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Mechanisms of TiO_2 NPs-induced phoxim metabolism in silkworm (*Bombyx mori*) fat body

J.S. Hu [a,1], F.C. Li [a,1], K.Z. Xu [a,1], M. Ni [a], B.B. Wang [a], J.H. Tian [a], Y.Y. Li [a], W.D. Shen [a,b], B. Li [a,b,*]

[a] School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu 215123, PR China
[b] National Engineering Laboratory for Modern Silk, Soochow University, Suzhou, Jiangsu 215123, PR China

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ABSTRACT

Silkworm is an important economic insect. Abuse of organophosphorus pesticides in recent years often leads to poisoning of silkworms, which significantly affects sericulture development by reducing silk production. Previous studies have shown that TiO_2 NPs can effectively mitigate the damages caused by organophosphorus pesticides in silk glands and nerve tissues. The fat body is an important metabolic detoxification organ of silkworms, but it is unknown whether TiO_2 NPs affect pesticide metabolism in fat body. In this study, we characterized the transcription of antioxidant genes and enzyme activity in fat body after TiO_2 NPs and phoxim treatments using transcriptome sequencing, real-time PCR, and enzyme activity assay. Transcriptome sequencing detected 10 720, 10 641, 10 403, and 10 489 genes for control group, TiO_2 NPs group, phoxim group, and TiO_2 NPs + phoxim group, respectively. The TiO_2 NPs + phoxim group had 705 genes with significantly differential expression (FDR < 0.001), among which the antioxidant genes thioredoxin reductase 1 and glutathione S-transferase omega 3 were significantly upregulated. In phoxim group, the expression levels of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase delta (GSTd), and thioredoxin peroxidase (TPx) were increased by 1.365 -fold, 1.335 -fold, 1.642 -fold, and 1.765 -fold, respectively. The level changes of SOD, CAT, GSTd, and TPx were validated by real time PCR. The contents of reactive oxygen species (ROS), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) were increased by 1.598 -fold, 1.946 -fold, and 1.506 -fold, respectively, indicating that TiO_2 NPs treatment can relieve phoxim-induced oxidative stress. To clarify the mechanism of TiO_2 NPs's effect, the transcription levels of P450 gene family were measured for the TiO_2 NPs + phoxim group; the expression levels of *CYP4M5*, *CYP6AB4*, *CYP6A8*, and *CYP9G3* were elevated by 2.784 -fold, 3.047 -fold, 2.254 -fold, and 4.253 -fold, respectively, suggesting that high expression of P450 family genes can enhance the metabolism of phoxim in the fat body. The results of this study indicated that TiO_2 NPs treatment promoted the transcriptional expression of the P450 family genes to improve the fat body's ability to metabolize phoxim and reduce phoxim-induced oxidative stress. This may be the main mechanism of TiO_2 NPs' mitigation of phoxim-induced damages in the fat body.

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1. Introduction

Silkworm is a Lepidopteran insect with a 5700 years of domestication history, and it plays an important role in mankind's economic and cultural lives [1,2]. In recent years, wide use of phoxim pesticide in agriculture and forestry often leads to mulberry contamination and silkworm poisoning, which seriously affects the healthy development of sericulture [3]. In China, the annual raw silk production is reduced by up to 30% due to pesticide contamination [4]. After phoxim poisoning, silkworm's nutrition metabolism, growth and development, and reproduction are all affected [5,6]. Fat body is an important insect organ with multiple physiological functions, including energy storage, detoxification, immunity, and hormone level regulation [7–9]. Nath B.S. and Bollenbacher W.E. studied insect fat body's role in phoxim pesticide metabolism [10,11]. The cytochrome P450 (CYP450) family proteins are important detoxification enzymes in the fat body, and their transcriptional levels have been shown to be closely correlated with pesticide metabolism [11–13]. Feng Tang et al. reported that CYP450 oxidases play an important role in cotton bollworm's pesticide resistance, with their activities being increased by 2.3-fold in resistant strains compared with the sensitive strains [14].

TiO_2 NPs are a new additive for silkworms that can effectively improve raw silk production [15,16]. It can also reduce phoxim-induced damages in silkworm nerves, silk gland, and midgut, and ease oxidative stress [17–19]. However, the mechanism of TiO_2 NPs on phoxim metabolism in fat body remains unknown.

* Corresponding author at: School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu 215123, PR China.
E-mail address: lib@suda.edu.cn (B. Li).
[1] These authors contributed equally to this study.

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2. Materials and methods

2.1. Chemicals

TiO_2 NPs (99.99%, analytical purity) was purchased from Hangzhou Wanjing New Material Ltd. TiO_2 NPs was first prepared as 5 g/L stock solution and diluted to 5 mg/L for experiments [18,20].

Phoxim [O,O-diethyl O-(alpha-cyanobenzylideneamino) phosphorothioate] was purchased from Sigma-Aldrich with a purity grade of 98.10%. First, acetone was added to the powder of phoxim to prepare for the stock solution (100 g/L), and working solution was prepared by dilution using double-distilled water (4 mg/L) [21].

2.2. Insects and treatments

Silkworms (Jingsong × Haoyue) were reared in the laboratory with a cycle of 12 h light/12 h dark until fifth instar larvae. Fifth instar larvae were divided into 4 groups with 100 silkworms in each group: control group, TiO_2 NPs group, phoxim group, and TiO_2 NPs + phoxim group. The control group and phoxim group silkworms were fed with clear water-treated mulberry leaves, while TiO_2 NPs group and TiO_2 NPs + phoxim group were fed with TiO_2 NPs-treated leaves; all silkworms were fed 3 times daily. On the 4th day, phoxim group and TiO_2 NPs + phoxim group silkworms were fed instead with phoxim-treated mulberry leaves. After 24 h, all silkworms were dissected to isolate fat bodies that were to be preserved at −80 °C [17].

2.3. RNA-Seq (quantification) analysis

The total RNA samples were first treated with DNase I to eliminate DNA contamination, and mRNAs were enriched using oligo(dT) magnetic beads (for eukaryotes). After being mixed with fragmentation buffer, mRNAs were fragmented into ~200 bp. First strand cDNA was synthesized using random hexamer-primers. Reaction buffer, dNTPs, RNase H, and DNA polymerase I were used for second strand synthesis. Double strand cDNA was purified with magnetic beads, followed by end repair and 3′-end single nucleotide A addition. Finally, sequencing adaptors were ligated into the fragments, which were enriched by PCR amplification. During the QC step, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System was used to qualify the sample library. The library products were sequenced using Illumina HiSeqTM 2000 in BGI Shenzhen Technology Services Ltd.

2.4. Total RNA isolation and sample preparation

Trizol reagent (Takara) was used to extract total RNA from the fat body of phoxim-fed and control silkworm larvae after 24 h. The total RNA was treated with DNase I to remove potential genomic DNA contamination. First-strand cDNA was synthesized with M-MLV reverse transcriptase and oligo (dT) primer.

2.5. Gene expression levels

Gene expression levels were calculated by using the RPKM method [22] (Reads Per kb per Million reads), and the formula is:

$$\mathrm{RPKM} = \frac{10^6 C}{NL/10^3}.$$

Here, RPKM(A) is the expression level of gene A; C is the number of reads that were uniquely aligned to gene A; N is the total number of reads that were uniquely aligned to all genes; L is the number of bases of gene A. The RPKM method eliminated the effect of differences in gene length and the sequencing discrepancy on the calculation of gene expression levels. Therefore, the RPKM values can be used for the direct comparison of gene expression levels. If one gene has more than one transcript, the longest one was used for expression level calculation and coverage.

2.6. Screen for differentially expressed genes (DEGs) between samples

The number of unambiguous clean tags from gene A was denoted as x; every gene's expression occupied only a small part of the library; and $p(x)$ closely followed the Poisson distribution.

$$p(x) = \frac{e^{-\lambda}\lambda^x}{x!} (\lambda \text{ is the real transcripts of the gene})$$

The total clean tag number of sample 1 is N1, and the total clean tag number of sample 2 is N2; gene A holds x tags in sample 1 and y tags in sample 2. The probability of gene A being expressed equally between two samples was calculated with:

$$2\sum_{i=0}^{i=y} \mathrm{p}(\mathrm{i}|x) \ \text{ or } \ 2 \times \left(1 - \sum_{i=0}^{i=y} \mathrm{p}(\mathrm{i}|x)\right) \left(\textit{if} \ \sum_{i=0}^{i=y} \mathrm{p}(\mathrm{i}|x) > 0.5\right)$$

$$p(y|x) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1 + \frac{N_2}{N_1}\right)^{(x+y+1)}}$$

P-value corresponds to the differential gene expression test. FDR (False Discovery Rate) is a method to determine the threshold of P-value in multiple tests. Theoretically, if we picked R differentially expressed genes but only S genes were really differentially expressed, the rest V genes were false positive. If the error ratio "Q = V/R" stayed below a cutoff (e.g. 1%), the FDR should be no larger than 0.01 [23]. We used "FDR ≤ 0.001 and the absolute value of $\log_2$ Ratio ≥ 1" as the threshold to determine the significance of gene expression differences. More stringent criteria with smaller FDR and bigger fold-change value may be used to identify DEGs.

2.7. Quantitative real-time PCR

All real time PCR primers were designed using Primer 5.0 software, and the internal reference gene was actin 3 (Table 1). Real-time qPCR was performed using the Via 7 Real-time PCR System (ABI) with SYBR

Table 1
Primer sequences used in quantitative RT-PCR.

Probe name	Gene	Primer sequences (5′-3′)	Produce size(bp)
CAT	CAT	ACTACGGAGTGCTGGACG TCGGGCTTGATGGTCTT	217
TPx	TPx	ACTACGGAGTGCTGGACG TCGGGCTTGATGGTCTT	217
GSTd	GSTd	GCATACGGCAAAGACGATT TGTCGGCTTTCTCCTGATC	163
SOD	SOD	TCCTCAAGCACCTACACC GATAGCCAATGATACACG	216
CYP6AE2	Cyp6AE2	CACGCTAGACTCTATCTGCTC ATACGATGCTTCCCATGTTT	101
CYP6AB4	Cyp6AB4	TTTGATAGTCCGAATGAATTCCGAC CATTAAGCCAAGGCGAGCA	132
CYP9G3	Cyp9G3	CAGCAAGAACATCGTGGAG ATTAACCTGGAGTCCGAAA	121
CYP4M5	Cyp4M5	TCCAAAGTTGTCGTTCATTC AGGAAACGCCCAAATCCTAT	127
CYP4L6	Cyp4L6	TCTCGCTGATGGACAACC GAGCATTCACTGACACCC	98
CYP306	Cyp306a1	TTTCAAACTGGTAAGCGGATGT CGACGGTTCCACGCATCT	142
CYP6A8	Cyp6A8	TTGGTCGTTCGATGTTG GGTTCGTCGGCTTGTAG	129
Actin 3	Actin 3	CGGCTACTCGTTCACTACC CCGTCGGGAAGTTCGTAAG	147

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