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SEQUENCE ANALYSIS OF INSECTICIDE RESISTANCE AND DETOXIFICATION RELATED GENES IN *Spodoptera littoralis*(LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) is a well-known as one of the most destructive agricultural lepidopterous pests. It is a true generalist species with a promiscuous feeding strategy which enables it to attack numerous economically important crops all year round including vegetables, ornamental plants, and cotton. Recently, chemical control has been commonly used to suppress populations of *S. littoralis*; however, a very large number of *insecticides have led*to the emergence of*resistance.* An extensive use of insecticides also has other side effects, including the elimination of non-targeted organisms, environmental damage and harm to human health. Genome-wide high-throughput technologies help developing resistance management strategies, especially identifying genetic mechanisms of resistance. The aim of the present study was to produce a de novo transcriptome for *S. littoralis* as a resource for current and future studies of this pest species by using next-generation sequencing. This resource was then used as a reference for identifying genes by encoding the target sites of insecticides currently in use for Egyptian cotton leaf worm control. To achieve this, a cDNA library was sequenced using 454 FLX Titanium Sequencing on the Roche platform which revealed good coverage of genes encoding insecticide target sites and detoxification enzymes using a manual annotation. Annotations of assembled sequences were carried out by BLASTx against NCBI non-redundant protein sequence databases using the software Blast2GO. The g*ene*sencoding enzymes*involved*in *insecticide* detoxification such as *Acetylcholinesterase, Cytochrome p450, Glutathione S-transferase* were characterized. Furthermore, a phylogenetic analyses based on three protein sequences were generated in order to give evolutionary insights into insecticide resistance gene families of *S. littoralis*.

Keywords: *Spodoptera littoralis, transcriptome, insecticide resistance, detoxification.*

INTRODUCTION

Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval), is a polyphagous pest that damages numerous economically substantial crops in countries around the Mediterranean Basin and in Southeast Asia (Guz *et al*., 2013). The use of insecticides to control this species cause important damage to ecosystems, development of resistance in agricultural pests and elimination of non-targeted organisms from nature. Insecticide resistance is a genetic change in response to selection through target site mutation, and also boosted metabolism by sequestering insect detoxification enzymes (Feyereisen, 1995; Karatolos *et al*., 2011).

Acetylcholinesterase (AChE), Cytochrome P450 (CYP) and Glutathione S-transferase (GST) are the key enzymes that are associated with insecticide resistance. AChE, is the primary enzyme terminates nerve impulses in insect central nervous system by hydrolysis of the neurotransmitter acetylcholine (Toutant, 1989). Inhibition of acetylcholinesterase causes death in insects. Two major insecticide families, organophospates and carbamates, have been developed to inhibit this enzyme in an irreversible way. CYPs are hemoprotein super families which are widely distributed in all aerobic organisms. Their main function is to oxidize various substrates and also to catalyze plenty of other reactions (Mansuy, 1998). Increased metabolic detoxification by CYP450 is one of the most common mechanisms of insecticide resistance primarily to pyrethroids (Scott, 1999). GSTs are a varied enzyme family which plays a central role in detoxification of xenobiotics and endogenous compounds (Salinas and Wong, 1999). GSTs have been implicated in resistance against organophosphorous in many insects (Dauterman, 1985; Oppernoorth, 1985). More recently, elevated levels of GST activity have also been reported in pyrethroid resistant insect species (Vontas *et al*., 2001, 2002; Ranson and Hemingway, 2005).

In this study, we determined cDNAs encoding Acetylcholinesterase (*SpliAChE-1*), Cytochrome p450 (*SpliCYP450*) and Glutathione S-transferase (*SpliGST*) found in the transcriptome analysis of *S. littoralis*. We also presented phylogenetic reconstruction and structural bioinformatics analyses for each putative protein and discussed their putative roles in insecticide resistance and detoxification mechanisms.

MATERIAL AND METHODS

S. littoralis culture was maintained on an artificial diet at 25 ± 1°C with 60% relative humidity and 16:8 h light-dark photoperiod (Sorour *et al.* 2011). Nervous system was dissected from last instar larvae using sterilized forceps under a dissection microscope in ice-cold phosphate buffered saline (pH 7. 4). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated RNA was treated with DNase-free (Ambion, Austin, TX, USA) using 1. 5 units/µg of total RNA. Quantification and integrity was assessed by using ethidium bromide-stained 1% agarose gel and Nanodrop ND-1000 spectrophotometry (Thermo Scientific, Waltham, MA, USA), with a cut-off value of 1. 8 for the A260: 280 ratio.

RNA was fragmented with a zinc chloride solution. Fragmented RNA was quantified using the Agilent 2100 Bioanalyzer system using PicoGreen dsDNA Assay Kit (Invitrogen). cDNA was synthesized using the cDNA Synthesis System Kit with random hexamer primers (Roche Applied Science, Indianapolis, IN, USA). The cDNA fragments were subjected to ligation to the sequencing adaptors provided with the GS FLX Titanium Rapid Library Preparation Kit (Roche Applied Science), and small fragments were removed with AMPure XP (Beckman Coulter, Fullerton, CA, USA). Sequencing was performed on a GS FLX platform with Titanium chemistry (Roche 454) using a Small region of a Pico Titer Plate (PTP) per library, following the manufacturer's instructions.

Annotations of assembled sequences were carried out by BLASTx against NCBI (National Center for Biotechnology Information) non-redundant protein sequence databases using the software Blast2GO (Conesa *et al.*,2005). The partial cDNA and deduced amino acid sequences were compared using the BLASTx tool and EXPASY. Sequences were aligned using the MUSCLE software. Phylogenetic trees were inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were performed with MEGA6 (Tamura *et al.*,2013).

RESULTS AND DISCUSSION

Nervous transcriptome analysis of *S. littoralis* led to the identification of putative SpliAChE-1, SpliCYP450 and SpliGST proteins. The length of the nucleotide sequence of *SpliAChE-1*, *SpliCYP450* and *SpliGST* cDNAs were 670 bp, 493 bp and 610 bp, respectively. These sequences contained putative open reading frames (ORF) of 390 bp encoding a 130 amino acid for SpliAChE, 492 bp encoding 164 aa for SpliCYP450 and 610 bp encoding 203 aa for SpliGST polypeptides. BLAST analysis showed that SpliAChE-1, SpliCYP450 and SpliGST have similarities with other known insect proteins. SpliCYP450 showed the highest homology to *Spodoptera litura* CYP450 with 99% identity while SpliAChE-1 showed 88% homology to *Helicoverpa armigera* and SpliGST showed 69% homology to *S. litura* GST.

The partial cDNA encoding *SpliAChE-1* includes abhydrolase domain in *S. littoralis* (Marchler-Bauer *et al.*,2015). Based on the alignment of amino acid sequences, phylogenetic trees were constructed for three putative proteins (Figure 1, 2, 3). The phylogenetic analysis of SpliAChE showed that SpliAChE was clustered together in Lepidopteran AChE clade (Figure 1). SpliAChE is phylogenetically close to *H. armigera* AChE which is also consistent with BLAST analysis.

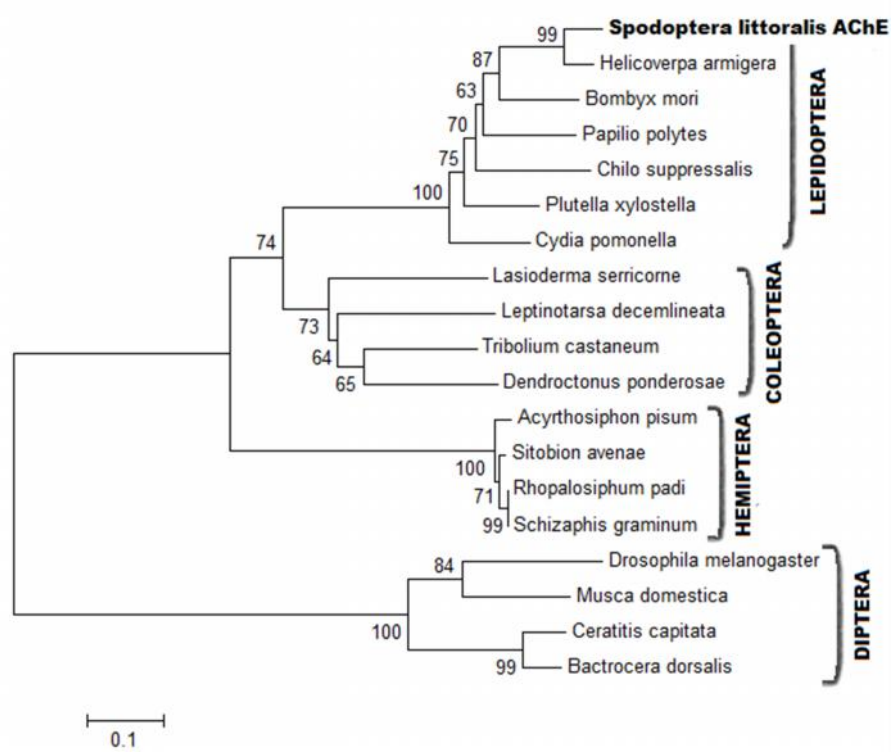

Figure 1. Phylogenetic analysis of putative AChE proteins from various insects. The species and GenBank accession numbers of the AChE sequences used to draw the phylogenetic tree are as follows: *Helicoverpa armigera* AAY59530. 1; *Bombyx mori* XP_012552768. 1; *Plutella xylostella* NP_001292470. 1; *Papilio polytes* XP_013148676. 1; *Cydia pomonella* ABB76667. 1; *Chilo suppressalis* AIY69049. 1; *Tribolium castaneum* NP_001280548. 1; *Lasioderma serricorne* ADA63843. 1; *Leptinotarsa decemlineata* AEI70751. 1; *Dendroctonus ponderosae* ENN79997. 1; *Sitobion avenae* AAV68493. 1; *Rhopalosiphum padi* AII01394. 1; *Acyrthosiphon pisum* XP_001948653. 1; *Drosophila melanogaster* AAL39345. 1; *Musca domestica* AAN06931. 1; *Ceratitis capitata* NP_001266363. 1; *Bactrocera dorsalis* AAO06900. 1.

The deduced sequence analysis of *SpliCYP450* includes specific P450 superfamily domain. Analysis reveals several conserved regions characteristic with other members of the P450 super family, such as the EXXR motif (EALR, position 387-390) located in the K helix, an oxygen-binding domain (proton transfer groove) ((A/G)Gx(E/D) T(T/S) as AGFET, position 329-333), and a microsomal cytochrome P450 motif PxxFxPE/DRF (PERF, position 444-452) (Werck-Reichhart and Feyereisen, 2000; Feyereisen, 2005) (Figure 2). Phylogenetic analysis of insect CYP450 showed that the SpliCYP450 was clustered closely with CYP450s from other members of Lepidoptera, including *S. litura, Spodoptera exigua, Mamestra brassicae, H. armigera* and *H. zea* (Figure 3).

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293 VAAGFATVEESAVGKKSADRVWSDEDLVAQAVLFFIAGFETVSTGMIFLLYELAVNPDVQ 352
353 ERLAQEIKEVDAKNGGKFDFNSIQNMVYMDMVVSEALRLWPPAVILDRMCTKDYNMGKPN 412
413 PKAEKDVILRKGTGVWIPAYAFHRDPQYFPNPDKFDPERFSEEN 456
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Figure 2. Deduced partial amino acid sequence of SpliCYP450. Putative conserved regions are shown with colorful frames.

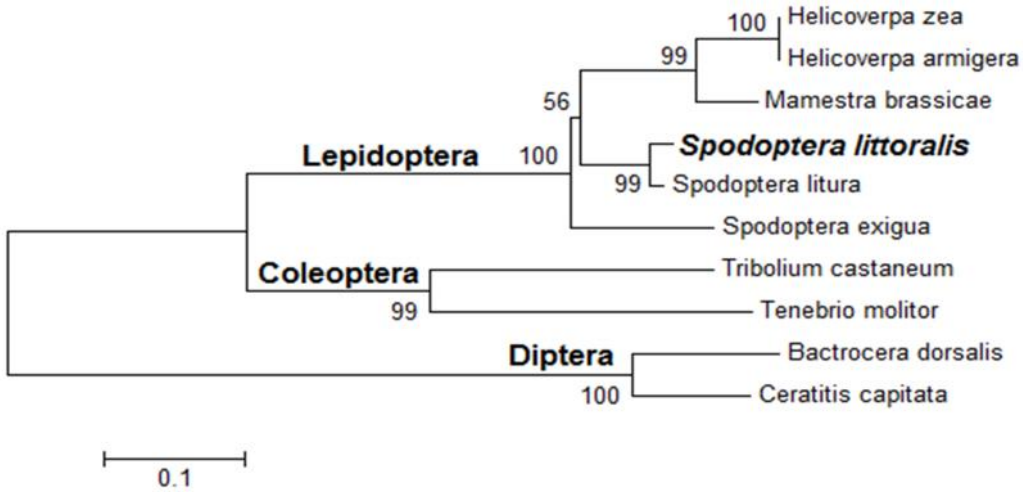

Figure 3. Phylogenetic analysis of putative CYP450 proteins from various insects. The species and GenBank accession numbers of the CYP450 sequences used to draw the phylogenetic tree are as follows: *Mamestra brassicae* AAR26518. 1; *Spodoptera litura* ACV88722. 1; *Spodoptera exigua* BAG71410. 1; *Helicoverpa zea* ABH09252. 1; *Helicoverpa armigera* AAV28704. 1; *Bactrocera dorsalis* AME15803. 1; *Ceratitis capitata* XP_012157753. 1; *Tribolium castaneum* NP_001034541. 1; *Tenebrio molitor* AKZ17702. 1.

Similar to other insect GSTs, SpliGST protein has a characteristic feature of glutathione binding region (G-site) and an electrophilic substrate binding region (H-site) conserved motifs that have an important role in GST activity (Deng *et al.* 2009). GSTs have been classified into seven main classes including delta, epsilon, omega, sigma, theta, zeta, and microsomal (Friedman, 2011). Among these delta and epsilon classes has been suggested to play significant roles in xenobiotic metabolism, particularly in insecticide detoxification (Ranson *et al.,* 2001, Ranson *et al.*, 2002, Meng *et al.*, 2015). For epsilon GSTs; there is a feature motif, RAVELTAK, found in *A. gambiae* (Ortelli *et al.*,2003). In epsilon SpliGST four amino acids were found conserved in this motif region (Arginine (R), Alanine (A), Leucine (L), Threonine (T)) (Figure 4). Based on the phylogenetic analysis and amino acid sequence comparison with epsilon GSTs from *Bombyx mori*, *Drosophila melanogaster*, *A. gambiae* and *Spodoptera litura* species, *SpliGSTs*were clustered into the Lepidopteran branch closely with epsilon-9 from *S. litura* (Figure 5).

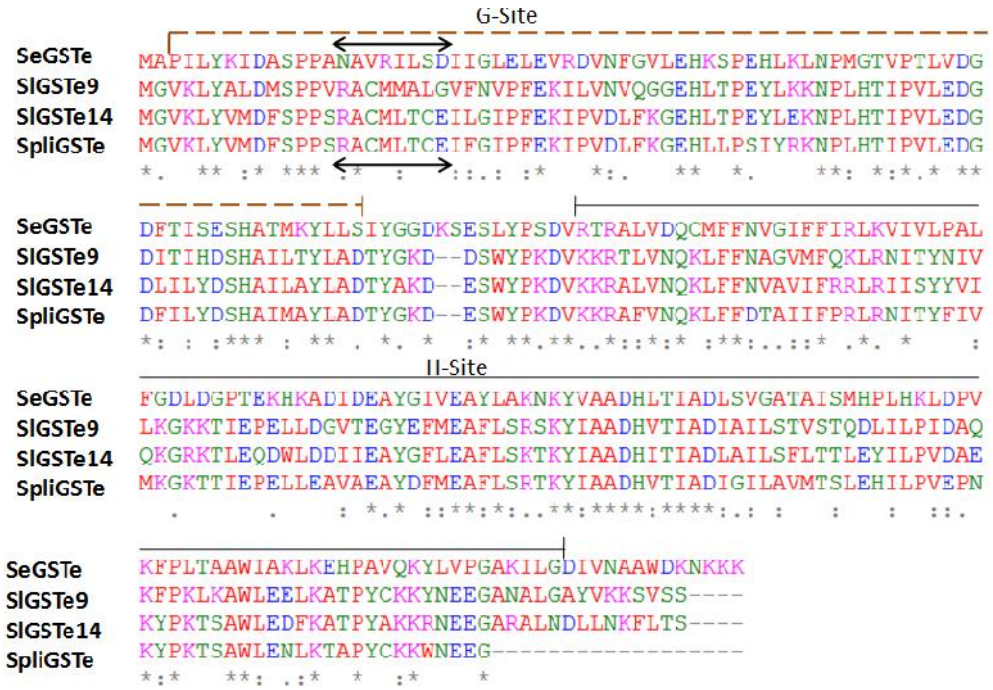

Figure 4. Multiple sequence alignment of SpliGST with other *Spodoptera* GSTs. The putative G-site and the H-site are indicated by dash and solid lines at the top of the panels, respectively. The specific motif of epsilon GST is implied with arrow. The accession numbers of the sequences used in the alignment are as follows: *S. exigua* (SeGSTe) AHB18378, *S. litura* (SlGSTe9) AIH07584, *S. litura* (SlGSTe14) AIH07589.

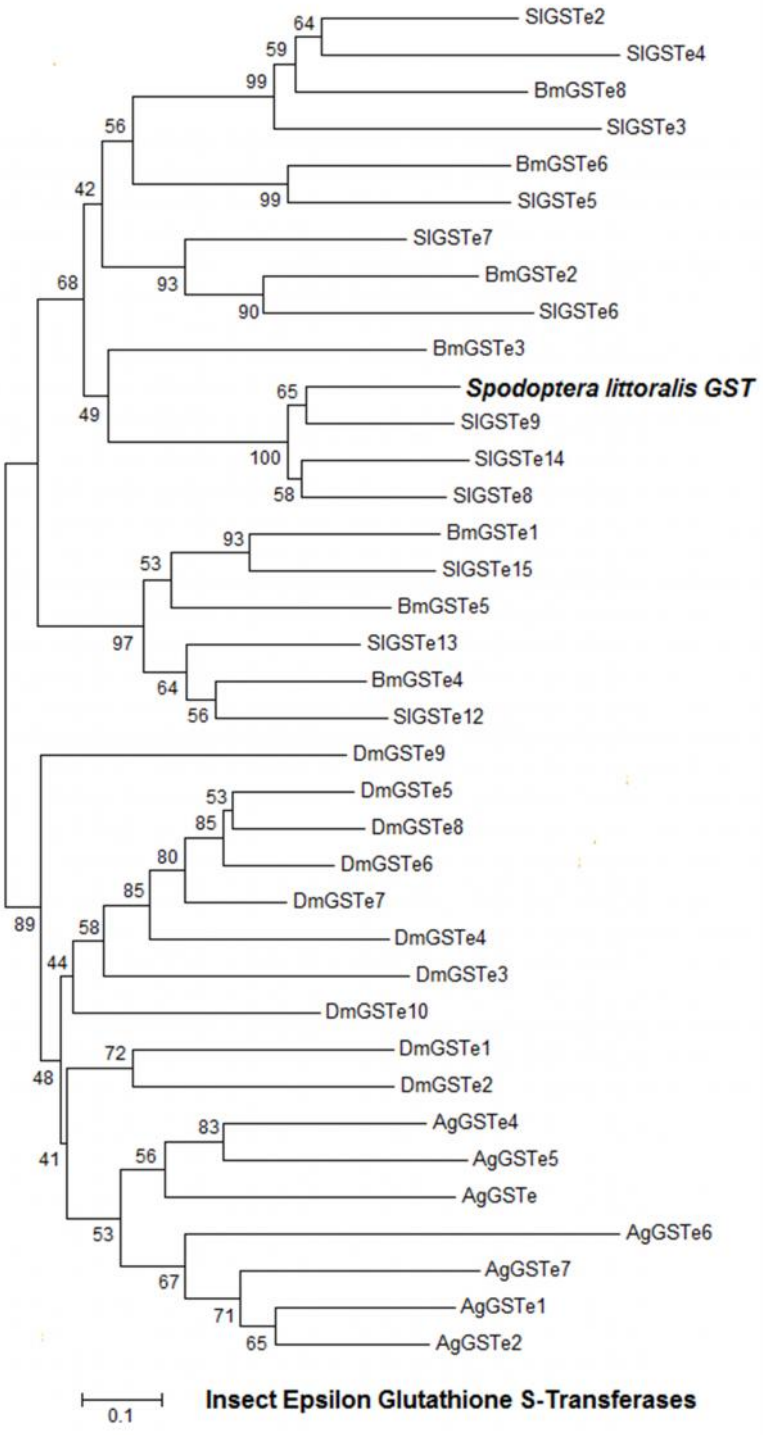
SlGSTe2
SlGSTe4
BmGSTe8
SlGSTe3
BmGSTe6
SlGSTe5
SlGSTe7
BmGSTe2
SlGSTe6
BmGSTe3
Spodoptera littoralis GST
SlGSTe9
SlGSTe14
SlGSTe8
BmGSTe1
SlGSTe15
BmGSTe5
SlGSTe13
BmGSTe4
SlGSTe12
DmGSTe9
DmGSTe5
DmGSTe8
DmGSTe6
DmGSTe7
DmGSTe4
DmGSTe3
DmGSTe10
DmGSTe1
DmGSTe2
AgGSTe4
AgGSTe5
AgGSTe
AgGSTe6
AgGSTe7
AgGSTe1
AgGSTe2
0.1
Insect Epsilon Glutathione S-Transferases

Figure 5. Phylogenetic analysis of putative GST proteins from various insects. The species and GenBank accession numbers of the GST sequences used to draw the phylogenetic tree are as follows: *Drosophila melanogaster* E1 (DmGST e1) NP_611323. 1; *D. melanogaster* E2 (DmGSTe2) NP_611324. 1; *D. melanogaster* E3 (DmGSTe3) NP_611325. 2; *D. melanogaster* E4 (DmGSTe4) NP_611326. 1; *D. melanogaster* E5 (DmGSTe5) NP_611327. 1; *D. melanogaster* E6 (DmGSTe6) NP_611328. 1; *D. melanogaster* E7 (DmGSTe7) NP_611329. 1; *D. melanogaster* E8 (DmGSTe8) NP_611330. 2; *D. melanogaster* E9 (DmGSTe9) NP_725784. 1; *D. melanogaster* E10 (DmGSTe10) NP_611322. 1; *Anopheles gambiae* E1 (AgGSTe1) XP_319969. 1; *A. gambiae* E2 (AgGSTe2) XP_319968. 3; *A. gambiae* E3 (AgGSTe3) XP_319972. 1; *A. gambiae* E4 (AgGSTe4) XP_319967. 1; *A. gambiae* E5 (AgGSTe5) XP_319966. 1; *A. gambiae* E6 (AgGSTe6) XP_001238234. 1; *A. gambiae* E7 (AgGSTe7) XP_319970. 3; *Bombyx mori* E1 (BmGSTe1) NP_001037197. 1; *B. mori* E2 (BmGSTe2) NP_001037420. 1; *B. mori* E3 (BmGSTe3) NP_001108466. 1; *B. mori* E4 (BmGSTe4) NP_001108460. 1; *B. mori* E5 (BmGSTe5) NP_001108464. 1; *B. mori* E6 (BmGSTe6) NP_001108465. 1; *B. mori* E8 (BmGSTe8)XP_004930497. 2; *Spodoptera litura* E2 (SlGSTe2) ACZ73898. 1; *S. litura* E3 (SlGSTe3) ACZ73899. 1; *S. litura* E4 (SlGSTe4) AIH07579. 1; *S. litura* E5 (SlGSTe5) AIH07580. 1; *S. litura* E6 (SlGSTe6) AIH07581. 1; *S. litura* E7 (SlGSTe7) AIH07582. 1; *S. litura* E8 (SlGSTe8) AIH07583. 1; *S. litura* E9 (SlGSTe9) AIH07584. 1; *S. litura* E12 (SlGSTe12) AIH07587. 1; *S. litura* E13 (SlGSTe13) AIH07588. 1; *S. litura* E14 (SlGSTe14) AIH07589. 1; *S. litura* E15 (SlGSTe15) AIH07590. 1.

CONCLUSIONS

In conclusion, we have identified three detoxification enzymes from *S. littoralis,* characterized the putative proteins and also reconstructed phylogenetic trees. The sequence data will provide a critical step toward a comprehensive functional characterization of resistance mechanism of the *S. littoralis*. Further functional analysis should be carried out to clarify insecticide detoxification mechanisms and to improve alternative insect pest management strategies against this hazardous pest.

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