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Triazophos up-regulated gene expression in the female brown planthopper, *Nilaparvata lugens*

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ABSTRACT

The widespread use of insecticides has caused the resurgence of the brown planthopper, *Nilaparvata lugens*, in Asia. In this study, we investigated an organo-phosphorous insecticide, triazophos, and its ability to induce gene expression variation in female *N. lugens* nymphs just before emergence. By using the suppression subtractive hybridization method, a triazophos-induced cDNA library was constructed. In total, 402 differentially expressed cDNA clones were obtained. Real-time qPCR analysis confirmed that triazophos up-regulated the expression of six candidate genes at the transcript level in nymphs on day 3 of the 5th instar. These genes encode *N. lugens* vitellogenin, bystin, multidrug resistance protein (MRP), purine nucleoside phosphorylase (PNP), pyrroline-5-carboxylate reductase (P5CR) and carboxylesterase. Our results imply that the up-regulation of these genes may be involved in the induction of *N. lugens* female reproduction or resistance to insecticides.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål), is one of the most problematic insect pests of rice in Asia. Currently, chemical control of *N. lugens* primarily relies on the application of various insecticides, such as organo-phosphorous, carbamate, pyrethroid and neonicotinoid insecticides. However, insecticide application gives rise to planthopper resurgence. Possible reasons include the destruction of the natural enemy community and the stimulation of pest reproductivity by sub-lethal dosages of some pesticides. In addition, insecticide application stimulates rice growth, which indirectly stimulates *N. lugens* feeding and reproduction (Wu et al., 2001a,b). However, the mechanisms of *N. lugens* resurgence due to insecticide stimulation are poorly understood.

As a broad-spectrum organo-phosphorous insecticide, triazophos has been extensively used for controlling *N. lugens* by farmers in China. Nevertheless, spraying triazophos on adults during the first generation of *N. lugens* resulted in a high density of the egg population and the resurgence of the second generation of *N. lugens* (Zhuang et al., 1999; Zhu et al., 2004). Ge et al. (2009) reported that triazophos caused a significant increase in protein and RNA content in the ovaries of *N. lugens* adult females, which suggested that resurgence caused by triazophos might be closely related to *N. lugens* female reproduction.

Although the ecological mechanisms on *N. lugens* resurgence have been extensively studied, the physiological mechanisms of this phenomenon are still not clear. In this study, we attempt to understand the mechanism of resurgence by studying the alterations in gene expression, which may be associated with the resistance or to the high rate of female reproduction caused by triazophos application. The ovary of *N. lugens* starts to mature 1 or 2 days after adult emergence (Noda et al., 2008). To gain complete gene expression information throughout the maturation process, we focused on the female nymphs before emergence (around day 3 of the 5th instar).

A triazophos-induced *N. lugens* nymph cDNA library was constructed by suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) to identify the up-regulated genes. A total of 402 cDNA clones expressed in response to triazophos treatment were obtained from the library. Real-time qPCR analysis confirmed that six genes were up-regulated following triazophos treatment before emergence of the female nymphs. These genes may be involved in *N. lugens* female reproduction or resistance to triazophos.

2. Materials and methods

2.1. Insects and insecticides

The brown planthopper, *N. lugens*, was originally collected from a rice field located at Los Banus, Laguna, Philippines. The nymphs were reared at 26 ± 1 °C on rice seedlings under a 16:8 h light:dark photoperiod. Technical triazophos (85%) was supplied by Xinnong

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Chemical Industrial Group Co. Ltd. (Taizhou, Zhejiang, China). On day 2 of the 4th instar, nymphs were treated with a sub-lethal dose of triazophos (LC_{10}).

2.2. Bioassay and insect treatment

Bioassays were undertaken using the microtopical application technique (Nagata, 1982). In this experiment, the nymphs on day 2 of the 4th instar were used as test insects. Triazophos was diluted to a series of concentrations in acetone. Under carbon dioxide anesthesia, a droplet (0.039 μl) of triazophos solution was applied topically to the prothorax notum of *N. lugens* nymphs with a hand microapplicator (provided by Nanjing Agricultural University). Thirty of the 4th instar nymphs were treated at each concentration, and every treatment was repeated three times. The control treatments used acetone only. The treated nymphs were reared in plastic dishes containing rice seedlings in a rearing incubator at 26 ± 1 °C with a 16:8 h light:dark photoperiod. The results were checked after 24 h. The LC_{10} values were determined by standard probit analysis, using the DPS software version 8.01 (Tang and Feng, 2007).

2.3. Construction of the subtracted cDNA library through SSH

The *N. lugens* nymphs on day 2 of the 4th instar were treated with acetone or a sub-lethal dose (LC_{10}) of triazophos, and the total RNA was extracted from the whole body on day 3 of the 5th instar (just before adult emergence) by using SV Total RNA Isolation System (Promega). Poly $(A)^+$ RNA was purified by using an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), and 2 μg of Poly $(A)^+$ RNA was used as the starting material for reverse transcription to construct the subtracted cDNA libraries. SSH was performed using a PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA). In keeping with the manufacturer's protocol, we designated the cDNA samples that contained specifically expressed transcripts: those that responded to triazophos were "testers," and the reference cDNA samples that responded to acetone were "drivers." An SSH library was constructed by performing hybridizations between the different treatments. The subtracted cDNA library was generated by inserting the differentially expressed cDNA fragments into pGEM-T Easy vectors (Promega, Madison, WI, USA) and transforming these vectors in JM109 competent cells. Aliquots (100 μl) of the transformation mixture were then spread on Luria-Bertani (LB) agar plates that contained 100 μg/ml ampicillin, 80 μg/ml X-gal and 50 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and were incubated at 37 °C overnight.

All subtractive clones were subjected to sequencing. The nucleotide and deduced amino acid sequence homologies were determined by searching the GenBank database using the BLASTX and BLASTN algorithm (http://www.ncbi.nlm.nih.gov/). GO annotation was analyzed by Blast2GO software (http://www.blast2go.org/). The COG annotation was performed using Blastall software against the cluster of orthologous groups database (ftp://ftp.ncbi.nih.gov/pub/COG/COG/).

2.4. Confirmation of differentially expressed genes by performing real-time qPCR

Because the quantity of *N. lugens* nymphs is very low, a mixture of 10 nymphs was collected as one sample. Total RNA was prepared from the pooled bodies of 10 *N. lugens* that were treated with triazophos or acetone as well as untreated samples at different stages of development starting from day 2 of the 4th instar (day 3 and day 4 of the 4th instar as well as day 1, day 2 and day 3 of the 5th instar) using the SV Total RNA Isolation System (Promega). For each treatment at different developmental stages, three replicates (each replicate contained 10 nymphs) were used for real-time qPCR. The RNA concentration was adjusted with DEPC H_2O to 1 μg/μl, and 1 μg of RNA was reverse transcribed in a 10 μl reaction system using the AMV RNA PCR Kit (TaKaRa). Real-time qPCR was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA, USA) using the SYBR *Premix Ex Taq* Kit (TaKaRa), according to the manufacturer's protocol. Two microliters of diluted first-strand cDNA (1/10) and 2 μl of no-template control (NTC) were used as templates in each 20-μl reaction mixture under the following conditions: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and at 60 °C for 34 s. The fluorescent signals yielded by the PCR products were detected by subjecting the products to a heat-dissociation protocol (temperature range, 60–95 °C) during the last step of each cycle. Following amplification, melting curves were constructed, and data analysis was performed by using the ABI 7500 system SDS software. Specific primer sets were designed for genes that encode *N. lugens vitellogenin*, *bystin*, *multidrug resistance protein* (*MRP*), *purine nucleoside phosphorylase* (*PNP*), *pyrroline-5-carboxylate reductase* (*P5CR*), *carboxylesterase* and *trypsin-like protein* (Table 1). As an internal control, the *N. lugens actin*3 gene (GenBank accession no. EU179850) was also analyzed using the following primers: 5′-TGGACTTTGAGCAGGAAATGC-3′ (forward primer) and 5′-ACGTCG-CACTTCATGATCGAG-3′ (reverse primer). The specificity of the primers was confirmed by using NCBI BLAST algorithms (http://www.ncbi.nlm.nih.gov/). The results were standardized to the expression level of the constitutive *N. lugens actin3*. An NTC sample was run to detect contamination and to determine the degree of dimer formation. A relative quantitative method ($\Delta\Delta C_t$) was used to evaluate quantitative variation.

3. Results

3.1. Classification of gene ontology (GO) and clusters of orthologous groups (COG) of subtractive cDNA clones

Following the SSH experiment, a total of 402 cDNA clones were isolated from the subtractive library made from triazophos-induced *N. lugens* nymphs. After removal of non-informative data (i.e., 5′ or 3′ non-coding regions and short sequences), the cDNAs were clustered into 96 genes (Table 2). To avoid false-positive clones, singlet genes (only one clone obtained) were deleted from GO and COG analysis. We used the WEGO tool (Web Gene Ontology

Table 1
Primers used in real-time qPCR for confirmation of differentially expressed genes.

N. lugens gene	Forward primer	Reverse primer
vitellogenin	5′ AAGAATGCTGGATCTCAGCGCAAGG 3′	5′CAACGAGGGTCTCACCATCAAAGTC 3′
bystin	5′ GACCCAGTTCTCGGACAACGGCAGT 3′	5′ CCAGCGGTGCGGTTCAGTGAGATAC 3′
multidrug resistance protein (*MRP*)	5′ ACAGTGGTCGGTTTGAAGCCATCCT 3′	5′ AACAGTGTTCCCTACCTGAACGCCA 3′
purine nucleoside phosphorylase (*PNP*)	5′ CCACCCTCTTCTTCAGCCATTCA 3′	5′ ACTTTCCGAGCCGCTTCTTCTCC 3′
pyrroline-5-carboxylate reductase (*P5CR*)	5′ CCGCAAACAAAAACAGAAGCACCTC 3′	5′ GCAAAACCTGCAATCAAAGATCGAC 3′
carboxylesterase	5′ ACAACTGTTATCAGGAGACCCGACT 3′	5′ CTGTTTCACTTCCAACACGAAGACC 3′
trypsin-like protein precursor	5′ CCTTGCTGTTTGCTTGGTGTTTCAC 3′	5′ CTGCTATCGCTGGCTACAATTTCCT 3′
actin3	5′ TGGACTTTGAGCAGGAAATGC 3′	5′ ACGTCGCACTTCATGATCGAG 3′

Table 2
Distribution of subtracted cDNA clones in the COG functional categories.

Gene name	Gi/accession no.	*E*-Value	Functional class	Code	Clone number
Nilaparvata lugens rRNA processing protein Ebp2	gi|225030990|gb|ACN79502.1|	5.84E−125	RNA processing/modification	A	2
Nasonia vitripennis similar to histone H2AV (H2A.F/Z)	gi|156554717|ref|XP_001605065.1|	1.63E−50	Chromatin structure/dynamics	B	4
Nilaparvata lugens cytochrome oxidase subunit I	gi|6752912|gb|AAF27934.1|	8.33E−107	Energy production/conversion	C	5
Lepidopsocidae sp. RS-2001 cytochrome c oxidase subunit III	gi|31126990|gb|AAK55292.2|	2.09E−29	Energy production/conversion	C	3
Nasonia vitripennis ATP synthase-beta	gi|229577383|ref|NP_001153366.1|	4.19E−54	Energy production/conversion	C	3
Nasonia vitripennis mitochondrial F1F0-ATP synthase subunit c isoform 4	gi|156551904|ref|XP_001606914.1|	1.88E−26	Energy production/conversion	C	2
Apis mellifera proteasome 26S subunit 4 ATPase CG5289-PA isoform 1	gi|66509032|ref|XP_623527.1|	1.68E−55	Energy production/conversion	C	3
Maconellicoccus hirsutus putative ADP/ATP translocase	gi|121543621|gb|ABM55522.1|	2.89E−23	Energy production/conversion	C	2
Nilaparvata lugens similar to PFTAIRE-interacting protein factor 1	gi|225031002|gb|ACN79508.1|	8.46E−122	Cell division/chromosome partitioning	D	2
Drosophila virilis GJ20697	gi|195381605|ref|XP_002049538.1|	2.04E−29	Amino acid transport/metabolism	E	1
Culex quinquefasciatus 4-hydroxyphenylpyruvate dioxygenase	gi|170036301|ref|XP_001846003.1|	5.45E−22	Amino acid transport/metabolism	E	2
Aedes aegypti pyrroline-5-carboxylate dehydrogenase	gi|108878912|gb|EAT43137.1|	1.98E−24	Amino acid transport/metabolism	E	4
Drosophila willistoni GK12188 (pyrroline-5-carboxylate dehydrogenase)	gi|195440458|ref|XP_002068059.1|	3.05E−09	Amino acid transport/metabolism	E	1
Tribolium Castaneum similar to pyrroline-5-carboxylate reductase	gi|91079134|ref|XP_975446.1|	2.06E−32	Amino acid transport/metabolism	E	7
Nasonia vitripennis similar to cystathionine gamma-lyase	gi|156547261|ref|XP_001601960.1|	1.02E−98	Amino acid transport/metabolism	E	3
Pediculus humanus corporis GMP synthase	gi|212512030|gb|EEB14877.1|	1.94E−43	Nucleotide transport/metabolism	F	1
Culex quinquefasciatus cytidine deaminase	gi|170032339|ref|XP_001844039.1|	9.77E−11	Nucleotide transport/metabolism	F	2
Pediculus humanus corporis purine nucleoside phosphorylase, putative	gi|212511558|gb|EEB14498.1|	6.97E−09	Nucleotide transport/metabolism	F	9
Nilaparvata lugens chitin deacetylase 5	gi|225030998|gb|ACN79506.1|	1.06E−78	Carbohydrate transport/metabolism	G	2
Pediculus humanus corporis hexokinase type, putative	gi|212514107|gb|EEB16480.1|	5.54E−06	Carbohydrate transport/metabolism	G	3
Oncometopia nigricans enolase	gi|53830714|gb|AAU95200.1|	5.04E−20	Carbohydrate transport/metabolism	G	4
Pediculus humanus corporis peritrophin-1 precursor, putative	gi|212514416|gb|EEB16744.1|	1.87E−14	Carbohydrate transport/metabolism	G	2
Nilaparvata lugens short chain dehydrogenase	gi|225031008|gb|ACN79511.1|	5.09E−43	Lipid transport and metabolism	I	3
Pediculus humanus corporis isovaleryl-CoA dehydrogenase	gi|212518302|gb|EEB20055.1|	3.05E−25	Lipid transport/metabolism	I	4
Tribolium castaneum acetyl-coa carboxylase	gi|189238375|ref|XP_969851.2|	1.96E−52	Lipid transport/metabolism	I	5
Homalodisca coagulata delta-9 desaturase 1	gi|46561748|gb|AAT01079.1|	9.21E−22	Lipid transport/metabolism	I	4
Nilaparvata lugens carboxylesterase	gi|209171174|gb|ACI42853.1|	9.10E−38	Lipid transport/metabolism	I	9
Apis mellifera similar to choline/ethanolamine kinase	gi|66541617|ref|XP_624492.1|	1.43E−06	Lipid transport/metabolism	I	2
Acyrthosiphon pisum ATP citrate lyase	gi|237874159|ref|NP_001153852.1|	9.36E−01	Lipid transport/metabolism	I	4
Nilaparvata lugens vitellogenin	gi|154799937|dbj|BAF75351.1|	8.12E−59	Lipid transport/metabolism	I	17
Galaxea fascicularis egg protein	gi|99653631|gb|BAE94663.1|	1.12E−02	Lipid transport/metabolism	I	1
Nilaparvata lugens ribosomal protein S15e	gi|225030988|gb|ACN79501.1|	1.61E−18	Translation/ribosomal structure	J	3
Nilaparvata lugens 16S ribosomal RNA	gi|225030984|gb|FJ810191.1|	2.24E−69	Translation/ribosomal structure	J	19
Nilaparvata lugens putative ribosomal protein S23	gi|225031004|gb|ACN79509.1|	7.31E−27	Translation/ribosomal structure	J	3
Nasonia vitripennis ribosomal protein L41 (Rpl41)	gi|209571496|ref|NM_001135907.1|	5.05E−23	Translation/ribosomal structure	J	4
Nilaparvata lugens mitochondrial ribosomal protein S11	gi|225030996|gb|ACN79505.1|	2.95E−80	Translation/ribosomal structure	J	2
Triatoma infestans ribosomal protein L11	gi|149898923|gb|ABR27976.1|	5.34E−25	Translation/ribosomal structure	J	5
Apis mellifera similar to mitochondrial ribosomal protein L15 CG5219-PA	gi|66543736|ref|XP_395827.2|	1.81E−06	Translation/ribosomal structure	J	2
Palloptera ambusta 12S ribosomal RNA gene, mitochondrial	gi|51039405|gb|AY573089.1|	4.80E−12	Translation/ribosomal structure	J	3
Triatoma infestans 60S ribosomal protein L8	gi|149689084|gb|ABR27873.1|	2.50E−22	Translation/ribosomal structure	J	1
Hister sp. APV-2005 ribosomal protein L18e	gi|70909737|emb|CAJ17294.1|	4.36E−56	Translation/ribosomal structure	J	5
Culex quinquefasciatus 40S ribosomal protein S27	gi|170038730|ref|XP_001847201.1|	1.55E−24	Translation/ribosomal structure	J	2
Nilaparvata lugens ribosome biogenesis protein	gi|225031006|gb|ACN79510.1|	1.49E−126	Translation/ribosomal structure	J	3
Acyrthosiphon pisum hypothetical protein LOC100159774	gi|240848641|ref|NP_001155412.1|	6.33E−23	Translation/ribosomal structure	J	1
Pediculus humanus corporis KH domain-containing protein C56G2.1	gi|242010558|ref|XP_002426032.1|	9.58E−08	Transcription	K	2
Candidatus Carsonella ruddii RNA polymerase beta-prime subunit	gi|225591853|gb|AAK55935.1|	9.32E−01	Transcription	K	4
Nasonia vitripennis similar to ATP-dependent RNA helicase	gi|156550342|gb|XP_001606676.1|	4.71E−18	Replication/recombination/repair	L	2
Aedes aegypti predicted membrane protein	gi|94469326|gb|ABF18512.1|	4.63E−13	Cell wall/membrane biogenesis	M	1
Himetobi P virus capsid protein precursor	gi|50251150|dbj|BAD27585.1|	2.31E−85	Cell wall/membrane/envelope biogenesis	M	2
Nasonia vitripennis similar to sorting and assembly machinery component 50 homolog (*S. cerevisiae*)	gi|156537145|ref|XP_001603591.1|	5.47E−06	Cell wall/membrane biogenesis	M	2
Pediculus humanus corporis 2-oxoglutarate dehydrogenase	gi|212511739|gb|EEB14632.1|	7.19E−59	Chaperones	O	6
Apis mellifera similar to CG8258-PA	gi|66544702|ref|XP_623832.1|	6.18E−18	Chaperones	O	2
Drosophila mojavensis GI20714 (calcium-transporting ATPase)	gi|195122746|ref|XP_002005872.1|	4.32E−43	Inorganic ion metabolism	P	4
Nilaparvata lugens trypsin-like protein precursor	gi|54792420|emb|CAH65684.1|	9.82E−16	Catabolism	Q	10
Apis mellifera similar to dipeptidyl aminopeptidase III CG7415-PC, isoform C	gi|66509442|ref|XP_395694.2|	2.07E−72	Catabolism	Q	2

Table 2 (*Continued*)

Gene name	Gi/accession no.	*E*-Value	Functional class	Code	Clone number
Acyrthosiphon pisum similar to pancreatic lipase related protein 1	gi|193587079|ref|XP_001947773.1|	8.50E−02	Catabolism	Q	3
Nasonia vitripennis similar to arginine/serine-rich splicing factor	gi|156545752|ref|XP_001605411.1|	2.56E−48	General function prediction	R	5
Nilaparvata lugens inter-alpha-trypsin inhibitor heavy chain H4 precursor	gi|225030986|ref|ACN79500.1|	3.01E−126	General function prediction	R	7
Graphocephala atropunctata putative translationally controlled tumor protein	gi|90819984|gb|ABD98748.1|	3.95E−39	General function prediction	R	5
Tribolium castaneum similar to CG3621 CG3621-PA	gi|91083977|gb|XP_975169.1|	4.02E−12	General function prediction	R	1
Tribolium castaneum similar to CG32019-PA	gi|189233817|ref|XP_971502.2|	3.30E−153	General function prediction	R	3
Drosophila yakuba GE16131	gi|194188049|ref|EDX01633.1|	1.84E−15	General function prediction	R	1
Acyrthosiphon pisum hypothetical protein LOC100164311	gi|242247223|ref|NP_001156187.1|	1.50E−22	Function unknown	S	2
Drosophila ananassae GF12803	gi|194754008|ref|XP_001959297.1|	1.00E−61	Function unknown	S	3
Nasonia vitripennis similar to BMKETTIN	gi|156555815|ref|XP_001602095.1|	3.96E−12	Function unknown	S	2
Aedes aegypti bystin	gi|157103337|ref|XP_001647934.1|	9.03E−25	Function unknown	S	9
Nasonia vitripennis similar to conserved hypothetical protein	gi|156537882|ref|XP_001608121.1|	3.05E−33	Function unknown	S	4
Acyrthosiphon pisum ACYPI008021	gi|239792066|dbj|BAH72417.1|	7.23E−12	Function unknown	S	2
Triatoma infestans hypothetical protein	gi|149898806|gb|ABR27865.1|	2.73E−07	Function unknown	S	1
Acyrthosiphon pisum similar to conserved hypothetical protein	gi|193657105|ref|XP_001951753.1|	3.86E−05	Function unknown	S	2
Pediculus humanus corporis conserved hypothetical protein	gi|212516403|gb|EEB18416.1|	3.78E−64	Function unknown	S	3
Acyrthosiphon pisum similar to AGAP005226-PA	gi193678941|ref|XP_001951641.1|	5.00E−12	Function unknown	S	1
Drosophila grimshawi GH22290	gi|193899755|gb|EDV98621.1|	1.36E−04	Function unknown	S	3
Drosophila persimilis GL18518	gi|194107191|gb|EDW29234.1|	5.36E−01	Function unknown	S	2
Tribolium castaneum similar to AGAP007135-PA	gi|189241070|ref|XP_001808548.1|	1.75E−25	Function unknown	S	3
Tribolium castaneum similar to CG10990 CG10990-PB	gi|91076522|ref|XP_973518.1|	3.33E−07	Function unknown	S	1
Drosophila virilis GJ12417	gi|194153718|gb|EDW68902.1|	4.62E−01	Function unknown	S	2
Anopheles gambiae str. PEST AGAP003993-PA	gi|158298263|ref|XP_318445.4|	5.19E−01	Function unknown	S	2
Riptortus clavatus JR-1	gi|2108224|gb|AAB72002.1|	3.34E−03	Function unknown	S	1
Nilaparvata lugens reovirus polypeptide	gi|540520|dbj|BAA03517.1|	4.09E−47	Function unknown	S	3
Maconellicoccus hirsutus putative 14-3-3 protein	gi|121543925|gb|ABM55627.1|	1.01E−48	Signal transduction	T	2
Tribolium castaneum similar to serine/threonine-protein phosphatase PP-V	gi|91091568|ref|XP_967314.1|	4.16E−62	Signal transduction	T	7
Nilaparvata lugens putative chemosensory protein CSP8	gi|215254084|gb|ACJ64054.1|	8.73E−34	Signal transduction	T	5
Apis mellifera similar to Hpr1 CG2031-PA isoform 1	gi|110760796|ref|XP_393145.2|	4.08E−08	Signal transduction	T	2
Acyrthosiphon pisum similar to integrin alpha FG-GAP repeat containing 2	gi|193582598|ref|XP_001951649.1|	6.39E−38	Signal transduction	T	1
Laodelphax striatellus mucin-like protein	gi|46360158|gb|AAS88902.1|	3.00E−13	Defense mechanisms	V	2
Acyrthosiphon Pisum similar to multidrug resistance protein	gi|193636681|ref|XP_001947434.1|	6.03E−09	Defense mechanisms	V	7
Nasonia vitripennis similar to lectin 4 C-type lectin	gi|156545611|ref|XP_001604870.1|	6.49E−18	Defense mechanisms	V	2
Nilaparvata lugens actin 1	gi|163883731|gb|ABY48093.1|	1.58E−45	Cytoskeleton	Z	3
Nilaparvata lugens myosin 2 light chain	gi|225031000|gb|ACN79507.1|	1.00E−43	Cytoskeleton	Z	4
Nilaparvata lugens alpha 2-tubulin	gi|225031010|gb|ACN79512.1|	1.04E−99	Cytoskeleton	Z	3
Drosophila melanogaster 60C beta tubulin	gi|860916|emb|CAA34726.1|	1.32E−36	Cytoskeleton	Z	2
Pediculus humanus corpori Titin	gi|212517000|gb|EEB18954.1|	6.61E−66	Cytoskeleton	Z	2
Nilaparvata lugens troponin I	gi|225030992|gb|ACN79503.1|	6.88E−70	Cytoskeleton	Z	4
Acyrthosiphon pisum ACYPI007392 (similar to troponin C)	gi|239790194|dbj|BAH71673.1|	4.53E−69	Cytoskeleton	Z	1
Bombyx mandarina flightin	gi|209922135|gb|FJ263955.1|	8.88E−01	Cytoskeleton	Z	2
No significant similarity with known proteins	No hit		No COG category		76
Total					402

List of *N. lugens* genes in triazophos-activated library. Gene name: annotation of the subtracted cDNA sequences was based on GenBank database using the BLASTX algorithm. Functional class and code: classes to which the sequences belong to, according to classification for COG (ftp://ftp.ncbi.nih.gov/pub/COG/COG). Number of clones: distribution of the cDNA clones corresponding to the given gene with available sequences in the subtracted library.

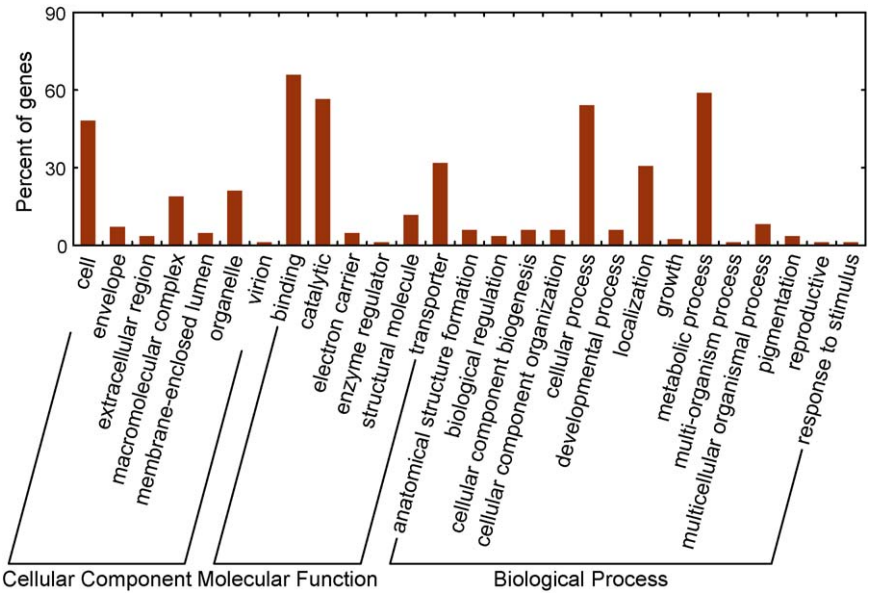

Fig. 1. Gene ontology classification of triazophos-induced genes in *N. lugens*. The genes are summarized in three main categories: cellular component, molecular function and biological process. The *Y*-axis indicates the percentage of a specific category of genes in that main category.

Annotation Plotting) for plotting the GO annotation results (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). In terms of GO classification, the sequences can be categorized into 27 functional groups (Fig. 1). In each of the three main categories (cellular component, molecular function and biological process) of the GO classification, "Cell," "Binding and Catalytic" and "Metabolic process and Cellular process" terms are dominant. The GO annotation results also include two viral genes encoding an *N. lugens* reovirus polypeptide and a Himetobi P virus capsid protein precursor, which are found in the cellular component category in the least abundance.

To further evaluate the effectiveness of the annotation process, we searched the annotated sequences for the genes involved in COG classifications by using Blastall software against the COG database. After removal of singlet genes, 289 sequences have a COG annotation using an *E*-value cut-off of 10^{-5}. Among the COG categories, the cluster for 'translation/ribosomal structure/biogenesis' represents the largest group (51 clones, 17.7%), followed by 'lipid transport/metabolism' (44 clones, 15.2%) and 'function unknown' (33 clones, 11.4%). Metabolism-related clusters including energy production/conversion, amino acid transport/metabolism, nucleotide transport/metabolism, carbohydrate transport/metabolism and secondary metabolites biosynthesis/catabolism are the most abundant group that together comprised 23.5% (68 clones) of the COG annotated sequences. The clusters for signal transduction, defense mechanisms and cytoskeleton shared similar abundance with individual metabolism-related clusters (3.8–6.2%). In contrast, RNA processing/modification, cell cycle control/cell division/chromosome partitioning, transcription and replication/recombination/repair are the smallest clusters, which together only comprised 2.77% of the clones in the COG categories (Fig. 2).

3.2. Determination of differentially expressed cDNAs

In order to determine the expression of triazophos-activated genes, especially in nymphs on day 3 of the 5th instar, we performed a time course analysis by real-time qPCR (Fig. 3). We focused on the genes encoding metabolic enzymes and female development-related proteins showing high abundance in the library and determined their transcript levels during various *N. lugens* developmental stages. The candidate genes had significant sequence homologies to *N. lugens vitellogenin*, *Aedes aegypti bystin*, *Acyrthosiphon pisum multidrug resistance protein* (*MRP*), *Pediculus humanus corporis purine nucleoside phosphorylase* (*PNP*), *N. lugens carboxylesterase*, *Aedes aegypti pyrroline-5-carboxylate reductase* (*P5CR*) and *N. lugens trypsin-like protein*, which may be involved in mechanisms of the resistance to insecticides or female reproduction of planthopper.

Transcripts of *N. lugens vitellogenin*, a gene with female-specific expression, were detected at extremely low levels within the 4th instar and on day 1 and day 2 of the 5th instar; however, transcript levels increased on day 3 of the 5th instar, a time point just before emergence (Fig. 3). Triazophos and acetone greatly up-regulated *vitellogenin* gene expression at this developmental stage, with the former induction looking stronger than the latter. The transcript levels for *MRP* slowly increased throughout the developmental process and attained the maximum level on day 3 of the 5th instar (Fig. 3). At this time point, the expression levels under triazophos treatment were notably higher than under acetone and no treatment, indicating that triazophos enhanced *MRP* expression around the emergence stage and that *MRP* expression is likely to be associated with adult emergence action. *Bystin* showed a similar expression pattern to *MRP*. Transcript levels gradually increased throughout the developmental process, and triazophos significantly up-regulated its expression on day 3 of the 5th instar (Fig. 3). The transcript levels of *P5CR* presented a mountain-like pattern during the 4th instar, and expression increased to an even greater extent on day 2 of the 5th instar (Fig. 3). *Carboxylesterase* transcripts were detected with the highest levels on day 4 of the 4th instar with triazophos stimuli, while its transcript levels did not change during the natural developmental process or with acetone treatment (Fig. 3). *PNP* gene expression was slightly up-regulated by triazophos from day 2 of the 4th instar and quickly increased to a peak on day 4 of the 4th instar. After a short decrease on day 1 of the 5th instar, *PNP* gene expression increased again on days 2–3 of the 5th instar (Fig. 3). The variation of *PNP* transcripts was not significant throughout the normal developmental stages. Acetone increased *PNP* gene expression, but the greatest increase in expression was with triazophos stimulation. Intriguingly, the expression of *trypsin-like protein* showed a distinct pattern from the above candidate genes. Transcript levels of *trypsin-like protein*

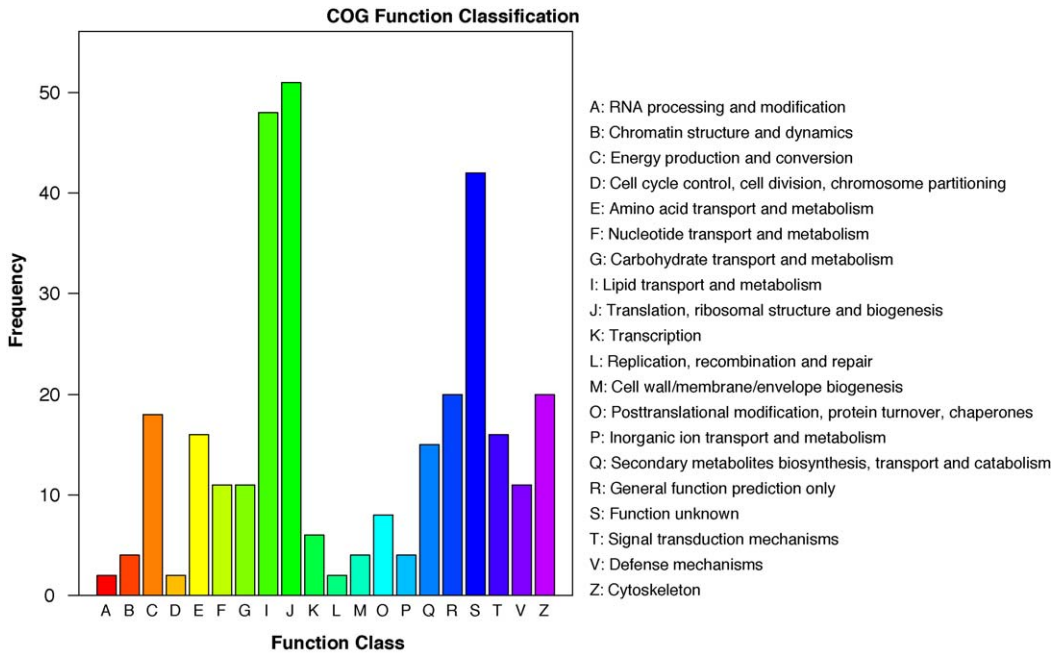

Fig. 2. Clusters of orthologous groups (COG) classification of triazophos-induced genes in *N. lugens*. The cDNA sequences were used for a blast search and annotation against the cluster orthologous groups database using an *E*-value cut-off of 10^{-5}. After removal of singlet genes, 289 sequences have a COG classification among the 20 categories.

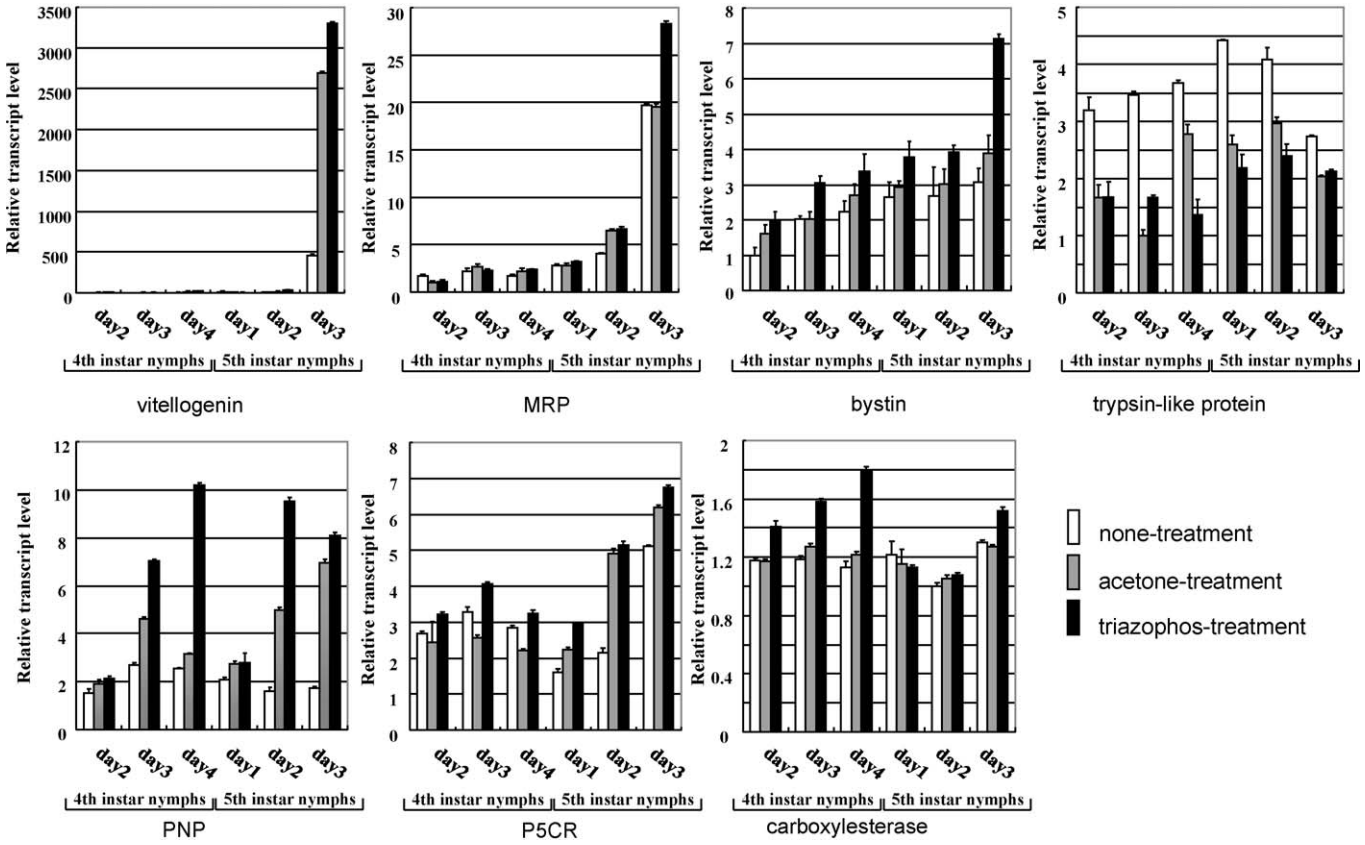

Fig. 3. Expression time course of triazophos-activated genes in *N. lugens*. The 4th instar nymphs on day 2 were treated with a sub-lethal dose (LC_{10}) of triazophos. Total RNA was extracted from the pooled whole bodies of 10 *N. lugens* that were treated with triazophos or acetone as well as untreated control samples at the indicated times. Two microliters of each 10-fold dilution from the first-strand cDNA (20 ng) reaction were analyzed in each real-time qPCR reaction. The reaction was performed with the specific primers for amplifying *N. lugens vitellogenin*, *bystin*, *MRP*, *PNP*, *P5CR*, *carboxylesterase* and *trypsin-like protein*. The relative expression levels of each gene at different time points were normalized using the C_t values that were obtained for the *actin3* amplifications run on the same plate. In each assay, the expression level is shown relative to the lowest expression level, which is arbitrarily set at one. The mean value ±SD was used for analysis of relative transcript levels for each time point using the $\Delta\Delta C_t$ method. The untreated, the acetone-treated and the triazophos-treated samples are shown on the left (white), middle (grey) and right (black), respectively.

were much lower with acetone and triazophos treatment compared to the untreated condition throughout the time course (Fig. 3), suggesting that acetone and triazophos inhibited *trypsin-like protein* gene expression.

Real-time qPCR analysis confirmed the up-regulated expression of the six candidate genes on day 3 of the 5th instar, the time point for constructing the triazophos-activated cDNA library. Among the genes, *vitellogenin*, *MRP* and *bystin* transcript levels were significantly up-regulated by triazophos when compared to acetone treatment. Increased transcript levels were also observed for *PNP*, *P5CR* and *carboxylesterase* at this time point. Triazophos inhibited *trypsin-like protein* expression, although a slightly increased transcript level was observed when compared to acetone treatment on day 3 of the 5th instar. In addition, triazophos consistently up-regulated *bystin*, *PNP* and *P5CR* gene expression throughout the tested developmental stages.

4. Discussion

The widespread application of various insecticides causes resistance to insecticides and resurgence of the planthopper. The mechanisms responsible for insecticide resistance are attributable to high enzyme activity, which metabolizes the compounds quickly (Noda et al., 2008); however, the resurgence of *N. lugens* is closely associated with the insecticide-induced increase in female productivity (Ge et al., 2009).

In this study, we focused on the identification of the genes encoding metabolic enzymes and female reproduction-related molecules because they may be involved in the resistance or resurgence mechanisms in *N. lugens*. Among the 402 cDNA clones detected in the subtracted library (Table 2), the transcripts for metabolic enzymes represented the highest abundance (127 clones in total, 31.6%).

In the gene distributions, the transcripts for *N. lugens vitellogenin* and *16S ribosomal RNA* showed the most abundance, at 4.2% (17 clones) and 4.7% (19 clones), respectively, in the library. Vitellogenin is a female-specific protein that is mainly synthesized extraovarially in the fat body and transported into the growing oocytes (Tufail and Takeda, 2009). It is well known that vitellogenin synthesis is under hormonal control; however, none of the literature has reported that insecticides promote vitellogenin production in insects. In this study, the significantly up-regulated expression of *N. lugens vitellogenin* was observed in nymphs on day 3 of the 5th instar after triazophos stimuli (Fig. 3), clearly indicating that triazophos increased *vitellogenin* gene expression before adult emergence. This result implies that triazophos might prompt female reproduction. In this study, we isolated the cDNA clones for the *N. lugens* 16S rRNA gene (GenBank accession no. FJ810191), which had an insert sequence (546 bp) showing 99% identities with an *Oryza sativa* resistance-related non-coding RNA (GenBank accession no. AY166969) that was induced after planthopper infestation. This result is identical to 16S rRNA genes identified in *N. lugens*, which shared 99% similarities with *O. sativa* resistance-related non-coding RNA (NCBI, accession no. AF158038 and AF158034). In addition, we identified the 16S rRNA sequence in the *N. lugens* genome, suggesting that *N. lugens* might integrate the *O. sativa* partial RNA gene sequence in the genome.

A similar expression tendency was seen for the genes encoding bystin and MRP. *N. lugens bystin* gene transcripts shared 80% sequence homology with *Aedes aegypti bystin* at deduced amino acid level. Bystin was found in the human placenta during early stages of pregnancy but disappeared after the 10th week of pregnancy (Suzuki et al., 1999). Aoki et al. (2006) demonstrated that bystin is essential for the survival of mouse embryos. Bystin expression started during hatching and increased in the expanded blastocyte. Furthermore, Fukuda et al. (2008) reported that bystin functions in ribosomal biogenesis, specifically in processing of 18S RNA in human cancer cells and mouse embryos. In *Drosophila*, maternal *bystin* is strongly expressed in cells exhibiting high levels of rRNA synthesis and ribosome production (Stewart and Nordquist, 2005). In this study, *N. lugens* bystin seemed to be involved in *N. lugens* development due to its tendency to increase expression slowly throughout the developmental process (Fig. 3). A significantly up-regulated expression on day 3 of the 5th instar by triazophos treatment implies that *bystin* may function in adult emergence or ovary maturation. The deduced amino acid sequence of *N. lugens MRP* is most similar to *Acyrthosiphon pisum MRP*, with which it shares 55% identity. MRP was first identified in a human small-cell lung cancer cell line where its overexpression conferred resistance to a large spectrum of drugs (Cole et al., 1992). Though *MRP* genes have been cloned from several insect species, *i.e.*, *Drosophila melanogaster* (Grailles et al., 2003), *Acyrthosiphon pisum* (NCBI, accession no. XM_001947399) and *Culex quinquefasciatus* (NCBI, accession no. XM_001866949), the function of the protein in insects remains unknown. In this experiment, *MRP* expression gradually increased under physiological conditions, and triazophos significantly increased its expression on day 3 of the 5th instar, implying a potential induction of resistance to triazophos.

The expression patterns of the other candidate genes were different. For example, *PNP* was constitutively expressed at low levels throughout the time course and was significantly up-regulated after triazophos stimuli. Acetone also enhanced *PNP* gene expression but not stronger than triazophos (Fig. 3). The purine pathway is indispensable in *Anopheles gambiae* and *Plasmodium* species, in which PNP functions in purine recycling and purine salvage (Taylor et al., 2007; Ting et al., 2008). As PNP activity is crucial for mosquito stage development and parasite survival (Ting et al., 2008), it has been regarded as a target for the development of therapeutic agents for blocking malaria transmission (Taylor et al., 2007). The purine pathway is not clear in *N. lugens*; however, purine synthesis may be related to *N. lugens* productivity as purine production may not be sufficient for its own developmental requirements. If this is true, the triazophos-induced increase in *PNP* expression may be one of the factors leading to *N. lugens* resurgence; however, further evidence is necessary to prove this assumption. P5CR is the last enzyme of the proline biosynthetic pathway (Hua et al., 1997), and the accumulation of proline is a striking metabolic response to stresses by a large number of organisms from bacteria to higher plants (Kishor et al., 1995). P5CR plays a crucial role in intermediary metabolism in *D. melanogaster* (Misener and Walker, 2001); however, its biological function in *N. lugens* is totally unknown. In this study, significant up-regulation of *P5CR* was observed on day 2 and day 3 of the 5th instar in triazophos-stimulated *N. lugens* (Fig. 3); however, it is currently difficult to assume whether this enzyme facilitates *N. lugens* productivity around emergence or contributes to triazophos resistance.

The *Carboxylesterase* gene expression pattern was somehow different. The transcript levels did not vary under normally physiological conditions, while triazophos significantly increased its expressions during the 4th instar and day 3 of the 5th instar. Small et al. (1998) reported that esterases in the resistant mosquito *C. quinquefasciatus* have a high affinity for organophosphates. In *N. lugens*, widespread resistance to organo-phosphorous insecticides is associated with elevation of carboxylesterase activity (Small and Hemingway, 2000). In this experiment, increased *carboxylesterase* expression may be associated with the activity of carboxylesterase or affinity to triazophos. The expression pattern of *N. lugens trypsin-like protein* was significantly different from the above candidate genes. Its transcripts showed high levels throughout the time course; however, triazophos down-regulated its expression. This may be a strategy of *N. lugens* that mitigated physiological

costs via inhibiting digestion action to maintain the insecticides resistance mechanisms.

In summary, *vitellogenin*, *MRP* and *bystin* shared similar expression patterns. Their expression was significantly up-regulated by triazophos treatment on day 3 of the 5th instar, which is a time point before emergence. Triazophos treatment also significantly up-regulated *PNP* gene expression, especially in the late stages of the 4th and 5th instar. These results imply that the up-regulation of gene expression by triazophos may be associated with *N. lugens* female reproduction or resistance to triazophos. Although it is not clear, these candidate genes may have the potential to induce resurgence via increasing female reproduction or resistance to triazophos. Our data provide clues that the resurgence mechanism may be due to triazophos-induced up-regulation of certain genes that affect *N. lugens* physiology.

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