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SILENCING THE EGYPTIAN COTTON LEAFWORM; *Spodoptera littoralis* (BIOSD.) CYTOCHROME P450 INHIBITS THE METABOLISM OF GOSSYPOL USING RNAI TECHNIQUE

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ABSTRACT

RNA interference is an effective means regulate gene expression in both vitro and vivo. P450 monooxygenase plays a vital role in adaptation to cotton defence compounds such gossypol; toxic to Lepidopteran pests. This gene is highly expressed in insect midgut and its expression correlates with larval growth when gossypol is included in diet. Commonly, the transcription levels of P450 monoxygenase enzymes in the insect midgut are increased in the presence of gossypol compound. The current study is therefore revealed the inhibition effect of siRNA of cytP450 on the Egyptian cotton leafworm; *Spodoptera littoralis* (Biosd.) by selective targeting of cytochrome P450 gene. Small sequence of siRNA was designed and was subjected to cotton leafworm larvae using two different assays; diet mixing assay and force feeding assay. In diet mixing assay; the larval mortality percentage was ranged between 6.7 to 60% with 20 to 300ng siRNAi/g diet but this mortality showed 23.3 to 53.35 % in force feeding assay respectively 72h after treatment. Also, changes in esterase enzyme level was proved in insect gut (0.08 to 0.19 ng) in treated and untreated larvae when they subjected to diet incorporating 300ng/g siRNAi. Thus, the current investigation revealed that dsRNA used in this study plays as key factor for growth inhibition of cotton leafworm larvae which may consider as pave to develop transgenic cotton plants express siRNAi sequence that could reduce cotton leafworm population without insecticides intervention.

INTRODUCTION

Cotton leafworm; *Spodoptera littoralis* (Biosd.) is one of the most important Lepidopteran insect pest attacks cotton crop and many other vegetable crops in Egypt and elsewhere. It causes severe damage to the small buds and the plant foliage that lead to a huge reduction in crop production. In addition, relying on chemical pesticides control method against this insect; has generated several problems including insecticide resistance; outbreaks of secondary pests by natural enemies; safety risks for humans and native animals: contamination of ground water; decreased biodiversity; and other environmental concerns. These problems and sustainability of programs based predominantly on conventional insecticides have stimulated increased interest in IPM. On the other hand, sustainable agriculture in the 21st century will rely increasingly on alternative interventions to chemical pesticides for pest management that are environmentally friendly and reduce the amount of human contact with pesticides.

Recently, RNAi has been exploited in plants for applications ranging from functional genomics to provision of valuable crop traits, such as resistance against viruses, bacteria and nematodes. However, it has not yet

been harnessed to defend crops against insects. dsRNA- mediated silencing of essential genes in insects can induce cessation of feeding and ultimately insect growth retardation and eventually insect death. Higher organisms, including insects, possess common machinery for sequence-specific gene silencing that is triggered by the presence of dsRNA (Hannon, 2002). This process is called RNA interference (RNAi) in animals and post-transcriptional gene silencing in plants (Baulcombe, 2004). In 1998, since dsRNA was discovered, it has been shown its ability to catalyse its own replication and synthesis many other RNA in the cell. In addition to its capacity to work as a key factor in gene silence, RNAi has been used as an effective tool in plants and insects (Wesley et al, 2001 and Aravin et al, 2001). But it requires an efficient uptake of dsRNA either by feeding or by topical application. It has been reported that ingestion of dsRNA can silence specific genes in the nematode *Caenorhabditis elegans* (Fire et al., 1998 and Timmons and Fire, 1998). Micro-injection of dsRNA into adults and late-instar larvae has been used to silence genes and study gene function in the coleopteran *Tribolium castaneum* (Tomoyasu and Denell, 2004). Similarly, in Lepidopteraninsect pest, dsRNA induced an RNAi response in *Spodoptera litura* upon injection, but not after ingestion (Rajagopal et al., 2002).

Despite the insect genes can be down regulated by injection of dsRNA or by oral administration of high concentrations of exogenously supplied dsRNA as part of an artificial diet (Winston et al., 2002), it would be interesting if an efficient method of delivering dsRNA to control pests under field condition. RNAi technology also used successfully to study the correlation between gossypol and larval growth in *Helicoverpa armigera* (Mao et al., 2007).

CYP4 genes family is present in insects and found to be important to this group of Eukaryotic organism. There are four large clades of insect P450 genes that existed before the divergence of the class insecta and that are also represented by CYP families in vertebrates namely; CYP2 clade, CYP3 clade, CYP4 clade and the mitochondrial P450 clade. In addition, P450 family genes in insects found to be significant group in order to protect the insect from harmful compounds like photochemicals exist in the plant's tissue. Among them, gossypol is one of several allelochemicals produced by plants for protectionism against insect infestations. Also, large families of these enzymes in insect *viz*., P450s, glutathione S-transferases and carboxylesterases found to detoxify plant chemical cocktails. Recently, those genes have become attractive targets for applying the ingestible dsRNA for managing insect resistance against plant allelochemicals or any other insecticides. In insects, RNAi is usually accomplished by injection of double-stranded RNA (dsRNA) (Carriere et al. 2003). Therefore, silencing P450 oxygenase group in the Egyptian cotton leafworm; *S. littoralis* via RNAi technology may reduce pest population under field condition by inhibiting its growth and resistance to the plant toxic photochemicals. This latter observation, combined with the demonstration of transgenic-encoded RNAi in plants has long prompted speculation that plants could be protected from herbivorous insects by engineering them to express dsRNAs targeting vital

insect genes (Fire et al,1998). Thus, the current study aimed to identify the siRNA sequence that inhibits P450 oxygenase enzymes exist in cotton leafworm pest.

MATERIALS AND METHODS

***1- Spodoptera littoralis* culture:**

Spodoptera littoralis (Biosd.) strain was reared at 25±1°C with 70-80% relative humidity under a L16: D8 photoperiod in the laboratory. This strain was reared for more than 12 subsequent generations under above conditions as described by Sehnal et al., (1976).

1- Overlay siRNA diet mixing bioassay against *Spodoptera littoralis*:

In this assay, the 4th instar larvae of *Spodoptera littoralis* one day old were used to test the effect of silencing monoxygenase enzyme by siRNAi sequence that designed before. Among of above siRNAi sequence, the initial bioassay data showed that, the first one was most effect siRNAi sequence that produced higher mortality. Thus, three serial concentrations of siRNA of the first one viz., 20ng, 100ng and 300ng were prepared using Phosphate buffered saline (PBS) P^{H} 7.0 treated with DEPC water. The cotton oil was extracted at room temperature and was used as a source of natural gossypol coming from plant. The amount of 250 µl of cotton oil was added to ten grams of semi synthetic artificial diet and the identified amount of siRNAi for each concentration was overlay, mixed well and divided into three divisions each represents one replicate. Three replicates were used for each concentration and ten larvae were released onto each. The negative and positive controls were also considered and performed. In the negative control, PBS buffer treated with DEPC water was added to ten grams semi synthetic diet, mixed well and was divided into three replicates. But in case of positive control, PBS buffer along with 250 µl cotton oil were mixed with ten grams of diet, mixed well and then divided into three replicates. All the experiments done through our project was kept under the condition of 26±1°C and 65-70% RH. The mortality observations were taken 72h after treatment.

1. Force feeding bioassay using siRNA:

One day old of 4th instar larvae of *S. littoralis* were force fed with 5µl of three different concentrations of siRNA *viz*., 20ng, 100ng and 300ng. PBS buffer DEPC treated water was used in order to prepare the above concentrations siRNA. The Larvae were fed on the semi synthetic diet mixed with 250µl of cotton oil, three replicates were used for each concentration and ten larvae were fed in each replicate. The larvae were then released onto normal diet. The experiment was kept under the same rearing condition mentioned above. In such experiment, negative control larvae were force fed on PBS buffer and then released on normal artificial diet but the positive control larvae were force fed on PBS buffer along with 250µl cotton oil and released on normal diet as well. The mortality observations were taken 72h after treatment.

2- Esterase activity assay:

The 5th instar larvae were anesthetized using CO_2 and kept for 10-15 min at -20 ^{0}C then force fed (injected). Each Larva was injected with 5µl total volume. Two different concentrations of siRNAi only were applied viz., 100ng and 300ng. The injection time was for 3 sec and the larvae of control treatment were injected with PBS buffer treated with DEPC water only. The negative and positive controls were also kept in order to compare them with the treated larvae. All larvae were kept in the insectary room at 26±1ºC 65-70% RH. The midgut was isolated from the larvae every 24h interval till 72 hr to measure the expression level of esterase enzyme. The dissected midgut was kept at -80ºC for further use. The isolated midgut were homogenized well with 500µl protein extract buffer (1M Sodium Acetate, 0.1% TritonX, 35mM Sucrose, 5mM DTT, PH 7.0) at the cold room then the samples were centrifuged using 10,000 rpm at 4ºC for 30 min. The supernatant was transferred into autoclaved eppendorf tubes. The amount of 10µl of each sample was added to 490µl protein extract buffer then 1ml of Bradford reagent was added in order to estimate the protein concentration. The sample was then measured using spectrophotometer at 595 OD. The reaction was prepared by taken an amount of 6.9µl of each sample and added to 0.99 ml of (50nM NaPO4, 10mM MgCl2, 0.1% NP4O, 50µM Napthyle acetate) buffer, mixed well then placed into cuvette for reading. Each sample was measured three times into the spectrophotometer, each time was considered as one replicate. The reading time of each was lasting for three min, and each reading was recorded every 30 seconds.

RESULTS AND DISCUSSIONS

The concept of the current study is that siRNA sequence when mixed with the semi synthetic diet, it targets the monoxygenase enzymes located in the insect midgut resulting that gossypol will be free to bind with the cell of insect midgut causes cell toxicity. Finally, the cell gets distribution thus the insect stops feeding and eventually dies (Fig. 1). When 4th instar larvae of *Spodoptera littoralis* fed on three different concentrations of siRNA (20, 100, 300ng/ g diet) mixed with diet incorporating cotton oil, the mortality % was increased at higher concentration of 300ngdsRNAi/g diet and reached to 60% after 72 h after treatment. Whereas, the controlled larvae; both fed on normal diet and diet mixed with cotton oil did not produce any mortality (Fig. 2). Thus, *S. littoralis* couldn't tolerate gossypol presents in cotton oil when dsRNAi added to silencing P450 gene. The mortality percentage was 53.35% when the larvae treated with dsRNA using force feeding method compared with that fed on diet incorporating dsRNA at the same concentration (300ng dsRNAi /gm diet), (Table 1 and Fig. 2). The LC_{50} of dsRNAi in diet mixing bioassay and force feeding assay were 212.59 and 266.67ng dsRNAi/g diet. The larval body weight was also severely affected when dsRNAi at various doses was subjected (Fig. 3). This indicated that the dsRNA sequence can be used as a potential technology against cotton leafworm. This data is in agreement with the data mentioned by Hannon, (2002) who reported that the higher organisms, including insects, have common machinery for sequence-specific

gene silencing that is triggered by the presence of dsRNA. Also, Mao et al., (2007) studied the correlation between gossypol and larval growth in *Helicoverpa armigera* using RNAi technology. The level of esterase activity was also varied when a fixed dose of dsRNAi subjected to larvae at different time intervals (Fig. 4). Esterase activity assay using the larvae midgut of *S. littoralis*, revealed high levels between untreaed and treated larvae in presence and absence of cotton oil only. When the concentration of diRNA increased, the level of esterase was decreased. But in the absence of of cotton oil, the treated and untreatreted lavae with diRNA were not affected (Fig. 3). In insect, metabolic resistance commonly involves over-expression of enzymes that are capable of detoxifying chemical insecticides or alterations in the amino acid sequences that cause modifications in the levels and activity of detoxifying proteins.

In this respect, Hemingway and Ranson (2000) reported that three major enzyme families are involved in this type of resistance; glutathione-S-transferases (GST), carboxylesterases and P450 cytochromes. Carboxylesterases involved in organophosphate, carbamate and pyrethroid resistance, while P450 cytochromes involved in the digestion of pyrethoids and detoxification of organophosphates, carbamates and any other toxic materials like xenobiotics. P450 cytochromes are a complex family of enzymes that are essential for metabolism of xenobiotics and have a role in the endogenous metabolism. They are involved in the metabolism of virtually all insecticides, including gossypol material that goes alongwith cotton leaf while feeding led to detoxification (Scott et al., (1998). The obtained results clearly explain and support our theory based on the gossypol is digested by cytochrome P450 in insect gut and when insect obtained dsRNA via semi synthetic diet. Thus, our data here can conclude the importance of RNAi technology to break down P450 cytochromes enzymes in detoxification process of gossypol which may lead to insect death and delay also insect resistance development to other insecticides (Sabourault et al., 2001). This is the first attempt to use dsRNA technology to control cotton leafworm and also, such technology is applicable for other major pests that attack major crops worldwide. This work could be used as a pave for creating a transgenic plant that control cotton leafworm pest under Egyptian fauna.

Table 1. Mortality percentages of *Spodoptera littoralis* larvae treated with siRNA by diet mixing and microinjection.

Treatments	Negative Control	Positive Control	20ng dsRNA	100ng dsRNA	300ng dsRNA
Diet mixing assay	0.0	0.0	6.7	46.6	60.0
Force feeding assay	0.0	0.0	23.3	33.3	53.35

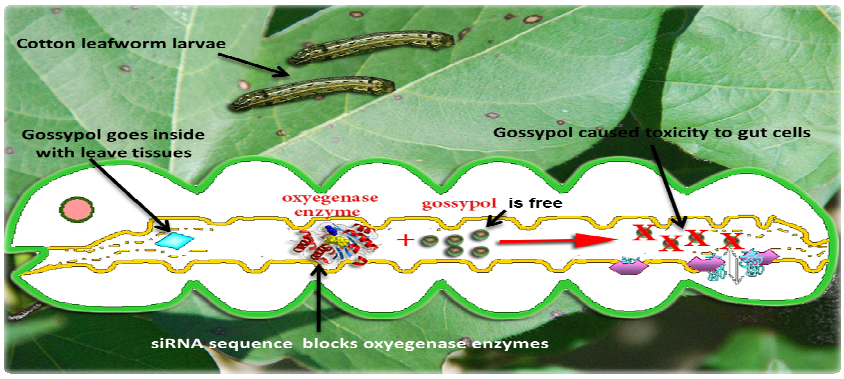

Fig. (1): Graphical diagram showed the proposed mode of action of siRNA sequence on cotton leafworm larvae. The gossypol is taken by the larva via mouthparts alongwith the tissues of cotton leaf. While siRNA sequence delivered with insect diet, it targets the monoxyegenase enzymes, so it blocks them. Simultaneously, the gossypol will be free in the insect gut resulting cell toxicity and eventually, insect death

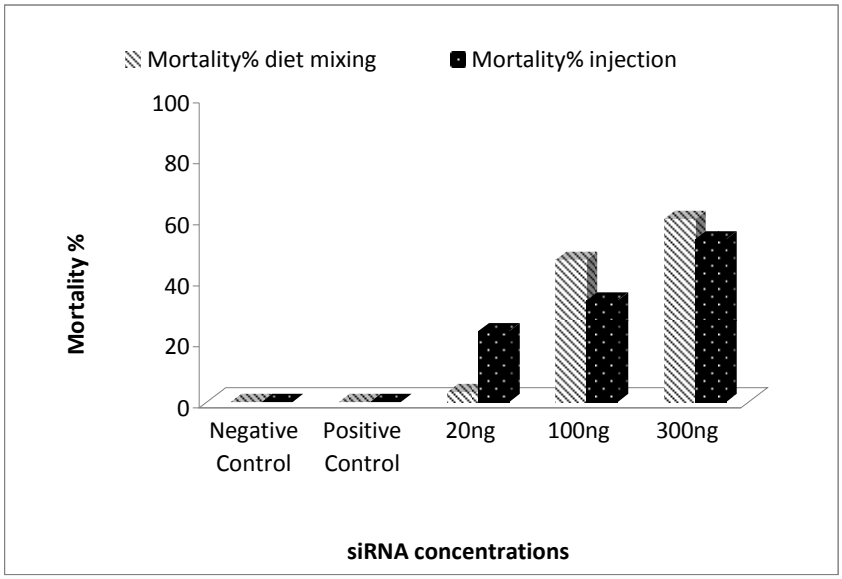

Fig. (2): Diet mixing bioassay for blocking monoxygenase enzyme:*S. littorlais* larval mortality percentage with using siRNA. Mortality difference between two different assays; diet mixing assay and microinjection assay (force feeding assay). Black column: Represents mortality % in microinjection method, White column: Represents mortality % in diet mixing assay

Fig. (3): Diet mixing assay, A: Represents larvae fed on diet mixed with cotton oil only, B: Represents larvae fed on diet mixed with cotton oil + siRNA sequence

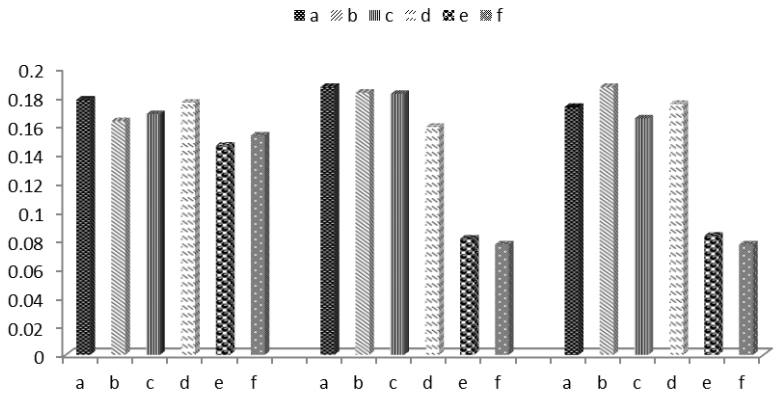

Fig. (4): Esterase activity assay from force feeding experiment:

The 5th instar larvae were anesthetized using CO_2 and kept for 10-15 min at -20 ^{0}C then injected. Each Larva was injected with 5µl total volume. a: Represents larvae injected with PBS buffer then released on normal diet (Negative control), b: Represents larvae injected with PBS buffer then released on diet mixed with cotton oil (Positive control), c: Represents larvae injected with 100ng/µl siRNA then released on normal diet, d: Represents

larvae injected with 200 ng/µl siRNA then released on diet mixed with cotton oil, e: Represents larvae injected with 300ng/µl siRNA then released on normal diet, f: Represents larvae injected with 100ng/µl siRNA then released on diet mixed with cotton oil. Esterase activity was measured 24, 48, and 72h after treatment for group 1, group 2 and group 3 respectively

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تثبيط السيتوكروم بى ٤٥٠ فى حشرة دودة ورق القطن الذى يوقف هضم الجيسيبول باستخدام تكنولوجيا الحمض النووى الريبوسومى

سعد موسى و هبة الله ابو المعاطى

وحدة بحوث بيوتكنولوجيا الحشرات – معهد بحوث وقاية النباتات

يلعب الحمض النووى الريبوسومى دورا هاما فى تنظيم عمل الجين داخل وخارج الخلية. كما تقوم مجموعة الانزيمات من النوع (بى ٤٥٠) بالقيام بدور هام فى عملية تأقلم حشرات حرشفية الاجنحة على المركبات الكيمائية السامة الموجودة بنبات القطن مثل مادة الجيسيبول التى تدخل معدة الحشرة. هذه المجموعة من الانزيمات (بى ٤٥٠) يتم افرازها بالمعى الوسطى للحشرة بكثرة ويتفق معدل وجودها مع معدل نمو وتطور اليرقة. وعموما يزداد مستوى افراز هذه المجموعة من الانزيمات فى معدة الحشرة فى حالة وجود مادة الجيسيبول. لذا هذه الدراسة تبحث التأثير التثبيطى لتكنولوجيا الـ siRNA لمجموعة ٤٥٠ على حشرة دودة ورق القطن عن طريق استهداف الجينات الخاصة بالسيتوكروم ٤٥٠.تم تصميم قطعة صغيرة من التتابع للحمض النووى الريبوسومى ثم أعطت للحشرة مع البيئة الصناعية فى معاملة وفى معاملة أخرى تم اعطائها للحشرة عن طريق الفم. عند خلطها بالبيئة الصناعية وصلت نسبة موت اليرقات الى ٦.٧ – ٦٠% مع تركيز ٢٠ ، ٣٠٠ نانوجرام (حمض نووى ريبوسومى) ولكن فى حالة اعطائها للحشرة عن طريق الفم وصلت نسبة موت اليرقات من ٢٣.٣ – ٥٣.٣٥% على التوالى مع نفس التركيزين السابقين وذلك خلال ٧٢ ساعة بعد المعاملة فقط. أوضحت النتائج أيضا أن هناك تغيرات فى مستوى انزيم الاستيريز ما بين اليرقات المعاملة والغير معاملة بالـ siRNA (٠.٠٨ – ٠.١٩ نانوجرام) عندما تغذت اليرقات على ٣٠٠ نانوجرام siRNA . لذا توضح هذه الدراسة أهمية الـ siRNA كعامل أساسى فى تثبيط نمو يرقات دودة ورق القطن التى تعتبر بداية هامة لانتاج نبات معدل وراثيا يحمل هذا الجين الذى يتسبب فى قتل الحشرة اذا ما تغذت عليه.