



# Analysis of persistent changes to $\gamma$ -aminobutyric acid receptor gene expression in *Plutella xylostella* subjected to sublethal amounts of spinosad

X.-H. Yin<sup>1\*</sup>, Q.-J. Wu<sup>2\*</sup>, Y.-J. Zhang<sup>2\*</sup>, Y.-H. Long<sup>1</sup>, X.-M. Wu<sup>1</sup>, R.-Y. Li<sup>1</sup>, M. Wang<sup>1</sup>, X.-L. Tian<sup>1</sup> and X.-G. Jiao<sup>3</sup>

<sup>1</sup>Institute of Crop Protection, Guizhou University, Guiyang, China

<sup>2</sup>Department of Plant Protection, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China

<sup>3</sup>College of Life Sciences, Hubei University, Wuhan, China

\*These authors contributed equally to this study.

Corresponding author: X.H. Yin

E-mail: xhyin@gzu.edu.cn

Genet. Mol. Res. 15 (3): gmr.15038782

Received May 16, 2016

Accepted June 3, 2016

Published July 25, 2016

DOI <http://dx.doi.org/10.4238/gmr.15038782>

Copyright © 2016 The Authors. This is an open-access article distributed under the terms of the Creative Commons Attribution ShareAlike (CC BY-SA) 4.0 License.

**ABSTRACT.** A multi-generational approach was used to investigate the persistent effects of a sub-lethal dose of spinosad in *Plutella xylostella*. The susceptibility of various sub-populations of *P. xylostella* to spinosad and the effects of the insecticide on the gene expression of  $\gamma$ -aminobutyric acid receptor (GABAR) were determined. The results of a leaf dip bioassay showed that the sensitivity of *P. xylostella* to spinosad decreased across generations. The sub-strains had been previously selected based on a determined LC<sub>25</sub> of spinosad. Considering that GABA-gated chloride channels are the primary targets of spinosad, the cDNA of *P. xylostella* was used to clone GABAR $\alpha$  by using reverse transcription-polymerase chain reaction (RT-PCR). The mature peptide

cDNA was 1477-bp long and contained a 1449-bp open reading frame encoding a protein of 483 amino acids. The resulting amino acid sequence was used to generate a neighbor-joining dendrogram, and homology search was conducted using NCBI BLAST. The protein had high similarity with the known GABAR sequence from *P. xylostella*. Subsequent semi-quantitative RT-PCR and real-time PCR analyses indicated that the GABAR transcript levels in the spinosad-resistant strain (RR, 145.82-fold) and in Sub1 strain (selected with LC<sub>25</sub> spinosad for one generation) were the highest, followed by those in the spinosad-susceptible strain, the Sub10 strain (selected for ten generations), and the Sub5 strain (selected for five generations). This multi-generational study found significant correlations between spinosad susceptibility and GABAR gene expression, providing insights into the long-term effects of sub-lethal insecticide exposure and its potential to lead to the development of insecticide-resistant insect populations.

**Key words:**  $\gamma$ -aminobutyric acid receptor gene; Multi-generation; *Plutella xylostella*; Spinosad; Sublethal effect

## INTRODUCTION

The spinosyns, as shown by studies using spinosad, have a wide range of activity against lepidopteran and dipteran insects (Besard et al., 2011). The unique mechanism of action of spinosad provides long-term protection against various insects, including Lepidoptera, Diptera, Hymenoptera, Thysanoptera, and Siphonaptera, in many crops (Salgado, 1998). Spinosad has been commercially used in China since 2002; initially, it was found to be very effective in controlling *Plutella xylostella* (Lepidoptera: Plutellidae) (Xue et al., 2005). Spinosyns act predominantly by creating a hyperexcitable nervous system in the targeted insect by stimulating nicotinic acetylcholine receptors. In *Drosophila melanogaster*, the particular site of action is the alpha6 subunit; it leads to changes in the behavior of  $\gamma$ -aminobutyric acid (GABA)-gated chloride channels. These changes in channel functioning lead to an increase in spontaneous muscle contractions, muscle movements and, ultimately, paralysis and death (Morandin et al., 2005; Watson et al., 2010). In addition to killing insects, the widespread use of insecticides might cause sub-lethal effects. This variability occurs because insects are exposed to varying concentrations of the insecticide, which can result from how the insecticides were applied and the duration between the time the insecticide was applied and insect exposure (Singh and Marwaha, 2000).

The eco-toxicological effects of spinosad on the fitness of various organisms have been extensively studied; these studies have focused on the effects after acute, short-term exposure (Schneider et al., 2004; Schneider and Üner, 2013; Khan et al., 2014). The results of such studies have shown that the survival, growth, and reproduction of insects and arthropods were altered significantly after sub-lethal exposure to this pesticide (Martínez-Villar et al., 2005; Ruiz et al., 2008; Duchet et al., 2011; Sayed and Sheikh, 2015). However, the majority of these studies only investigated the effects of sub-lethal exposure on a single generation. Whether such sub-lethal exposure to pesticides can lead to changes over multiple generations is currently not known. Hence, in this study, the trans-generational approach was used (Yin

et al., 2009). Using a method that can elucidate the influence over several generations is required to better understand the effects of persistent, sub-lethal doses in native populations and ecological communities (Vogt et al., 2007; Kolarevic et al., 2012).

One mechanism for adaptation includes introduction of advantageous alterations in mRNA transcription levels caused by exposure to sub-lethal concentrations of insecticides (Frankham, 2005; Beggel et al., 2012; Bauer et al., 2013). Only a few empirical studies have investigated the persistent effects of sub-lethal amounts of insecticides on gene expression in insect populations. In this study, how multi-generational gene expression changes as a function of sub-lethal spinosad concentrations in a *P. xylostella* population was determined, with a particular focus on the effects of the expression of the  $\gamma$ -aminobutyric acid receptor in the stressed population over ten generations.

## MATERIAL AND METHODS

### Materials

#### *Insect Stock Culture*

An insecticide-susceptible colony of *P. xylostella* was obtained from the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences (Beijing, China). The founder colony was divided into two groups: the SS strain was not exposed to insecticides, whereas the other group - the sub-strain - was exposed to spinosad at LC<sub>25</sub>. Larvae hatched and were raised on cabbage plants (*Brassica oleracea* L. var. Jingfeng 1) in an insectary at constant temperature (25°C), relative humidity (60 ± 2%), and photoperiod (8:16, dark:light). When the larvae reached adulthood, they were maintained on an additional nutritional supplement consisting of 10% sugar solution.

#### *Chemicals*

Spinosad [Success 2.5 g (AI)/100 g] was purchased from Dow AgroSciences and diluted to sub-lethal concentrations for both selection and bioassays.

### Methods

#### *Bioassays and selection*

To evaluate the susceptibility of *P. xylostella* to Spinosad, we used a leaf-dip bioassay (Yin et al., 2009). Five concentrations of Spinosad, diluted with distilled water, were used; a control of distilled water was set. Triton X-100 (0.05%, v/v) served as an emulsifier for all experimental and control solutions. Cabbage leaf discs 30 mm in diameter were submerged for 10 s in either experimental or control solution and air-dried at room temperature. *P. xylostella* were starved for approximately 2 h; subsequently, 15 larvae at the 3rd instar were placed in a plastic cup (7 x 7 cm) that had two leaf discs. Each of the two discs was treated with the same spinosad concentration. All the tested concentrations were examined across four replicates. The cups were covered and kept at 25°C with a light:dark cycle of 16:8 h. Larval deaths were noted after 24, 48, and 72 h. Probit analysis was performed using POLO (LeOra Software,

1997) to evaluate the concentration-mortality data. Abbott's formula (Abbott, 1925) was used to correct the death rates for each probit analysis.

The bioassay results allowed for the selection of a 48-h  $LC_{25}$  concentration for all following experiments. The leaf-dip method was used to select successive sub-strains. For each generation, 3rd instar larvae were fed cabbage leaves that had been dosed with the  $LC_{25}$ -equivalent of spinosad. Gene expression analysis was performed at generation one (Sub1), generation five (Sub5), and generation ten (Sub10). The resistant strain (RR) was derived from the susceptible strain (SS) and was selected with spinosad for more than 20 consecutive generations. SS was maintained without selection in the laboratory. RR and SS *P. xylostella* were used as the control group. The susceptibility ( $LC_{50}$ ) to spinosad in all strains was checked every five generations. The susceptible strain has an equivalent  $LC_{50}$  as that of the wild strains, whereas the susceptibility of RR was reduced after selection. Some living 4th instar larvae of every generation were collected, instantly frozen using liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

### ***Molecular cloning of Px-GABAR***

#### ***Isolation of total RNA and reverse transcription***

The Trizol™ method (Invitrogen, USA) was used to obtain total RNA from 4th instar diamondback moth larvae (50 mg) according to manufacturer instructions. The resulting RNA pellet was then dissolved in TE buffer (30  $\mu\text{L}$ ). Next, 1  $\mu\text{L}$  RNA solution was used in conjunction with the TaKaRa™ cDNA Synthesis Kit (TaKaRa, China) to synthesize cDNA. Both the Trizol method™ and cDNA synthesis kit were used in accordance with manufacturer instructions.

#### ***Primer design and PCR***

The sequences of all degenerate primers were based on the conserved amino acid sequences of the fragment and mature peptide cDNA of the GABA  $\alpha$  receptor gene reported for *P. xylostella*; all sequences were retrieved from GenBank with the accession number AY973948. The forward primers were 5'-TGTGAAATTCAGTTCGTACG-3' and 5'-GGGGTGGCATGTTTGGAG-3' and the reverse primers were 5'-ACCATTACGAAGCATGTACC-3' and 5'-AAAGGCGATTGGCGCTTA-3' for the fragment and mature peptide cDNA of GABA $\alpha$  receptor gene, respectively.

The aforementioned degenerate primers and a PCT-200 thermocycler were used to perform polymerase chain reaction (PCR). The thermocycling program for the fragment cDNA of the GABA $\alpha$  receptor gene was as follows: 3 min at  $95^{\circ}\text{C}$  (denaturation), 30 cycles of 1 min at  $94^{\circ}\text{C}$  (denaturation), 1 min at  $55^{\circ}\text{C}$  (annealing), and 1 min at  $72^{\circ}\text{C}$  (extension). The program concluded with a final 10-min extension at  $72^{\circ}\text{C}$ . For the mature peptide cDNA of the GABA $\alpha$  receptor gene, thermocycling conditions were as follows: 10 min at  $94^{\circ}\text{C}$  (denaturation), 35 cycles of 1 min at  $94^{\circ}\text{C}$  (denaturation), 1 min at  $58^{\circ}\text{C}$  (annealing), and 1 min at  $72^{\circ}\text{C}$  (extension). The program concluded with a final 10-min extension at  $72^{\circ}\text{C}$ .

The PCR products of all the fragment and mature peptides were separated using 2.0% agarose gel electrophoresis. Resulting bands were purified using a Universal DNA Purification Kit (Tiangen Biotech, China); subsequently, they were ligated to the pGEM-T Easy Vector Systems (Promega, China). Vectors were then transformed into competent *Escherichia coli*

DH5 $\alpha$  cells according to manufacturer instructions. Positive clones were sequenced using an ABI PRISM 3730 DNA sequencer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China).

### ***Sequence analysis***

BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to determine any sequence homology, and any conserved sequences were then analyzed using Conserved Domains (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). From these analyses and their corresponding secondary structures, we then predicted transmembrane (TM) segments ([www.cbs.dtu.dk/services/TMHMM](http://www.cbs.dtu.dk/services/TMHMM)). The hydrophathy profile of the consensus amino acid sequence of Px-GABAR was analyzed using ProtScale (<http://web.expasy.org/cgi-bin/protscale/protscale.pl>).

### ***Expression of Px-GABAR in the sublethal treated multi-generation***

Semi-quantitative RT-PCR and real-time PCR were performed to determine the expression of Px-GABAR in the sub-lethal exposed multi-generation *P. xylostella*. Next, 900 ng of template RNA from 4th instar larvae of *P. xylostella* was used for the synthesis of 10  $\mu$ L of the first cDNA strand. All cDNAs were synthesized using a QuantScript RT Kit (Tiangen Biotech, China); they were then used as the template for PCR amplification as well as for subsequent Px-GABAR identification. The 18S rRNA was used as an internal control to normalize sample loading. The primers used for Px-GABAR were 5'-TGTGAAATTCAGTTCGTACG-3' (forward) and 5'-ACCATTACGAAGCATGTACC-3' (reverse). The primers used for 18S rRNA were 5'-CGGCTACCACATCCAAGG-3' (forward) and 5'-GCTGGAATTACCGCGGCT-3' (reverse).

### ***Semi-quantitative RT-PCR***

The reaction mixes contained 1  $\mu$ L first-strand cDNA, 5  $\mu$ L 10X reaction buffer, 2  $\mu$ L dNTPs (10 mM each), 2.5 U Taq polymerase (TRANS Gen, China), 5  $\mu$ L each Px-GABAR primer 10  $\mu$ M in a total volume of 50  $\mu$ L. Thermocycling protocol was as follows: 95°C for 4 min, 2 cycles of 94°C, 55°C, and 72°C for 1 min each, followed by 72°C for 10 min on a T-Gradient Thermoblock (Biometra, Germany). Next, 5  $\mu$ L each 18S rRNA primer (10  $\mu$ M) was added to the above reaction mixture and again subjected to 33 cycles of the PCR. Resulting PCR products were separated on a 2.0% agarose gel and visualized using ethidium bromide. Image master VDS software was used for analysis.

### ***Real-time PCR***

Real-time PCR was conducted on an iQ<sup>TM</sup>5 (Bio-Rad, Hercules, CA, USA) by using 2  $\mu$ L cDNA (final concentration, 100 ng/reaction) in a total volume of 25  $\mu$ L with 0.5  $\mu$ L 0.2  $\mu$ M of each Px-GABAR or 18S rRNA primer and 12.5  $\mu$ L QuantiTectSYBR<sup>®</sup> Green PCR Master Mix (Qiagen, China). The thermocycling program used was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 10 s, 61°C for 30 s, and 72°C for 30 s or at 80°C for 15 s.

The cycle number where the sample fluorescence was greater than that of the

background was termed the crossing point and was determined using a Bio-Rad iQ™ 5 Standard Edition (v2.0). After amplification, melting curve analysis was conducted to exclude any incidence of nonspecific amplification and/or primer-dimer formation. Normalized CT values were obtained using an 18S rRNA standard and the raw threshold cycle (CT) values (Fu et al., 2013). These normalized values and  $2^{-\Delta\Delta C_t}$  method were used together to calculate the relative expression level of each sample (Livak and Schmittgen, 2001). All real-time PCR data were obtained from independent experiments conducted in triplicate.

### Data analysis

Data are reported as means  $\pm$  standard deviation. Statistical significance was tested using one-way ANOVA to compare treatment differences. All statistical tests were conducted using a commercially available statistical package (SPSS 13.0, SPSS Inc.). P values of less than 0.01 were considered to be statistically significant.

## RESULTS

### SS and sub-strain susceptibility to spinosad

In this study, the toxic effects of spinosad on the susceptible, resistant, and treatment strains of *P. xylostella* obtained by selecting these populations by exposing them to a sub-lethal concentration of spinosad for different generations were determined. The sensitivity of the 3rd instar larvae to spinosad was determined using the leaf-dip method after 48 h (Table 1). During the duration of the experiment, the toxicity of spinosad on SS remained relatively constant. The range of 48 h-LC<sub>50</sub> was 0.27-0.65 mg/L, the fluctuation of which was within the 95% confidence interval. The LC<sub>25</sub> for SS was 0.21 mg/L. This concentration was then used as the sub-lethal concentration for the subsequent experiments. When the 3rd instar larvae of SS were treated with 0.21 mg/L spinosad for 48 h, the corrected larval mortality was 24.58%. This finding ascertained that the LC<sub>25</sub> concentration selected for use was appropriate. The LC<sub>50</sub> and LC<sub>25</sub> values for SS and Sub5 strains were nearly the same, even though the slope increased from 1.37 to 1.49. However, the LC<sub>50</sub> of the Sub10 strain was 1.63-times greater than that of SS. Since no overlap between their 95%CI values was noted, this difference was considered to be statistically significant ( $P < 0.05$ ). Furthermore, the regression curve slope increased by 21.9%, indicating that the individuals from the strains that were exposed to sub-lethal concentrations of spinosad showed increased homogeneity with an increased in the number of cycles of selection. RR was showed reduced susceptibility to spinosad after selection, with a resistance ratio of 145.82; it was used as the contrast population in the experiment.

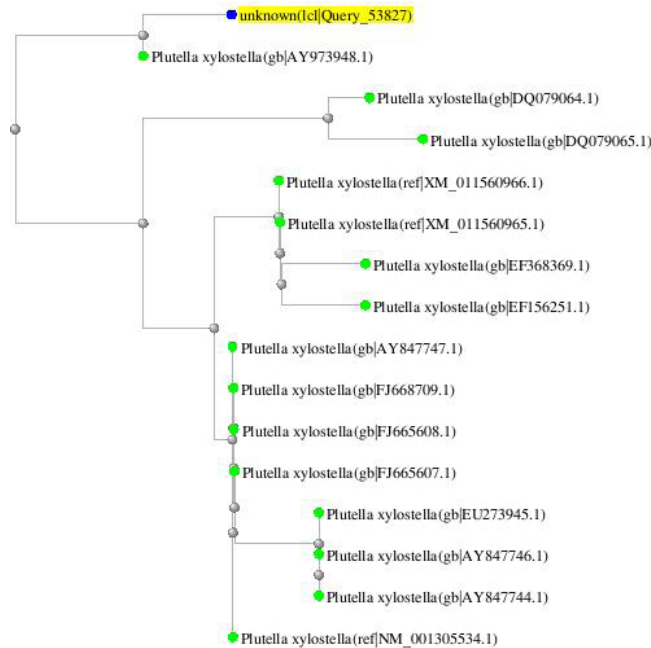
### Molecular cloning of Px-GABAR cDNA

Degenerate primers were generated for the conserved region of the GABAR gene by using the CLUSTAL alignment of previously reported GABAR genetic sequences of *P. xylostella*. These primers were used with the 4th instar larval template cDNA to yield a 248-bp fragment. This fragment was then cloned into the pGEM-T Easy Vector Systems and sequenced. A BLAST search revealed significant homology between this sequence and the *P. xylostella* GABAR gene sequences (Figure 1).

**Table 1.** Susceptibility of different treatment strains of *Plutella xylostella* to spinosad (48 h).

Strains	Bioassay time	N <sup>e</sup>	Slope ± SE	LC <sub>50</sub> (95%CI <sup>f</sup> ) (mg/L)	χ <sup>2</sup>	LC <sub>25</sub> (mg/L)
SS <sup>a</sup>	October, 2013	360	1.72 ± 0.07	0.27 (0.19-0.38)	4.10	0.11
SS	May, 2014	360	1.37 ± 0.07	0.65 (0.48-0.88)	3.68	0.21
Sub5 <sup>b</sup>		360	1.49 ± 0.05	0.65(0.52-0.83)	3.57	0.23
SS	October, 2014	360	1.38 ± 0.04	0.62 (0.50-0.77)	1.31	0.26
Sub10 <sup>c</sup>		360	1.67 ± 0.05	1.06 (0.82-1.37)	2.88	0.42
SS	May, 2015	360	1.37 ± 0.07	0.28 (0.20-0.38)	3.30	0.12
RR <sup>d</sup>		360	1.62 ± 0.05	40.83 (13.81-96.10)	2.47	18.64

<sup>a</sup>SS = Spinosad susceptible strain. <sup>b</sup>Sub5 = LC<sub>25</sub> spinosad-selected SS strain for five generations. <sup>c</sup>Sub10 = LC<sub>25</sub> spinosad-selected SS strain for 10 generations. <sup>d</sup>RR = spinosad resistant strain. <sup>e</sup>Number of larvae tested. <sup>f</sup>Confidence interval.



**Figure 1.** Neighbor-joining analysis performed using BLAST pairwise alignments, indicating the relationships between the known *Plutella xylostella* GABA receptor subunits. Sequences with differences greater than 0.05 were excluded from the analysis.

Specific primers were designed to determine the mature peptide of the GABAR-encoding gene. The mature peptide Px-GABAR cDNA was 1477-bp long. This sequence was compared to those in other insects by using a ClustalW alignment program (v2.0). The deduced Px-GABAR sequence had a high degree of similarity to the GABAR sequences identified in *P. xylostella*, *Heliothis virescens*, *Apis mellifera*, and *Ceratitis capitata*, in which the homology of Px-GABAR with the GenBank accession No. AY973948 was 100%. The homology comparison between the amino acid sequences of AY973948 GABAR and Px-GABAR gene was performed using the DNAMAN software. The results indicated that the homology was 99.80%, and 3 bases were located in the same position, which were replaced by 599 C-T, 829 G-A, and 1323 G-A (Figure 2).

ss	GCAGTTC AAGGTGCTCGGCCATCGCCAGCGGGCCATGGAATCTCCCTTACTACA	605
AY973948	GCAGTTC AAGGTGCTCGGCCATCGCCAGCGGGCCATGGAATCTCCCTTACTACA	605
Consensus	gcagttcaagggtgctcggccatcgccagcggggccatggaaatctccct actaca	
ss	GGAAATTA CTCAAGACTGGCTTGTGAAATACAGTTCGTACGTTCAATGGGATACT	660
AY973948	GGAAATTA CTCAAGACTGGCTTGTGAAATACAGTTCGTACGTTCAATGGGATACT	660
Consensus	ggaaat tactcaagactggcttgtgaaatacagttcgtacgttcaatgggatact	
ss	ATCTTATTCAAATTTATATTCCTTCGGGGCTTATTGTTATTATATCTGGGTATC	715
AY973948	ATCTTATTCAAATTTATATTCCTTCGGGGCTTATTGTTATTATATCTGGGTATC	715
Consensus	atcttattcaaatttatattcttcggggcttattgttattatctgggtatc	
ss	GTTCTGTTGAACCGGAACGCCACGCCCTGCCCGAGTTTCCTTGGGCGTGACGACA	770
AY973948	GTTCTGTTGAACCGGAACGCCACGCCCTGCCCGAGTTTCCTTGGGCGTGACGACA	770
Consensus	gttc tggttgaac cggaac gccac gccctgcccagtttccttggg cgtgacgaca	
ss	GTGTTGACGATGACGACGCTAATGTCGTCCACCAACGCTGCTCTACAAAAATAT	825
AY973948	GTGTTGACGATGACGACGCTAATGTCGTCCACCAACGCTGCTCTACAAAAATAT	825
Consensus	gtgttgacgatgaccacgcta atgtcgtccacc aacgctgctctacaaaaat	
ss	CCTCGTC AAGTCTATAGACGTATATCTGGGTACCTGCTTCGTCATGETGTTTGC	880
AY973948	CCTCGTC AAGTCTATAGACGTATATCTGGGTACCTGCTTCGTCATGETGTTTGC	880
Consensus	cct cgtcaagtc tatagacgtatattctgggtacctgcttcgtcatggtgtttgc	
ss	CCACGTCGTCGGCTGACGATCTGGTCTTCGCGGGAGGATAAATAGAGGCAT	1375
AY973948	CCACGTCGTCGGCTGACGATCTGGTCTTCGCGGGAGGATAAATAGAGGCAT	1375
Consensus	cc acgtcgtgctgacgatctgggtcctcggggaggataa atagagggc at	
ss	TTATACAATCCACTTATTTTTCCACAAAGTTAACCATGGCATTCAATCCGATAA	1430
AY973948	TTATACAATCCACTTATTTTTCCACAAAGTTAACCATGGCATTCAATCCGATAA	1430
Consensus	ttatacaatccacttattttccacaaagttaaccatggcattcaatccgataa	
ss	TAGTCGTTCAAAGCTAGATATTGCATAGTAAGCGCCAATCGCCTT	1476
AY973948	TAGTCGTTCAAAGCTAGATATTGCATAGTAAGCGCCAATCGCCTT	1476
Consensus	tagtcgttcaaaagctagatattgc atagtaagc gccaatcgcctt	

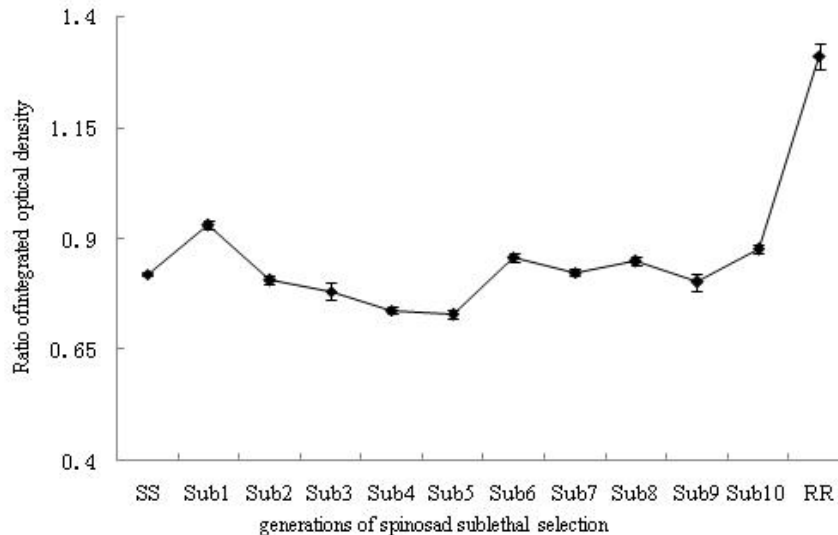
Figure 2. GABAR  $\alpha$  subunit nucleotide sequence alignment obtained using the DNAMAN software.

The ORF of the gene began at the 28th nucleotide and was 1449 bp in total length. The entire sequence encoded a 483-amino-acid-long protein having a calculated molecular weight of approximately 54.16 kDa and a pI of 8.96. The predicted GABAR $\alpha$  encoded an amino acid sequence consisting of the four transmembrane domains, i.e., YYLIQIYIPSGLIVISWVFWL, VSLGVTTVLTMTTLMSTNAALP, VYLGTCFVMVFASLLEYATVGYM, and RIVFPVCFVCFNLMYWIILHVS.

### Expression of the Px-GABAR transcript in different generations of spinosad sublethal selection

Semi-quantitative RT-PCR was used to 1) determine whether Px-GABAR transcripts were present and 2) estimate their approximate expression levels. Varying expression levels of Px-GABAR were identified in the 4th instar larvae of the different generations following spinosad sub-lethal selection. These levels were then compared with those obtained from the internal 18S rRNA control. The transcript levels of Px-GABAR in RR were abundant; these strains had the highest level, whereas the levels were slightly lower in Sub1, SS, Sub2, or Sub6 strains and markedly lower in Sub5 (Figure 3).



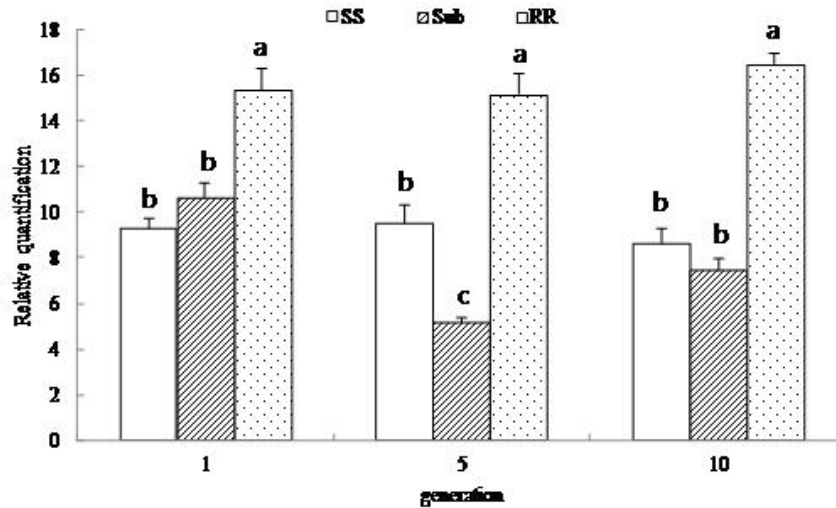


**Figure 3.** Ratio of integrated optical density of GABAR to 18SrRNA expression levels in different strains of *Plutella xylostella*.

A more precise determination of the expression level of Px-GABAR in different generations of spinosad sub-lethal selection populations was achieved by using real-time PCR. The dissociation curves of the experimental versus 18S rRNA controls were analyzed, and a single melting peak was observed. The presence of a single peak suggested the presence of a specific signal, which included both the target sequences and internal control. No fluorescence signal was noted in any of the negative control samples, validating that the extraction procedure and DNaseI treatment were effective to eliminate all genomic DNA from the tested RNA samples. The relative level of Px-GABAR in RR was significantly higher than those in the SS and Sub1 strains ( $F_{2,6} = 18.743$ ,  $P = 0.003$ ), and the difference in the latter two strains was not significant. Relatively low expression of Px-GABAR was found in both the Sub5 (0.56-fold) and Sub10 strains (0.86-fold). Significant differences were noted among SS, Sub5, and Sub10 strains ( $F_{2,6} = 54.019$ ,  $P < 0.001$ ); RR strain showed the highest expression, followed by that in SS strain, and the lowest expression was noted in Sub5. The relative level of Px-GABAR in RR was significantly higher than those in SS and Sub1 strains ( $F_{2,6} = 34.099$ ,  $P < 0.001$ ), and the difference in the expression level between SS and Sub1 strains was not significant (Figure 4). The results of RT-PCR analysis of Px-GABAR expression were consistent with those found using semi-quantitative RT-PCR. Moreover, evaluation of the expression profiles revealed that Px-GABAR mRNA levels changed across generations following sub-lethal spinosad selection. These data indicate that Px-GABA receptor functioning might be related to the susceptibility of *P. xylostella* populations to spinosad.

## DISCUSSION

Our findings clearly showed that the effects of sub-lethal insecticide exposure are distinct across generations. The results obtained for the first generation corroborated



**Figure 4.** Quantitative RT-PCR of Px-GABAR transcript levels found in the 4th instar larvae treated with spinosad. A method for determining the relative expression level was used to evaluate transcript abundance. This method was based on the difference in threshold cycle (CT) values between Px-GABAR and 18S rRNA. Px-GABAR transcript levels were determined at 1, 5, and 10 generations after an initial spinosad treatment in the SS, sub, and RR strains. All data were obtained from a representative assay, which was the mean of three identical replicates  $\pm$  SD. Different letters indicate statistically significant difference ( $P < 0.01$ ) between groups.

those obtained from a pilot experiment. Later generations showed clear changes, and the susceptibility and GABAR gene expression levels changed in response to the stressor. The phenomenon of increasing adaptation of insect and arthropod populations has been observed after exposure to chemical stressors, including tributyltin (Vogt et al., 2007) and ammonia (Kolarevic et al., 2012) and might be a general response to chemical stress. Results from the present study indicated that spinosad sensitivity of the 3rd instar larvae of *P. xylostella* declined as the sub-lethal selection cycles increased. The 3rd instar larvae of the Sub10 strain showed a significant decrease in susceptibility to the stressor (Table 1). This finding is in accordance with the initial data, which were evaluated to determine the appropriate sub-lethal concentration of spinosad for this study (Yin et al., 2009). Although increased variation was noted at several of the selected time points between both the replicates and generations, this finding provides evidence for the alterations that occur in populations being exposed to sub-lethal concentrations of an insecticide over multiple generations and affect the overall fitness of an organism, providing a better understanding of the entire process.

Insecticides are known to predominantly affect the insect GABAR. As such, any mutations in the GABAR subunits are likely to result in increased insecticide resistance (French-Constant et al., 1993a,b; Le Goff et al., 2005; Li et al., 2006). Spinosyn A is the active ingredient in the insecticide spinosad that has a unique mechanism of action: it does not specifically interact with many of the common insecticide targets, including nicotinic or GABA receptors (Orr et al., 2009). Thus, obtaining a better understanding of its physiological and pharmacological attributes by cloning and characterizing the various GABA receptor subtypes is necessary. This might allow the elucidation of the mechanisms of insecticide resistance (Shang et al., 2009).

Our results indicated that the ORF amino acid sequence of the Px-GABAR gene was 1,449-bp long and encoded a 483-amino-acid-long protein containing four transmembrane domains. This result was consistent with that obtained from the sequence analysis of the GABA  $\alpha$  receptor gene of *P. xylostella* and *Spodoptera exigua* (Zhou et al., 2006; Shang et al., 2009).

The findings of the present study show that exposure to a sub-lethal concentration of externally applied spinosad on *P. xylostella* over multiple generations changed with time. The expression of GABAR decreased in Sub5, whereas it suddenly increased from Sub10 to RR. This finding was similar to the changes in LC<sub>25</sub> shown in Table 1. Studies investigating the effects of TBT across multiple generations of *Chironomus riparius* revealed that TBT increased the tolerance toward the stressor in the TBT-exposed group, but did not alter genetic variability (Vogt et al., 2007). However, exogenous compounds are known to affect gene expression levels, which is associated with the binding element within the promoter that interacts with these exogenous compounds (Tan et al., 2014). Diamide insecticide concentration is known to depend on the relative ryanodine receptor expression levels, as found in *P. xylostella* larvae. Thus, the receptor expression gradually increased with increasing levels of diamide (Sun et al., 2012). Similarly, the constitutive over-expression of metabolic genes has been shown to be more important for insect survival than for the activation of members of these gene families after a brief treatment of a lethal spinosad concentration (Willoughby et al., 2006). The ubiquitous reduction of *dADAR* gene expression increased spinosad sensitivity, whereas reduction of *dADAR* expression in either cholinergic neurons or muscle led to a decrease in spinosad susceptibility (Rinkevich and Scott, 2012). Further, considerable evidence suggests that the expression of pest insect orthologues of Da6 in various species can rescue the spinosad response phenotype, indicating the relevance of this receptor for resistance monitoring in the field (Perry et al., 2015). Although whether GABAR is a direct target site of spinosad is not known, available evidence supports that the expression of the GABAR gene is related to the sub-lethal concentration selection generation; the differences in the expression levels of this gene were a kind of rescue response to spinosad. This relationship might be a direct relationship or an indirect cascade reaction to *P. xylostella* susceptibility (Kolarevic et al., 2012).

The results of our study provide evidence for the importance of GABA receptors to insecticide resistance in *P. xylostella*. Future studies are warranted to further elucidate the mechanism underlying this phenomenon. In addition, our results indicate that an adaptive process occurs in response to a sub-lethal exposure of spinosad that could potentially lead to emergence of chemical-resistant insect populations over time.

## ACKNOWLEDGMENTS

We thank Dr. YouJun Zhang and the staff at the Department of Plant Protection Laboratory, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, for supplying the susceptible strain of *Plutella xylostella* and for the valuable information on its culture and handling. This work was funded by grants from the National Natural Science Foundation of China (#31460481 and #21267007), Agricultural Research Projects of the Science and Technology Department of Guizhou Province [(2010)3066, (2012)3010], and the Introduction of Talents Scientific Research Project of Guizhou University [(2008)030].

## REFERENCES

- Abbott WS (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18: 265-267. <http://dx.doi.org/10.1093/jee/18.2.265a>
- Bauer M, Greenwood SJ, Clark KF, Jackman P, et al. (2013). Analysis of gene expression in *Homarus americanus* larvae exposed to sublethal concentrations of endosulfan during metamorphosis. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 8: 300-308. <http://dx.doi.org/10.1016/j.cbd.2013.07.002>
- Beggel S, Werner I, Connon RE and Geist JP (2012). Impacts of the phenylpyrazole insecticide fipronil on larval fish: time-series gene transcription responses in fathead minnow (*Pimephales promelas*) following short-term exposure. *Sci. Total Environ.* 426: 160-165. <http://dx.doi.org/10.1016/j.scitotenv.2012.04.005>
- Besard L, Mommaerts V, Abdu-Alla G and Smagghe G (2011). Lethal and sublethal side-effect assessment supports a more benign profile of spinetoram compared with spinosad in the bumblebee *Bombus terrestris*. *Pest Manag. Sci.* 67: 541-547. <http://dx.doi.org/10.1002/ps.2093>
- Duchet C, Mitie Inafuku M, Caquet T, Larroque M, et al. (2011). Chitobiase activity as an indicator of altered survival, growth and reproduction in *Daphnia pulex* and *Daphnia magna* (Crustacea: Cladocera) exposed to spinosad and diflubenzuron. *Ecotoxicol. Environ. Saf.* 74: 800-810. <http://dx.doi.org/10.1016/j.ecoenv.2010.11.001>
- Frankham R (2005). Genetics and extinction. *Biol. Conserv.* 126: 131-140. <http://dx.doi.org/10.1016/j.biocon.2005.05.002>
- French-Constant RH, Rocheleau TA, Steichen JC and Chalmers AE (1993a). A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature* 363: 449-451. <http://dx.doi.org/10.1038/363449a0>
- French-Constant RH, Steichen JC, Rocheleau TA, Aronstein K, et al. (1993b). A single-amino acid substitution in a  $\gamma$ -aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. *Proc. Natl. Acad. Sci. USA* 90: 1957-1961. <http://dx.doi.org/10.1073/pnas.90.5.1957>
- Fu W, Xie W, Zhang Z, Wang S, et al. (2013). Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). *Int. J. Biol. Sci.* 9: 792-802. <http://dx.doi.org/10.7150/ijbs.5862>
- Le Goff G, Hamon A, Bergé JB and Amichot M (2005). Resistance to fipronil in *Drosophila simulans*: influence of two point mutations in the RDL GABA receptor subunit. *J. Neurochem.* 92: 1295-1305. <http://dx.doi.org/10.1111/j.1471-4159.2004.02922.x>
- Khan HAA, Akram W and Shad SA (2014). Genetics, cross-resistance and mechanism of resistance to spinosad in a field strain of *Musca domestica* L. (Diptera: Muscidae). *Acta Trop.* 130: 148-154. <http://dx.doi.org/10.1016/j.actatropica.2013.11.006>
- Kolarevic J, Takle H, Felip O, Ytteborg E, et al. (2012). Molecular and physiological responses to long-term sublethal ammonia exposure in Atlantic salmon (*Salmo salar*). *Aquat. Toxicol.* 124-125: 48-57. <http://dx.doi.org/10.1016/j.aquatox.2012.07.003>
- LeOra Software (1997). POLO-PC: Probit and Logit analysis. LeOra Software, Berkeley, CA.
- Li A, Yang Y, Wu S, Li C, et al. (2006). Investigation of resistance mechanisms to fipronil in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 99: 914-919. <http://dx.doi.org/10.1093/jee/99.3.914>
- Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>- $\Delta\Delta C_t$</sup>  Method. *Methods* 25: 402-408. <http://dx.doi.org/10.1006/meth.2001.1262>
- Martínez-Villar E, Sáenz-De-Cabezón FJ, Moreno-Grijalba F, Marco V, et al. (2005). Effects of azadirachtin on the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). *Exp. Appl. Acarol.* 35: 215-222. <http://dx.doi.org/10.1007/s10493-004-5082-6>
- Morandin LA, Winston ML, Franklin MT and Abbott VA (2005). Lethal and sub-lethal effects of spinosad on bumble bees (*Bombus impatiens* Cresson). *Pest Manag. Sci.* 61: 619-626. <http://dx.doi.org/10.1002/ps.1058>
- Orr N, Shaffner AJ, Richey K and Crouse GD (2009). Novel mode of action of spinosad: Receptor binding studies demonstrating lack of interaction with known insecticidal target sites. *Pestic. Biochem. Physiol.* 95: 1-5. <http://dx.doi.org/10.1016/j.pestbp.2009.04.009>
- Perry T, Somers J, Yang YT and Batterham P (2015). Expression of insect  $\alpha 6$ -like nicotinic acetylcholine receptors in *Drosophila melanogaster* highlights a high level of conservation of the receptor:spinosyn interaction. *Insect Biochem. Mol. Biol.* 64: 106-115. <http://dx.doi.org/10.1016/j.ibmb.2015.01.017>
- Rinkevich FD and Scott JG (2012). Reduction of *dADAR* activity affects the sensitivity of *Drosophila melanogaster* to spinosad and imidacloprid. *Pestic. Biochem. Physiol.* 104: 163-169. <http://dx.doi.org/10.1016/j.pestbp.2012.06.008>
- Ruiz L, Flores S, Cancino J, Arredondo J, et al. (2008). Lethal and sublethal effects of spinosad-based GF-120 bait on the tephritid parasitoid *Diachasmimor phalngicaudata* (Hymenoptera: Braconidae). *Biol. Control* 44: 296-304. <http://dx.doi.org/10.1016/j.biocontrol.2007.10.022>
- Salgado VL (1998). Studies on the mode of action of spinosad: symptoms and physiological correlates. *Pestic. Biochem.*

- Physiol.* 60: 91-102. <http://dx.doi.org/10.1006/pest.1998.2332>
- Sayed AE and Sheikh E (2015). Comparative toxicity and sublethal effects of emamectin benzoate, lufenuron and spinosad on *Spodoptera littoralis* Bois. (Lepidoptera: Noctuidae). *Crop Prot.* 67: 228-234. <http://dx.doi.org/10.1016/j.cropro.2014.10.022>
- Schneider MI, Smagge G, Pineda S and Viñuela E (2004). Action of insect growth regulator insecticides and spinosad on life history parameters and absorption in third-instar larvae of the endoparasitoid *Hyposoter didymator*. *Biol. Control* 31: 189-198. <http://dx.doi.org/10.1016/j.biocontrol.2004.04.013>
- Shang QL, Liang P and Gao XW (2009). Cloning, developmental and tissue-specific expression of  $\gamma$ -aminobutyric acid (GABA) receptor alpha2 subunit gene in *Spodoptera exigua* (Hübner). *Pestic. Biochem. Physiol.* 93: 1-7. <http://dx.doi.org/10.1016/j.pestbp.2008.07.006>
- Singh JP and Marwaha KK (2000). Effects of sublethal concentrations of some insecticides on growth and development of maize stalk borer, *Chilo partellus* (Swinhoe) larvae. *Shashpa* 7: 181-186.
- Sun L, Cui L, Rui C, Yan X, et al. (2012). Modulation of the expression of ryanodine receptor mRNA from *Plutella xylostella* as a result of diamide insecticide application. *Gene* 511: 265-273. <http://dx.doi.org/10.1016/j.gene.2012.09.027>
- Tan Y, Xiao L, Sun Y, Zhao J, et al. (2014). Sublethal effects of the chitin synthesis inhibitor, hexaflumuron, in the cotton mirid bug, *Apolygus lucorum* (Meyer-Dür). *Pestic. Biochem. Physiol.* 111: 43-50. <http://dx.doi.org/10.1016/j.pestbp.2014.04.001>
- Vogt C, Nowak C, Diogo JB, Oetken M, et al. (2007). Multi-generation studies with *Chironomus riparius*--effects of low tributyltin concentrations on life history parameters and genetic diversity. *Chemosphere* 67: 2192-2200. <http://dx.doi.org/10.1016/j.chemosphere.2006.12.025>
- Watson GB, Chouinard SW, Cook KR, Geng C, et al. (2010). A spinosyn-sensitive *Drosophila melanogaster* nicotinic acetylcholine receptor identified through chemically induced target site resistance, resistance gene identification, and heterologous expression. *Insect Biochem. Mol. Biol.* 40: 376-384. <http://dx.doi.org/10.1016/j.ibmb.2009.11.004>
- Willoughby L, Chung H, Lumb C, Robin C, et al. (2006). A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and phenobarbital. *Insect Biochem. Mol. Biol.* 36: 934-942. <http://dx.doi.org/10.1016/j.ibmb.2006.09.004>
- Xue JX, Qiao J and Zhong CX (2005). Toxicity of spinosad to *Plutella xylostella* (L.) and its natural enemies. *Chin. J. Eco Agric.* 13: 161-163.
- Yin XH, Wu QJ, Li XF, Zhang YJ, et al. (2009). Demographic changes in multigeneration *Plutella xylostella* (Lepidoptera: Plutellidae) after exposure to sublethal concentrations of spinosad. *J. Econ. Entomol.* 102: 357-365. <http://dx.doi.org/10.1603/029.102.0146>
- Zhou XM, Wu QJ, Hu MY, Zhang YJ, et al. (2006). Cloning and expression characteristics of cDNA fragment of GABA  $\alpha$  receptor gene in diamondback moth (*Plutella xylostella*). *Chin. Acta Hort. Sin.* 33: 300-305.