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cDNA Representational Difference Analysis of Deltamethrin-Resistant and –Susceptible Strains of Diamondback Moth

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Abstract.- The diamondback moth (*Plutella xylostella* L.), a universal insect pest of cruciferous crops, has shown an extraordinary capacity to resist synthetic pesticides. Identification of genes associated with insecticide resistance has been a valuable tool to understand the mechanisms of resistance to insecticides. cDNA representational difference analysis (cDNA RDA) was performed to screen the upregulated genes in deltamethrin-resistant samples compared with the controls (susceptible strain). The fourth instar larve of deltamethrin-resistant and –susceptible strains of diamondback moth were selected for this study. The cDNA of resistant strain was used as tester amplicon, the driver amplicon was from the susceptible strain. After three rounds of substractive hybridization, we obtained six differentially expressed genes. Comparison between our results and data in the GenBank showed that one differential sequence had high homology with ribosomal protein gene L7 (*RpL7*). To validate the result, *RpL7* was cloned and *RpL7* expression levels between the resistant samples and the controls were detected using real-time quantitative PCR (qPCR). The results showed that mRNA level of *RpL7* was significantly higher in deltamethrin-resistant samples than in the controls. There are no direct reports indicating the upregulated gene *RpL7* is associated with insecticide resistance, further studies are still needed.

Keywords: cDNA RDA, insecticide resistance, *Plutella xylostella*, ribosomal protein L7, qRCR.

INTRODUCTION

The diamondback moth (*Plutella xylostella* L.) is one of the most destructive insect pests of cruciferous crops throughout the world (Bautista *et al.*, 2009), It has developed growing resistance to various insecticides, especially to the pyrethroid insecticides such as deltamethrin. Deltamethrin is one of the most frequently and widely used pyrethroids against a broad spectrum of insect pests of economically important crops. However, the long term use of deltamethrin led to a series of physiological and biochemical changes in insect pests, which ultimately resulted in pests' resistance to deltamethrin. It was previously reported that cuticular penetration, metabolic resistace and target site resistance are all the possible mechanisms for insecticide resistance. Enhanced activity of cytochrome P450 (Daborn and Yen, 2002; Shen *et al.*, 2003), the insensitivity of acetylcholinesterase (Kwon *et al.*, 2012) and point mutations in the sodium channels (Lee and Soderlund, 2001) are the main causes in pesticide resistance.

Studies showed that the resistance from a pest was related to a variety of mechanisms and various genes (Pedra *et al.*, 2004; Vontas *et al.*, 2005; Liu *et al.*, 2007). Complicated molecular network must have been involved in the generation and maintenance of the insecticide resistance. It's especially important to illustrate the metabolic resistance from the expression level, differentially expressed genes of interest should be identified, cloned and characterized.

To study the differentially expressed genes in cells or an organism under a given treatment, several techniques have been invented, such as mRNA differential display, suppression substrative hybridization (Yokota *et al.*, 2004) and cDNA representational difference analysis (cDNA RDA). Unlike other techniques, cDNA RDA, a technique combining substrative hybridization and PCR, provides us with some advantages of being simple, fast, and most importantly, sensitive. The experimental conditions can also be optimized (Jacob *et al.*, 2000; Pastorian *et al.*, 2000). Consequently, it becomes more and more popular in

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finding the differentially expressed genes between two complex cDNA populations. In this study, cDNA RDA was used to isolate the upregulated genes in deltamethrin-resistant *Plutella xylostella*. To verify the reliability of cDNA RDA results, the abundantly expressed gene *RpL7* was selected to detect the mRNA level between the deltamethrin-resistant samples and the controls using real-time fluorescent quantitative PCR.

MATERIALS AND METHODS

Plutella xylostella

The *P. xylostella* was originally obtained from Huaxi cabbage fields (Guiyang, China). Toxicity evaluation showed that it was a deltamethrin-susceptible strain (LD50 0.55 ug / larva). It was reared in our laboratory as the standard susceptible strain in this study.

The deltamethrin-resistant strain of *P. xylostella* was established and maintained by our laboratory selection (Liu *et al.*, 1995), with continuous exposure of deltamethrin to the susceptible strain generation by generation. The larvae of both strains are reared on 3 days old radish sprouts and maintained at 27 ± 1□, with a photoperiod of 16 h light and 8 h dark.

RNA isolation and cDNA synthesis

Ten deltamethrin-susceptible and –resistant strains of diamondback moth were chosen for RNA extraction using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The yield, concentration and purity of total RNA were determined using NanoDrop™ 1000 spectrophotometer (Thermo, USA) by measuring their absorbance at 260nm and 280nm. The integrity of total RNA was evaluated by 1.5% agarose gel with ethidium bromide staining. First-strand cDNA was generated using PrimeScript® RT reagent Kit (Takara, Japan) according to the manufacturer's recommendations and then the second-strand cDNA was synthesized. Double-stranded cDNAs were analysed on 1% agarose gel.

Oligonucleotides for cDNA RDA

The oligonucleotide sequences used for representational difference analysis were as follows (Lammens *et al.*, 2009):

T1-HhaI-10: 5'-CTCCCTCGGA-3';
T1-HhaI-24: 5'-GCAACTGTGCTATCCGAGGGAGCG-3';
T2-HhaI-10: 5'-CCTGGTAGAT-3';
T2-HhaI-24: 5'-CCGACGTCGACTATCTACCAAGCG-3';
D-HhaI-10: 5'-CGGTCAGAGG-3';
D-HhaI-24: 5'-AGCACTCTCCAGCCTCTGACCGCG-3'.

cDNA representational difference analysis

cDNAs of deltamethrin-susceptible and –resistant strains were digested with HhaI (NEB, USA). After phenol-chloroform extraction, the digested fragments from both strains were ligated to the adaptor T1-HhaI-10/24. These fragments were amplified by PCR using T1-HhaI-24 primer to generate driver and tester amplicons. The tester and driver amplicon were digested again by HhaI to remove the adaptor, and then only tester amplicon was ligated to the adaptor T2-HhaI-10/24. The tester (50 ng) and driver amplicons were mixed together in the ratio of 1:100 to perform the first round of substractive hybridization. The mixture was phenol-chloroform extracted, ethanol precipitated and then resuspended in 5 ul hybridization buffer (30 mM EPPS, pH 8.0; 30 mM EDTA; 1 mM NaCl; $\rho = 0.1$ g/ml PEG8000). After 98□ for 5 min and 67□ for 22 h, the products were diluted with TE, then 10 cycles amplication was performed. After phenol-chloroform extraction and isopropanol precipitation, the PCR products were diluted in TE. After treatment with 20 U mung bean nuclease at 37□ for 20 min, the products were then purified as mentioned above, finally resuspended in 20 ul TE. Additional PCR was performed with 30 cycles of 95°C for 1 min, 72°C for 3 min. The purified products were the first round differential products. Two more rounds of cDNA RDA were carried out with the identical method as mentioned in the first round. The subtraction ratio between the tester and driver amplicon were 1:500, 1:1000, with the adaptor of the tester amplicon T2-HhaI-24, D-hhaI-24, respectively.

The final difference products were purified using Nucleic Acid Purification Kit (Axygen, USA), then linked up to pMD® 19-T vector (Takara, Japan). Ligation mixture were transformed into *Escherichia coli* DH5α competent cells and cultured on LB plate containing ampicillin (100 mg/ml), IPTG (24 mg/ml)

and X-Gal (20 mg/ml). Fifteen positive clones were screened randomly and sequenced. The sequence alignment analysis was made with sequences in the GenBank, DDBJ and EMBL databases.

Cloning and sequencing of ribosomal gene RpL7 from diamondback moth

RNA extraction and cDNA synthesis from deltamethrin-resistant and –susceptible strains were conducted as described above. *RpL7* was amplified using a pair of specific primers: 5'-CGCAGCACAATGGTTGCCACTACAG-3' and 5'- GCTTGAACGGCCACAGGAAGTTACT-3'. The PCR reaction was carried out using 5 pmol of each primer under the following condition: initial denaturation at 95□ for 3 min, followed by 30 cycles of 95□ for 30 s, 60□ for 30 s and 72□ for 1 min with a final 72□ extension for 10 min. The amplified products were checked by 1% agarose gel electrophoresis. After purification, the products were transformed into *E. coli* DH5α competent cells and sequenced.

Sequence analysis of ribosomal gene RpL7

The predicted amino acid sequence was analyzed using Basic Local Aligment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). RpL7 protein molecular mass, isoelectric point (pI) and signal peptide were predicted by ExPASy (http://www.expasy.org/). The potential subcellular localization was predicted with the psort online server (http://psort.hgc.jp/form2.html). Multiple sequence aligments of DNA and amino acids were carried out using the ClustalX program (Jeanmougin *et al.*, 1998), and the phylogentic tree was constructed by the neighbour-joining method using MEGA 4.0 software (Tamura and Dudley, 2007). The sequences of RpL7 used in this study were from *Plutella xylostella*, *Spodoptera frugiperda*, *Papilio polytes*, *Bombyx mori*, *Manduca sexta*, *Papilio xuthus*, *Danaus plexippus*, *Euphydryas aurinia*, *Agriotes lineatus*, *Tribolium castaneum* and *Scarabaeus laticollis*.

Real-time fluorescent quantitative PCR

Ribosomal protein L7 was selected for qPCR analysis to verify the reliability of the cDNA-RDA data. Total RNAs were extracted from both strains using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The purity and concentration of total RNA were determined by spectrophotometry. The RNA integrity was observed on a 1.5% agarose gel. RNA (1 ug) was synthesised into cDNA, and qPCR was carried out on the Rotor-Gene Q (Qiagen, Germany) using the SYBR® Premix Ex Taq™ (Takara, Japan) according to the manufacturer's protocols. The housekeeping gene GAPDH was used for normalisation. Forward and reverse primers were 5'-AAGCACCGCAAGAGGAGGGA-3' and 5'-CGGATACGGATGACAAAGGC-3' for RpL7, respectively; 5'-TTCCAATACGACTCCACCCAC-3' and 5'-CCCTTTCAGAGAAGACAGCGA-3' for GAPDH, respectively. The 20 ul PCR mixture contained SYBR Green PCR mix, forward and reverse primers, and diluted cDNA. The following program was employed: 95□ for 5 min, followed by 40 cycles of 95□ for 15 s and 60□ for 1 min. Each sample was carried out in triplicate. All analysis was repeated three times using independent purified RNA samples. Gene expression levels were calculated and determined following the method described by Livak and Schmittgen (2001).

Statistical analysis

Data are expressed as mean ± standard error (SE) and were analyzed by *t*-test using SPSS PASW Statistics version 18.0. $p < 0.05$ was considered statistically significant.

RESULTS

cDNA RDA detects differential products

To identify the upregulated genes in diamondback moth for obtaining the deltamethrin-related genes, cDNA RDA was performed. As shown in Figure 1, band smear enriched between 100 and 500 bp on a agarose gel. The results of consecutive subtractive hybridization showed that the more the number of hybridization rounds, the less the difference products found.

Cloning, sequencing and sequence similarity search

It was found that six different gene fragments were successfully sequenced after random screening and sequencing. All these six gene fragments were

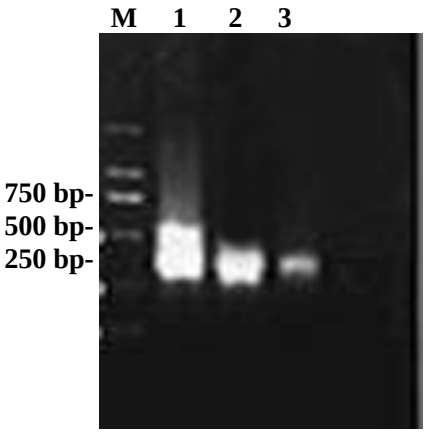

Fig. 1. The bands obtained from three rounds of hybridisation. M: marker; 1-3: First, Second and third round of hybridisation products, respectively.

taken to the NCBI for sequence similarity search. One of them had high homology with the known *RpL7* gene deposited in the database while others did not have a match. Figure 2 showed the gene fragment of *RpL7* and its amino acid sequence deduced by DNAStar. The nucleotide of *RpL7* fragment was compared with the sequences of other species stored in the database. The results showed that the fragment was 89% identical to the corresponding regions of *Papilio xuthus* and *Manduca sexta*, 88% identical to *Bombyx*, 87% identical to *Spodoptera frugiperda*.

Cloning and characterization of RpL7

There was only one distinct band with a predicted size amplified by RT-PCR in the deltamethrin-resistant and –susceptible strains (Fig. 3). After sequencing, a sequence of 672 bp was obtained, with an ORF of 642 bp encoding a predicted protein of 213 amino acid residues. The start codon ATG and stop codon TAA were underlined, and the predicted nuclear localization signal sequence is shaded (Fig. 4). The predicted isoelectric point of RpL7 was 11.69 and molecular mass was 24.55 KD. Alignment of the deduced ribosomal protein L7 amino acid sequence by Clustal X software showed the relative conservation of ribosomal protein L7 in different species. Blast analysis of RpL7 amino acids among *Plutella xylostella* and other species showed that RpL7 of *P. xylostella* has 87% identity with RpL7 of *S. frugiperda*, 85% identity with *M. sexta* and 84%

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AAGAGGAAAACCATCTTCAAGAGGGCCGAGGCTTACGTCAAGGAATACCGCATCAGGGAGCGAGATGAGATCAGGCTAGCCAGACAAGCACGCAAC
 K  R  K  T  I  F  K  R  R  A  E  E  A  Y  Y  V  K  E  Y  R  I  R  E  E  R  D  E  E  I  R  L  A  R  R  Q  A  R  N
AACGGCAACTACTATGTTCCCGGAGAAGCCAAATTGGCCTTTGTCATCCGTATCCGTGGTATCAACCAGGTCTCACCTAAGGTCCGCAAAGTGCTG
 N  G  N  Y  Y  V  P  G  E  E  A  K  L  L  A  F  V  I  R  I  R  R  G  I  N  Q  V  S  P  K  K  V  R  K  V  L
CAGCTGTTCCGCTTGCGGCAGATCAACAACGGCGTGTTCGTGCGCCTCAACAAGGCCACCGTCAACATGATGCGGATCGCCGAA
 Q  L  F  R  L  R  Q  I  N  N  G  V  F  V  R  L  N  K  A  T  V  N  M  M  R  I  A  E
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the differentially expressed cDNA fragment.

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CGCAGCACAATGGTTGCCACTACAGCAAAGAAGCCCGTGAAGGGTAAGGAGGACAGCAAGAAGCTGCCCGCAGTCCCCGAGTCAGTGCTCAAGCACCGCAAGAGGAGGGAGGCTCTCCGC [120]
      M  V  A  T  T  A  K  K  P  V  K  G  K  E  D  S  K  K  L  P  A  V  P  E  S  V  L  K  H  R  K  R  R  E  A  L  R
ACCAGACGTGTTCAGGTCACCCTCAAGAAGGCGCACAGCGGCACTCAAGAAGGAAAACTATCTTCAAGAGGCCGAGGCTTACGTCAAGGAATACCGCATCAGGAGCCGAGATGAGATC [240]
T  R  R  V  Q  V  T  L  K  R  R  R  T  A  A  L  K  K  R  K  T  I  F  K  K  R  R  A  E  A  Y  Y  K  E  Y  R  I  R  E  R  D  E  I
AGGCTAGCCAGACAAGCACGCAACACGGTAACTACTATGTTCCCGGAGAAGCCAAATTGGCCTTTGTCATCCGTATCCGTGGTATCAACCAGGTCTCACCTAAGGTCCGCAAAGTGCTG [360]
R  L  A  R  Q  A  R  N  N  G  N  Y  Y  V  P  G  E  A  K  L  A  F  V  I  R  I  R  G  I  N  Q  V  S  P  K  V  R  K  V  L
CAGCTGTTCCGCCTGCGGCAGATCAACAACGGCGTGTTCGTGCGCCTCAACAAGGCCACCGTCAACATGATGCGCATCGCCGAGCCCATACATCACGTGGGGTACCCCAACCTGAAGAG [480]
Q  L  F  R  L  R  Q  I  N  N  G  V  F  V  R  L  N  K  A  T  V  N  M  M  R  I  A  E  P  L  H  H  V  G  V  P  Q  P  E  E
CGTGCGCGAGCTGGTCTACAAGCGCGGGTTCGCCAAGGTGAAGGTCAGCGAGTGCCGATCACCTCCAACACCATCGTGGAGGACAAGGCTCGGCAACAAGGACATCATCTGTGTGGAGGA [600]
R  A  R  A  G  L  Q  A  R  V  R  Q  G  E  G  S  A  S  A  D  H  L  Q  H  H  R  G  G  Q  A  R  Q  Q  G  H  H  L  C  G  G
CCTCATCCACGAGATCTTCACCGTTGGGGACAAGTTCAAGTACGCCAGTAACTTCCTGTGGCCGTTCAAGCA [672]
P  H  P  R  D  L  H  R  W  G  Q  V  Q  V  R  Q  *
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Fig. 4. The nucleotide and deduced amino acid sequences of *P. xylostella RpL7* gene. The deduced amino acid sequence is presented below the nucleotide sequence in a single letter. The bipartite nuclear localization signal sequence is shaded.

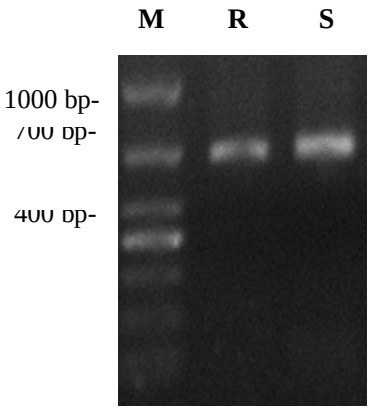

Fig. 3. Amplification of *RpL7* gene in diamondback moth. M: marker 1000, R: *RpL7* in deltamethrin-resistant strain, S: *RpL7* in deltamethrin-susceptible strain.

similarity with *P. polytes*, *D. plexippus* and *E. aurinia*. The phylogenetic tree was shown using neighbor-joining method (Fig. 5), which provided us with two kinds of information: the branching pattern and branch length. The branch pattern analysis showed the molecular phylogenetic relationships of RpL7 of *P. xylostella* and other species. The results indicated that Lepidoptera insects have the common ancestry than other three species.

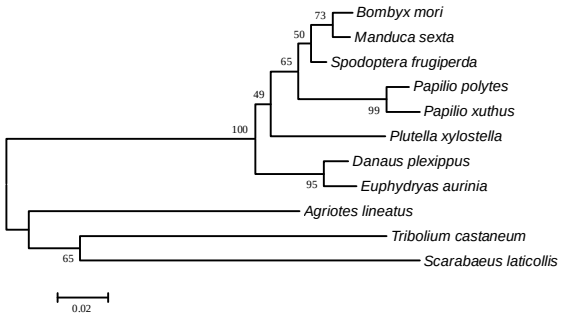

Fig. 5. The phylogentic tree obtained by NJ method based on the RpL7 amino acid sequences showed the relationships of RpL7 between *P. xylostella* and other species. Bootstrap values with 1000 replicates are given along the branches.

RpL7 is upregulated in deltamethrin-resistant diamondback moth

Quantitative real-time PCR was performed to

assess the expression of *RpL7* in fourth larvae of both strains. The relative expression of *RpL7* in deltamethrin-susceptible strain was considered as background level or 1, and the result showed that the expression of *RpL7* was 2.03-fold higher in resistant larvae compared with susceptible larvae of diamondback moth (Fig. 6). This result along with the RDA result indicated that RpL7 expression was upregulated in the deltamethrin-resistant strain of diamondback moth.

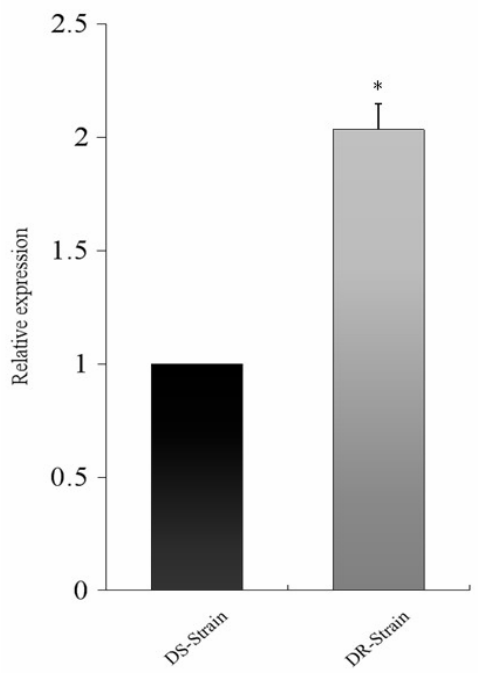

Fig. 6. Quantitative real-time PCR analysis for investigation of *RpL7* expression in deltamethrin-resistant and –susceptible diamondback moth. Results are expressed as mean ± standard error (SE). The relative expression of *RpL7* in DS-strain was considered as background level or 1. * $p < 0.05$. The experiment was repeated three times. DS-Strain: deltamethrin-susceptible strain; DR-Strain: deltamethrin-resistant strain.

DISCUSSION

We investigated the genes that were differentially expressed in deltamethrin-resistant diamondback moth after high-dose deltamethrin exposure using the cDNA RDA method. Since the deltamethrin-exposed diamondback moth originated from the unexposed parent population, they were thus similar in genetic background. Therefore, any cDNA sequences with differential expression obtained could reflect resistance from high dose deltamethrin toxicity. The tester amplicon was from the deltamethrin-induced population of diamondback moth. The cDNA RDA results revealed that *RpL7* was found upregulated in the drug-induced strain. qPCR was also performed to validate the veracity of cDNA RDA results. Our data showed that *RpL7* mRNA level was higher in diamondback moth induced by deltamethrin than the control samples, indicating the effective method and the reliable results in this study.

At present, molecular level for the study of the diamondback moth's resistance to insecticide has been carried out. It was reported that the resistance genes P450, glutathione-s-transferase, and a family of enzymes known to play an important role in cellular detoxification, were cloned and characterized; but insecticide resistance is a multi-gene phenomenon, other genes associated with pesticide resistance still need to be found and analyzed (Hemingway *et al*., 2004; Wu *et al*., 2004). The deltamethrin-related genes identified in our study were not identical with previous identified genes for resistance. Various factors, such as different cell types, different genomic techniques or the samples derived from different developmental phases and so on, can affect the experimental findings. To the best of our knowledge, this is the first report that *RpL7* may be a deltamethrin-related gene.

The eukaryotic ribosome is composed of about 80 different ribosomal proteins and four rRNA moleculars, its primary function is protein synthesis (Kannan *et al*., 2012), and the ribosomal proteins are highly conserved from yeasts to mammals (Lecompte *et al*., 2002; Klein *et al*., 2004). In addition to the assembly of ribosome and participation in protein synthesis, a number of ribosomal proteins were recently reported to have other extraribosomal functions. Therefore, it would be of great importance to rediscover and evaluate the function of ribosomal proteins. Studies have shown that some ribosomal proteins are differentially expressed in tumor cells compared with normal cells (Kondoh *et al*., 2001; Wang *et al*.,

2006; Han *et al*., 2012). Ribosomal proteins related to antibiotic resisitance were reported (Björkman *et al*., 1999). Ribosomal protein S4 gene was found overexpressed in deltamethrin-resistant strain from *Culex pipiens pallens* in the previous study (Hu *et al*., 2007). He *et al*. (2009) found that *L22* gene was closely associated with deltamethrin resistance in *Culex pipiens pallens*.

A number of studies have demonstrated that deltamethrin could induce genotoxic and immunotoxic effects (Ismail *et al*., 2012). In this study, we found three amino acids mutation in ribosomal protein RpL7 after diamondback moth's continuous exposure to deltamethrin. Whether the mutation confers some resistance to deltamethrin still need further research. During deltamethrin metabolism, Reactive oxygen species (ROS) were generated and caused oxidative stress. overproduction of ROS could do oxidative damage to biological macromolecules (Li *et al*., 2007). One important mechanism of drug resistance is the increasing ability of DNA damage repair (Kim *et al*., 2000). Many studies have demonstrated that ribosomal protein S3 is closely related to DNA damage repair (Sandigursky *et al*., 1997; Hegde *et al*., 2009). This study showed that RpL7 has high basic amino acids with 23.5% basic residues (lysine and arginine), and only 7.5% acidic amino acids. This highly basic feature suggests that it may be a nucleic acid binding protein (Cohen *et al*., 2008). Some ribosomal proteins have already been reported to function as gene expression regulators. Through removing inhibition of p53 inhibitors by ribosomal protein L5 and L11, transcription factor p53 was activated (Dai and Lu, 2004; Horn and Vousden, 2008). Chen *et al*. (2007) also demonstrated that S7 overexpression increases p53 transactivational activities. p53 can either prevent cell cycle of the damaged cells until repair completed, or directly involved in the damage repair (Smith *et al*., 2000). It is possible that through elevating DNA repair ability of RpL7, diamondback moth acquired deltamethrin resistance to a certain degree.

There is a common problem that insects resistant to insecticides are less fit in the free environment, it is a phenomenon which was called the fitness cost of resistance (Babiker, 2009). As a ribosomal protein, high expression of RpL7 may influence the synthesis of a subset of proteins. We postulate that *RpL7*-mediated insecticide resistance may be a result of increased translation of proteins which can confer protection against inverse factors. The altered translational profile may enhance the fitness of the organisms against harmful chemicals such as deltamethrin. However, once they live in an insecticide-free environment, the genotype of resistance is meaningless to them, the defects of this resistance phenotype may emerge, and overexpressed genes such as *RpL7* may become the price of resistance fitness.

In summary, our study firstly reported that ribosomal protein gene *RpL7* is upregulated in deltamethrin-resistant strain of diamondback moth using cDNA RDA, Further study showed that *RpL7* expression was significantly increased in the deltamethrin-resistant diamondback moth. There are no reports showing its upregulation is associated with insecticide resistance. It is necessary to further investigate the role of *RpL7* in deltamethrin resistance in our future study.

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