Transgenic Canola Plants Expressing cry1Ia5 Gene Are Resistant to (Agrotis Ipsilon) Using Sonication Assisted Agrobacterium Mediated Transformation (SAAT) Technique

Etr H.K. Khashaba (ater_hk@yahoo.com)  
PPRI: ARC Plant Protection Research Institute  
https://orcid.org/0000-0001-9688-6886

Reda E. Moghaieb  
Cairo University Faculty of Agriculture

Sawsan S. Youssef  
Cairo University Faculty of Agriculture

Sayed Moraad  
PPRI: ARC Plant Protection Research Institute

Research Article

Keywords: Agrotis ipsilon, transgenic canola, cry1Ia5 gene, digestive enzymes, detoxification enzyme

DOI: https://doi.org/10.21203/rs.3.rs-190901/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

The aim of this work was to transfer the cry1Ia5 gene into the genome of two commercial canola cultivars using Sonication assisted Agrobacterium mediated transformation system and to test its toxicity against A. ipsilon. The integration and expression of the cry1Ia5 gene into the plants genome was confirmed using PCR, Southern blot and Northern blot analyses. The insecticidal activity of transgenic plants against A. ipsilon was studied. The 2nd and 4th A. ipsilon larval instars were fed on leaves from five transgenic lines (L1, L2, L3, L4 and L5) for one week of T1 plants. Transgenic L5 was found to be more toxic to the 2nd larval instar causing 60% mortality on the 3rd day followed by 100% on the 4th day post feeding. While the other transgenic lines L2, L3, L4 caused 60, 20, 80% mortality respectively on the 6th day, whereas L1 did not show any observed toxicity to A. ipsilon. Digestive and detoxification enzyme activity were examined in the body of 2nd instar of A. ipsilon, fed on bt canola, showing significantly lower trypsin, protease, lipase, esterase and acetylcholinesterase activities and significantly increase Glutathione-S-transferase and Chitinase activities than non-bt fed larvae. Whilst the 4th larval instar fed on bt canola showed cannibalism as change in the behavior. The results of the study imply that transgenic canola lines obtained varying in toxicity against A. ipsilon which could be used to construct new canola cultivars resistant to A. ipsilon.

Introduction

Vegetable oils have long been an essential component of diet of humans. Today, the four main crops producing vegetable-oil are oil-palm, soybean, rapeseed and sunflower, respectively (USDA- FAS 2014). As an oil crop, rapeseed (Brassica napus) is widely used worldwide. It is also used as protein-rich livestock feed, as a source of biodiesel, lamp oil, soap, and plastics, an effective heavy metal hyper accumulator plant, as an excellent rotating crop for soil management, (Saeidnia and Gohari, 2012). One solution to improve the quality and quantity of canola products is the transfer of useful properties such as insect resistance and herbicide resistance through genetic transfer techniques (Tarinejad et al., 2013).

Because of transfer of low gene copy numbers and the transgene stability, Agrobacterium tumefaciens that contributes to gene technology is considered popular for genetic manipulation (Travella et al., 2005; Lee and Zhang, 2014). As the most effective protein insecticides used in agriculture, Bt delta-endotoxins coded by Cry genes of Bacillus thuringiensis are well known. These toxins are widely used as bacterial preparations, to control varied pest insects that are sprayed on field plots (Entwistle et al. 1993, Kuvshinov et al. 2001). Under field conditions these preparations are costly and degrade rapidly. Cry genes have been transformed into various plant species to grow insecticidal crops to solve these issues.

Selvapandiyan et al. (1998), cloned Cry1Ia5 gene from an Indian isolate of B. thuringiensis and characterized it (EMBL accession number Y08920). Cry1Ia5 provided full defense against the neonate stage larvae of H. armigera, When expressed in tobacco plants via nuclear transformation, Moghaieb (2010), tested the transformation of rice plants with Cry1Ia5 against the third larval instars of Chilo agamemnon and concluded that it provides a good protection against the stem borer of rice.

The present study describes an effective protocol for the development of transgenic canola plants expressing the cry1Ia5 gene resistant to black cutworm (A. ipsilon), which is one of the Egypt's major canola pests. The insecticidal activity produced by the transgenic plant against the larvae of A. ipsilon was examined.

Materials And Methods

Plant materials

Two canola genotypes, namely Serw3, Serw4 were kindly provided by Field Crop Institute, Agricultural Research Center, and Ministry of Agriculture – Egypt.

Canola seeds were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite, and rinsed in sterile distilled water. The sterilized seeds were germinated in flasks on 0.7% agar (w/v). The cultures were incubated at 25°C under a 16/8 h day/night photoperiod (1000-Lux) for subsequent uses in transformation (Moghaieb et al. 2006, 2014).
Bacterial strain

Professor V.S. Reddy (International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India) kindly given Agrobacterium tumefaciens strain LBA4404 containing the pBI-121 Cry1Ia5 plasmid (Selvapandiyan et al., 1998). The Agrobacterium was grown overnight in 30 ml of LB medium (Luria and Burrow, 1955) containing 50 µg/ml Kanamycin at 28°C (Fig. 1).

Sonication assisted Agrobacterium mediated transformation (SAAT)

The hypocotyl explants (0.5 cm in length) were isolated from 5-day-old canola seedlings (cultivars: Serw4 and Serw3) and were subjected to sonication for 5 seconds using Sonicor (model No./SC/152, N.Y) water bath, then immersed in bacterial suspension for 5 minute following Moghaieb et al. 2014 stated protocol. The explants were subsequently blotted on sterilized filter paper, placed onto a co-cultivation medium, which consisting of MS medium (Murashige and Skoog, 1962) supplemented with 1mg/l 2, 4-D and then incubated under dark conditions. The explants were transferred to supplemented shoot induction medium with 4.5 mg/l BA in addition to 50 mg/l kanamycin sulfate. The plates were parafilm- sealed and incubated at 26°C under a 16/8-h light/dark photoperiodic regime (1000-Lux).

Molecular conformation of the transformation events:

Complete DNA was extracted from both of bt transformed and non-transformed plants according to the method previously defined by Rogers and Bendich (1989) to confirm the stable incorporation of the bt gene into the resulting plantlet genome. Genomic DNA subjected to PCR analysis using 35S promoter specific primers, The sequence of forward and reverse primers 5'- AAA GGA AGG TGG CTC CTA CAA AT-3' and 5'-CCT AGT AAA GTA AAC CTC TCC-3', respectively. The reaction mixture (20µl) contained 10ng DNA, 200µM dNTPs, 1µM of each primer, 0.5 units of Taq polymerase (Thermo Fisher Scientic Inc.) and 10-X Taq polymerase buffer. Samples were heated to 94°C for 5 min, then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72°C. Via agarose gel electrophoresis, the PCR products were separated by and visualized with ethidium bromide. Plants were acclimatized and transferred in to the growth chamber to collect the T1 seeds that showed positive result for 35S promoter.

Southern blotting analysis. Southern analysis was performed from genomic DNA of positive plants for the 35S promoter. In 2 % agarose gels, the digested DNA was separated, blotted to a nylon membrane following standard protocols (Sambrook et al. 1989). As a probe, the PBI-121-Cry1Ia5 plasmid was used. Labeling of the probes, hybridization and detection were performed using the Biotin Chromogenic Detection kit #K0661, #K0662 according to instructions directed by the manufacturer (Thermo Fisher Scientific Inc.).

RNA dot blot and Northern blot analysis. Following the technique of Chrigwin et al. (1979), Total RNA was isolated from leaf samples of both the transgenic T1 and control plants. Labeling of the probe, hybridization and detection was carried out using an RPN 3450 Gene Image kit in compliance with the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Insect bioassays on transgenic canola plants expressing Cry1Ia5

According to procedure of Parrott et al. (1994), detached leaf-insect bioassays have been carried out on T1 plants. Petri dishes held single, detached two leaf discs were removed from the oldest non-senescing leaf on each plant, and were placed on moist filter paper in a Petri dish and the 2nd and the 4th larvae instars from black cutworms (Agrotis ipsilon) were placed on each plate of transgenic lines and non-transgenic control line with replicate plates. Under a 23-h photoperiod, the larvae were allowed to eat the plant material for 7 days and every day all the insects were subjected to weight. At the end of each 7-d experiment, insect survivorship data were obtained. After insects were collected and frozen at the end of the experiment, total protein determination and shift in enzyme activates in larval were measured.

Total protein determination in larvae (Protein assay)

Larvae were chilled on ice. The reaction was made according to method of Bradford (1976) and updated by Spector (1978). As standard protein, bovine serum albumin (BSA) was used. Spectrophotometer was used to read the reaction (absorbance) at 595
nm. Protein concentrations in mg/ml were determined from a standard curve of absorbance of known concentrations of bovine serum albumin.

**Change in enzyme activities**

According to Moghaieb et al. (2019) *Agrotis ipsilon* larvae have been used for hemolymph collection and enzymes extraction preparation. For the processing of hemolymph, Prolegs were cut and the hemolymph was dripped into microtubes. From each individual, approximately 25µl of hemolymph was obtained.

1. **α-Esterase assay**

As described in Asperen (1962) with minor modifications, esterase activity in the samples was determined spectrophotometrically. The mixture contained 1ml of 0.54 mM α- naphthylacetate in PB and 200µl of the sample. The concentration of α- naphthyl produced during the reaction was calculated spectrophotometrically at the wave length of 555 nm. 200µl of α-NA solution and 50µl of stain were included.

2. **Acetyl cholinesterase (AChE)**

AchE activity was measured according to Simpson et al. (1963). The reaction mixture contained 200µl enzyme solution, 500µl 0.067 M phosphate buffer (pH 7) and 500µl AchBr (3mM). The test tubes were incubated at 37ºC for 30 min. 1ml of alkaline hydroxylamine chloride was applied to the test tubes. Then 500µl of HCl was added. The mixture allowed standing for 2 min. then 500µl of ferric chloride solution was added and mixed well and was assayed colorimetric at 515 nm. The decrease in AchBr due to hydrolysis by AchE was observed.

3. **Glutathione S-transferase (GST)**

Enzyme activity was estimated following the procedure of Habig et al. (1974) using 1-chloro-2, 4 dinitrobenzene (CDNB), and GSH as substrates. The absorbance level shift was noted at 340nm. Routine assays consisted substrate buffer of 1.82ml (100 mM potassium phosphate buffer pH 6.5), 40µl from 70 mM 1-chloro-2, 4 dinitrobenzene (CDNB) dissolved in 2 ml 95% ethanol to obtain final concentration (1.4mM); 40µl 70mM reduced glutathione as a cofactor in 2ml distal water to be 1.4mM.

4. **Trypsin assay**

As defined by Erlanger et al. (1961) the activity of trypsin was calculated using alpha-benzoyldl- arginine-p-nitroanilide (BapNA) 4mM N α-benzoyl-DL-arginine-p-nitroanilide (BapNA) in 7% Dimethylformamide (DMF) was diluted with 50mM Trizma hydrochloride (pH 8.5) then incubated with the sample for 30 min. at 37ºC. At 410 nm, the rate of nitroaniline formation was calculated.

5. **Chitinase activity**

According to Ishaaya and Casida (1974), colloidal chitin was prepared. The reaction mixture with some modifications, according to Bade and Stinson (1981) consisted of 1ml phosphate buffer (0.2 M, pH 6.5), 200µl of 0.5% colloidal chitin and 200µl enzyme solution. After 1.5 hour incubation at 37ºC, enzyme activity was terminated by boiling test tube. Undigested chitin was sedimented at 8.000 rpm by centrifugation for 15 min. The supernatant was taken for determination of N- acetylglucosamine produced as a result of chitin digestion by the sensitive method of Waterhouse et al. (1961).

6. **Lipase determination**

As defined by Tsujita et al. (1985), lipase assay was carried out. Thirty microlitre of gut extracts and 100µl of p-nitrophenyl butyrate (PNPB, 27 mM), as substrate were incorporated, mixed thoroughly and added at 37ºC (Obtained by preliminary test). After 10 min, 100µl of distilled water were applied to each tube and absorbance was read at 405 nm. (Zibaee and Fazeli-Dinan, 2012)

**Statistical Analysis**

In order to compare the protein content and enzyme expression levels between the control and the treated ones, biochemical data was subjected to a one– way analysis of variance (ANOVA) levels of statistical significance were determined at < 0.05. Observed
differences between the control and the treated ones were evaluated by independent t-test (SPSS version 9.0).

**Results**

The well proliferated explants derived from two canola cultivars (Serw3 and Serw4) were subjected to sonication for 5 seconds, in order to produce canola plant resistant to the black cutworm (*A. ipsilon*). According to the protocol defined by Moghaieb et al. (2014), high frequency of transgenic shoots production results. Before the co-cultivation with *A. tumefaciens* strain LBA4404, the explants were moved to MS medium containing 1mg/l 2,4-D for callus induction (Moghaieb et al., 2006). Followed by regenerating media containing 4.5 mg/l BA for two weeks during the selection culture; the explants were sub-cultivated into fresh medium containing 50 µg/ml kanamycin. Table (1) revealed the transformation percentages were 25 and 36% for serw3 and serw4 respectively.

Serw4 T0 plants that can live at 50 µg/ml kanamycin containing medium were subjected to PCR analysis using primers specific to the 35 S-promoter to confirm the stable incorporation of the T-DNA into the putative transgenic plantlet genomes because serw4 gives the highest transformation percentage. Figures (2) showed that a clear band at 250 bp was observed only in transgenic plants, but no such bands were seen in the non-transformed controls under similar conditions. In the presence of 50 mg/l kanamycin sulfate, the transformation efficiency of the two tested canola cultivars after selection was 25 and 36%, with a total of 30 and 90 transgenic plants derived form the cultivars Serw3 and Serw4, respectively. Using Southern analyses, the integration of the *Cry1a5* gene in the T0 plant genomes was confirmed. As indicated in figure (2), the Southern analysis data suggest that single or two copy of the transgene found in most of the transgenics.

The expression of *Cry1a5* gene was investigated by northern blot analyses using the T1 plants to detect transcripts that hybridized directly to *Cry1a5* gene derived probe and accumulated in the transgenic T1 plants which could not be detected in the non-transformic plants (Fig. 3)

**The Insect Bioassay to transgenic canola plants expressing the *Cry1a5* gene:**

Selected northern positive T1 plants (showing normal morphology and growth) were assessed for to black cutworm (*A. ipsilon*) resistance and control plants were also included according to Kuvshinov et al. (2001). Transgenic plants were put on a moistened filter paper disc in a 90 mm-diameter Petri dish and infested with two larvae of black cutworm per Petri dish. Then Petri dishes were incubated at 26°C in the dark and, leaf cuttings from the same transgenic line were supplied twice a day for one week.

The insect weight increased from line 1–5 until the 5th day after feeding with transgenic material and then substantial decrease in their weight was observed compared to the control feeding insects (Fig. 4) which increased in weight with days of experiment.

The data obtained for the insecticide activity of five transgenic lines showed that the transgenic line 5 was more toxic to the 2nd larval instar of black cutworm (*Agrotis ipsilon*). On the 3rd day it resulted in 60% mortality followed by 100% on the 4th day. The other transgenic lines L2, L3, L4 triggered 60, 20, 80 percent mortality respectively on the 6th day and then the mortality rate for line 2 increased to 80% on day 7 the (Figs. 5 & 6), where no mortality was shown by the control plants. This suggests that transgenic canola plants have been toxic to *Agrotis ipsilon*, and may serve as genetic resource for canola resistance to insects.

**Changes in the Protein content in the 2nd larval instar fed on transgenic canola**

From the 2nd larval instar that had been fed on transgenic canola plants expressing the *Cry1a5* gene from five transgenic lines and from the control plants, total protein was extracted and analyzed. It should be noted that significantly lower protein levels have been observed in *Agrotis ipsilon* fed on *Bt* transgenic canola lines compared with the control canola (P < 0.0001) (Fig. 7). Compared with the other lines and the control, L5 displayed the greatest decrease in protein content which was associated with decreasing in the insect’s weight.

**Activities of the digestive enzymes in the 2nd larval instar fed on transgenic canola contain *Cry1a5* gene:**

**Trypsin:**
In most insects, Serine proteinases, such as trypsin, chymotrypsin, are the key digestive alkaline endopeptidases and are essential both in the solubilizing and activating of Bt protoxins (Oppert, 1999; Terra and Ferreira (2005)). The proteinases like trypsin derived from the larvae that fed on transgenic lines showed substantial decrease from that fed on control line (P < 0.0001) Fig. (8), and the decrease observed in L2 and L5 which was associated with the decrease in the protein content of both lines.

**Protease:**

As insecticidal agents, Proteases are logical candidates for use. Proteolytic enzymatic action can target and destroy essential proteins and tissues to an extent that mortality would result (Harrison and Bonning 2010). As shown in Figure (8), The protease derived from larvae fed on transgenic canola lines significantly decreased compared with that fed on the control line (P < 0.0001).

**Lipase:**

Lipids in insects are involved in many physiological functions like molting during larval and adult development (Kawooya and Law, 1988), reproduction (Majumder and Sengupta, 1979), energy supplement during starvation (Ziegler, 1991) and immunity. In the 2nd instar of *Agrotis ipsilon* fed on Bt transgenic canola lines, lipase activity decreased compared to fed on the control canola (P = 0.0001), and the reduction was more pronounced in insects fed on line 4 and line 5 as the weight of the insect decrease in those two lines(Fig. 8).

**Activities of protection and detoxification enzymes in the 2nd larval instar fed on transgenic canola contain Cry1ia5 gene:**

**Estrase:**

In conferring or leading to insecticide resistance in insects, esterase enzymes play a significant role (Claudianos et al. 1999; Taskin and Kence, 2004). Enzyme synthesis reduction is due to the direct impact of toxicants on the synthesis and can be used as a marker for resistant individuals in populations of *B. tabaci* (Wool and Greenberg 1991, Kurappasamy et al., 2001).

The data presented in Figure (9) Suggest that the $\alpha$- esterase concentration in the larvae fed transgenic leaves from line 1 and line 2 compared to the control was reduced. Compared to the control, while the insects fed on transgenic lines 3, 4 and 5 showed an increase in the enzyme activity. These results support our mortality's data or higher insecticidal activity that was found in transgenic lines 3, 4 and 5 (Fig. 9).

**Acetylcholinesterase (AChE):**

Acetylcholinesterase (AChE) is an essential enzyme that terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (ACh) in the nervous system of insects (Soreq and Seidman, 2001).

In the 2nd larval instar which fed on transgenic plants, the AChE activity reduced compared to the control (P < 0.0001). In larvae fed on line 4 and line 5, the reduction was more remarkable (Fig. 9). And this could clarify that the percentage of mortality on line 4 and 5 was higher than the other lines (Fig. 9).

**Glutathione-S-transferase:**

Current data suggest substantial increase in the GST extracted from larvae fed on bt canola relative to control canola (P < 0.0001), while line 1 decreased compared to the other lines (Fig. 11). Insect fed on transgenic line 5 displayed higher GST content followed by line 4 then line 3 and suggesting that these insects appear to increase GST concentration in their body for detoxify the Bt toxin from their body. These data is confirmed by the data provided in (Fig. 9) whereas transgenic line 3, 4 and 5 show higher mortality percentage.

**Chitinase:**

The activity of chitinase extracted from larvae fed on Bt expressing plants, increase extremely significant compared with fed on the control (P < 0.0001). In But line 1 and 3 displayed a decline in chitinase activity because of the insects in the two lines molt
earlier than all the lines and the wild type (Fig. 9).

The analysis of variance of different enzymes is shown in Table (2). The differences between insects that were supplied by transgenic material from different lines and the control plants were extremely significant for total protein contents, trypsin, protease, lipase, α-esterase, AchE, GST and chitinase activity.

**Evaluation of Cry1a5 expressing canola plants against the 4th larval instar of black cut worm**

The killing and consumption of all or part of a conspecific is called Cannibalism (Polis 1981, Elgar and Crespi 1992). Many density-dependent and density independent factors can influence.

Compared to that fed on the non transgenic plant material the 4th larval instar consumed much less transgenic leaves in the present study. The larvae started to fast and their behavior changed and began to cannibalize on the other larvae found in the same Petri dishes in the presence of canola leaves. In order to prevent the stress imposed by bt toxin, some larvae were forced to pupate, but the other insect did not pupate began to feed on the insect's pupa (Fig. 10).

In the body of the 4th instar larvae fed on transgenic plant leaves, there is an increase in the esterase activity measured. The data indicate that this enzyme activity was increased in the larvae fed on bt expressing plants compared to the control, which led to increased resistance to the bt toxin as esterase enzyme considered to be detoxifying enzyme and is essential in the resistance to insecticides and plant secondary substances (Klowden, 2007).

**Discussion**

Several plant transformation methods have been recorded for crop improvement program and can be applied to generate transgenic plant species, including agrobacterium and biolistic gun (John, 1997; Moghaieb 2010, 2014). In term of optimizing in vitro tissue culture and gene transformation, *Brassica napus* serves as suitable host for several new characteristic in is highly genotype based (Mashayekhi et al., 2008; Burbulis et al., 2010). Plants transformed by bt genes have varying degrees of defense against various insects. (Chen et al. 2007, Rahman et al. 2007)

The present study indicate that the expression of the *Cry1a5* gene was confirmed by Southern, RNA dot blot and northern blot analyses using T0 and T1 plants.

The mortality percentage of the five transgenic was checked on black cut worm and found that L5 was more toxic to larvae causing mortality % up to 100% on the 4th day followed by L2, L3, L4 causing 60, 20, 80% mortality on the 6th day as the copy number in Southern blot indicated, while the control showed 0 % mortality and these data agrees with findings of Cao et al. (2008) as four *Cry1C Brassica juncea* plants showed complete resistance to susceptible *Plutella xylostella* with 100% mortality. Also the finding agrees with Moghaieb (2010), when using the transgenic rice lines expressing *Cry1a5* gene against rice stem borer (*Chilo Agamemnon*) and recorded mortality rate of 100% four days post treatment, where as our findings disagree with Silva et al. (2016) as the Bt transgenic soybean had no impact on *S. cosmioides* as there is up to 80 % of larval survival.

The Changes in the Protein content in the 2nd larval instar of *A. ipsilon* fed on transgenic canola was lower than that fed on control plants and this decreases in haemolymph protein content could be caused by DNA and RNA synthesis inhibition, Mitlin et al. (1977) reported that there were also results consistent with Guo et al. (2011) in boll weevils treated with chitin synthesis inhibitors as there were significantly lower protein levels observed in *Spodoptra exigua* fed on Bt cotton for 1 h than those for 4, 6 and 24h.

The decrease in digestive enzymes like Trypsin was agreed with finding of Li et al. (2004) as the specific activity of trypsin-like proteinases in the soluble fraction of Bt-resistant *O. nubilalis* larvae was substantially lower than those of the susceptible larvae fed on diets containing doses of Bt subsp. *Kurstaki*. Guo et al., (2011) also found that the activity of trypsin in *S. exigua* after being fed on Bt cotton and non-Bt cotton for 24 h decreased significantly. The lipase activity was substantially lower in the experiment result of the use of bt expressing plants convenient with Zibaee et al. (2010), as that *B. thuringiensis* and plant
extracts lowered the amount of lipase activity, also in accordance with Guo et al. (2011) who noticed decrease in lipase in S. exigua fed on Bt cotton for different times compared with those on control cotton for the same periods.

We conclude that the concentration of esterase enzyme has been decreased by analyzing the activities of defense and detoxification enzymes in the 2nd larval instar fed on transgenic Cry1a5 canola plants. Abuldahab et al. (2011), agree with our data finding that the activities of α – and β – esterases have decreased in larvae treated with B. thuringiensis israelensis and the enzymatic activities reduction were highly significant.

Acetylcholinesterase (AChE) demoralization results in excessive accumulation of ACh, leading to hyperactivity and eventually paralysis and death (Soreq and Seidman 2001). This explains the decrease in (AChE) in larvae fed on line 4 and line 5 which Guo et al. (2011) agreed with as the AChE activities were significantly lower in S. exigua fed on Bt cotton compared with those fed on non-Bt cotton for the same periods.

Glutathione-S-transferase (GST) enzymes play an important role in detoxification and protection against xenobiotic compounds including insecticides. Shuanyang et al., (2001) found that after 24 hours of feeding the American white moth larvae with transgenic poplar, the activity of (GST) increased significantly and concluded that the detoxification enzyme mechanism in the midgut was inhibited and further toxic reaction of Bt protein could occur, this in line with our findings as there is substantial increase in the GST in insects fed on Bt plants.

Chitinases are enzymes that in many species are widely distributed and play roles in immunity and protection, digestion, pathogenicity and moulting of arthropods (Arakane and Muthukrishnan, 2010). Data showed that the activity of chitinase extracted from larvae fed on Bt expressing plants compared with fed on the control increase extremely significant and this agrees with Rashad et al. (2015) who worked with four insecticidal compounds on the 4th instar larvae of Pink bollworm and found that chitinase enzymes were increased in moderate percentages in both Teflubenzuron and V. lecanii compound although these disagree with the conclusion of Al-shannaf et al. (2012) that chitinase activity was increased in H. armigera as compared to the biopesticides, uses chlorfluazuron and pyriproxyfen as insect growth regulators.

The propensity to cannibalize, include many factors like, developmental asynchrony with host plants, sex or genetic relationships of the participants, food quantity or quality limitations, and abiotic environmental factors such as humidity and high temperatures. (Richardson et al. 2010).

In the study, the change in the behavior of 4th larval instar of black cut worm was studied to find that the insects’ began to cannibalize on the other insects in the presence of the canola leaves confirming the finding of Richardson et al. (2010), and this finding need some study to understand either bt plants have negative effects on the behavior of the targeted insects or not. Our findings explains the rise in the esterase enzyme as it is known to be a detoxifying enzyme due to insect’s resistance to insecticides as postulated by Klowden (2007).

Conclusion

The present study indicated that SAAT transformation technique has the potential for producing transgenic canola lines varying in toxicity against A. ipsilon. These transgenic materials can be used to construct new canola cultivars resistant to A. ipsilon to be used in IPM programs and to minimize the use of chemical pesticides.

Abbreviations

SAAT: Sonication assisted Agrobacterium mediated transformation

AChE: Acetylcholinesterase

GST: Glutathione-S-transferase

ANOVA: analysis of variance
**Declarations**

**Funding:**

This research did not receive any specific grant from funding agencies in public, commercial, or not-for-profit sectors.

**Competing of interests**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Availability of data and materials**

All datasets are presented in the main manuscript.

**Authors' contribution**

Etr H. K. Khashaba: participated sufficiently in the work and manuscript writing.

Reda E.A. Moghaieb: experimental design, analysis, interpretation of data and manuscript writing.

Sawsan S. Youssef: participated in manuscript editing.

Sayed A. Morad: participated in the part of insect culture and bioassay.

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Acknowledgements**

We would like to thank Dr. V.S. Reddy (International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India) for providing the cry1la5 gene, Prof. Dr. Sanaa A. Ibrahim (plant protection research institute, ACR) for intellectual support to this research.

**References**

Abuldahab F, Najlaa F, Abozinadah Y, Al Haiqi NS (2011) Impact of Bacillus thuringiensis β – exotoxin to some biochemical aspects of Musca domestica (Diptera: Muscidae) Journal of Bacteriology Research. 3(6):92-100.

Al-shannaf HM, Mead HM, Al-Kazafy HS (2012) Toxic and Biochemical Effects of Some Bio insecticides and Igrs on American Bollworm, Helicoverpa armigera (hüb.) (noctuidae: lepidoptera) in Cotton Fields. J. Biofertil and Biopestici. 3:1.
Arakane Y, Muthukrishnan S (2010) Insect chitinase and chitinase-like proteins. Cell. Mol. Life Sci. 67: 201–216. as genetic markers. Nucleic Acids Res 18:6531-6535.

Asperen VK (1962) A study of housefly esterases by means of a sensitive colorimetric method. J. Insect Physiol. 8: 401-416.

Bade ML, Stinson A (1981) Biochemistry of insect differentiation. A system for studying the mechanisms of 122 chitinase activity in vitro. Archs. Biochem. Biophyscs. 206: 213-221.

Bradford MM (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding Analytical Biochemistry. 72: 248-254.

Burbulis N, Blinstrieiune A, Kupriene R (2010) Genotypic and growth regulator effects on shoot regeneration from hypocotyl and stem segment explants of spring rapeseed (Brassica napus L.). Journal of Food Agriculture and Environment. 8: 634-637.

Cao J, Shelton AM, Earle ED (2008) Sequential transformation to pyramid two Bt genes in vegetable Indian mustard (Brassica juncea L.) and its potential for control of diamondback moth larvae. Plant Cell Reports. 27:479–487.

Chrigwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. 18:5294-9.

Claudianos C, Russell RJ, Oakeshott JG (1999) The same amino acid substitution in orthologous esterases confers organophosphate resistance in the housefly and a blowfly. Ins. Biochem. Mol. Biol. 29: 675–686.

Elgar M A, Crespi B J (1992) Ecology and evolution of cannibalism. pp. 1–12.

Entwistle PF, Cory JS, Bailey MJ, Higgs S (1993) Bacillus thuringiensis, an environmental biopesticide: theory and practice, Wiley, Chichester. (Eds.).

Erlanger BF, Kokowski N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271– 278.

Guo J, Wu G, Wan F (2011) Temporal allocation of metabolic tolerance to transgenic Bt cotton in beet armyworm, Spodoptera exigua (Hübner). Life Sciences China. 54 (2): 152–158.

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130–7139.

Harrison RL, Bonning BC (2010) Proteases as Insecticidal Agents Toxins. 2:935-953.

John ME (1997) Cotton crop improvement through genetic engineering. Crit. Rev. Biotech., 17:185-208.

Klowden MJ (2007) Physiological systems in insects. Oxford, Elsevier Inc. Press. pp. 497–499.

Kurappasamy R, Ecantheziyan C, Parasarthi K (2001) Inhibitory effects of Annona squamota(Annonaceae) on the digestive enzymes of Pheropsophus hilaris (fabr.) (Coleoptera: Carabidae). Environment and Ecology. 19: 584-587.

Kuvshinov V, Kolvi K, Kanerva A, Pehu E (2001) Transgenic crop plants expressing synthetic Cry9Aa gene are protected against insect damage. Plant Science. 160: 341–353.

Lee H, Zhang ZJ (2014) Agrobacterium-mediated transformation of maize (Zea mays) immature embryos. Methods in Molecular Biology. 1099: 273-280.
Li H, Oppert B, Higgins RA, Huang F, Zhu K Y, Buschman LL (2004) Comparative analysis of proteinase activities of Bacillus thuringiensis resistant and susceptible Ostrinia nubilalis (Lepidoptera: Crambidae) Insect Biochemistry and Molecular Biology. 34: 753–762.

Luria SE, Burrows JW (1955) Hybridization between Escherichia coli and Shigella. J. Bacteriol. 74:461-76.

Majumder, UK, Sengupta A (1979) Triglyceride composition of chrysalis oil, an insect lipid. J. American. Oil Chem. Soc. 56: 620-623.

Mashayekhi M, Shakib AM, Ahmad-Raji M, Ghasemi Bezd K (2008) Gene transformation potential of commercial canola (Brassica napus L.) cultivars using cotyledon and hypocotyls explants. Afr. J. Biotechnol. 7: 4459–4463.

Mitlin N, Wiygul G, Haynes JW (1977) Inhibition of DNA synthesis in boll weevils (Anthonomus grandis Boheman) sterilized by Dimilin. Pest. Biochem. Physiol.7:559–63.

Moghaieb REA, Khashaba EH, Abdel Azzim AM (2019) The toxicity of Cry1a5 transgenic soybean plants against Spodoptera littoralis. Journal of Plant Protection Research. 59(2): 185–192.

Moghaieb REA, EI Awady MA, EI-Mergawy RG, Youssef SS, EI-Sharkawy AM (2006) A reproducible protocol for regeneration and transformation in canola (Brassica napus L.). African Biotechnology Journal. 5: 143-148.

Moghaieb REA, Mohammed EHK, Youssef SS (2014) Genetic diversity among some canola cultivars as revealed by RAPD, SSR and AFLP analyses. 3 Biotech. 4:403–410.

Moghaieb REA, Mohammed EHK, Youssef SS, El-Sharkawy AMA (2010) Comparing the efficiency of sonication assisted Agrobacterium -mediated and particle bombardment for the production of transgenic canola plants. International Journal of Advanced Research 2(10): 200-208.

Moghaieb REA (2010) Transgenic rice plants expressing Cry1a5 gene are resistant to stem borer (Chilo agamemnon). GM Crops. 1(5): 288-293.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473 - 379.

Oppert, B (1999) Protease Interactions with Bacillus thuringiensis. Archives of Insect Biochemistry and Physiology 42:1–12.

Parrott WA, A11 JN, Adang MJ, Bailey MA, Boerma HR, Stewart CN (1994) Recovery and evaluation of soybean (Glycine max Merr.) plants transgenic for a Bacillus thuringiensis var. kurstaki insecticidal gene. In Vitro Cell Dev. Biol. 30: 144-149.

Polis GA (1981) The evolution and dynamics of intraspecific predation. Annu. Rev. Ecol. Syst. 12:225–251.

Rahman M, Rashid H, Shahid AA, Bashir K, Husnain T, Riazuddin S (2007) Insect resistance and risk assessment studies of advanced generations of basmati rice expressing two genes of Bacillus thuringiensis. Electronic Journal of Biotechnology 10 (2): 240–251. DOI: 10.2225/vol10-issue2- fulltext-3

Rashad, AM, EI- Khayat, EF, Abd-El Zaher, TR, Shams El- Din, AM and Salim, HS (2015) Biochemical Studies of Some Pesticidal Formulations against Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae). American-Eurasian J. Agric. & Environ. Sci. 15 (3): 303-307.

Richardson ML, Mitchell RF, Reage PF, Hanks LM (2010) Causes and Consequences of Cannibalism in Non carnivorous Insects. Annu. Rev. Entomol. 55:39–53.

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. Plant Mol. Biol. 5:69–76.
Saeidnia S, Gohari AR (2012) Importance of Brassica napus as a medicinal food plant. Journal of medicinal plants research. 6(14): 2700-2703.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press. New York.

Selvapandiyan A, Reddy VS, Anand PK, Tewari KK, Bhatnagar RK (1998) Transformation of Nicotiana tabacum with a native Cry1a5 gene confers complete protection against Heliothis armigera. Molecular Breeding. 4: 473–478.

Shuangyang D, Huai-ye L, Xue-feng, Zhi-yi Z (2001) Effects of two kinds of transgenic poplar on protective enzymes system in the midgut of larvae of American white moth. Journal of Forestry Research. 12(2): 119-122.

Silva GV, Buenob AF, Bortolottoa OC, dos Santosc AC, Fernandes AP (2016) Biological characteristics of black armyworm Spodoptera cosmioides on genetically modified soybean and corn crops that express insecticide Cry proteins. Revista Brasileira de Entomologia 60: 255–259.

Simpson R, Riordan J, Vallee B (1963) Functional tyrosyl residues in the active centre of bovine pancreatic carboxypeptidase A. Biochem. 2: 616-622.

Soreq H, Seidman S (2001) Acetylcholinesterase – new roles for an old actor. Nat. Rev. Neurosci. 2: 294–302.

Tarinejad RA, Nezami P, Dawoud M, Jafari M (2013) Response to tissue culture in four canola cultivars through hypocotyl explants. World Applied Sciences Journal. 24 (1): 76-83.

Taskin V, Kence M (2004) The genetic basis of malathion resistance in housefly (Musca domestica L.) strains from Turkey. Russ. J. Genet. 40: 1475-1482.

Terra WR and Ferreira C (2005) Biochemistry of digestion. In: Gilbert Li, Iatrou K And Gill SS (Eds), Comprehensive Molecular Insect Science, Oxford: Elsevier 4: 171– 224.

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. Plant Cell Reports 23: 780-789.

Tsujita T, Ninomiya H, Okuda H (1985) p-Nitrophenyl butyrate hydrolyzing activity of hormone-sensitive lipase from bovine adipose tissue. Journal of Lipid Research. 30: 997-1004.

USDA- FAS (2014) Oilseeds and Products Annual 2014.Gain Report Number: RS1421.

Wool D, Greenberg E (1991) Esterase activity in whiteflies (Bemisia tabucrz) in relation to insecticide resistance. Ent. exp. and appl. 57: 251-258.

Zibaee I, Bandani AR, Sendi JJ, Talaei-Hassanloei R, Kouchaki B (2010) Effects of Bacillus thuringiensis var. kurstaki and medicinal plants on Hyphantria cunea Drury (Lepidoptera: Arctiidae). Invertebrate Survival Journal. 7: 251-261.

Zibaee A, Fazeli-Dinan M (2012) Purification and characterization of a digestive lipase In Naranga Aenescens Moore (Lepidoptera: Noctuidae). SOAJ Entomological Studies 1: 33-48.

Ziegler R (1991) Changes in lipid and carbohydrate metabolism during starvation in adult Manduca sexta. J. Comp. Physiol. 161: 125-13.

Tables

Table 1. The transformation frequencies of the two canola cultivars transformed with Cry1a5 gene.
| Cultivar | No. of calli | No. of regenerated shoots | No. of Kan.+ regenerated plants | Molecular conformation using PCR primer specific to 35S promoter | Transformation % |
|----------|--------------|----------------------------|---------------------------------|---------------------------------------------------------------|-----------------|
| Serw-3   | 400          | 120                        | 30                              | 30                                                            | 25              |
| Serw-4   | 400          | 250                        | 90                              | 90                                                            | 36              |

Table 2. Analysis of variance showing the differences in the enzyme activity between five transgenic canola lines expressing the *Cry1a5* gene against black cutworm (*A. ipsilon*).

| S.V     | d.f | Total protein | Trypsin | Protease | Lipase | α-estrases | AchE | GST | Chitinase | F 0.05 |
|---------|-----|---------------|---------|----------|--------|------------|------|-----|-----------|--------|
|         |     | MS            | MS      | MS       | MS     | MS         | MS   | MS  | MS        |        |
| Replicates | 2   | 0.1354ns      | 2.793ns | 34.802ns | 944.22ns | 277.72ns   | 3.921ns | 4.324ns | 208.39ns   | 4.1    |
| Treatment | 5   | 10.327**      | 33.413**| 1522.2** | 13258**| 24940**    | 42.305**| 272.48**| 122593**  | 3.32   |
| Error   | 10  | 0.4714        | 1.237   | 25.329   | 746.69 | 823.26     | 1.029 | 2.434| 1464.4    |        |
| Total   | 17  |               |         |          |        |            |       |     |           |        |

Ns= non significant, ** = highly significant