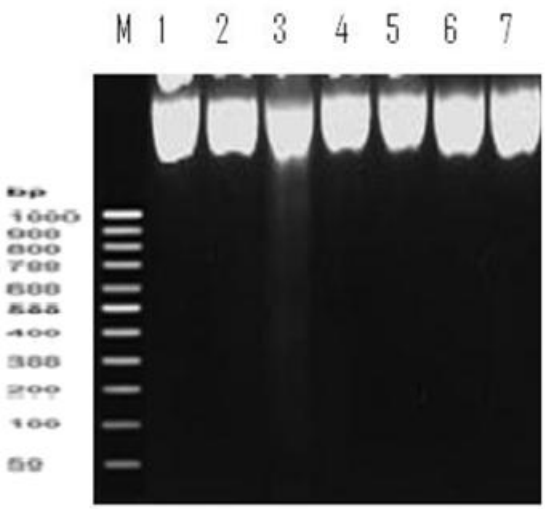


Plate (1): Shows the DNA ladder in the 1st lane while the other lanes contain the PCR products of the vira-like chitinase gene for the studied groups all at the same length.

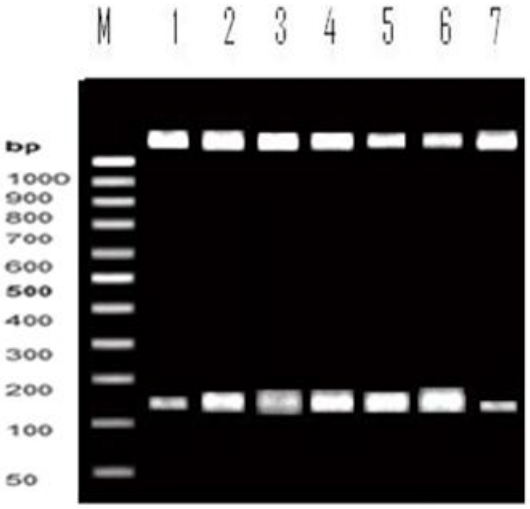


Plate (2): Represents that SpeI restricted the vira-like chitinase gene of all groups at the different time intervals post treatments with the same length 137pb and 1081pb

1218bp Plate (1). The restricted fragments resulted from the digestion of vira-like chitinase of all groups of treated and untreated larvae with the endonuclease SpeI are represented in (Plate 2). The enzyme cut gene one time and resulted in two fragments of 137 and 1081 bp in length for all treatments.

EcoRI endonuclease digests the gene of the treated larvae with LC_{50} and LC_{90} of flufenoxuron and LC_{90} of tebfenozide (lan 3,4 and 7, respectively) after 6 hrs post treatment (Plate 3) into three fragments of 129, 334 and 755 bp in length, remaining different treated groups have the similar restricted fragments as normal with lengths 334pb and 884pb. Plate (4) showes that the endonuclease enzyme EcoRI digests the vira-like chitinase gene of the treated larvae with sub lethal doses LC_{50} and LC_{90} of flufenoxuron and tebufenozide after 12 hrs, lanes (3-4-6-7). Resulted in three bands with lengths 129pb, 334pb and 755pb, remaining different treated groups have the similar restricted fragments as normal with lengths 334pb and 884pb. Plate (5) shows the PCR-RFLP products of the studied gene of the different groups after 24&48hrs post treatment, which restricted with the enzyme EcoRI. It cut the gene in three restricted fragments. These restricted fragments are equal in the different groups with lengths 129pb, 334pb and 755pb (lanes 2 -7). The enzyme BbsI cut the vira-like chitinase gene of the treated larvae with LC_{50} and LC_{90} of flufenoxuron as well LC_{90} of tebufenozide after 6 hrs post treatment (Plate 6) lanes (3-4-7). Producing three bands these bands with lengths 60pb, 134pb and 1024pb, remaining groups and normal has the same lengths 60pb and 1158pb. The digestion of the gene with the enzyme BbsI after 12hrs post treatment showed (plate7) three distinct bands presented in lanes (3-4) at lengths 60pb, 134pb and 1024pb as a result of the restriction of the gene of the treated larvae with doses LC_{50} and LC_{90} of flufenoxuron. The gene of LC_{50} and LC_{90} tebufenozide treated larvae was restricted into three distinct bands in lanes (6-7) at lengths 60 pb,324 pb and 834pb. Digestion of Vira-like chitinase gene after24&48hrs with the enzyme **Bbs**I (plate 8) showed that, three distinct bands were presented in lane 2 at lengths 60pb, 134pb and 1024pb as a result of the restriction of vira-like chitinase gene of the treated larvae with the sub lethal dose LC_{25} of flufenoxuron. In lanes (3- 4) there were four bands at lengths 60pb, 134pb, 488pb and 536 pb represents the result of restriction of the gene of LC_{50} and LC_{90} treated flufenoxuron larvae. The last three sub lethal doses of the tebufenozide have the same restricted fragments when cut with the same enzyme at the lengths 60pb, 324pb and 834pb and represented in lanes (5-6-7).

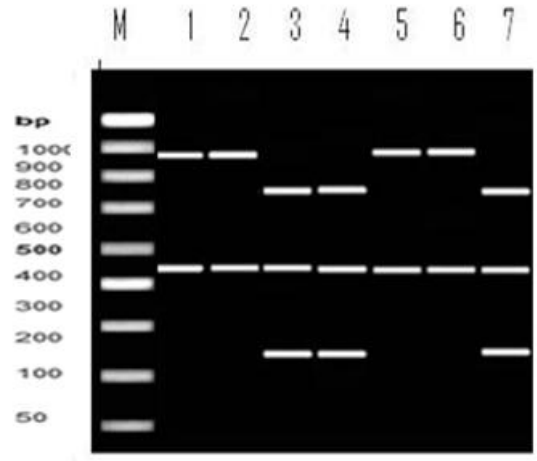


Plate (3): The digestion of enzyme EcoRI to the vira-like chitinase gene of all groups at 6hrs. post treatment

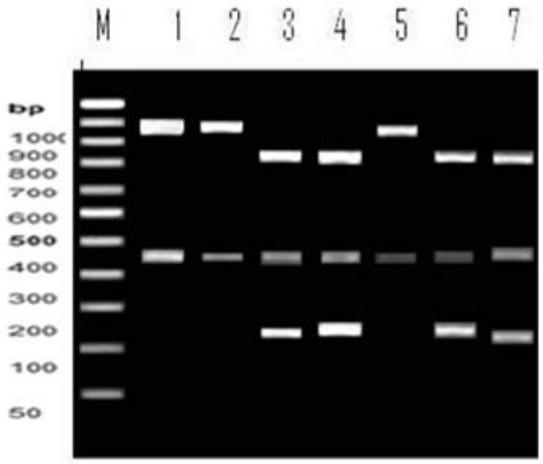


Plate (4): the digestion of enzyme EcoRI to the vira-like chitinase gene of all groups at 12hrs. post treatment

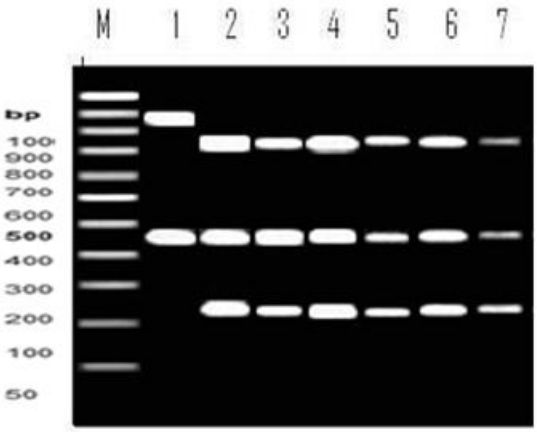


Plate (5): Shows the digestion of enzyme EcoRI to the vira-like chitinase gene of all groups at 24 &48 hrs post treatment

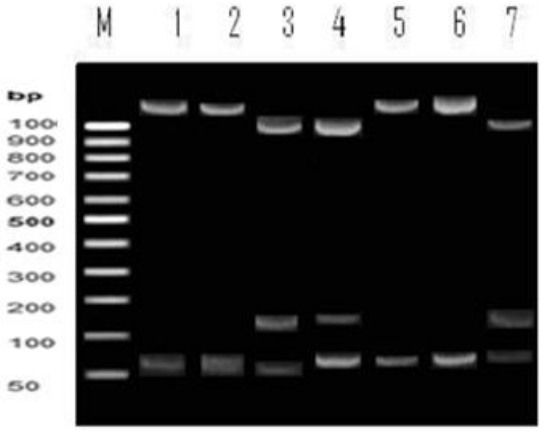


Plate (6): The digestion of enzyme BbsI to the vira-like chitinasegene of all groups at 6hrs. post treatment

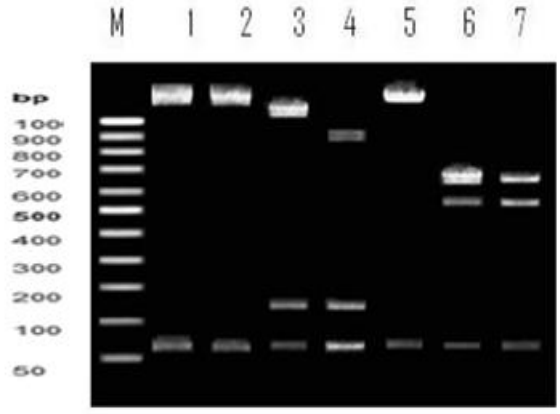


Plate (7): The digestion of enzyme BbsI to the vira-like chitinase gene of all groups at 12hrs. post treatment.

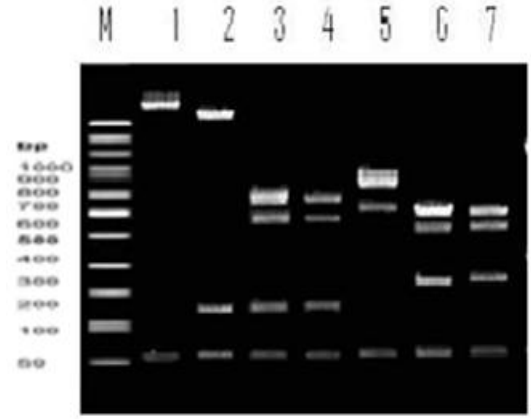


Plate (8): Shows the digestion of enzyme BbsI to the vira-like chitinase gene of all groups at 24&48 hrs. post treatment

M Marker
1 Norma
2 LC25 of Flufenoxuron
3 LC50 of Flufenoxuron
4 LC90 of Flufenoxuron
5 LC25 of Tebufenozide
6 LC50 of Tebufenozide
7 LC90 of Tebufenozide

The alignments of the vira-like chitinase gene sequences and the encoded amino acids for the studied groups were obtained by CLC Main Workbench program (version 5.5). Five groups of mutated vira-like chitinase gene resulted from the treatments of the 2nd larval instar with the different sub lethal doses of the used IGRs at the different times intervals post treatments in addition to the normal one. 1st group (G1) includes the treated larvae with the following doses LC_{25} of flufenoxuron and tebufenozide after 6&12 hrs ,respectively post treatments as well as LC_{50} of tebufenozide after 6 hrs. The 2nd group (G2) includes the treated larvae with the LC_{90} of tebufenozide after 6 hrs post treatment. The 3rd group (G3) includes the treated larvae with the following doses, LC_{50} and LC_{90} of flufenoxuron after 6&12 hrs post treatments, and LC_{25} of flufenoxuron after 24&48 hrs post treatments. The 4th group (G4) includes the treated larvae with the following doses, LC_{50} and LC_{90} of tebufenozide after 12, 24 and 48 hrs post treatments and LC_{25} of tebufenozide after 24& 48 hrs post treatments. The 5th group (G5) includes the treated larvae with the following doses, LC_{50} and LC_{90} of flufenoxuron after 24& 48 hrs post treatments (Table 2).

As the yielding protein of the mutated vira-like chitinase gene has been changed, the RNA secondary structure also is expected to be changed. For each studied group, the RNA secondary structure stems nucleotide sequences, positions and free energy were recorded as well as the distinct secondary structure was drawn using a computer based online program known as gene bee RNA secondary structure prediction (Table 3 and Plates: 9-13).

Table (2): groups of mutated vira-like chitinase gene resulted from the treatments of the 2nd larval instar with the different sub lethal doses of the used IGRs at the different times intervals post treatments

DNA sequence					Treated groups
Restriction enzymes	Amino acid position	Amino acid change	Nucleotide change position	Nucleotide change	
EcoRI *Bbsl*					G1
EcoRI *Bbsl*	65	I ▶ T	194	T ▶ C	G2
EcoRI *Bbsl*	65 156	I ▶ T L ▶ F	194 468	T ▶ C G ▶ C	G3
EcoRI *Bbsl*	156	L ▶ F	384 468	T ▶ C G ▶ C	G4
EcoRI *Bbsl*	65 156 244	I ▶ T L ▶ F G ▶ R	194 468 730	T ▶ C G ▶ C G ▶ C	G5

Table (3): Free energies of the predicted RNA structure of the different studied groups.

Groups				
G5	**G4**	**G3**	**G1 1 G2**	**N**
TOTAL NUMBER OF STEMS				
40	**42**	**39**	**40 1 41**	**40**
	Kkal/mol		**Free energy**	
-203.9	**-194.8 1 -187.8**		**-202.7 1 -202.6**	**-202.7**

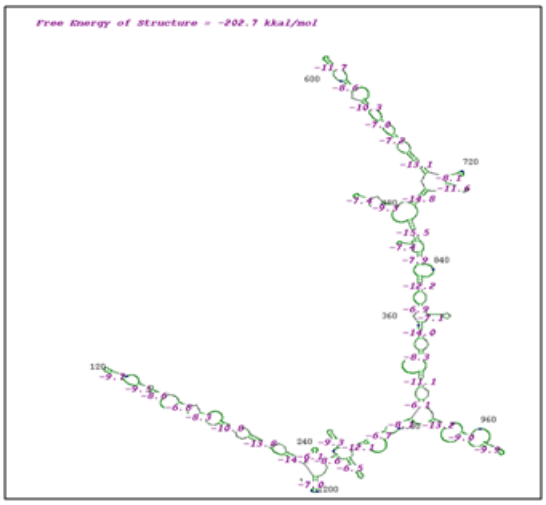


Plate (9): The structure of the predicted RNA structure of the normal structure

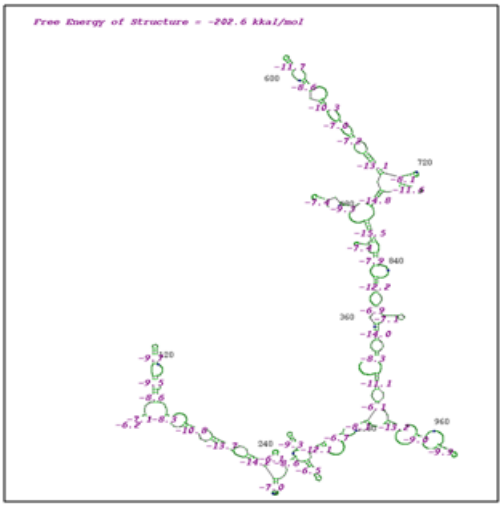
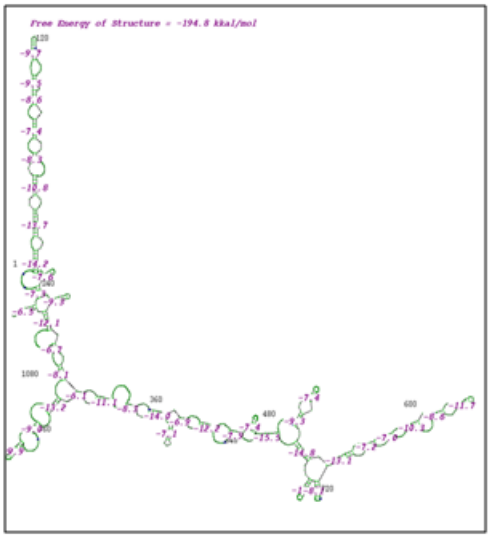


Plate (10): The structure of the predicted RNA of 2nd group.



Plate(11): The structure of the predicted RNA structure of 3rd group

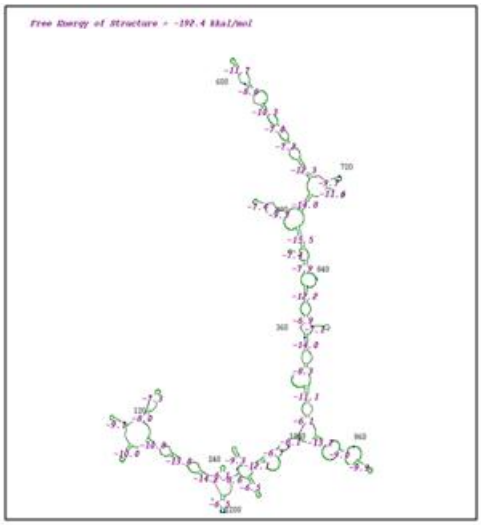


Plate (12): The structure of the predicted RNA structure of 4th group

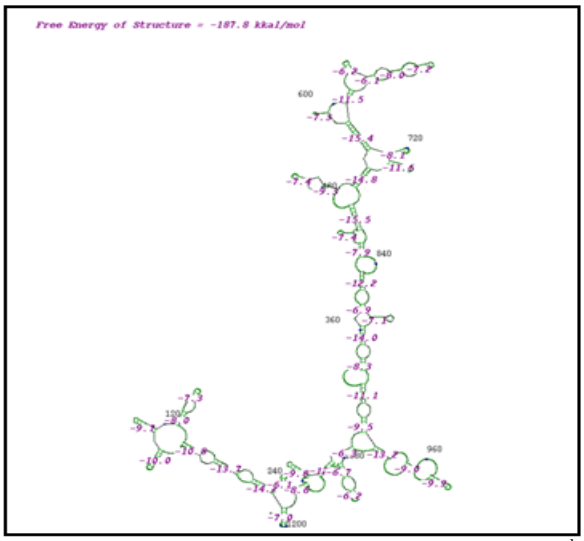


Plate (13): the structure of the predicted RNA structure of 5th group.

IV. Discussion

Chitin synthesis inhibitors found to be effect on the vira-like chitinase gene which responsible for producing chitinolytic enzyme work in remodeling chitinous structures known as glycanohydrolase, catalyze the hydrolysis of [β- (1-4) glycoside] bonds of chitin polymers and oligomers (Konodo et al., 2002). These compounds involved in chitin degredation. It is also effect on the gene which responsible for production of glycolytic enzyme, triosephosphate isomerase. This enzyme involved in catalyzes the inter conversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The alimentary canal is lined with cuticle (fore and hind gut) which consisted of chitin, proteins, lipids and hydrocarbons. Thus the alimentary canal of the treated larvae is the first position to be affected with these compounds, as well as the mid gut (peritrophic membrane), chitinases seem to be involved in the formation, perforation and degredation of the midgut peritrophic matrix, which protect the gut epithelium from damaging factors (Shen and Jacobs-Lorena, 1997 and Filho et al., 2002).

This study showed a different mutation in the vira-like chitinase gene depending on the dose of tested IGRs (tebufenozide and flufenoxuron) and the time post-treatment. flufenoxuron induced gene malformations. The same results were obtained by Bakr et al. (2014). The authors studied the vira-like chitinase gene mutations induced by lufenuron (match 5%) on the 2nd instar larvae of *S. littoralis.* Their results showed significant changes in the sequences of the treated groups which were dose and time dependants. Treatment of the 2nd instar larvae wit LC_{25} of flufenoxuron and tebufenozide not induced any gene mutation before 24 h after treatment. While Digestion of Vira-like chitinase gene with EcoRI or BbsI after treatment of insects with LC_{50} and LC_{90} of flufenoxuron showed gene mutation directly after 6h. Many authors cited that Flufenoxuron was relatively more toxic than Diflubenzuron on the third instar larvae of S. exigua (Rao and Subbaratnam 2000) and 2nd and 4th instars larvae of *S. littoralis* (Anwar and Abd El-Mageed, 2005).

The degradation of cuticular chitin by chitinases is a vital step prior to ecdysis and metamorphosis (Kim et al., 1998). Kramer et al. 1993 reported that the chitinase gene of *Manduca sexta* was most highly expressed in epidermal and gut tissues during the larval-pupal metamorphosis and its transcription was stimulated by ecdysterone (20E) and inhibited by a JH mimic. The chitinase might be involved in remodelling of the integument during metamorphosis.

These mutations which induced by flufenoxuron and tebufenozide increased with increasing in change in RNA secondary structure. According to these changes the minimum free energy and number of stems were changed. A viral-like chitinase was found to be up-regulated. RNA plays many diverse roles in biology, including catalyzing peptide bond formation) Hansen et al., 2002), catalyzing RNA splicing (Doudna and Cech 2002), localizing protein and flagging development (Lagos-Quintana et al., 2001).

The stability of the RNA secondary structure is quantified as the amount of free energy being released or used by the forming base pairs. The stability increases according to the number of GC versus AU and GU base pairs and the number of base pairs in a hairpin loop region. The number of unpaired bases decreases the stability of the structure such as interior loops, hairpin loop or bulges (Mohsen et al., 2009).The stability of the secondary structure depends on the amount of free energy released to form the base pairs. Thus, the more negative the free energy of a structure is, the more stable a particular sequence is formed. This structure is called the MFE secondary structure (Layton and Bundschuh 2005).

The above mentioned facts about the secondary structure could be represent a molecular explanation to the malformations appears in the present study.

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