Characterization of *Spodoptera litura* (*Lepidoptera: Noctuidae*) Takeout Genes and Their Differential Responses to Insecticides and Sex Pheromone

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Spodoptera litura (*S. litura*) is one of the most serious agricultural insect pests worldwide. Takeout (TO) is involved in a variety of physiological and biochemical pathways and performs various biological functions. We characterized 18 *S. litura* TO genes and investigated their differential responses to insecticides and sex pheromones. All predicted TO proteins have two Cysteines that are unique to the N-terminal of the TO family proteins and contain four highly conserved Prolines, two Glycines, and one Tyrosine. The expression levels of seven TO genes in the male antennae were higher than those in the female antennae, although the expression levels of 10 TO genes in the female were higher than those in the male. We investigated the effects of the sex pheromone and three insecticides, that is, chlorpyrifos (Ch), emamectin benzoate (EB), and fipronil (Fi), on the expression levels of the TO genes in the antennae. The results showed that the insecticides and sex pheromone affect the expression levels of the TO genes. One day after the treatment, the expression levels of SlTO15 and SlTO4 were significantly induced by the Ch/EB treatment. Two days after the *S. litura* moths were treated with Fi, the expression of SlTO4 was significantly induced (28.35-fold). The expression of SlTO10 changed significantly after the Ch and EB treatment, although the expression of SlTO12 and SlTO15 was inhibited by the three insecticides after two days of treatment. Our results lay a foundation for studying the role of TO genes in the interaction between insecticides and sex pheromone.

**Key words:** *Spodoptera litura*, sex pheromone, chlorpyrifos, emamectin benzoate, fipronil
et al. 2007, Du et al. 2003, Justice et al. 2003, Saito et al. 2006). The TO genes were expressed in the head, the fat body and other parts of the male fruit-fly but not in the female fruit-fly (Dauwalder et al. 2002). TO affects male courtship behavior by processing sex-biased signals (Dauwalder et al. 2002). TO affects male courtship behavior by processing sex-biased signals (Dauwalder et al. 2002). The TO gene in the termite Reticulitermes flavipes, deviate, is expressed in various tissues, such as the thorax and head, and is also involved in trail-following behavior (Schwinghammer et al. 2011). Hagai et al. speculated that juvenile hormone (JH) mediates the regulation of TO genes via the development-related factors of the Italian honey bee Apis mellifera (Hagai et al. 2007). The expression level of the TO gene in Adenophora glauca was significantly higher than that in migratory adults (Zhu et al. 2008). The TO gene in Drosophila is also involved in the regulation of longevity (Bauer et al. 2010). The TO gene in Locusta migratoria manilensis plays crucial roles in the transformation from mutual exclusion to mutual attraction as the population increases from a low density to a high density (Guo et al. 2011). The receptivity of the chemical receptors in Helicoverpa armiger is influenced by the JH (Angioy et al. 1983), suggesting that the TO genes may endogenously perform the hormonal functions of lipophilic ligands that act through hydrophobic receptors in lymphocytes and are released into helper cells.

**Spodoptera litura** (Lepidoptera: Noctuidae, S. litura) is a widely distributed crop pest that has a significant impact on the productivity of economic crops (Meagher et al. 2008). The resistance and cross-resistance of S. litura to insecticides make it a more difficult pest to control (Rehan and Freid 2014). Identifying the correlation between the TO gene and insecticides can lay a foundation for the future development of a strategy to control S. litura. Sex pheromones are the key to the courtship behavior of S. litura (Lin et al. 2015). It has also been reported that the TO gene is associated with courtship behavior (Dauwalder et al. 2002). Therefore, studying the correlation between the TO genes and the sex pheromone is important for the understanding of the function of this protein family. The mating ratio of male and female diamondback moths, that is, Plutella xylostella, is decreased in an avermectin-resistant strain, indicating that the mating behavior of P. xylostella is affected by the insecticide application and the resistance generated (Xu et al. 2010). Volatiles from host plants also contribute to the response of Spodoptera exigua to sex pheromones (Deng et al. 2004). The electroantennogram (EAG) responses of Choristoneura occidentalis and Orgyia pseudotsugata to sex pheromones and a mixture of sex pheromones and insecticides were significantly different. Following a treatment of sex pheromones, the EAG response is high, although the EAG response to the sex pheromone/insecticide mixture was significantly lower than that in the control group, indicating that the insecticides affected the response to the sex pheromone (Sower and Shorb 1985). To control the pest both short term and long term, the pheromone and the insecticide are sometimes used together (Trimble et al. 2001). Insecticides have been shown to affect the courtship and sex pheromone communication systems in insects (Dallaire et al. 2004, Wei and Du 2004, Clark and Haynes 1992, Yang and Du 2003). Changes in the sex pheromone communication system are correlated with insecticide resistance in moths (Xu et al. 2010, Elsayed et al. 2001). Sub-lethal doses of the insecticide malathion decrease the localization of females by sex pheromone (Zhou et al. 2005, Elsayed et al. 2001). In addition, it has been reported that insecticides could affect olfactory function even in vertebrate, such as in salmon (Moore and Waring 1996).

It has been reported that the TO gene is highly expressed in the antennae of Aedes aegypti (Bobbot and Vogt 2005), and previous studies have also shown that the TO gene is highly expressed in D. melanogaster and Phormia regina (Vanahan et al. 2012, Fujikawa et al. 2006). Our recent work revealed that the TO genes in Nilapavarta lugens (N. lugens) are male-biased and regulated by JH signaling (Lin et al. 2017). However, the role of the TO family genes in the cross interaction between insecticides and the sex pheromone remains unclear. Here, we studied the potential role of the TO family genes by measuring their expression levels in the antennae of male and female S. litura adults and the changes in the expression of the TO genes in response to insecticides and the sex pheromone.

**Materials and Methods**

**Sequence Analysis**

Eighteen S. litura TO gene sequences were obtained from previously reported transcriptome data (Feng et al. 2015), which were named TO1-18. All sequences were deposited in GenBank. The accession numbers are: SlituTO1 (MF196295), SlituTO2 (MF196296), SlituTO3 (MF196297), SlituTO4 (MF196298), SlituTO5 (MF196299), SlituTO6 (MF196300), SlituTO7 (MF196301), SlituTO8 (MF196302), SlituTO9 (MF196303), SlituTO10 (MF196304), SlituTO11 (MF196305), SlituTO12 (MF196306), SlituTO13 (MF196307), SlituTO14 (MF196308), SlituTO15 (MF196309), SlituTO16 (MF196310), SlituTO17 (MF196311), SlituTO18 (MF196312). A Blast search was performed in NCBI. Maximum Likelihood (ML) was used to construct a phylogenetic tree using previously identified 92 TO genes from eight species along with 18 SlituTO genes using MEGA6 (Hall 2013). The SlituTO genes were translated into amino acid sequences, and the sequences were aligned using ClustalW2 (Larkin et al. 2007). A WebLogo sequence alignment map was generated at http://weblogo.berkeley.edu/logo.cgi.

**Biological Materials and Experimental Treatment**

Male and female S. litura pupae were purchased from Jiyan Baiyun Industrial Co., Ltd. (Henan, China). The male S. litura used in the insecticide treatment experiment and the sex pheromone treatment experiment were trapped using traps (SL02-SP, main components: Z9, Z11-14: Oac, Z9, Z12-14: OAc), which were a gift from Ningbo Newcomb Biotechnology Company (Ningbo, China). The trapped male S. litura adults were incubated with 10% sucrose for 24h. The moths were divided into the control group, chlorpyrifos group, emamectin Benzoate group, fipronil group, and sex pheromone group. The control group was treated with 10% sucrose water; the insecticide treatment was performed by adding 1 ppm (Haynes 1988) of the insecticide in 10% sucrose water; and the sex pheromone group was treated with 10% sucrose water, the moths were placed in a plastic basket and PVC lure was placed outside the basket. The antennae of the surviving insects were cut 1 day and 2 days after the treatment. Three sets of biological replicates were established in each group.

**RNA Extraction, cDNA Synthesis and qRT-PCR**

The antennae were cut and ground in TRizol (Takara, Dalian, China), extracted using chloroform, precipitated using isopropanol, washed with ethanol, and finally dissolved in DEPC. The PrimeScript RT-PCR Kit (Roche, Shanghai) was used for the reverse transcription with a total of 20 μL in the following three steps: 1) 1 μg of total RNA, 1 μL of OligdT, and RNA free Water to 13 μL were added in a 65 °C water bath for 10 min; 2) 4 μL of Buffer, 2 μL of dNTP, 0.5 μL of the inhibitor, and 0.5 μL of Transcriptase were added in a 55 °C water bath for 30 min; and 3) the solution was
placed in an 85°C water bath for 5 min. The SYBR FAST qPCR Master Mix2 (KAPA) was used for the qRT-PCR with a total volume of 20 μL as follows: 10 μL of 2 × KAPA SYBR FAST qPCR Master Mix 2 Universal, 0.4 μL each (10 μM), ≤ 20 ng of cDNA, 0.4 μL of 50 × ROX High, and PCR-grade water to 20 μL. The qRT-PCR reaction conditions were as follows: 95°C for 3 min, 95°C for 3 s, and 55°C for 20 s (40 ×). The primers are listed in Table 1. RPL10 was used as the reference gene (Lu et al. 2013).

Data Analysis
The qRT-PCR data of the TO gene expression levels in the antennae of male and female S. litura adults were analyzed using the 2^(-ΔΔCt) method (Livak and Schmittgen 2001). The TO gene expression in the S. littura female antennae was analyzed by SPSS20.0 (IBM-SPSS, Somers, NY) using Student’s t-test. The effects of the insecticides and sex pheromones on the expression of the TO gene in male S. littura were analyzed using a single factor ANOVA analysis, and the LSD method was used for multiple comparisons. The figures were generated using OriginPro 9.1.

Heat Map
The heat map was generated with HemI 1.0 using the data of the heat map was generated using OriginPro 9.1.

Results
Sequence Analysis of the TO Family Genes in S. littura
Eighteen TO family genes were identified by analyzing previously reported S. litura transcriptome data (Feng et al. 2015) and named SlituTO1–SlituTO18. We translated the 18 S. littura TO gene sequences into amino acids, and a phylogenetic tree was generated by comparing 92 TO genes from other eight species with the TO genes from S. littura (Fig. 1). SlituTO8, SlituTO11, SlituTO6, SlituTO17, SlituTO12, and SlituTO16 clustered together, and they are highly homologous intraspecies (Fig. 1). SlituTO1, SlituTO4, SlituTO8, SlituTO10, SlituTO11, Slitu12, and SlituTO18 have the highest homology with the TO genes from B. mori (Bm), which indicates that these TO genes are conserved, at least in Lepidoptera.

We also compared the TO genes using WebLogo (Fig. 2). There are two highly conserved Cys (100%) in the N-terminus; three highly conserved amino acids, that is, Proline (100%), Glycine (100%), Tyrosine (100%), in the middle; and a highly conserved Glycine (100%) in the C-terminus (Fig. 2).

Differential Expression Levels of the TO Genes Between Male and Female S. littura
The expression levels of the TO genes in the antenna of the female and male adults are shown in Figure 3. The expression level of the genes in the females is normalized to that in the males. There was no significant difference in the expression of SITO2 between the male and the female (Fig. 3). There were seven SITO genes that were more highly expressed in the male than in the female, and three of these genes were the most highly expressed genes, that is, SITO6 (94.2-fold), SITO10 (115.3-fold), and SITO12 (167.2-fold). Ten TO genes were more highly expressed in the female than in the male, and five of the TO genes, that is, SITO4, SITO7, SITO11, SITO17, and SITO18, were more highly expressed by more than threefold (Fig. 3).

Effects of the Insecticides and the Sex Pheromones on the Expression Levels of the TO Genes
We assessed the expression levels of 18 TO genes in male and female S. littura 1 day and 2 days after the insecticide treatment and the sex pheromone treatment.

Most of the genes (11 of 18) are not changed or only have minor changes after the insecticide or sex pheromone treatment (< fourfold, Supp Table 2 [online only], Supp Fig. 1 [online only]). However, the remaining seven genes have relatively higher fold changes after the insecticide treatment or the sex pheromone treatment (> fourfold, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression level of SITO6 was significantly decreased 1 day after the chlorpyrifos treatment, although the expression level of SITO12 and SITO15 significantly increased. The expression levels of SITO6, SITO10, and SITO12 were significantly decreased after a 1-day

Table 1. Primers used in the qRT-PCR

| Gene     | 5' primer                  | 3' primer                  |
|----------|----------------------------|----------------------------|
| SlituTO1 | TGGCATACTACATATATCCCTCA    | GTCCCTGGAAAGTGACCTTTGA     |
| SlituTO2 | GATGGAGAGAACAGCTTGGAAG     | GCTAATGATGCGCTTGACTA       |
| SlituTO3 | GACTACATCATGAGAGGCG       | GTTGAAGAGTTGGAAGGATG       |
| SlituTO4 | ACAAGATCAACCCAGACACAG     | CATTCCAAGTGGCTACTCTTTT     |
| SlituTO5 | CGAGTGAGAAAGGAGGAG         | GAACGTAGTGGCTTCATAGTTTT    |
| SlituTO6 | TATGCGCTTCTCATTCCAAAG     | CACCTCTTCTTCCAGTTTTCCG     |
| SlituTO7 | GGAGTCCTCAGGTCCTCATAAG   | AAGTCTGCTGCTCATTGAAC       |
| SlituTO8 | CAACCTGGATTCTCATCTCAT     | GTGTTCTCACCTCTTCCACA       |
| SlituTO9 | CGAGCCAGATGTCTATA         | TCTCTAGATGCTGCTGACA        |
| SlituTO10| TGGTGGACCTCACAGGTGAGA     | ATGAGGATTTTAGAGAGGCG       |
| SlituTO11| GAAACGGACCTGGATCTTTAAG    | CAACTTTTCACCAAGGAGG        |
| SlituTO12| GTGACGAGGACACCTTACTAT     | CGATTTCCCAGTTCTCTTCT       |
| SlituTO13| ATACACGCTTGACACTGCTGTTT   | GCATCTGAGAAGGAGTCTGCT      |
| SlituTO14| GCTACGGCTCTAAGAGTAGTAG    | ACCGTCTTCTGCTAGGTTCT       |
| SlituTO15| TAAAACCTCGAGGACAGATCT     | AACACTAGTGGCAGCTTTTCA      |
| SlituTO16| CTTGTCGATCGTGAGTGGAAA    | ATCTGCTACCCAGGAGG           |
| SlituTO17| GTAGACGCTGTCATTTCAACCAA  | GAAGTATAGACACCAGGCTGCG     |
| SlituTO18| TCTGCAATACGCCCCTGACTA    | GAAACGTGCTACTTTCCAGTTTCT   |
| SlituRPL10| GACCTGGGTAAGAGAAG        | GATGACATGGGAATGTGAC         |
treatment with emamectin benzoate (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]), although the expression levels of \( \text{SlTO4}, \text{SlTO9}, \text{and SlTO15} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression levels of \( \text{SlTO2}, \text{SlTO6}, \text{and SlTO10} \) were significantly decreased 1 day after the treatment with fipronil, although the expression of \( \text{SlTO15} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression levels of \( \text{SlTO2}, \text{SlTO7}, \text{SlTO10}, \text{SlTO12}, \text{and SlTO17} \) were significantly decreased 1 day after the sex pheromone treatment, although those of \( \text{SlTO1}, \text{SlTO8}, \text{SlTO9}, \text{and SlTO13} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]).

The expression levels of \( \text{SlTO2}, \text{SlTO11}, \text{SlTO12}, \text{SlTO15}, \) and \( \text{SlTO18} \) were significantly decreased after the 2-day treatment with chlorpyrifos (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]), although the expression level of \( \text{SlTO10} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression levels of \( \text{SlTO3}, \text{SlTO12}, \text{SlTO15}, \) and \( \text{SlTO18} \) significantly decreased after the 2-day treatment with emamectin benzoate (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]), although the expression levels of \( \text{SlTO1}, \text{SlTO4}, \text{SlTO5}, \text{and SlTO10} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression of \( \text{SlTO2}, \text{SlTO3}, \text{and SlTO12} \) significantly decreased after the 2-day treatment with fipronil, although the expression levels of \( \text{SlTO4} \) (28.5-fold), \( \text{SlTO5}, \text{and SlTO7} \) significantly increased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]). The expression levels of \( \text{SlTO3}, \text{SlTO6}, \text{SlTO11}, \text{and SlTO13} \) significantly increased after the 2-day treatment with the sex pheromone, although the expression levels of \( \text{SlTO1}, \text{SlTO7}, \text{SlTO9} \) significantly decreased (Fig. 4, Supp Table 2 [online only], Supp Fig. 1 [online only]).

**Discussion**

The TO proteins are highly conserved and contain N-terminal Cys that influence the disulphide bond formation (Dauwalder et al. 2002, Du et al. 2003, Fujikawa et al. 2006, Sarovblat et al. 2000, So et al. 2000, Larkin et al. 2007). Moreover, the TO proteins contain four highly conserved amino acids, two Glycines, one Proline, and
one Tyrosine (Fig. 2). The role of the TO protein family remains unknown (Chamseddin et al. 2012, Saito et al. 2006, Fujikawa et al. 2006, So et al. 2000). The TO family genes in S. litura are closely related to those in B. mori, which indicates that the TO gene in the two Lepidoptera species had a high homology.

By searching previously reported studies, we analyzed the bias of the TO genes expressed in the male and female of species, including D. melanogaster, N. lugens, B. mori, and P. regina (Supp Table 1 [online only]). There are three types of TO genes as follows: male biased, female biased, and not sex biased. In S. litura, 10 TO genes are female biased, 7 TO genes are male biased, and 1 TO gene has no bias. However, in the other three species, most of the TO genes are actually male biased (Supp Table 1 [online only]). The expression profiles of the TO genes in D. melanogaster and N. lugens are male biased (Sarovblat et al. 2000, Lin et al. 2017, Dauwalder et al. 2002), whereas here, we found that the expression levels of the TO family genes in S. litura are different from those in D. melanogaster and N. lugens (Supp Table 1 [online only], Fig. 3). The expression levels of the TO family genes in female S. litura adults are higher than those in the male, suggesting that the sex bias of the TO family genes is different in different species. As shown in Supp Table 1 [online only], the sex bias of the TO family genes is similar in S. litura.

![WebLogo alignment of Takeout protein sequences. Alignment of 18 Takeout proteins from S. litura. The x-axis represents the amino acid position. The y-axis (bits) represents the relative proportion of the amino acid at one position. The height of the logo varied inversely with the variability at the position.](image-url)
Fig. 3. Difference in the expression levels of the Takeout genes between S. litura male and female adults. M: Male; F: Female; Student's t-test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Fig. 4. Heatmap showing the influence of three insecticides and the sex pheromone on the expression levels of 18 Takeout genes in S. litura male adults after a 1 d and 2 d treatment. 1 d: expression changes in TO genes in male S. litura after 1 d of insecticide or sex pheromone treatment; 2 d: expression changes in TO genes in male S. litura after two days of insecticide or sex pheromone treatment; CK: control; Ch: chlorpyrifos; EB: emamectin benzoate; Fi: fipronil; P: sex pheromone. Hierarchical average linkage was used as the clustering method.
and *B. mori*, which is likely because these two species belong to Lepidoptera and is consistent with the phylogenetic analysis (Fig. 3). Interestingly, we found that three *S. littoralis* TO genes, that is, SITO6, SITO10, and SITO12, are expressed only in the male; however, we have not identified TO genes that are expressed specifically in the female (Fig. 3). In fact, we found that the fold-change of all TO genes that were highly expressed in the female are less than fourfold.

The insecticide treatments showed that certain *S. littoralis* TO genes, such as SITO4, SITO10, and SITO15, are highly induced by at least one insecticide with a fold-change > 4. SITO3, SITO4, SITO6, SITO10, SITO12, SITO13, and SITO15 have a high degree of fold-change (Supp Table 2 [online only]). Altogether, SITO6 and SITO13 are mainly responsive to the sex pheromone treatment; SITO3, SITO4, SITO12, and SITO15 are mainly responsive to the insecticide treatment, and SITO10 is responsive to both the insecticide and sex pheromone treatment with a high degree of fold-change (Supp Table 2 [online only]). Moreover, the expression changes in SITO10 after the treatment with the insecticides were similar to those observed after the sex pheromone treatment.

We recently showed that the *N. lugens* TO genes are regulated by the JH signaling pathway (Lin et al. 2017), but the mechanism by which the TO family genes regulate physiological functions remains unclear. Moreover, an understanding of the role of the TO genes in the insecticide-induced gene expression and inhibition of the TO genes could help us understand the role of the TO family genes in vivo. Future works investigating the function of the TO genes in vivo are required.

Insecticides and sex pheromones are directly and indirectly linked via physiological and biochemical pathways. The application of sex pheromones decreases the pest population and achieves a long-term pest population decrease; it is also an important pest management tool for controlling several notorious insect pests (Witzgall et al. 2010, Hirano 1980). In addition, the application of insecticides lowers the trapping efficiency of the sex pheromone lure. In addition, understanding the potential interaction between insecticides and trapping males might be correlated; if the interaction between insecticides and trapping males is high, then the sex pheromone lure will not be effective.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

**Authors’ Contributions**

X.L. and L.Z. designed the research study, analyzed the data, and wrote the paper. X.L., L.Z., and Y.J. performed the experiments.

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