THE INSECTICIDAL ACTIVITY OF THE FUSED PROTEIN HA-ASTL/GNA AGAINST *SPODOPTERA LITTORALIS* AND *SITOPHILUS ORYZAE*

Mervat R. Diab[1]*, Ebtissam H.A. Hussein[2] , Mahmoud M. Ahmed[3] and Ahmed Mohammed[1]

[1]*Agriculture Genetic Engineering research Institute, Agriculture Research Center, 9 Gamaa St., Giza, Egypt.
[2]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.
[3]Department of Zoology and Agricultural Nematology, Faculty of Agriculture, Cairo University, Giza, Egypt.

Abstract

The fusion technology was exploited in the field of insect control as a trial to enhance the insecticidal activity of certain toxic proteins against different pests. The astacin like metalloprotease toxin was fused to the GNA snowdrop lectin in the same frame and expressed in Pichia pastoris. The synergistic effect of astl and GNA was examined on the *S. littoralis* larvae and *S. oryzae* adults. The mortality percentages of the fused protein (Ha-astl/GNA) "1000 µg/ml " after 4 days, were 78.6%± 4.16 and 71.66% ±3.51 for first and second spodoptera larval instars, respectively. At the same concentration "1000 µg/ml ", the mortality of the fused protein on *S. oryzae* adults was 49.3±2.08%.

Introduction

Many of the insecticidal peptide toxins have been isolated from spider venoms and their activity has been demonstrated. Astacin-like metalloproteases alongside with phosolipases-D and the inhibitor cystine knot (ICK) peptides represent three major toxin families within the brown spider venom gland as these toxins enable interactions with range of molecular targets (Trevisan-Silva *et al.* 2010 and Chavez-Moreira *et al.,* 2019). Astacins play a major role in the peptide processing, degradation of ploypeptides, activation of growth factors, processing and degradation of extracellular molecules, developmental process, tissue differentiation, embryonic hatching, loss of cellular adhesion and proteolytic activity on proteins such as fibronectin, fibrinogen and gelatin (Dumermuth *et al.,* 1991, Bond and Beynon 1995, Mohrlen *et al.,* 2004 and Da Silveira *et al.*, 2007).

The fusion technology has been exploited to resume the oral insecticidal activity of spider venom (Hardy *et al.*, 2013). The fusion protein technology uses a carrier protein allowing spider venom toxins to be orally effective. This technology was exploited in many previous investigations particularly with snow drop GNA lectin protein . Fitches *et al.* (2012) demonstrated that the fusion technology enhanced the toxicity of some spider

neurotoxins acting on the insect central nervous system (CNS). The ω -hexatoxin-Hv1a (Hv1a) is highly toxic to the cabbage moth, Mamestra brassicae, larvae upon injection while it shows insignificant effect per os. Hv1a acts on voltage-gated calcium channels at CNS.Hv1a gene was fused in the same frame with snowdrop lectin (GNA) coding sequence and its toxicity against M. brassicae larvae was determined orally. The oral effect of the chimeric protein showed about =80% mortality of the larvae. They pointed out that GNA acts as a "carrier" by traversing the epithelial cells of the midgut transferring the toxin into the insect hameolymph. Moreover, Pyati *et al.* (2014) achieved a 100% mortality by injecting 20 µg of MODHv1a/GNA.

Pests have been conventionally controlled by chemical insecticides. Chemical insecticides were first introduced in the 1940s (Aktar *et al.*, 2009 and Chattopadhyay *et al.*, 2017). They represent a quick and inexpensive solution to the growing insect pest problems (Pan-UK, 2003; Khan and Law, 2005 and Thapa *et al.*, 2017). However, extensive use of agrochemicals has led to adverse environmental effects (Cooper and Dobson, 2007), soil and water toxicity (Tayade *et al.*, 2013), severe harmful effect on the natural enemies (Singh *et al.*, 2014) and development of pest resistance to chemical insecticides (Siegwart *et al.*, 2015 and Vaschetto and

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Author for correspondence : E-mail: mervatdiab10@gmail.com

Beccacece, 2019).

In recent years, bioinsecticides have been applied as effective pest control agents (Hajek, 2004; Chandler *et al.*, 2011; Lacey *et al.*, 2015; Silva *et al.*, 2018 and King, 2019). The bioinsecticides are natural organisms or their metabolic products that can be employed for the control of insect pests.

Cotton leaf worm, Spodoptera littoralis, is one of the most destructive agricultural lepidopteran pests that can damage many economical crops such as cotton, maize, tomato and vegetables (Salama *et al.*, 1970). The larvae of *S. littoralis* feed on the leaves, fruits, flowers, buds and bolls of cotton leaving them useless (Bishara, 1934).

Sitophilus oryzae (*S. oryzae*) is one of the most dangerous insect pests on stored grains such as rice, wheat and their products (Baloch, 1992). *S. oryzae* infects the stored grains and causes loss of weight, reduction in the nutritional value and increasing the infection with other mites and fungi, therefore the commercial value of the stored grains decreases (Madrid *et al.*, 1990).

Material and Method

Snow drop lectin (GNA) assembly

The gene encoding the catalytic domain of lectin in the snowdrop (Galanthus nivalis agglutinin, GNA) protein, was synthesized by in silico designing ten overlapping fragments (Fig. 1). Each fragment was composed of two complementary single stand 25-35 oligonucleotide strip (Table 1). Each strip had extra few nucleotides on the 3'end to be used as sticky ends which help in ligating the neighboring fragments with each other. Each two single stranded strips were annealed together in 1X annealing buffer (10 mM Tris, pH 7.5 - 8.0,50 mM NaCl and 1 mM

Fig. 1: Strategy for synthesizing and assembling the lectin gene fragments

EDTA) at 95°C for 5 min, then the temperature was decreased gradually till reaching the RT. The annealed fragments were ligated together using the sticky ends as follows: The fragments were purified and their concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Equal concentration of each two neighbor fragments was mixed together and allowed to anneal as previously described. After compiling these ten fragments in one single frame, one µl T4 DNA ligase enzyme (ThermoScientific, Cat.# EL0011) was added to fill the gaps between the annealed fragments through formation of phosphsphodiester bonds. The ligation reaction was conducted overnight at 4°C in 1X ligase buffer. The final fragment was amplified by PCR using the lec 1F and lec 10'R specific primer set (Table 1).The full length GNA lectin fragment was cloned into the pGEM-T- easy vector and subjected to sequencing. The nucleotide sequence of GNA lectin fragment was then aligned with the original sequence using the NCBI-BLAST tool to confirm the accuracy and integrity of the nucleotides sequence. The names and the sequences of primers used to synthesize the lectin coding gene were shown in Table 1.

Cloning of the GNA lectin gene in yeast expression vector

The GNA catalytic domain was amplified using the specific primers GNASEF1/GNAXR1 (Table 2). Then, digestion of the amplified fragment and the vector pPICZaA was performed using 15 units of each of the restriction enzymes EcoR1 and Xba1. The digestion reaction was incubated for 4 hours at 37°C. Then, the digested fragment was ligated to the digested pPICZaA vector at a 4:1 ration. The ligation reaction was carried out using 10 units of T4 DNA ligase enzyme (Promega) in 1x ligase buffer and incubated at 4°C overnight.

The recombinant plasmid was then transformed into TOP10 F' bacterial competent cells. The transformed cells were spread on LB agar plates containing 100mg/ml of Zeocin antibiotic and incubated at 37°C overnight.

Identification of the positive clones was performed by PCR screening. A number of grown colonies were dissolved separately in 5 µl ddH$_2$O. Two µl of the solution were employed as a template in the PCR reaction using universal primers "Alfa factor and 3'AOX1" and the specific primers, GNASEF1/GNAXR1. The PCR products were resolved on agarose gel. The remaining 3 µl of the solution of each of the putative positive clones were inoculated into 5ml LB broth medium and incubated in a shaking incubator at 150 rpm overnight at 37°C to extract the recombinant DNA.
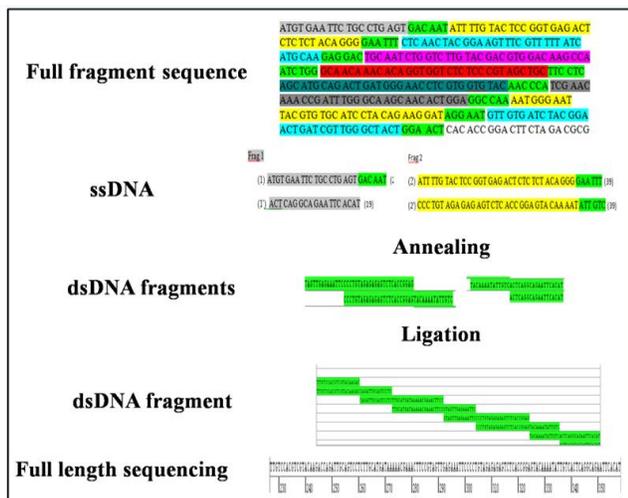
Table 1: Names and sequences of the 10 lectin oligonucleotides

Primer Name	Primer sequence 5'-3'
Lec 1 F	ATGT GAA TTC TGC CTG AGT GAC AAT
Lec 1' R	ACT CAG GCA GAA TTC ACAT
Lec 2 F	ATT TTG TAC TCC GGT GAG ACT CTC TCT ACA GGG GAA TTT
Lec 2' R	CCC TGT AGA GAG AGT CTC ACC GGA GTA CAA AAT ATT GTC
Lec 3 F	CTC AAC TAC GGA AGT TTC GTT TTT ATC ATG CAA GAG GAC
Lec 3' R	TTG CAT GAT AAA AAC GAA ACT TCC GTA GTT GAG AAA TTC
Lec 4 F	TGC AAT CTG GTC TTG TAC GAC GTG GAC AAG CCA ATC TGG
Lec 4' R	TGG CTT GTC CAC GTC GTA CAA GAC CAG ATT GCA GTC CTC
Lec 5 F	GCA ACA AAC ACA GGT GGT CTC TCC CGT AGC TGC TTC CTC
Lec 5' R	GCA GCT ACG GGA GAG ACC ACC TGT GTT TGT TGC CCA GAT
Lec 6 F	AGC ATG CAG ACT GAT GGG AAC CTC GTG GTG TAC AAC CCA
Lec 6' R	GTA CAC CAC GAG GTT CCC ATC AGT CTG CAT GCT GAG GAA
Lec 7 F	TCG AAC AAA CCG ATT TGG GCA AGC AAC ACT GGA GGC CAA
Lec 7' R	TCC AGT GTT GCT TGC CCA AAT CGG TTT GTT CGA TGG GTT
Lec 8 F	AAT GGG AAT TAC GTG TGC ATC CTA CAG AAG GAT AGG AA
Lec 8' R	ATC CTT CTG TAG GAT GCA CAC GTA ATT CCC ATT TTG GCC
Lec 9 F	TGTT GTG ATC TAC GGA ACT GAT CGT TGG GCT ACT GGA ACT
Lec 9' R	AGT AGC CCA ACG ATC AGT TCC GTA GAT CAC AAC ATT CCT
Lec 10 F	CAC ACC GGA CTT CTA GA CGCG
Lec 10' R	CGCG TCT AGA AGT CCG GTG TGA GT TCC

Table 2: List of primers used for the cloning of the fusion of Ha-astl and GNA in pSK+ vector and in yeast expression vector

Primer Name	Primer sequence 5'-3'
For Ha-astl	
astlHEF	AAG TAA GCT TGA ATT CAC TCA GAT GAG ATG GAC C
astlPR	CGCG CTG CAG GTT CTT GGC CAC CAT GGT GAG
For GNA	
GNASEF1	TGT GAG CTC GAA TTC ATG TGC CTG AGT GAC AAT AT
GNAX R1	GCG GCG GCC GC CTC GAG TCC GGT GTG AGT TCC
GNAPF	A TTC CTG CAG TGC CTG AGT GAC AAT ATT T
GNAXR	CGCG TCT AGA AGT CCG GTG TGA GT TCC

Cloning of Ha-*astl/GNA* fused sequence in yeast expression vector

Cloning strategy of the fused fragment was planned as follows; firstly the active domain sequence of the astacin like metalloprotease of H. adansoni (*Ha-astl*) (accession no. MN453831) was cloned into pSK+ and then the active domain sequence of the lectin gene (GNA) was subcloned downstream Ha-astl in the same frame, as an intermediate cloning step, forming the recombinant plasmid pSK+Ha-astl/GNA. Secondly, the fused Ha-astl/GNA fragment was subcloned into the yeast expression vector pPICZαA.

(1) Cloning of the fused Ha-astl/GNA in pSK⁺ vector

The fragment encoding the active sequence of *Ha-astl* was amplified using the astl HEF and astlPR primers (Table 2). Both the pSK⁺ vector and the fragment were digested with 15 units of *HindIII* for 4 hours at 37°C and purified by ethanol precipitation. Then, 15 units of *PstI* were added and the reaction was incubated for 2 hours at 37°C . The digestion reactions was ethanol precipitated. Then, ligation of the *Ha-astl* fragment and the linearized pSK+ was performed in a ratio of 4:1 using 10 units of T4 DNA ligase enzyme (Promega) and incubation at 4°C overnight.

The ligated DNA was transformed into TOP10F' bacterial competent cells. The transformed cells were spread on LB agar plates and incubated at 37°C overnight. Putative positive colonies were identified by PCR screening using the specific primers and the universal primers M13 FWD and M13 RVS. The positive clones were inoculated into 5ml LB broth medium to be used for extraction of the pSK⁺ astl plasmid by the Wizard miniprep kit (Promega, USA, Cat# A1460).

The fragment encoding the active domain of snowdrop lectin (GNA) was amplified using the GNAPF and GNAXR specific primer set (Table 2). The amplified

PCR fragment and pSK+Ha-astl plasmid were double digested using 15 units of each of the restriction enzymes *Pst1* and *Xba1* and incubation for 4 hours at 37°C. The digestion reactions were followed by ligation between the digested fragment and vector in a ratio of 4:1 using 10 units of T4 ligase enzyme (Promega) and incubation at 4°C overnight. The transformation and screening procedures were performed as mentioned above.

Sub-cloning of the astl/GNA fused sequence into the yeast expression vector pPICZαA

The pSK+astl/GNA plasmid DNA was used as a template to amplify the astl/GNA fused fragment using the astlHEF and GNAXR primer set. The cloning procedure of the astl/GNA fragment into pPICZαA plasmid was carried out by digestion using 15 units of each of the restriction enzymes "Xba1 and EcoR1" for 4 hours at 37°C. Then, the ligation between the digested fragment and the vector was performed with a 4:1 ration using 10 units of T4 DNA ligase and the reaction was incubated at 4°C overnight

The ligated DNA was transformed into TOP10F' bacterial competent cells that were then spread on LB agar plates supplemented with 100mg/ml Zeocin and incubated at 37°C overnight. Putative positive colonies were identified by PCR analysis using the specific primers and the universal primers. The positive clones were inoculated into 5ml of LB broth medium and used to extract the p PICZaAastl/GNA plasmid by the Wizard miniprep kit (Promega, USA, Cat ≠ A1460).

Transformation and screening of the pPICZαA/GNA and the pPICZαAastl/GNA plasmids into the yeast "*Pichia pastoris*"cells

An amount of 3-5µ g of pPICZaA/GNA or pPICZaAastl/GNA plasmid was digested by BstX1 (Thermoscientific, Cat# FD1024) at 37°C overnight. The plasmid DNA was purified then transformed into the yeast Pichia pastoris "KM71H" competent cells. The transformed cells were incubated at 28°C for 2-3 days on YPDS medium plates containing 100mg/ml of Zeocin. Then, the colonies were transferred to YPD plates and incubated for another two days to grow.

A number of colonies was screened by two rounds of PCR reactions; firstly using the universal primers Alfa factor and 3'AOX1. Secondly, the nested PCR was conducted using the specific primers.

Confirmation of the cloned genes by sequence analysis

To confirm the positive cloning and accuracy of the cloned DNA sequence fragments, all aforementioned cloned fragments were subjected to sequence analysis using the Big TriDye sequencing kit (ABI Applied Biosystems) by the facility of Macrogen, Korea.

Expression of the GNA and astl/GNA proteins

A single colony of the transformed KM71H yeast was inoculated in 25 ml of minimal glycerol medium ±histidine (MGYH) (1.34% yeast nitrogen base YNB, 1% glycerol, 4×10^{-5}% biotin and ±0.004% histidine) in a 250 ml flask. The culture was grown at 28-30°C in a shaking incubator (200 rpm) until the intensity of the culture reached an OD_{600}= 2-6 on. The cells were harvested at the log phase growth by centrifugation at 5000 rpm for 5 min at RT. The supernatant was decanted and the cell pellet was resuspended in 200 ml minimal methanol±histidine (MMH) (1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) to reach an OD_{600}=1. The nozzle of the flask was capped with many layers of tissue to reduce the evaporation of the methanol.

To induce the protein expression, the cells were further incubated to continue growth. To maintain induction an equal volume of the inducer, 100% methanol, was added every 24h to reach a final concentration of 0.5% methanol. The expression level was analyzed from day one to day four to determine the optimal harvest time post induction. The expression level depends on the nature of expressed protein. The supernatant was used to detect the secreted proteins.

Detection of gene expression

Three different methods ,i.e., Coomassie SDS – PAGE, Western Blot and ELISA, were used to analyze the expression of the GNA and the fused Ha-astl/GNA proteins according to Green and Sambrook (2012).

Assessment of the toxicity of the astl and astl/GNA proteins

Two different insect pests namely *Spodoptera littoralis* and *Sitophilus oryzae* were employed to assess the toxicity of the astl/GNA protein after 96 hours and fourteen days, respectively.

For all bioassays, both larvae and adults were treated in the same manner, using three concentrations (100, 500 and 1000 µg/ml) of expressed culture supernatant, three replicas, each replica contains 25, 20 larvae for first and second instars of Spodoptera littoralis and 25 adults for Sitophilus oryzae. In addition to the control "expressed culture supernatant of native pPICZαA" and the expressed "pPICZαA-GNA".

Statistical analysis

Statistical analysis of the mortality data was performed with Student's t-test in the Excel program.

Results

Snow drop lectin (GNA) assembly

GNA lectin gene was assembled by synthesizing 20 single stranded DNA oligo nucleotide strips. Each two strips were complementary to each other with extra few nucleotides at the 3' ends to be used as overhang to bind with the following neighbor strip. After annealing each two complementary strips, the double strand fragments with overhangs (sticky ends) were allowed to bind with the following fragment. Finally, the gaps were filled using the T4 DNA ligase enzyme through formation of the phosphsphodiester bonds between each two fragments.

Fig. 2 illustrates the electrophoretic separation of five out of the ten annealed fragments are shown in. The fragments were purified and their concentrations were measured. An equal concentration of two neighboring fragments was mixed together and allowed to anneal. After annealing all fragments in order, the T4 DNA ligase enzyme was added to fill the gaps. The assembled ten fragments were used as a single DNA template to amplify the *GNA lectin* gene using the specific primer set "Lec 1F/Lec 10'R" (Fig. 3A). The amplified fragment was then cloned into the pGEM-T- easy vector and screened by PCR to indentify the positive clones (Fig. 3B). The putative positive clones were confirmed by six different combinations of GNA primers as follows: Lec 2F, Lec 9R ; Lec 3F, Lec 8R ; Lec 4F, Lec 7R ; Lec 5F, Lec 10R; Lec 1F, Lec 10R and Sp6,T7 promoter (Fig. 3C). The PCR positive clones were subjected to DNA sequencing. The sequence was compared with the original GNA lectin sequence using the NCBI-BLAST
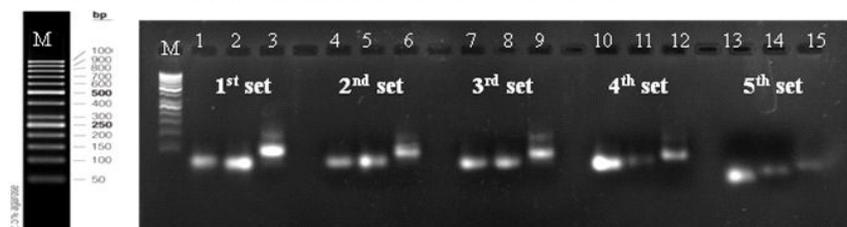
Fig. 2: Electrophoretic separation of the ssDNA and dsDNA for GNA fragments. Lanes 1,2,4,5,7,8,10,11,13 and 14 : the ss oligonuleotides of the 1st ,2nd ,3rd ,4th and 5th sets. Lanes 3,6,9,12 and 15: the dsDNA fragment of the 1st ,2nd ,3rd ,4th and 5th sets. M: 50 bp DNA ladder (Thermoscientific).

tool to ensure the accuracy and the order arrangement of the nucleotides sequence of the synthetic *GNA lectin* gene.

Cloning, transformation and expression of the GNA lectin gene in the pPICZαA

The fragment of GNA catalytic domain was amplified using the specific primers GNASEF1and GNANX R1. The amplified GNA lectin fragment and the pPICZαA

vector were double digested with EcoR1 and Xba1 then ligated to generate the recombinant plasmid pPICZαA-GNA. The ligation reaction was transformed into TOP10F'. The grown colonies carrying the pPICZαA-astl plasmid were screened by PCR analysis using the universal primers "alfa factor and 3'AOX1"followed by a nested PCR using the specific primer set "GNASEF1and GNANX R1" (Fig. 4).

The positive colonies containing the pPICZαA-GNA were expressed by methanol induction and the expressed proteins were analyzed by SDS-PAGE electrophoresis gel, ELISA and Western blot analysis. The protein band was hardly visible on SDS-PAGE gel. While, the positive values (0.25) for the samples were detected at OD 450 using ELISA comparing with the control (0.04) as shown in (Fig. 5 A). However, the putative positive colonies of the pPICZαA-GNA and pPICZαA-astl/GNA were confirmed by western blot analysis (Fig. 5 B).

Fusion of the astl and the GNA lectin active domains

The fusion was performed through two steps. The first step was cloning of the active domain of the astl in the pSK⁺ vector to create the pSK⁺Ha-astl recombinant plasmid. Then, the *GNA* fragment was subcloned in the pSK⁺-astl vector to generate the pSK⁺Ha-astl/GNA recombinant plasmid.

Cloning of the active domain of Ha-astl in pSK⁺vector

The 486 bp encoding the *astl* was amplified using the primer set "astlHEF/astlPR". Both the pSK⁺ vector and the purified PCR product were double digested with *EcoR1* and *Pst1* (Fig. 6A). Positive clones were screened by PCR analysis using the universal primers (M13FWD, M13RVS) and the specific primers, astlHEF/astlPR (Fig. 6B).

Further confirmation of the positive clones was performed by PCR analysis using different combinations of primers, *i.e.*, "M13FWD/M13RVS", "T3/T7 promoter", "M13F/ astlXR ", "astlHEF /M13RVS" and " astlHEF/astlPR" (Fig. 6C) . Then, the selected clones were confirmed by sequencing.

Cloning of the GNA fragment in the pSK+ -astl vector

The fragment encoding the GNA lectin was amplified by PCR analysis using the primer set "GNAPF/GNAXR". The PCR product and pSK+ -Ha-astl vector
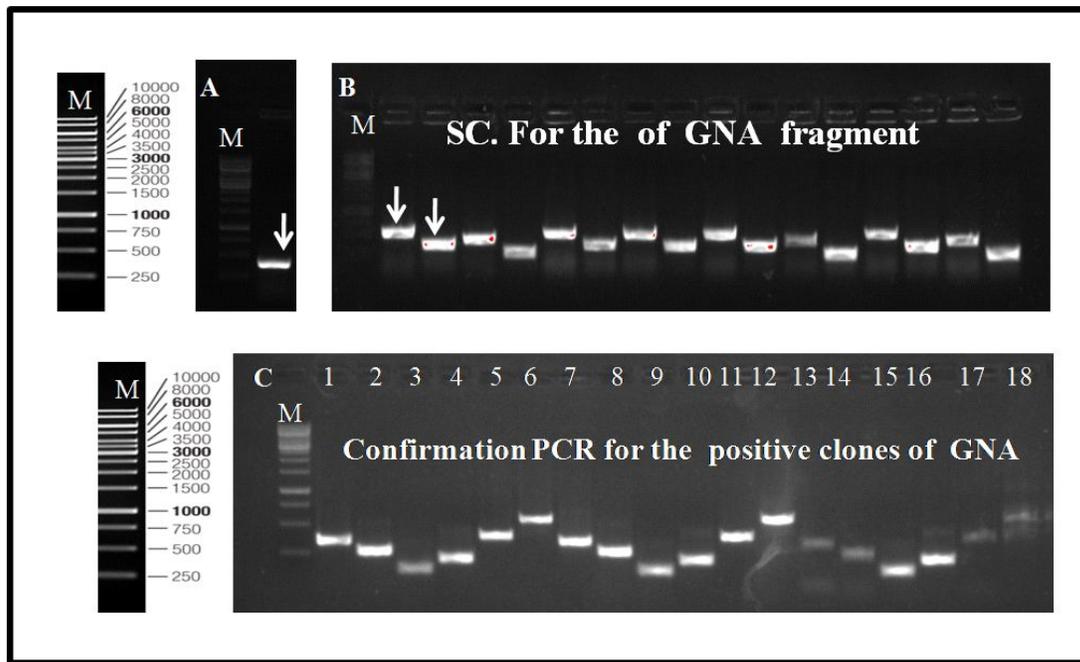
Fig. 3: (A) PCR amplification of the *GNA lectin* gene. (B) Screening of some transformed clones for GNA gene by universal primers (Sp6,T7 promoter) then specific primers (F1, R10). The white arrows refer to the expected positive bands amplified by universal and specific primers, respectively. (C) Confirmation PCR for the positive clones of GNA for three positive clones using specific and universal primers (Sp6,T7 promoter), respectively. Lanes 1-6 for the first clone, lanes 7-12 for the second clone and lanes 13-18 for the third clone. M: marker 1kb DNA ladder (Thermoscientific).
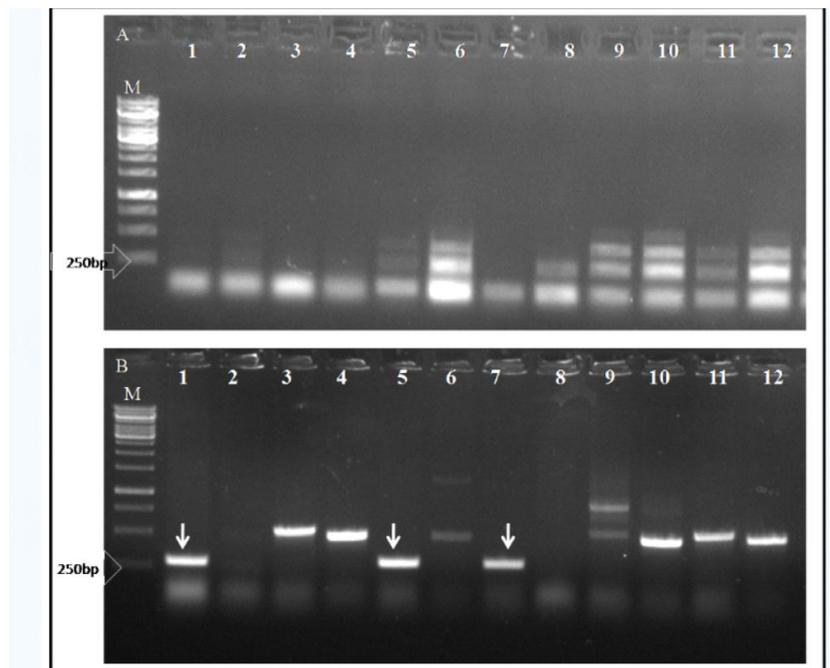
Fig 4: (A) PCR screening of 12 putatitive transformed yeast colonies carrying the pPICZαA-active domain of GNA by the universal primers (alfa factor and 3'AOX1) (B) Nested PCR for the colonies using GNASEF1and GNANX R1 primers. The expected positive colonies were referred by white arrows. M: marker 1kb DNA ladder (Thermoscientific)

were double digested with Pst1 and Xba1 (Fig. 7A) and ligated together with T4 DNA ligase enzyme then transformed into TOP10F'. The colonies were screened by PCR analysis using the universal primers "M13FWD/ M13RVS" (Fig. 7B). Two positive clones were confirmed by PCR analysis using different primer combinations, *i.e.,* "M13FWD/M13RVS", "astlHEF/GNAXR", "M13FWD/ GNAXR" and "astlHEF/M13RVS" (Fig. 7C).
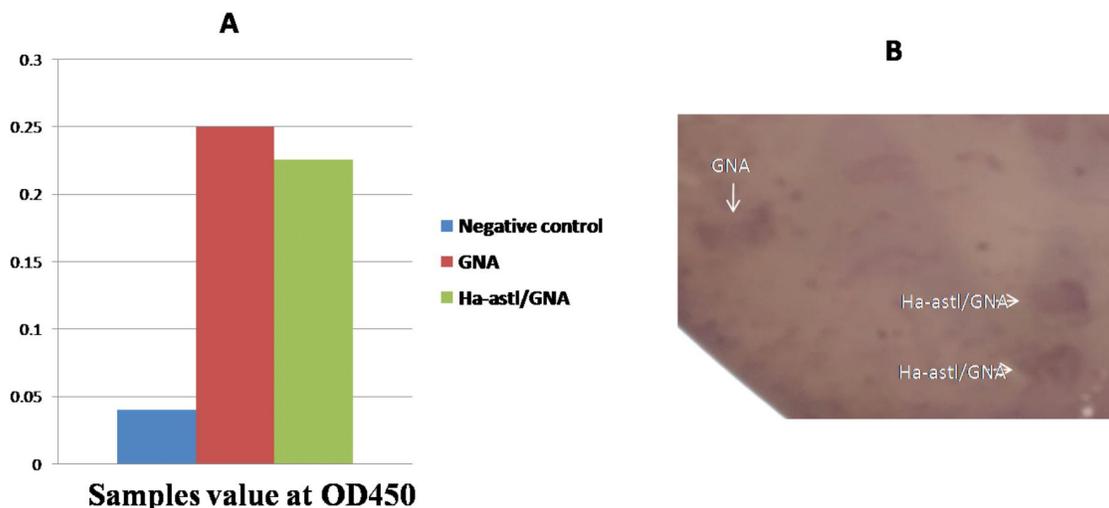
Fig 5: (A) Histogram of the sandwich ELISA test showing the average values of negative control, GNA and the pPICZαA-Ha-astl/GNA at OD 450. (B) Western blot analysis for the induced yeast colonies containing the recombinant vectors pPICZαA-GNA and pPICZαA-astl/GNA
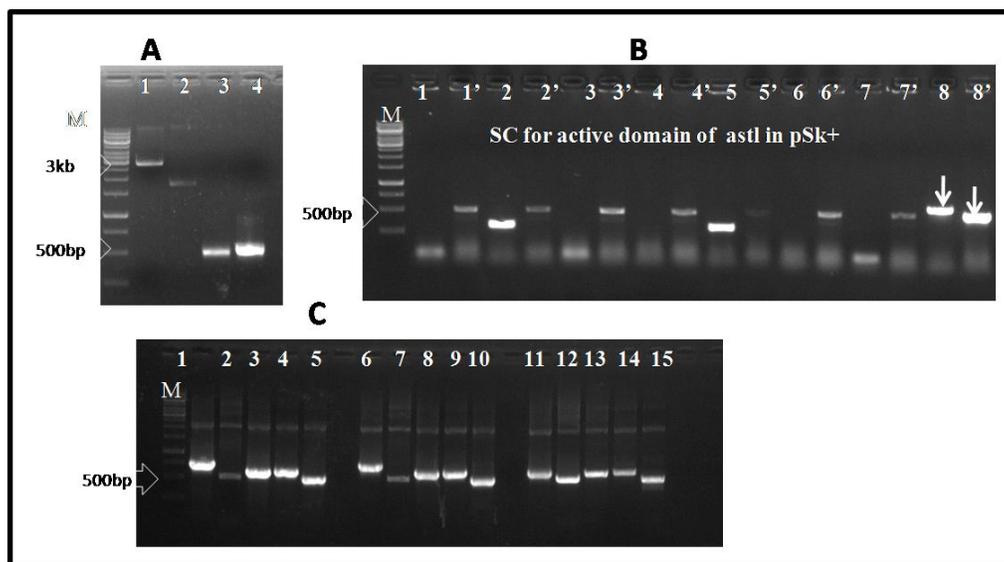
Fig 6: (A) Restriction digestion analysis for the pSK⁺vector (lane:1) and active domain of Ha-astl (lane: 3) by *EcoR1* and *Pst1*. Lanes 2 and 4: undigested pSK⁺ vector and undigested active domain of Ha-astl. (B) PCR screening for the active domain of Ha-astl in the putative pSK⁺ recombinant vectors using universal and specific primers . The white arrows refer to the selected positive clone. (C) Confirmation PCR for three positive clones by different primer combinations , "M13FWD/M13RVS", "T3/T7 promoter", "M13F / astlXR " , "M13RVS / astlHEF " and "astlHEF / astlPR " . Lanes 1-5 : first clone, lanes 6-10: the second clone and lanes 11-15: the third clone. M: marker 1kb DNA ladder (Thermoscientific).

Cloning of the fusion astl/GNA in the yeast expression vector pPICZαA

The fused fragment "Ha-astl/GNA" was amplified using the primer set "astlHEF / GNAXR" (Fig. 8A). The PCR product and the pPICZαA were double digested with EcoR1 and Xba1 restriction enzymes. PCR screening was performed using the universal primers "Alfa factor/3'AOX1" (Fig. 8B). Then, three positive clones were confirmed using different combinations of primers,

i.e., Alfa factor/ 3'AOX1, astlHEF / GNAXR, alfa factor/ GNAXR and astlHEF/ 3'AOX1 (Fig. 8C).

Transformation of the fused fragment "astl/GNA" in the yeast "Pichia pastoris" "KM71H strain"

The pPICZαA-astl/GNA was restriction digested with the enzyme Bstx1 (Fig. 9A) and transformed in the competent cells of the yeast strain KM71H. The yeast colonies were screened by two sequential PCR rounds. The first PCR was performed using "alfa factor/3'AOX1"
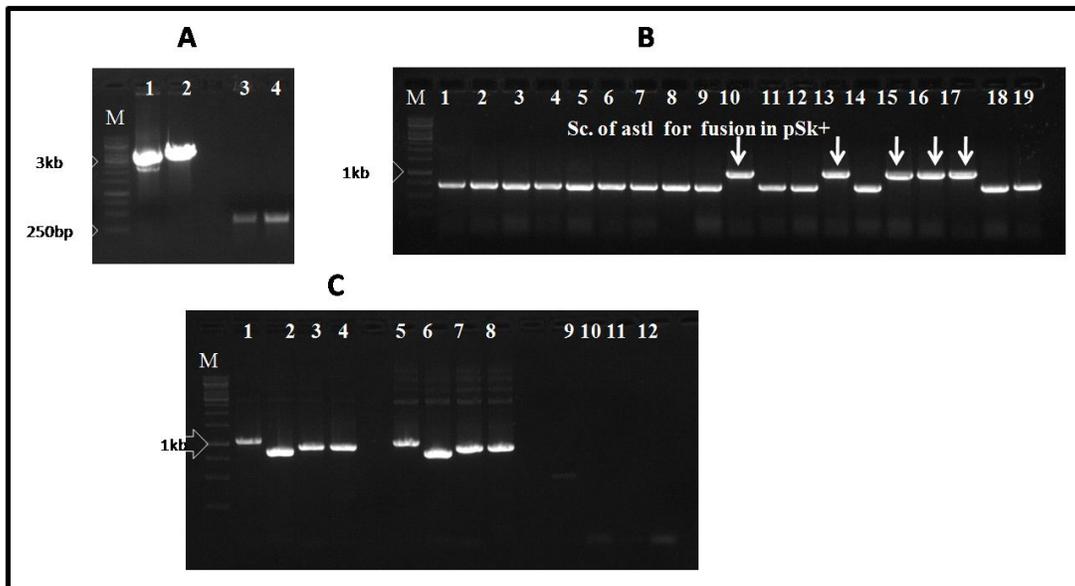
Fig. 7: (A) Restriction digestion analysis for the pSK⁺vector (lane 1), pSK⁺vector –astl vector (lane 2) and GNA fragments (lanes 3 and 4) by *Pst1* and *Xba1* restriction enzymes. (B) PCR screening for the fusion of the astl/GNA in the pSK⁺vector using universal primers M13FWD, M13RVS . The white arrows refer to the positive clones. (C) Confirmation PCR for two of the positive clones of the fusion in pSK⁺ using different primer combinations, "M13FWD/M13RVS, astlHEF/GNAXR, M13FWD/ GNAXR and astlHEF/ M13RVS". lanes 1-4: first clone, lanes 5-8: the second clone and lanes 9-12: negative controls. M: marker 1kb DNA ladder (Thermoscientific)
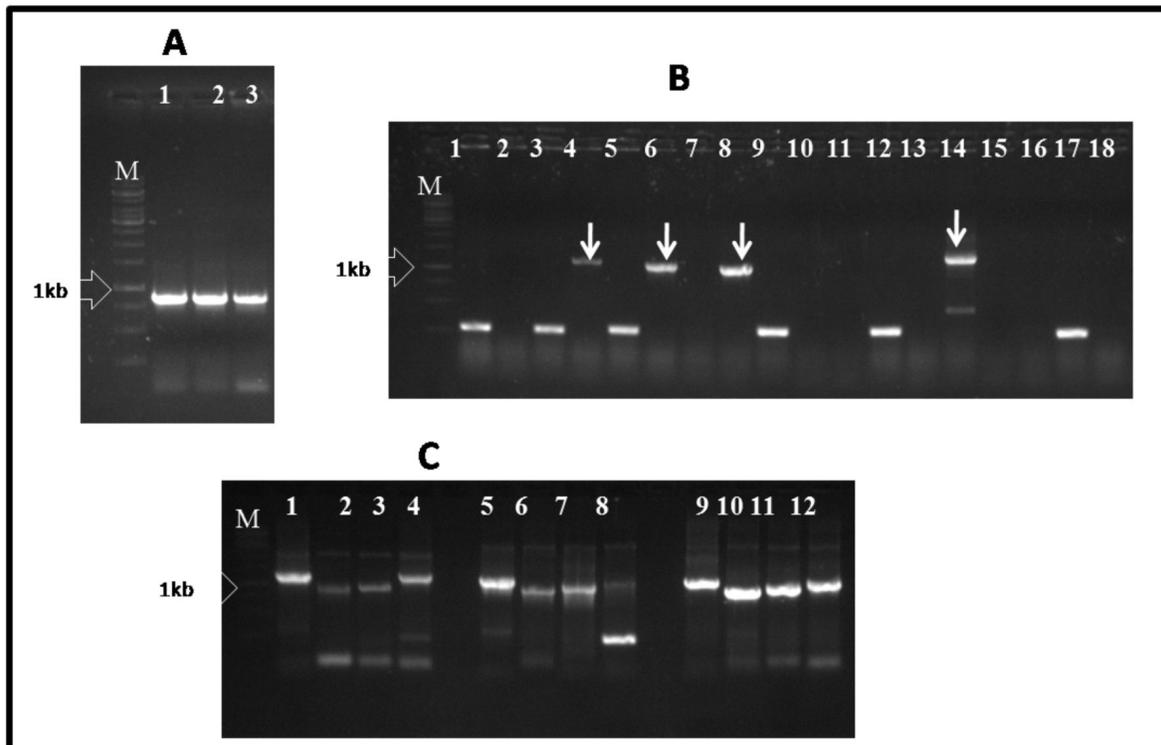
Fig. 8: (A) PCR analysis for the fused fragment "astl/GNA" using the primers, astlHEF/GNAXR. (B) PCR screening for the fused fragment "astl/GNA" in the pPICZαA vector using the universal primers "Alfa factor and 3'AOX1". The white arrows refer to the positive clones. (C) Confirmation PCR for three of the positive clones for the fused fragment "Ha-astl/GNA" in pPICZαA using different combinations of primers, *i.e.*, Alfa factor/ 3'AOX1, astlHEF / GNAXR, alfa factor/GNAXR and astlHEF/ 3'AOX1. Lanes 1-4: the first clone, lanes 5-8: the second clone and lanes 9-10: the third clone. M: marker 1kb DNA ladder (Thermoscientific).
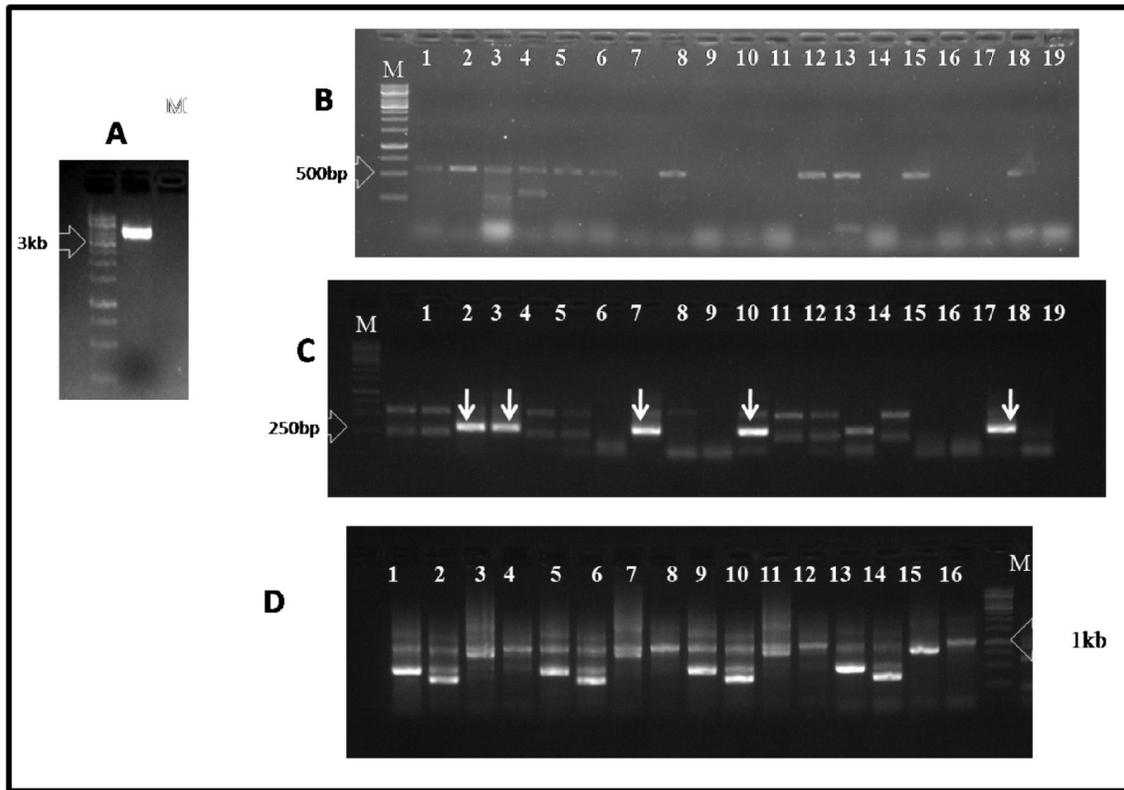
Fig. 9: (A) Restriction digestion analysis of the pPICZαA-astl/GNA using the enzyme Bstx1. (B) PCR screening for the pPICZαA-astl/GNA in yeast colonies "first round" using alfa factor and 3'AOX1 primers. (C) Nested PCR for the pPICZαA-astl/GNA in yeast colonies "second round" using the astlF1 and Lec 5R primers. The white arrows refer to the positive clones. (D) Confirmation PCR for four positive clones for pPICZαA-astl/GNA in yeast colonies using astlHEF/ astlPR , GNAPF/ GNAXR, astlHEF/ GNAXR and astlHEF/3'AOX1. 1-4: the first clone, 5-8: the second clone, 9-12: the third clone and 13-16: the fourth clone. M: marker 1kb DNA ladder (Thermoscientific).
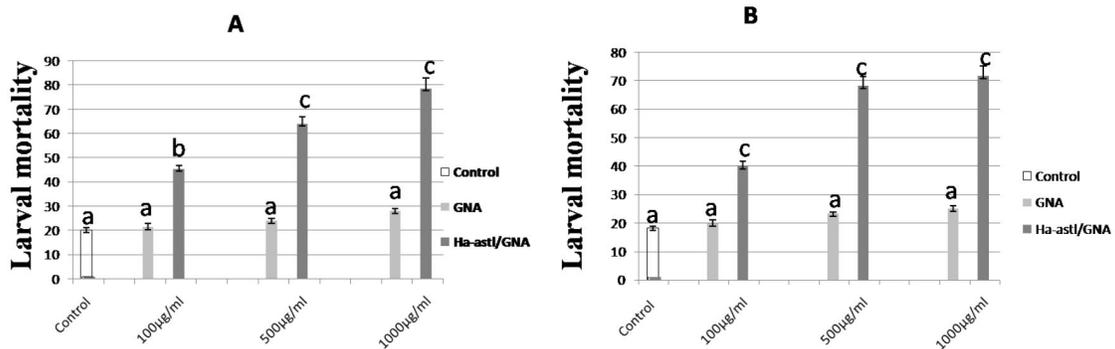
Fig. 10: Histograms revealing the mortality ratio of the first instar (A) and the second instar (B) of Spodoptera littoralis after 96 hours using three concentrations (100, 500, 1000 μg/ml) of GNA or fused (Ha-astl/GNA) proteins comparing with the control (expressed native pPICZαA protein). Error bars represent ±SE. Within the same protein concentration different letters indicate highly significant (c) at P>0.01

primers (Fig.9B). Then, 2μl of the PCR product were employed as a template in the second PCR round performed using the primers "astlF1/Lec 5 R". The size of the amplified fragment in the nested PCR for the fused fragment astl/GNA was 319 bp (144 bp of astl and 175 bp of GNA lectin. Positive clones of the fused fragment are shown in Fig. (9C).

Four positive colonies were further analyzed by PCR reactions using different primer combinations; "astlHEF/ astlPR, GNAPF/GNAXR, astlHEF/ GNAXR and astlHEF/3'AOX1". All reactions revealed the PCR product at the expected size thus, confirming the positive cloning (Fig. 9D).

Expression of the fused fragment astl/GNA in the
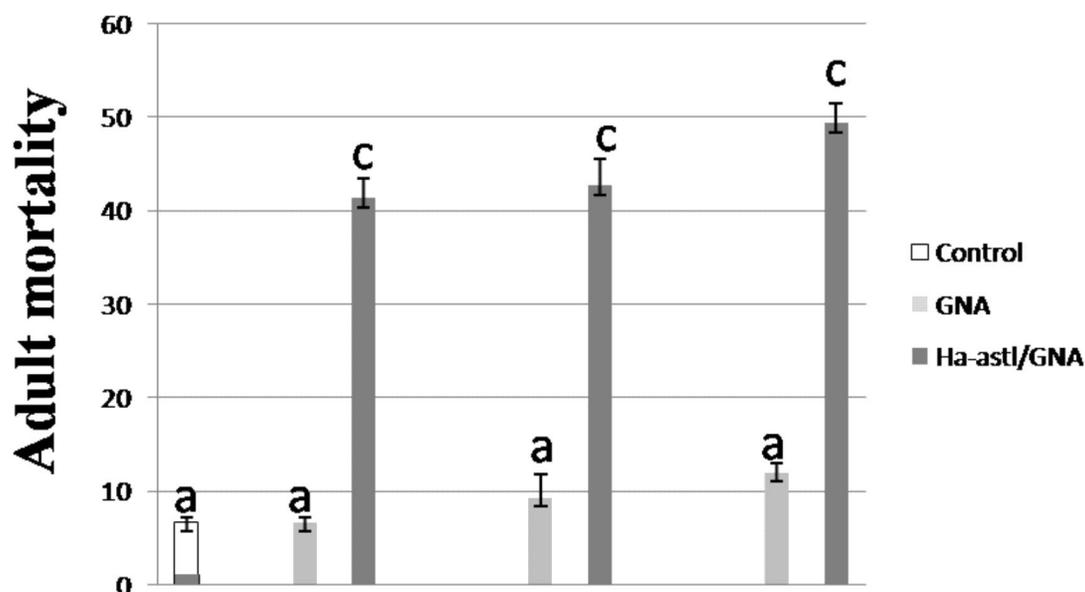
Fig. 11: Histogram revealing the mortality ratio of the adults of Sitophilus oryzae after fourteen days using three concentrations (100, 500, 1000 µg/ml) of GNA or fused (Ha-astl/GNA) proteins comparing with the control (expressed native pPICZαA protein). Error bars represent ±SE. Within the same protein concentration different letters indicate highly significant (c) at P>0.01.

yeast "Pichia pastoris" "KM71H strain"

The expression of the fused astl/GNA lectin gene was induced by methanol. Then, the expressed proteins were analyzed by SDS-PAGE electrophoresis gel , ELISA (Fig 5A) and western blot analysis (Fig. 5B).

The insecticidal activity of the Ha-astl/GNA proteins on Spodoptera littoralis

The toxic efficacies of the fused Ha-astl/GNA proteins taken per os was evaluated on the first and second instars of S.littoralis larvae using three different concentrations, *i.e.*, 100, 500, 1000 µg/ml of the protein. The oral activity was investigated by feeding the larvae on treated castor leaves which were replaced daily for four days. While, the control larvae were fed on treated castor leaves with the supernatant of native pPICZαA for the same period of time. In addition, three different concentrations of GNA lectin protein, *i.e.*, 100, 500, 1000 µg/ml were tested on the S.littoralis larvae using the same procedure. The larval mortality ratio showed daily increment during the four day experiment.

As expected, the highest insecticidal effect was recorded for the larvae fed for 4 days on the fused protein at the highest concentration (1000 µg/ml). The mortality percentages were 78.6%± 4.16 and 71.66% ±3.51 for first and second larval instars, respectively (Fig. 10A and B). The statistical analysis of the mortality ratio showed significant (b) and highly significant (c) effects of the proteins against the control larvae at (p>0.05) and (p>0.01), respectively. However, no significant mortality was recorded between the control and the various concentrations of GNA lectin.

The insecticidal activity of Ha-astl and the fused protein Ha-astl/GNA on *S.oryzae*

The toxicity of the fused proteins was evaluated on the adults of *S. oryzae* by per os intake alongside the control (native pPICZαA) and the GNA protein.

Experimental adults were fed on treated wheat seeds that were previously immersed in the protein solution for 30 min, then excess solution was filtered and the seeds were allowed to air dry. Three concentrations, *i.e.*; 100, 500, 1000 µg/ml were tested for each protein with three replicates and 25 adults per replica. The adult mortality was recorded two weeks post-treatment. As shown in (Fig. 11) the mortality ratio for the adults treated with the three fused protein (Ha-astl/GNA) concentrations, *i.e.*, 41.3%±2.0, 42.6%±2.8 and 49.3%±2.0, respectively.

The statistical analysis showed that the difference in the mortality ratio was highly significant (c) at (p>0.01) for all the concentrations of the fused (Ha-astl/GNA) proteins compared to the control. While, the difference in the mortality ratio between the insects treated with the different concentrations of the GNA lectin protein and the control were insignificant.

Discussion

Crop productivity is threatened by insects attack. Control of insect damage is considered one of the most challenging aspects for plant production. Application of

chemical insecticides is increasingly imposing environmental pollution. Therefore, the continuous search for new alternative ecofriendly bioinsecticides is of paramount importance to control insects. Recently, the insecticidal activity of various peptide toxins derived from spiders was examined on different insect pests from different orders.

Astacins may play a role in the prey paralysis and increase the permeability of peritrophic matrix therefore, Astacins (enhancins) have an important role in insect control (Lepore *et al.*, 1996). Fusion protein technology was exploited to use plant GNA snowdrop lectin as a 'carrier' protein allowing proteins such as spider venom toxins to act as orally delivered biopesticides. Previous studies of using recombinant fusion proteins combining GNA snowdrop lectin linked either to the insect neuropeptide (Manse-AS), to an insect spider venom neurotoxin (SFI1) or to scorpion neurotoxin (ButaIT) have demonstrated that GNA can be utilised as a transporter to deliver linked peptides to the larval haemolymph (Fitches *et al.*, 2002; 2004 and 2010). Also, the Hv1a/GNA fusion protein showed oral insecticidal activity against insects from different orders (Fitches *et al.*, 2012). Clearly, the site effect of these toxins is insect nervous system and GNA allowed these toxins to traverse the insect gut epithelium and access its sites of action, producing an orally active insecticidal protein. The toxicity of the Ha-astl/GNA fused protein was assessed against *Spodoptera littoralis* and *Sitophilus oryzae* and revealed a moderate mortality ratio towards the two different insects. Cotton leafworm larvae were slightly higher sensitive to the fused protein than the rice weevils adults. These two pests belong to two different orders which raise questions; do these two orders react differently to GNA fused proteinα or is it species-dependentα

Action of astacin metalloproteases is more likely on insect tissues rather than nervous system, thus, it does not require reaching heamolymph to be activated as other neurotoxins.

In conclusion, the present study represents an evidence that the GNA fusion with metalloprotease showed a moderate mortality ratio against two important agriculture pests. Thus the fused protein Ha-astl/GNA may play an important role in insect control field or participate as an agent in integrated pest management programs as a new biopesticide.

Acknowledgments

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