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Effects of methoxyfenozide on ecdysone receptor complex expression in larval Asian gypsy moth

Abstract: As a target of insect growth regulators, the ecdysone receptor complex binds ecdysone agonists to regulate gene transcription, development, and growth. To understand the response of *Lymantria dispar* to methoxyfenozide, we studied receptor complex transcription profiles following larval exposure to methoxyfenozide, and observed important variations amongst post-embryonic stages. After the exposure of 2nd and 3rd instar larvae to LC_5 and LC_{20} methoxyfenozide for 72 h, the transcription levels of ecdysone receptor complex genes were mainly upregulated in 2nd instar larvae, but downregulated in 3rd instar larvae. These results provide expression characteristics of the ecdysone receptor complex in *L. dispar* larvae after methoxyfenozide exposure.

Keywords: Expression profile, ecdysone receptor, *Lymantria dispar*, methoxyfenozide, stress

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1 Introduction

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Erebidae), is a highly destructive pest of forests worldwide, and a cooperative approach has been implemented for their control, including the use of natural predators, *Lymantria dispar* nuclear polyhedrosis virus (LdNPV), transforming insecticidal genes in plants, and chemical control [1]. Concerns over environmental and vertebrate safety have resulted in a paradigm shift away from the development of neurotoxic, broad-spectrum insecticides towards more environmentally friendly pest control agents, such as insect growth regulators (IGRs). IGRs interfere with the growth and development of insects at physiological, biochemical, and molecular levels [2-4].

A class of ecdysteroids, mainly 20E, regulates insect molting and metamorphosis. The action of 20E is mediated via an ecdysone receptor. The functional ecdysone receptor complex is a heterodimer of two proteins, ecdysone receptor (EcR) and ultraspiracle (USP), a homolog of the mammalian retinoic acid receptor (RXR) [5-7]. Both EcR and USP have been demonstrated to be phosphoproteins, which play an important role in regulating not only the ligand- and DNA-binding ability of the EcR/USP complex, but also in EcR/USP complex-mediated gene transcription. Upon binding to the EcR/USP complex in epidermal cells, 20E activates a cascade of transcriptional events that leads to molting. Methoxyfenozide has been reported as an effective agent to control lepidopterous pest insects instead of highly toxic pesticides [8].

Recently, our studies have determined the effects of methoxyfenozide on the development and growth in *L. dispar* larvae, and also studied detoxifying (carboxylesterase, mixed function oxidases, glutathione S-transferase) and protective enzymes (superoxide dismutase, catalase, peroxidase) [9-12]. However, little is known about the EcR/USP complex response to methoxyfenozide. In this study, we investigated the transcriptional profiles of target receptors in response to methoxyfenozide based on a characterization of the ecdysone receptor complex in *L. dispar* larvae.

2 Materials and methods

2.1 Insect treatment

Methoxyfenozide toxicity in *L. dispar* was determined by the leaf-dipping method described by Moores et al. with

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modifications [13]. Briefly, a stock of methoxyfenozide (98%, Dow Chemical Co., USA) was prepared in acetone and serially diluted with distilled water containing 0.05% (v/v) Triton X-100 and 1% acetone for bioassays. Birch (*Betula* sp.) leaves were dipped in methoxyfenozide solutions for 10 s and then placed in the shade to air dry. Bioassays were carried out by exposing 30 *L. dispar* larvae to methoxyfenozide-treated leaves. Control larvae were treated with distilled water containing 0.05% (v/v) Triton X-100 and 1% acetone. Each concentration was replicated at least three times, and the mortality was assessed 48 h after the treatment at 25°C. LC_{50}, LC_{20}, and LC_5 values were calculated by POLO software (LeOra Software Inc., Cary, NC). Second- and third-instar larvae were treated for 48 h with LC_5 (1.573 mol/L) and LC_{20} (4.575 mol/L) of methoxyfenozide (2nd instar larvae) and for 48 h with LC_5 (2.090 mol/L) and LC_{20} (6.898 mol/L) of methoxyfenozide (3rd instar larvae). Live and healthy larvae were collected from each replicate at 6, 12, 24, 48, and 72 h and stored at -80°C for RNA extraction.

2.2 Polygenetic analysis

Amino acid sequences of the EcR and USP proteins were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). To construct proteins in the polygenetic tree, we used CLUSTALX 1.83 software to align *L. dispar* EcR and USP with those of insect species at the level of the deduced amino acid sequence [14]. Polygenetic trees were generated using the neighbor-joining method and bootstrapped with 1000 replicates to evaluate the branch strength of the tree in MEGA 5.1 software [15].

2.3 Quantitative real-time PCR (qRT-PCR) analysis

Total RNA of *L. dispar* larvae was isolated at different developmental stages (eggs, larvae, pupae, and adults), and was treated with DNaseI to remove any residual DNA. Approximately 0.5 μg of total RNA was reverse-transcribed into cDNA using 1 μM of oligodeoxythymidine primer. The synthesized cDNAs were diluted to 100 μL with sterile water and used as a template for real-time PCR. Four EcR genes and one USP gene from the transcriptome library were selected to study the response to insecticides. The primer sequences are listed in Table 1. Real-time RT-PCR was performed with an MJ Opticon™2 machine (Bio-Rad, Hercules, CA, USA). The *actin* and *TUB* genes were chosen as internal controls to normalize the amount of total RNA present in each reaction. The reaction mixture (20 μL) contained 10 μL of SYBR Green real-time PCR master mix (Toyobo), 10 μM each of forward and reverse primers, and 2 μL of cDNA template (equivalent to 100 ng of total RNA). The amplification was performed with the following cycling parameters: 94°C for 30 s, followed by 45 cycles at 94°C for 12 s, 60°C for 30 s, 72°C for 40 s, and 1 s at 82°C for plate reading. A melting curve was generated for each sample at the end of each run to assess the purity of the amplified products. Real-time PCR was carried out in triplicate biological repeats to ensure the reproducibility of the results. The clone expression levels were calculated from the threshold cycle according to the delta-delta CT method [16].

3 Results

3.1 Characteristics of the EcR receptor genes

Four EcR (U27633, U4289, U5567, and U11596) genes and one USP (U23169) gene were identified in the *L. dispar* transcriptome by BlastX searches of the Nr and Swiss-Prot protein databases (Table 1). The cDNA length of U23169, U27633, U4289, U5567, and U11596 were 1796 bp, 856 bp, 425 bp, 283 bp, and 1488 bp, respectively. The similarities between U4289, U11596 and U23169 were found to be 20.44% and 18.27%, respectively, while the similarity between U11596 and U4289 was 4.38% (Table 2). Among these EcR receptor complexes, the open reading frame (ORF) of *USP* (U23169) was 1395 bp, which encoded a deduced polypeptide of 464 amino acids, with a predicted molecular mass of 52.48 kDa and a pI of 8.41.

3.2 Polygenetic analysis

According to the identities of the EcR receptor genes in 22 insect species, polygenetic trees of 48 ecdysone receptor genes were reconstructed (Figures 1 and 2). The U5567 and U11596 sequences of *L. dispar* EcR receptor genes were clustered into one group and were relatively close to the EcR genes of *Omphisa fuscidentalis* (ABS00249.1), *Choristoneura fumiferana* (AAC36491.2), *Danaus plexippus* (EHJ66465.1), *Papilio xuthus* (BAM20285.1), and *Plodia interpunctella* (AAR84611.1). The U27633 and U4289 sequences of *L. dispar* EcR receptor genes were clustered into a group with *Spodoptera litura* (AFK27932.1). Among the six USP genes, the U23169 sequence of the *L. dispar* USP gene was clustered into a group with *Manduca sexta* (AAB64234.1).

Table 1: Real-time PCR primers of EcR and USP genes.

Gene ID	Primer sequence (5'-3')	Product size (bp)	Gene description	E-value	Subject ID	Identity
U23169	R:AAGAATTACCCTCCGAACCATC F:CACGCCAAACATTTCTGATACC	236	ultraspiracle protein [*Spodoptera frugiperda*]	1e-161	AAM54495.1	93%
U27633	F:GCCGATATGTCCAATGCTAAAG R:GTACGAAATCGGATACGTGGTA	227	ecdysone receptor [*Helicoverpa armigera*]	1e-21	ABN11286.1	79%
U4289	F:GTTCAAGTGAGGTGATGATGTTG R:CCGGTCGGTCTGAGAATATAAC	228	ecdysone receptor [*Helicoverpa armigera*]	1e-78	ABN11286.1	96%
U5567	F:TACCGGGAGCTATGGTTATGT R:TCCGATTTAGGTGTAGTCGGT	214	ecdysone receptor B1 isoform [*Orgyia recens*]	3e-21	BAC44997.1	93%
U11596	F:GTCTCACACAACGGACATCA R:CTTGCTTCGCCATCTGTACTA	230	ecdysone receptor A [*Spodoptera exigua*]	4e-176	ADK66917.1	91%
TUB	F: AATGCAAGAAAGCCTTGCGCCT R: ATGAAGGAGGTCGACGAGCAAA	223	—	—	—	—
Actin	F: AGAAGCACTTGCGGTGGACAAT R: ACCTGTACGCCAACACTGTCAT	252	—	—	—	—

Table 2: Sequence identities among the five EcR receptor proteins (%).

Gene	U4289	U5567	U11596	U23169
U27633	7.02	8.77	5.26	7.02
U4289		2.13	4.38	20.44
U5567			10.64	11.70
U11596				18.27

3.3 Developmental expression profiles of the ecdysone receptor

Transcription profiles of the four EcR and one USP genes were identified in *L. dispar* during different developmental stages (Figure 3). Compared to the egg stage, the expression levels of U23169 and U4289 were downregulated during the developmental stages. The relative expression level of U27633 showed downregulation during the 1st, 4th, and 5th instar larvae, as well as the pupae and male adults, while they were upregulated in 2nd, 3rd, and 6th instar larvae and female adults. The transcriptional expression levels of the U5567 gene were mostly downregulated in other developmental stages. The U11596 gene expression level was similar to U5567 except for its expression in 5th instar larvae.

3.4 Expression profiles of the ecdysone receptor in response to sublethal methoxyfenozide levels

The expression profiles of ecdysone receptor genes in 2nd and 3rd instar *L. dispar* were investigated under the LC_5 and LC_{20} of methoxyfenozide stress during 72 h (Figures 4–7). For the 2nd instar gypsy moth larvae under sublethal methoxyfenozide stress, the U23169 and U27633 genes were induced under LC_5 concentration of methoxyfenozide for 72 h with 8.91- and 4.36-fold peak expression compared to unexposed larvae. However, the U11596 gene was downregulated 0.11-fold under an LC_5 concentration of methoxyfenozide during 72 h compared to unexposed larvae at the 24-h time point. The U4289 and U5567 genes showed similar levels of downregulated expression in response to the LC_5 concentration of methoxyfenozide at

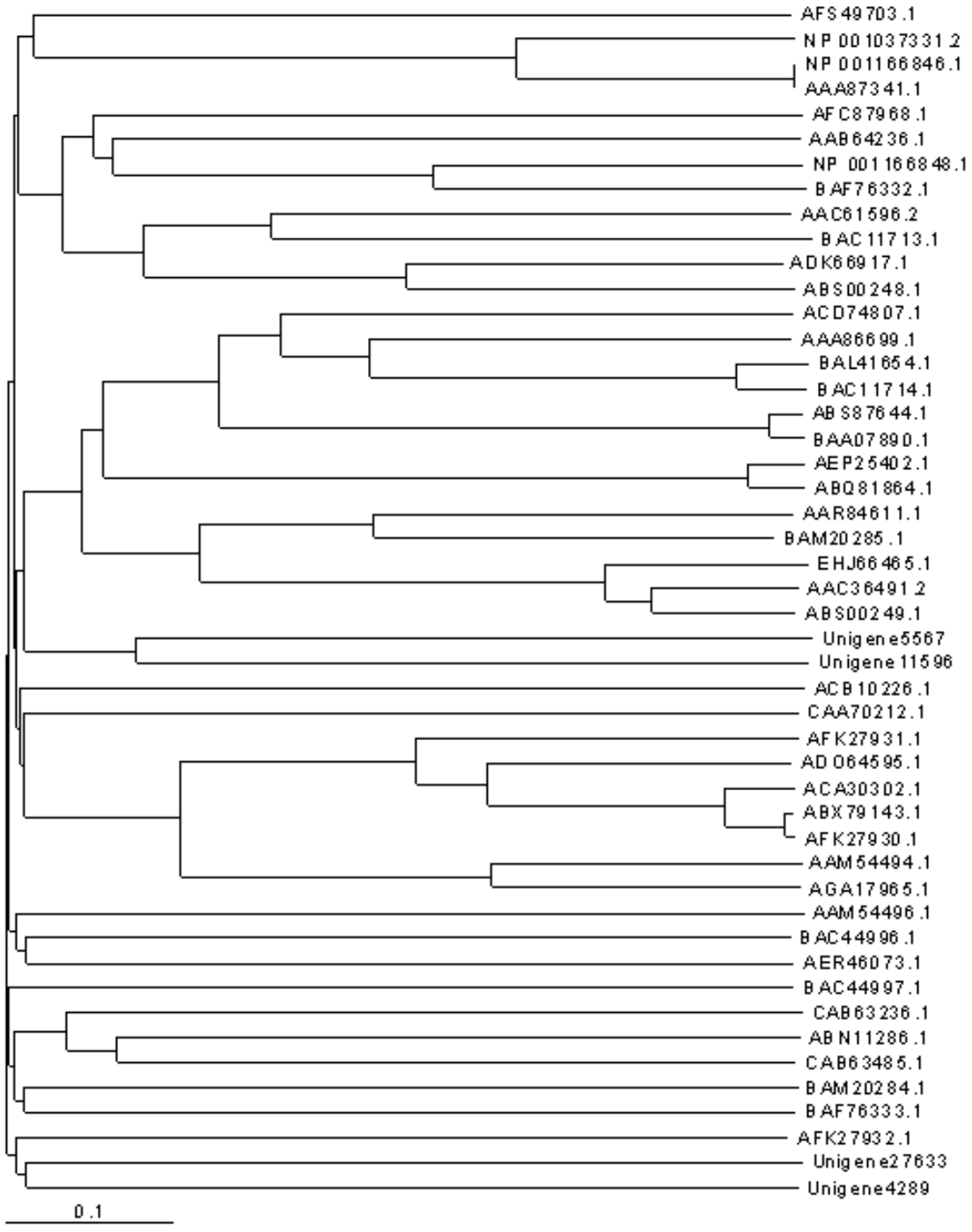

Figure 1. Polygenetic trees of EcR proteins. Insect EcR proteins are from *Bombyx mori* (NP_001037331.2, NP_001166846.1, NP_001166848.1, AAA87341.1, BAF76332.1,ABS87644.1, BAA07890.1, BAF76333.1); *Chilo suppressalis* (BAC11714.1, BAC11713.1); *Scirpophaga incertulas* (BAL41654.1); *Choristoneura fumiferana* (AAC61596.2); *Spodoptera litura* (ABX79143.1, AFK27930.1, AFC87968.1, AFK27932.1); *Manduca sexta* (AAB64236.1); *Spodoptera exigua* (ACB10226.1, ACA30302.1, AFK27931.1, ADK66917.1); *Helicoverpa armigera* (ABN11286.1, ACD74807.1); *Junonia coenia* (CAB63485.1); *Bicyclus anynana* (CAB63236.1); *Orgyia recens* (BAC44996.1, BAC44997.1); *Agrotis ipsilon* (AGA17965.1); *Plodia interpunctella* (AAR84611.1); *Spodoptera littoralis* (ADO64595.1); *Danaus plexippus* (EHJ66465.1); *Plutella xylostella* (AEP25402.1, ABQ81864.1, AFS49703.1); *Choristoneura fumiferana* (AAC36491.2); *Omphisa fuscidentalis* (ABS00249.1, ABS00248.1); *Papilio xuthus* (BAM20285.1, BAM20284.1); *Trichoplusia ni* (AAM54496.1); *Sesamia nonagrioides* (AER46073.1).

Figure 2. Polygenetic trees of USP proteins. USP proteins are from *Choristoneura fumiferana* (AAC31795.1); *Plutella xylostella* (ADA61199.1, AEP25407.1, ABQ81862.1); *Scirpophaga incertulas* (BAL41655.1); *Chilo suppressalis* (BAC53670.1); *Manduca sexta* (AAB64234.1); *Lymantria dispar* (LdUSP, U23169).

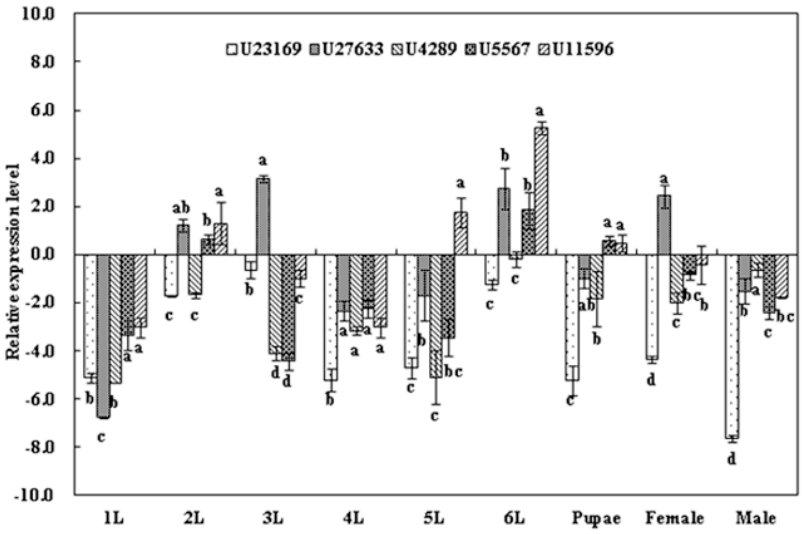

Figure 3. Transcription profiles of EcR and USP genes identified in Lymantria dispar during different developmental stages. All of the relative expression levels were log2 transformed. 1L-6L represent 1st-6th instar larvae. Means followed by the same letter above histograms at the same time point are not significantly different according to Tukey's HSD test (P = 0.05).

the 6-h and 48-h time points. After the 2nd instar gypsy moth larvae were subjected to LC_{20} of methoxyfenozide, the U23169, U27633, U4289 and U5567 genes showed high expression levels compared to the LC_5 concentration stress at 72 h. However, compared to the LC_5 concentration stress, the U11596 gene was also induced under high concentration stress, although their expression levels were downregulated at 12–48 h.

Interestingly, the U23169, U27633, U4289, and U5567 genes were mostly downregulated under sublethal LC_5 and LC_{20} concentrations of methoxyfenozide at 72 h, but the U11596 gene was upregulated by 3.55-fold compared to the unexposed larvae at 72 h of LC_5 concentration stress.

4 Discussion

The non-steroidal ecdysone agonist methoxyfenozide (RH-2485) has shown significant activity against lepidopterous and coleopterous pest insects by inducing a precocious lethal molting [4]. Our previous studies have indicated that

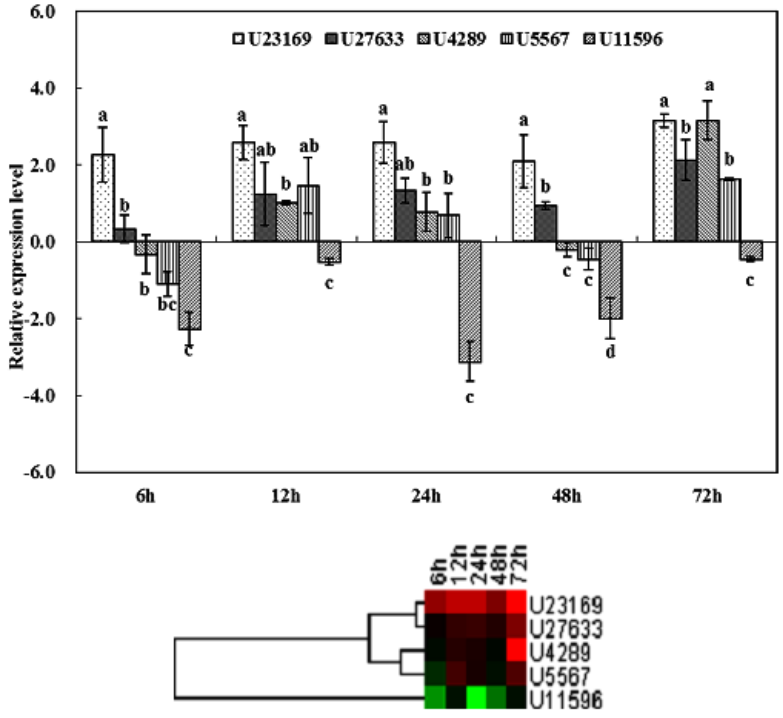

Figure 4. Transcription profiles of EcR and USP genes in 2nd instar larvae in response to the LC_5 sublethal concentration of methoxyfenozide. All of the relative expression levels were log2 transformed. Means followed by the same letter above histograms at the same time point are not significantly different according to Tukey's HSD test (P = 0.05).

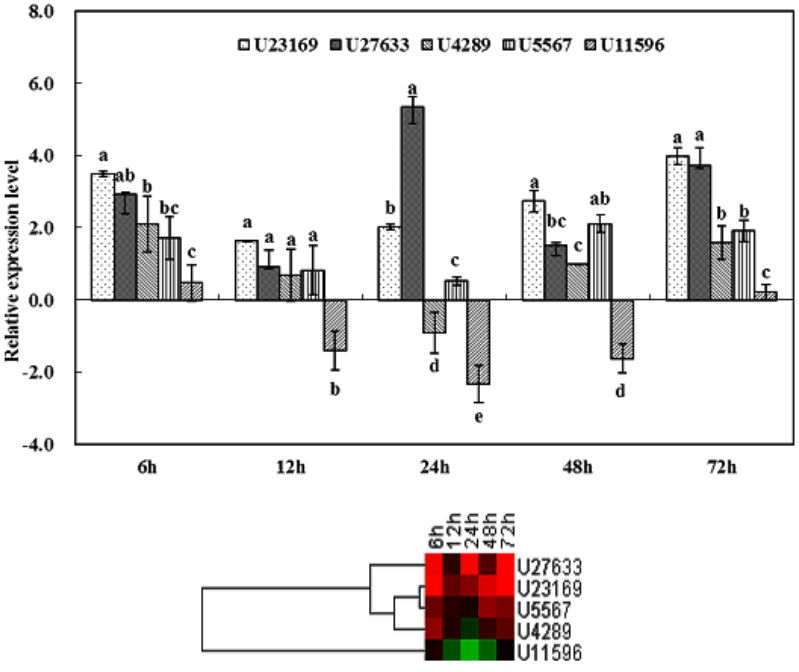

Figure 5. Transcription profiling of EcR and USP genes in 2nd instar larvae in response to the LC_{20} sublethal concentration of methoxyfenozide. All of the relative expression levels were log2 transformed. Means followed by the same letter above histograms at the same time point are not significantly different according to Tukey's HSD test (P = 0.05).

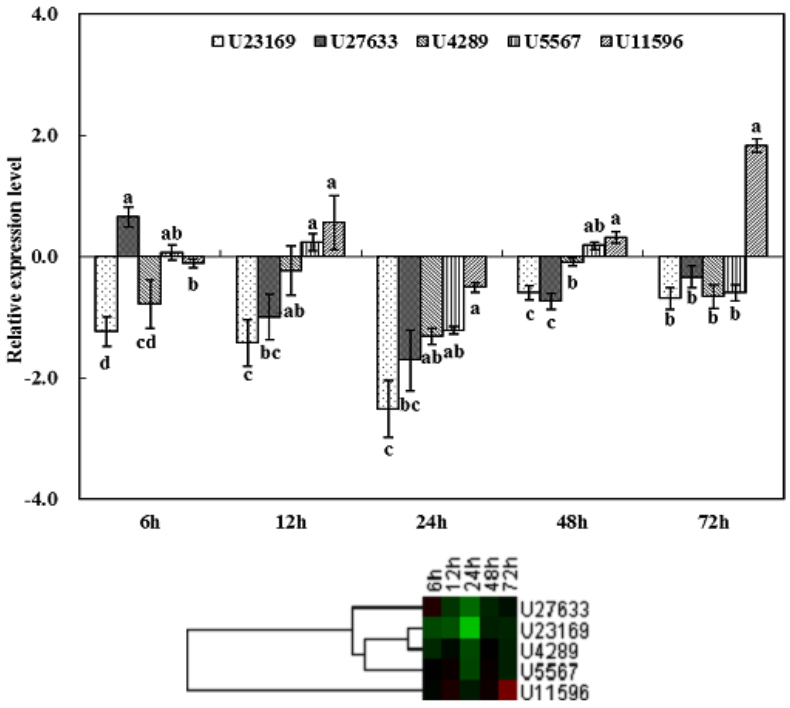

Figure 6. Transcription profiling of EcR and USP genes in 3rd instar larvae in response to the LC_5 sublethal concentration of methoxyfenozide. All of the relative expression levels were log2 transformed. Means followed by the same letter above histograms at the same time point are not significantly different according to Tukey's HSD test (P = 0.05).

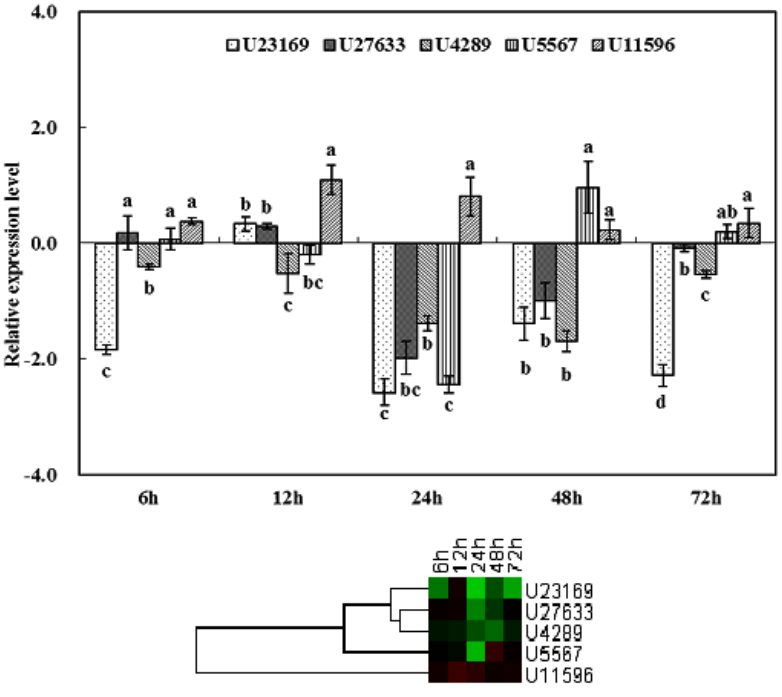

Figure 7. Transcription profiling of EcR and USP genes in 3rd instar larvae in response to the LC_{20} sublethal concentration of methoxyfenozide. All of the relative expression levels were log2 transformed. Means followed by the same letter above histograms at the same time point are not significantly different according to Tukey's HSD test (P = 0.05).

LC_{10} and LC_{30} of methoxyfenozide significantly decreased insect development and resulted in morphological abnormalities in 62.53% of all individuals [9]. Furthermore, methoxyfenozide could produce the effects of protective enzymes, phenoloxidase (PO), superoxide dismutase (SOD), peroxidase (POD), and of detoxifying enzymes, such as carboxylesterase (CarE), MFO O-demethylase (MFOD), and glutathione S-transferase (GST), to disrupt normal physiological metabolism [10,11,17]. After the 4th instar larvae were fed with methoxyfenozide, specific proteins were produced in the hemolymph, midgut and epidermal tissue (as detected by SDS-PAGE), which might interfere with the normal physiological metabolism and epidermal formation of *L. dispar* [17]. In this study, we further identified the ecdysone receptor genes and studied their expression profiles in response to methoxyfenozide. In total, four ecdysone receptor genes and one ultraspiracle gene were found in *L. dispar* larvae. These ecdysone receptor genes showed distinct developmental expression profiles in response to insecticide stress. The ecdysone receptor isoforms and the ultraspiracle gene showed different expression levels during different developmental stages. Compared to the pre-embryonic stage, the ultraspiracle gene was downregulated during all post-embryonic stages. However, the ecdysone receptor isoforms showed distinct expression patterns during the developmental stages. For example, the isoform U27633 of the ecdysone receptor gene had its lowest expression level in lst instar larvae, while isoform U11596 had its highest expression during the egg stage. These results indicate that *L. dispar* might trigger the expression of different ecdysone receptors in response to different insecticide stresses.

For 2nd instar larvae, the U23169 and U27633 genes were mainly induced while the U11596 gene was inhibited by LC_5 and LC_{20} levels of methoxyfenozide. Interestingly, the ecdysone receptor complex genes in 3rd instar larvae were mostly downregulated by exposure to LC_5 and LC_{20} of methoxyfenozide for 72 h. These results showed that the ecdysone receptor complex expression levels were affected by methoxyfenozide in a dose-dependent manner. The EcR complex included different isoforms as indicated by different responses to methoxyfenozide. This information could help with the development of new non-steroidal ecdysone agonists to control pest insects and delay the development of resistance.

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References

[1] Cao C.W., Liu G.F., Wang Z.Y., Yan S.C., Ma L., Yang C.P., Response of the gypsy moth, *Lymantria dispar* to transgenic poplar, *Populus simonii × P. nigra*, expressing fusion protein gene of the spider insecticidal peptide and *Bt*-toxin C-peptide, J. Insect Sci., 2010,10:200

[2] Boudjelida H., Bouaziz A., Soin T., Smagghe G., Soltani N., Effects of ecdysone agonist halofenozide against *Culex pipiens,* Pestic. Biochem. Physiol., 2005, 83:115–123

[3] Tang B.Z., Sun J.Y., Zhou X.G., Gao X.W., Liang P., The stability and biochemical basis of fufenozide resistance in a laboratory-selected strain of *Plutella xylostella*, Pestic. Biochem. Physiol., 2011, 101:80–85

[4] Zibaee A., Bandani A.R., Malagoli D., Methoxyfenozide and pyriproxifen alter the cellular immune reactions of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) against *Beauveria bassiana*, Pestic. Biochem. Physiol., 2012, 102:30–37

[5] Yao T.P., Segraves W.A., Oro A.E., McKeown M., Evans R.M., *Drosophila* Ultraspiracle modulates ecdysone receptor function via heterodimer formation, Cell, 1992, 71:63–72

[6] Yao T.P., Forman B.M., Jiang Z., Cherbas L., Chen J.D., et al. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes, Nature, 1995, 366:476–479

[7] Thomas H.E., Stunnenberg H.G., Stewart A.F., Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and Ultraspiracle, Nature, 1993, 362:471–475

[8] Sun X., Barrett B.A., Fecundity and fertility changes in adult codling moth (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide, J. Econ. Entomol., 1999, 92(5):1039–1044

[9] Liao Y.Z., Yan S.C., Cao C.W., Liu D., Effect of low dosages of methoxyfenozide on the development of *Lymantria dispar* Linnaeus, Acta Phytophylacica Sinica, 2009, 36(6):555–560

[10] Yan J.M., Liao Y.Z., Yan S.C., Cao C.W., Feng C.F., Effects of methoxyfenozide (RH-2485) on the activities of detoxifying enzymes and protective enzymes in *Lymantria dispar* (Lepidoptera: Lymantriidae), J. Northeast Forest. Univ., 2010, 38(11):112–114

[11] Liao Y.Z., Yan S.C., Li X.P., Cao C.W., Effect of methoxyfenozide on the activities of protective enzymes in larvae of *Lymantria dispar*, Scientia Silvae Sinicae, 2011, 47(9):93–99

[12] Zhong H., Cao C.W., Xue Y., Yan S.C., Effects of methoxyfenozide on the activities of mixed-Function Oxidases and Amidase in *Lymantria dispar* (Lepidoptera: Lymantriidae), J. Northeast Forest. Univ., 2013, 41(5):143–146

[13] Moores G.D., Gao X.W., Denholm I., Devonshire A.L., Characterization of insensitive acetylcholinesterase in the insecticide-resistant cotton aphid, *Aphis gossypii* Glover (Homoperta: Aphidedae). Pestic. Biochem. Physiol., 1996, 56:102–110

[14] Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acid Res., 1997, 25:4876–4882

[15] Tamura K., Dudley J., Nei M., Kumar S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0, Mol. Biol. Evol., 2007, 24:1596–1599

[16] Pfaffl M.W., Horgan G.W., Dempfle L., Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res., 2002, 30:36

[17] Liao Y.Z., Yan S.C., Cao C.W., Liu D., Effect of methoxyfenozide on activities of detoxifying enzymes and expression of proteins in *Lymantria dispar* larvae, Scientia Silvae Sinicae, 2012, 48(8):99–105