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*Full Length Research Paper*

# A proteomic analysis of short-term exposure to fipronil in larvae of *Plutella xylostella*

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**This study aimed at analyzing the short-term physiological response of *Plutella xylostella* L. (Diamondback moth), an insect pest of vegetables, to fipronil using two-dimensional gel electrophoresis and mass spectrometry. Protein expression in this insect was observed at 8,16 and 24 h after a fipronil treatment. Differential expression was examined for 20 protein spots, among which nine spots were down-regulated, seven spots were up-regulated and four spots were expressed only in the treated group. 17 protein spots were successfully identified, including proteins that participated in the immune response and in other metabolic pathways, cytoskeletal proteins and molecular chaperones. Differences in the expression of these proteins suggest that *P. xylostella* created general changes in the immune response, metabolic level and many other characteristics in response to the pesticide. This study could facilitate our better understanding of the physiological response of *P. xylostella* to pesticides.**

**Key words:** Diamondback moth, fipronil, toxicity response, two-dimensional gel electrophoresis.

## INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.), is one of the most important pests of cruciferous vegetables (Talekar and Shelton, 1993). This insect pest is difficult to control as it can increase its tolerance to many kinds of synthetic insecticides and bioinsecticides rapidly (Takeda et al., 2006). When exposed to insecticides, the behavior and metabolic changes of this insect would lead to various responses to insecticides. The responses may involve multiple physiological changes which cannot be assigned to only a single gene or protein.Among the types of insecticides used to control *P. xylostella*, fipronil remains one of the most frequently reported insecticides. Fipronil is the first member of the phenylpyrazole insecticide. Its mode of action involves the disruption of chloride ion flow by either interacting at the gamma-aminobutyric acid (GABA)-gated chloride channel/ionopore complex of the central nervous system, or by potently blocking the glutamate-induced chloride ion flow (Zhao et al., 2004). Most studies on fipronil focused on gene mutations and physiological changes including detoxifying enzyme activities of *P. xylostella* (Li et al., 2006; Mohan and Gujar, 2003). However, the understanding of *P. xylostella* biochemical and molecular mechanisms in response to fipronil exposure remains limited.

Proteomics has emerged a powerful method for gaining insight to different physiological changes at cellular levels and has been applied in several insect studies (Scharlaken et al., 2007; Zhou et al., 2008; Pauchet et al., 2009). Differential proteomics which is mainly intended to identify proteins that are up- or down-regulated during normal status and specific status, has been used in many essays including induced immunity, resistance mechanism and pathology (Sharma et al., 2004; Nguyen et al., 2008; Song et al., 2008; Jin et al., 2010).

In this study, the molecular basis of the toxicity of fipronil against the diamondback moth using a differential

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**Abbreviations**: **2-DE**, Two-dimensional gel electrophoresis; **MALDI-TOF-MS/MS**, matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry; **IEF**, isoelectric focusing; **IPG**, immobilized pH gradient; **PI**, isoelectric point; **TPX**, thioredoxin peroxidase.

proteomics approach, and the protein response to the fipronil treatment were identified through two dimensional polyacrylamide gel electrophoresis (2-DE) and MALDI-TOF MS/MS. The objective of this research was to develop a comprehensive mechanical understanding of the toxicity response of this insect species to the fipronil.

## MATERIALS AND METHODS

### Insects rearing

In this study, we used a susceptible strain of *P. xylostella*, which has been maintained in the laboratory for over six years without exposure to insecticides. This strain was previously collected from the vegetable field of Fuzhou, Fujian, China in July 2004. The *P. xylostella* larvae were reared with radish, *Raphanus sativus* L., seedlings at 25 ± 1°C, 65 to 75% RH, a photoperiod of 16:8 h (L:D), and 10% honey water was provided as additional nutrition for the adults.

### Bioassay

The technical fipronil (95% purity) was provided by Zhejiang Yongnong Biosciences Co. Ltd. To do the bioassay, six concentrations of fipronil (0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg/L) were prepared with distilled water containing 0.1% (v/v) Tween-20. Then the leaf-dip method was used in this study (Li et al., 2006). Cabbage, *Brassica oleracea* (L.), leaf discs of 6.5 cm in diameter were cut and dipped in one of the insecticide solutions for 10 s. Control discs were treated with 0.1% Tween-20 solution only. The leaf discs were then dried at room temperature for about 2 h.

Each treated leaf disc was placed in a plastic Petri dish with ten *P. xylostella* third instar larvae and kept at 25 ± 1°C. Three replicates were set for each concentration. Mortality was assessed after 24 h. Middle lethal concentration ($LC_{50}$) and 95% confidence intervals were then calculated using a probit regression analysis in SPSS 15.0 (Tao and Wu, 2005). The $LC_{50}$ value of the fipronil to the third-instar larvae was found to be 0.24 mg/L. This concentration was then used in all later proteomic and protein analyses.

### Treatment of the insects

For protein extraction, the same aged day third instar larvae (body length about 4 mm) were used. A solution containing the $LC_{50}$ (0.24 mg/L) of fipronil was prepared and sprayed onto fresh insecticide-free radish seedlings. After air-dried for about 2 h, 200 to 300 third instar larvae which has been starved for about 2 h were transferred into the seedlings. Surviving larvae were randomly collected at 8, 16 and 24 h after treatment and were frozen in liquid nitrogen for protein extraction. The larvae in the control group were fed on radish seedlings sprayed with clean water and collected at 0 h. This experiment was repeated three times.

### Protein preparation and extraction

Proteins were prepared according to the methods described by Cilia et al. (2009). After freezing in liquid nitrogen, the whole larvae were grounded into a powder and added directly to 10% trichloroacetic acid (TCA) in acetone containing 0.7% β-mercaptoethanol (ME) (1 g insect tissue:10 ml TCA-acetone w/v), and then transferred to 15 ml centrifuge tube and vortexed for 5 min. Furthermore, the proteins were allowed to precipitate overnight at -20°C. Precipitated protein was centrifuged at 20,000 × *g* for 30 min at 4°C. The precipitate was washed with 80% acetone followed by cool pure acetone and air-dried for about 15 min and then stored at -70°C until essay.

Protein lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% Bio-Lyte pH 3 to 10) was added to the rough protein powder at a concentration of 1 mg/20 µl. To increase solubility, the centrifuge tube containing the protein sample was floated in a sonicating water bath containing cold water. The resulting mixture was centrifuged at 20,000 × *g* for 30 min at 18°C. The supernatant was subjected to 2-DE. Protein quantification was undertaken according to Bradford (1976).

### Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out using a PROTEAN IEF isoelectric focusing system (Bio-Rad) with 17 cm gel strips (pH 5 to 8, linear, Bio-Rad). Protein samples (about 2 mg) were loaded during the rehydration step (12 h, 50 V). The electrophoresis parameters were as follows: 20°C, rehydration at 50 V for 12 h, 250 V for 0.5 h, 1000 V for 1 h, 4000 V for 1 h, 4000 to 10,000 V gradients for 5 h and 10,000 V for a total of 60,000 VHS. Before SDS-PAGE, the IPG strips were equilibrated for 15 min in an equilibration buffer [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT], followed by a further 15 min in another equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT. After transferring the gel strips onto vertical 12% SDS-PAGE gels, electrophoresis was performed at 18°C. Gels were stained with Coomassie Brilliant Blue (CBB) R-250.

Quantitive analysis of images was performed with PDQuest version 7.3 (Bio-Rad, Hercules, CA, USA) according to the protocol provided by the company. The gel images were normalized by total valid spot intensity. To study the expression changes in detail, the control gels were set as reference to be compared qualitatively and quantitatively with the treatment group. Comparison of the intensity abundance between the control and treatment groups (three replicate samples for each group) was performed using the Student's test. Protein spots in which the expression level was 2-fold or more than the control were selected as significant changes.

### In-gel digestion

20 protein spots of interest were manually excised from the CBB-stained gels and then transferred into V-bottom 96-well microplates pre-loaded with 100 µl of a 50% acetonitrile (ACN)/25 mM ammonium bicarbonate solution per well. After being detained for 1 h, the gel plugs were dehydrated with 100 µl of 100% ACN for 20 min and then thoroughly dried in a SpeedVac concentrator (Thermo Savant, USA) for 30 min. The dried gel particles were rehydrated at 4°C for 45 min with 2 µl trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate per well and then incubated at 37°C for 12 h. After trypsin digestion, the peptide mixtures were extracted with 8 µl extraction solution [50% ACN/0.5% trifluoroacetic acid (TFA)] per well at 37°C for 1 h. Finally, the extracts were dried under the protection of $N_2$ atmosphere. The peptides were eluted with 0.8 µl matrix solution [α-cyano-4-hydroxy-cinnamic acid (CHCA) in 0.1% TFA, 50% ACN] before spotting onto the target plate. The samples were allowed to air-dry.

### MALDI-TOF-MS/MS analysis and database searching

Protein analysis was performed using a 4700 MALDI-TOF/TOF proteomics analyzer (Applied Biosystems, Foster City, CA, USA).

Peptides were evaporated with a UV laser at 355 nm. They were then accelerated by 20 kV injection pulse. Spectra were calibrated with trypsin-digested myoglobin as internal standards. All acquired spectra of the samples were processed using the 4700 Explore™ software (Applied Biosystems) in default mode. The parent mass peaks that ranged from 700 to 3200 Da, and had a minimum S/N of 20 were selected for MS/MS analysis.

Combined MS and MS/MS spectra were submitted to MASCOT (Matrix Science, London, UK) by GPS Explorer software (Applied Biosystems, USA). A search was performed for each protein in both the NCBInr database and the EST database. The search parameters used were as follows: Taxonomy of Metazoa (Animals), trypsin digestion with one missing cleavage, no fixed modifications, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. According to the MASCOT probability analysis ($p < 0.05$), only significant hits were accepted.

#### Diamondback moth EST analyses

Peptide sequences obtained from the earlier mentioned proteomic analyses were subjected to a search for corresponding gene in an expressed sequence tags (EST, single-pass cDNA sequences) database of *P. xylostella*, in our laboratory. The genome sequence of *P. xylostella* remained incomplete; thus, the information provided in the NCBI protein database is limited. The use of the EST database can increase the protein identification rate. The reliability of identification from EST database was validated in previous article (Sharma et al., 2004; Xie et al., 2009). This database was prepared from eggs, larvae, pupae and adults of *P. xylostella* by mRNA extraction, cDNA synthesis, cloning into plasmids and sequencing the insert DNA. Each of over 100,000 EST nucleotide sequences was translated into six open reading frames and the translated amino acid sequences were searched for the peptide sequences from the proteomic analyses. The matched EST sequences were subjected to a search for corresponding protein in the public database (NCBI) to confirm the nature of assigned protein.

## RESULTS

### 2-DE map and different expression of proteins

2-DE was undertaken to compare the proteome patterns between the control and the treatment groups of *P. xylostella* by fipronil with time points of analysis. Over 600 spots were detected in each 2-D gel stained with CBB, using the control and the 8, 16, 24 h treatments (Figure 1). The comparative analysis of the 2-DE maps of *P. xylostella* larvae proteins using PDQuest software resulted in a total of 20 differential protein spots. The spot match rate of gels was about 70%. A two-fold change was observed between the treatment and control groups. These spots were indicated in their corresponding positions on the 2-DE map (Figure 2) and the results of spot quantitative analysis are expressed as mean ± SD (Figure 3). Nine spots were down-regulated: 1, 2, 7, 9, 10, 13, 14, 15 and 17. Among them, spot 7 was absent in the map after the 24 h fipronil treatment and spot 17 was not expressed after the 16 h fipronil treatment. 11 spots were up-regulated: 3, 4, 5, 6, 8, 11, 12, 16, 18, 19 and 20. Among them, spots 12, 16, 18 and 20 were expressed only in the treatment group. Changes in protein profiles only in the treatment group. Changes in protein profiles were most obvious at 8 h as the short-term response to fipronil.

### Protein identification

Using NCBInr protein database and the EST database (171, 262 EST sequence data) of *P. xylostella* in our laboratory, using Perl program to translate nucleotides to amino acids, 17 proteins were identified and they showed homology to hypothetical proteins from different organism (identification rate: 85%) (Table 1).

The theoretical and experimental molecular mass (Mr) and isoelectric point (pI) data were not always in agreement. These discrepancies could be attributed to post-translational modifications (especially phosphorylations and glycosylations), multiple protein isoforms and different protein compositions utilized by *P. xylostella* homologs of the reported proteins (Untalan et al., 2005).

Based on their physiological functions, the identified proteins were classified into the following categories: stress-responsive proteins, metabolism-associated proteins, ATP transfer-associated proteins and other proteins (Figure 4).

## DISCUSSION

At present, there have been relatively fewer attempts made to apply proteomic approaches to study insect adaptation and response to toxic substances. The short-term response of *P. xylostella* to fipronil induced changes in proteins that are involved in the stress-responsive proteins, multiple metabolic pathways, ATP transfer and others. Our results suggest that such a pesticide may have multiple effects on *P. xylostella*.

### Proteins involved in stress response

In this study, four proteins involved in stress response were affected in *P. xylostella* by fipronil. Spot 18 was identified as heat shock protein 19.9 (HSP19.9), a family of small stress-induced proteins under physiological and stress conditions. According to some researchers, the expression of heat shock proteins may adversely affect feeding and reproduction in *Liriomyza sativa* (Huang et al., 2009). After treatment with chlorfenapyr, the gene expression levels of *hsp70*, *hsp20.7* and *hsp19.7* in a cell line of *Mamestra brassicae* increased remarkably (Sonoda and Tsumuki, 2007). In this study, HSP19.9 expression was up-regulated in the fipronil-treated group, but no expression at the protein level was detected in the control group. The increase in expression of *hsp* was postulated to maintain homeostasis under stress induced by exposure to fipronil.

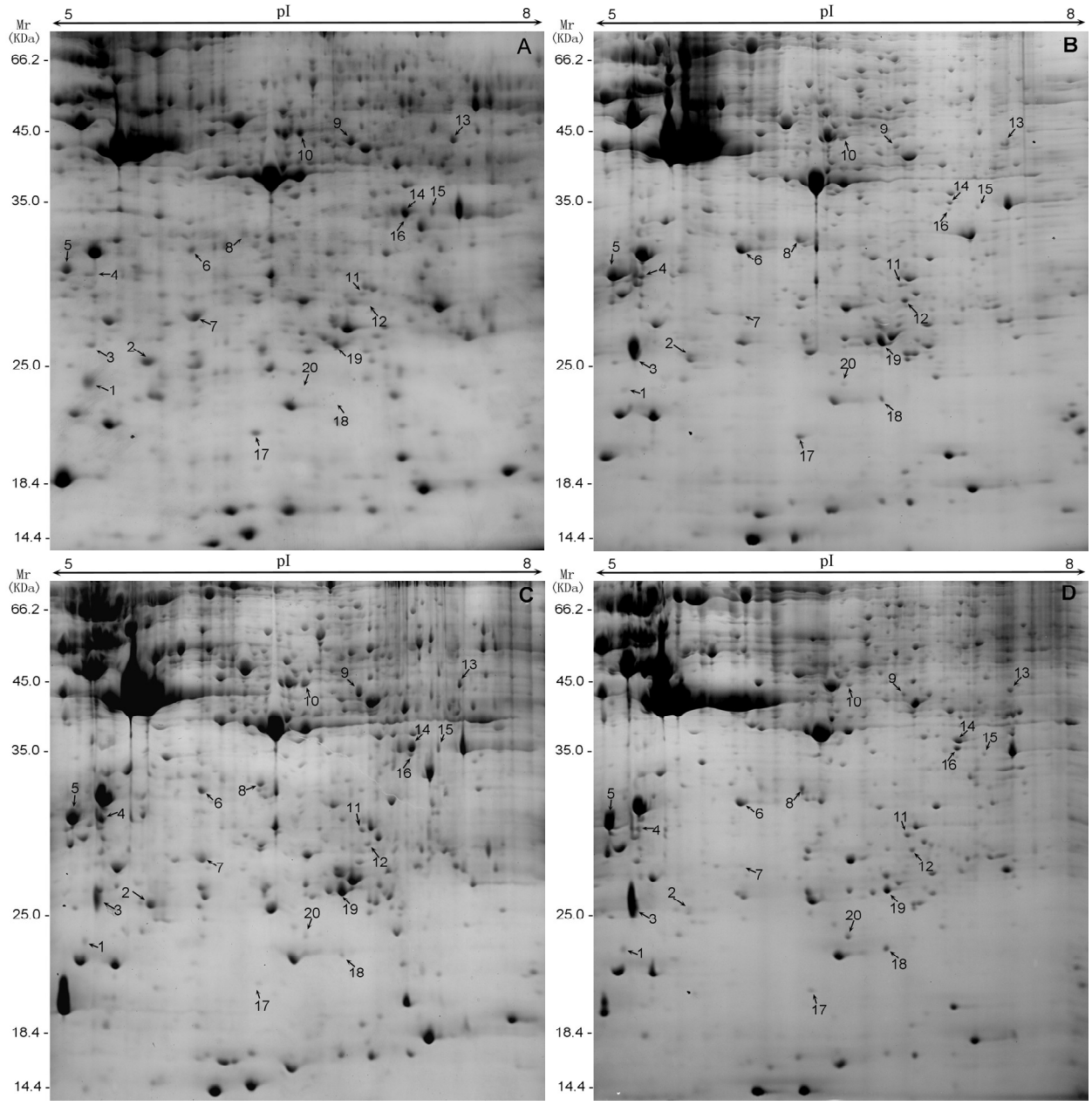


**Figure 1.** 2-DE analysis of proteins identified with coomassie brilliant blue staining from (A) control, (B) fipronil-treated *P.xylostella* after 8 h of treatment, (C) fipronil-treated *P.xylostella* after 16 h of treatment, and (D) fipronil-treated *P.xylostella* after 24 h of treatment.

Thioredoxin peroxidase (TPX, spot 19) was up-regulated in the fipronil-treated group. This type of peroxide reduces oxidation in the body using thioredoxin as its only hydrogen donor. Thus, it is called TPX and it belongs to the peroxiredoxin (PRX) anti-oxidative group of enzymes (Hu et al., 2010). The main functions of TPX are detoxification, resistance against oxidative stress and adjustments of hydrogen peroxide-mediated signal transduction as well as immune reactions (Collins et al., 2010). In silkworms, TPX plays a protective role in coping with the oxidative stress caused by extreme temperatures or viral infections (Lee et al., 2005). Two forms of TPX in aphids were up-regulated after parasitism treatments (Nguyen et al., 2008). In this study, the expression of TPX was up-regulated. The phenomenon postulate that, the elimination of free radicals is beneficial and it improves the detoxification ability of cells.

C-type lectin, a $Ca^{2+}$-dependent carbohydrate-binding protein, is an inherent immune component that is normally present in insects. The transcriptional level of C-type

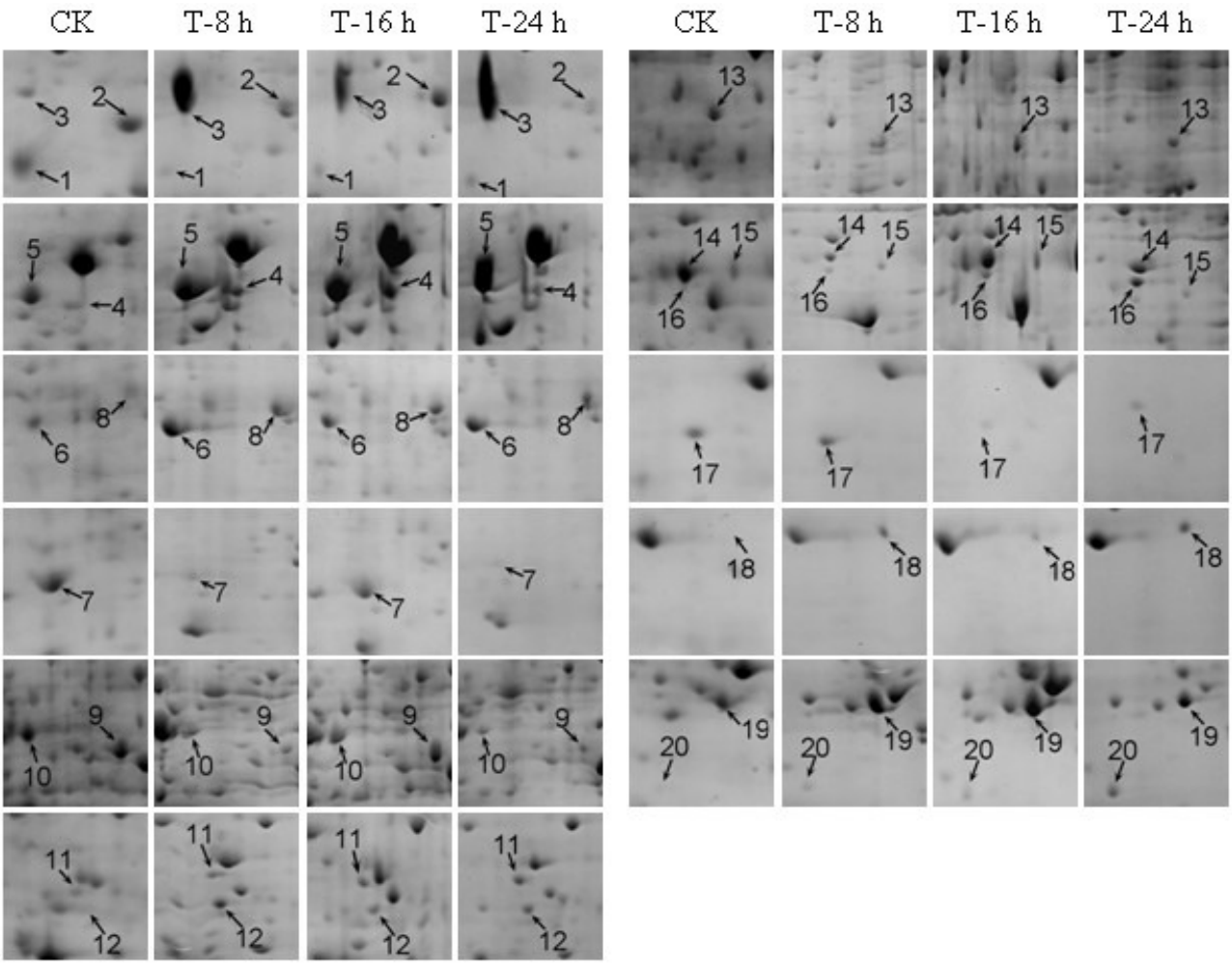


**Figure 2.** Close-up views of representative protein spots that were differentially regulated in *P. xylostella* in the control and after fipronil $LC_{50}$ treatment for 8, 16 and 24 h.

lectin in the larval body was markedly increased after *Heliothis virescens* has been bacterially infected (Shelby and Popham, 2009). The change of lectin levels was reported after silkworms were infected by microorganisms (Liu et al., 2009). Bacteria and viruses can induce lectin expression in the blood corpuscles of cotton bollworms (Chai et al., 2008). Lectin as a pattern recognition receptor in *Drosophila* larvae, regulates blood corpuscle cysts and causes melanization (Ao et al., 2007).

In this study, C-type lectin (spot 12) was expressed only in the treatment group and this might be due to an external induction caused by fipronil.

Spot 5 was identified as a trypsin III precursor. This protein belongs to the serine protease family, which plays important roles in pathogenicity and signal transduction in cells. The serine proteinases, HP12, HP14, HP19, HP21 and HP22 in the hemolymph of tobacco hornworm were up-regulated 24 h after bacteria infection (Wang et al., 2006). The serine proteases have been found to play an important role in the silkworm defense system and could be triggered by the stimulations (Liu et al., 2009).

In this study, the up-regulation of proteins in the fipronil-treated group indicate that the proteins also participated in the fipronil response in *P. xylostella*.

## Proteins involved in metabolic pathways

In this study, more than 30% of all identified proteins were related to multiple metabolic pathways.

Two enzymes involved in carbohydrate metabolism were affected in *P. xylostella* by fipronil. With respect to the tricarboxylic acid cycle (TCA cycle), isocitrate dehydro- genase (spot 13) was down-regulated. Down-regulation of this protein was also observed in *Pseudomonas aeruginosa* in the stress of toxic Cr (VI) (Kılıç et al., 2009). Spot 8 was identified as similar to CG8199, it encodes the pyruvate dehydrogenase, and it was up-regulated. The data show that the influence of insecticide on insects is a complex process. Inorganic

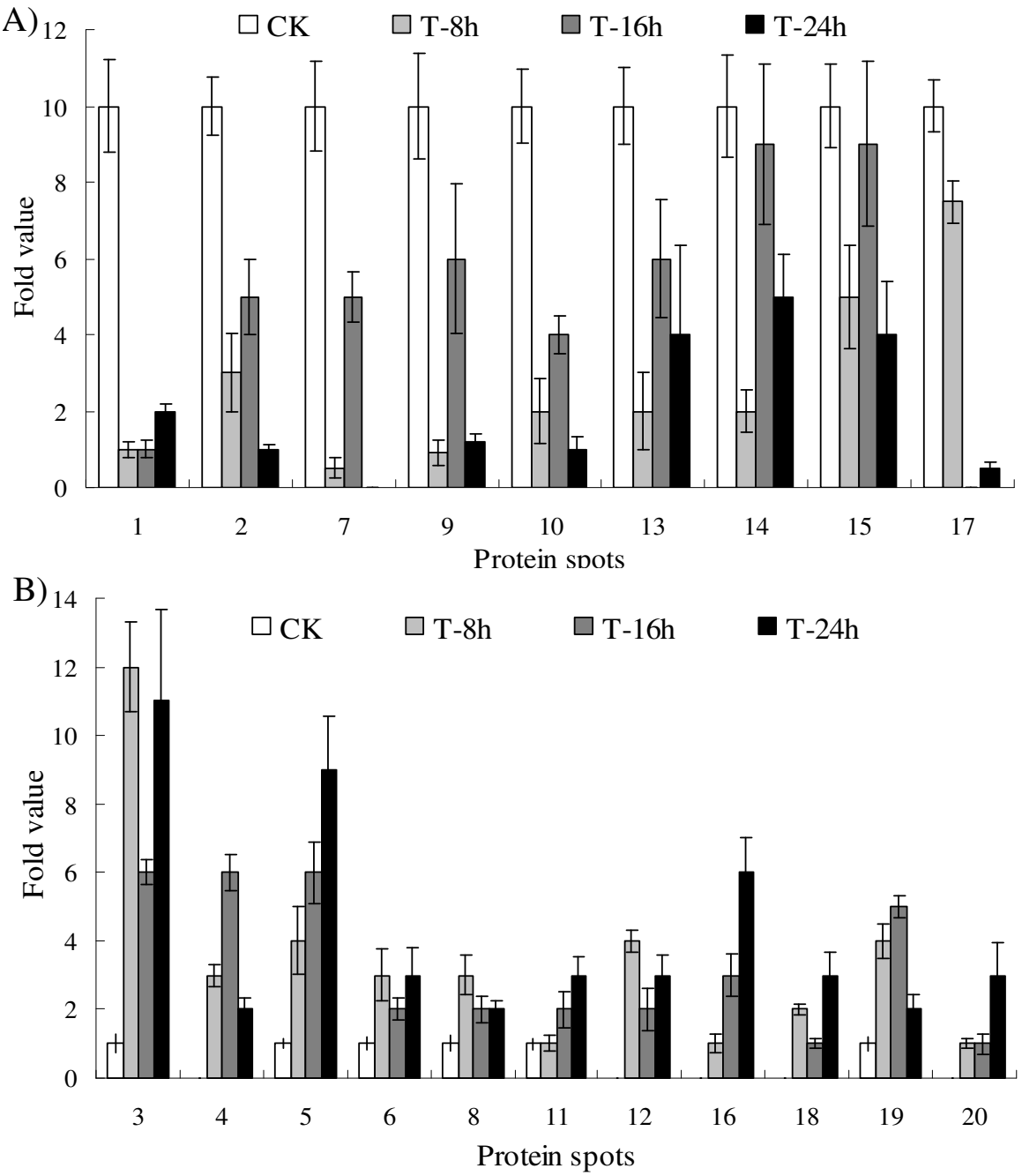


**Figure 3.** Quantification of the protein expression. (A) Proteins showing decreased expression; (B) proteins showing increased expression. Values are shown as mean ±SD.

pyrophosphatase (spot 4) was involved in lipid metabolism; it is an enzyme that catalyzes the conversion of one molecule of pyrophos- phate to two phosphate ions in the early steps of lipid degradation. This protein was up-regulated after fipronil treatment. Up-regulation of this protein has also been reported in tritrophic interactions among *Macrosiphum euphorbiae* aphids, their host plants and endosymbionts (Francis et al., 2010). In this study, a down-regulation of amino acid metabolism-associated proteins was observed. Homogentisate 1,2-dioxygenase (spot 9) belongs to the dioxygenase family; it is the key enzyme in the pathway for the catabolism of tyrosine and phenylalanine (Amaya et al., 2004). The expression of homogentisate 1,2- dioxygenase gene was shown to be changed in *Exophiala lecanii-corni* in the presence of ethylbenzene and may be responsible for the

**Table 1.** Identification of the differentially expressed proteins in the third-instar. *P. xylostella* larvae by MALDI-TOF-TOF/MS

| Spot number | Experimental Mw (kDa) / pI | Theoretical Mw (kDa) / pI | Accession number / homologous protein | Accession number / homologous protein to translated EST | Possible function | Sequence coverage | Protein score | Total ion score |
|---|---|---|---|---|---|---|---|---|
| 1 | 22.8 / 5.2 | 25.8 / 6.42 | | gi\|260908020 / DUF233 protein *[Heliothis virescens*] | Haemolymph juvenile hormone binding protein | 34 | 232 | 163 |
| 2 | 24.8 / 5.6 | 20.3 / 5.56 | | gi\|53884350 / $H^+$ transporting ATP synthase subunit d [*Bombyx mori*] | ATP synthase subunit d | 24 | 118 | 96 |
| 3 | 25.5 / 5.2 | 21.6 / 6.34 | | gi\|91082303 / PREDICTED: similar to F38B2.4 [*Tribolium castaneum*] | Adenylate kinase | 70 | 394 | 261 |
| 4 | 29.0 / 5.3 | 37.8 / 6.16 | | gi\|190622259 / GF11243, *[Drosophila ananassae*] | Inorganic pyrophosphatase | 28 | 71 | 53 |
| 5 | 29.0 / 5.4 | 28.1 / 5.57 | - | gi\|53883735 / trypsin Ⅲ precursor [*Sesamia nonagrioides*] | Trypsin-like serine protease | 36 | 106 | 55 |
| 6 | 30.0 / 5.8 | | - | - | | | | |
| 7 | 26.5 / 5.8 | | - | - | | | | |
| 8 | 31.0 / 6.3 | 48.8 / 6.48 | - | gi\|183979313 / similar to CG8199 [*Papilio xuthus*] | Pyruvate dehydrogenase | 31 | 110 | 66 |
| 9 | 41.0 / 6.8 | 48.2 / 6.38 | gi\|108869866/homo-gentisate 1,2-dioxygenase [*Aedes aegypti*] | | Homogentisate dioxygenase | 17 | 112 | 94 |
| 10 | 44.0 / 6.5 | 47.5/ 5.83 | gi\|10441583 / pxS-adenosyl-L-hom-ocysteine hydrolase [*Plutella xylostella*] | | S-adenosyl-L-homocysteine hydrolase | 17 | 237 | 174 |
| 11 | 28.0 / 6.9 | 33.1/ 9.82 | - | gi\|53883837 / ADP/ATP translocase [*Manduca sexta*] | adenine nucleotide translocase | 33 | 56 | |
| 12 | 26.9 / 6.9 | 25.8 / 6.08 | gi\|114052520 /lectin 4 C-type lectin [*Bombyx mori*] | | C-type lectin | 35 | 117 | 85 |

**Table 1.** Continue.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 13 | 44.0 / 7.4 | 27.0 / 7.16 | gi\|161088612 / isocitrate dehydrogenase [*Taygetis virgilia*] | | isocitrate dehydrogenase | 30 | 161 | 98 |
| 14 | 34.5 / 7.2 | 32.9/5.80 | - | gi\|193917509 / GI10501 [*Drosophila mojavensis*] | S-adenosylmethionine-depend-ent methyltransferases | 22 | 103 | 33 |
| 15 | 33.0 / 7.3 | 34.7 / 6.08 | | gi\|189566292 / phosphoribosyl pyrophosphate synthetase [*Bombyx mori*] | Pribosyltran Superfamily | 39 | 106 | 49 |
| 16 | 31.5 / 7.2 | 15.0/6.92 | - | gi\|87248327 / fumarylacetoacetase [*Bombyx mori*] | fumarylacetoacetate hydrolase | 30 | 105 | 47 |
| 17 | 22.2 / 6.2 | 17.0 / 6.17 | gi\|153792659 actin-depolymerizing factor 1 [*Bombyx mori*] | | Actin | 47 | 188 | 111 |
| 18 | 22.7 / 6.7 | 19.9 / 6.53 | gi\|112983420 / heat shock protein hsp 19.9 [*Bombyx mori*] | | Chaperonin | 27 | 104 | 75 |
| 19 | 31.3 / 6.7 | 21.5 / 6.36 | | gi\|254013135/ thioredoxin peroxidase [*Helicoverpa armigera*] | Typical 2-Cys PRX subfamily | 33 | 106 | 50 |
| 20 | 23.2 / 6.5 | | - | - | | | | |

ring cleavage step in the degradation pathway (Gunsch et al., 2006). Fumarylacetoacetase (spot 16) was also involved in the tyrosine catabolism pathway. The expressions of these proteins were changed in *P. xylostella* after fipronil treatment. It may indirectly influence energy metabolism. Phosphoribosyl pyrophosphate (PRPP) synthetase (spot 15) belongs to the phosphoribosyl transferase family and is considered as a link between carbon and nitrogen metabolism (Jiménez et al., 2008). In this study, PRPP synthetase was down-regulated in the fipronil-treated group. These data suggested that *P. xylostella* may be influenced in various metabolisms after treated with fiponil.

## Proteins involved in ATP production and transfer

The expression of the $H^+$-transporting ATP synthase subunit d (spot 2) in the larvae of *P. xylostella* was markedly decreased at 24 h after treatment with fipronil. However, this effect was not evident at the early time after the treatment. Some pesticides such as deltamethrin and acephatemet can inhibit oxidative phosphorylation in insect mitochondria (Li et al., 2006). Spot 11 was identified as ADP/ATP translocase (adenine nucleotide translocases, ANT), an important component of ADP/ATP carrier. Spot 3 was identified as similar to F38B2.4. The protein function based on specific homology domains is

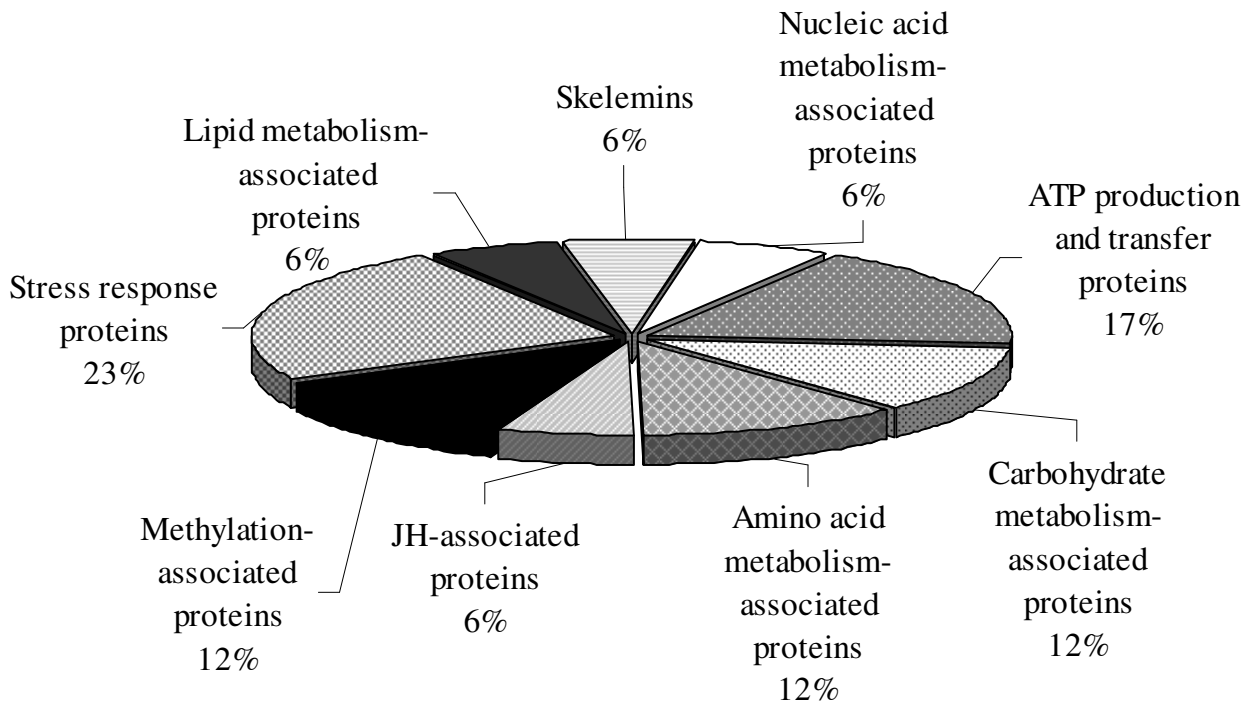


**Figure 4.** Functional categories of differentially expressed proteins of *P.xylostella* in response to fipronil.

adenylate kinase (AKs). The AKs are phylogenetically widespread, and involved in energy metabolism, energy transfer and homeostasis of cellular adenine nucleotide composition (Liu et al., 2009). The Aks can provide ADP to the ANT. In our study, two proteins were up-regulated after treatment with fipronil.

**Other proteins**

Spot 1 was identified as DUF233 whose domain was juvenile hormone binding protein (JHBP). The binding of JH to JHBPs was predicted to play a role in regulating JH levels, or to provide a means of transport for JH (Davies and Chapman, 2006). The juvenile hormones (JHs) regulated a diverse array of insect developmental and reproductive processes. Insecticides may affect the hormone receptor family, interference of endogenous hormones, thus changing the normal development of insects. The down-regulation of proteins in the fipronil-treated group indicated that *P. xylostella* could develop a shorter larva life stage when it responds to insecticides.

Spot 14 was identified as GI10501, which is the homolog with S-adenosylmethionine-dependent methyl-transferases (SAM methyltransferases). Spot 14 and spot 10 (pxS-adenosyl-L-homocysteine hydrolase, SAHH) were involved in methyl reaction in organisms. Increased expression of SAHH in *P. xylostella* was reported after bacterial invasion (Eum et al., 2007). When plant antiviral reactions, by expressing the antisense RNA for SAHH in transgenic tobacco plants, were studied, the transgenic tobacco plants that was constitutively expressing an antisense SAHH gene showed resistance to infection by various plant viruses (Masuta et al., 1995). SAHH has become a target for the design of drugs. Its inhibitor has demonstrated multiple antiviral and anti-parasitic functions (Yuan et al., 1999). In our experiment, the expression of methylation-association proteins was suppressed, which represented a physiological response of *P. xylostella* to fipronil.

In this study, the expression of actin-depolymerizing factor 1 (ADF, spot 17) was down-regulated in the fipronil-treated group. This protein is known to bind actin monomers and filaments *in vitro*, and it is important in regulating the aggregation of actin and the rapid movement of actin. Parasitic bees were discovered for ADF that was up-regulated and down-regulated in response to fluctuating temperatures and constantly low temperatures, respectively (Colinet et al., 2007). Pyrethroid toxin may result in the disruption of its normal function in the cytoskeleton in *Bactrocera dorsalis* (Jin et al., 2010). The observed change in the ADF expression may be related to the expression of actin, and can affect the physiological status of cell.

The study of the physiological responses of *P. xylostella* to a widely used insecticide, such as fipronil, provides a possibility to understanding its response to molecular mechanisms. By using 2-DE and MALDI-TOF MS/MS techniques, we successfully identified 20 proteins that may be functional in the response of the species to the

insecticide. In this study, the molecular function and biological roles of differently expressed proteins were predicted based on specific homology domains shared with proteins from other insect species. As expected, an increase in several proteins was clearly linked with immune response of *P. xylostella*.

However, the applied methods could only detect relative abundant proteins that underwent major changes in their expression levels in response to insecticides (at the applied time points). Many important regulatory proteins are less abundant and tend to undergo changes in their expression in response to stimulation, but they may not be detected by gel-based or mass spectrometry related proteomic techniques, such as GABA receptor.

The knowledge of insect physiological mechanisms responsible for insecticides has progressed substantially, but remains not fully understood. The development of proteomics techniques contributed much in helping to refine and enhance our understanding of the regulation of various biosynthesis pathways in insects in relation to the change of environmental conditions (Francis et al., 2006). The metabolic-associated proteins involved in the short-term response, expressed different changes, such that some proteins grew up and the others grew down. The phenomenon embodying the short-term response of *P. xylostella* to fipronil in multiple metabolic is complicated. However, the stress-responsive proteins were all up-regulated, including several proteins with predicted immune functions. The up-regulated proteins indicate that the species resisted the toxin of pesticides by regulating the organism immune system.

Other genetic studies have examined the evolution of resistance of the insect to specific insecticides such as abamectin, and it showed that this resistance can be the results of genetic differentiation (Tang and Bi, 2003; Zhou et al., 2010). In this case, we were able to examine the short term changes in function of a lethal critical level which is usually far less than the doses used to understand resistance evolution. However, such analyses can facilitate our understanding of the toxicology of pesticides.

The entire protein complement, expressed at a given time has been concerned with the isolation of individual proteins for use as markers of the targets of drug action (Kwak and Lee, 2005). Most likely, these changes reflect a global cellular response that cannot be solely attributed to a specific protein but probably involve in multiple physiological effects. This work reveals that the fipronil stress response is a complex process. Some up-regulated or down-regulated proteins observed in this study provided new insights for future studies on the mechanism of pesticide function and degradation in *P. xylostella*.

Most proteins identified in our study have been reported for the first time at the proteomic level as fipronil response. One of the future works is to verify the physiological roles of these proteins.In conclusion, this study reveals the functions of certain proteins and genes that are involved in the process of rapid toxicity response. Using the proteomic technology, we can better understand how this network of proteins is involved in defense to chemical or biological agents and this can serve as a reference for future studies on the physiological and biological role of protein expressions in *P. xylostella*. The analysis of responsive proteins will aid the development of pest controlling techniques.

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