Identification of genes differentially expressed during larval molting and metamorphosis of *Helicoverpa armigera*

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**Abstract**

**Background**: Larval molting and metamorphosis are important physiological processes in the life cycle of the holometabolous insect. We used suppression subtractive hybridization (SSH) to identify genes differentially expressed during larval molting and metamorphosis.

**Results**: We performed SSH between tissues from a variety of developmental stages, including molting 5th and feeding 6th instar larvae, metamorphically committed and feeding 5th instar larvae, and feeding 5th instar and metamorphically committed larvae. One hundred expressed sequence tags (ESTs) were identified and included 73 putative genes with similarity to known genes, and 27 unknown ESTs. SSH results were further characterized by dot blot, Northern blot, and RT-PCR. The expression levels of eleven genes were found to change during larval molting or metamorphosis, suggesting a functional role during these processes.

**Conclusion**: These results provide a new set of genes expressed specifically during larval molt or metamorphosis that are candidates for further studies into the regulatory mechanisms of those stage-specific genes during larval molt and metamorphosis.

**Background**

Molting is a physiological process common to all ecdysozoan animals, including nematodes and arthropods, during which the old exoskeleton, or cuticle, is shed and replaced by a new exoskeleton. The life cycle of the holometabolous insect is characterized by a series of molts: larval molts, during which the larva progresses from one instar to the next, and metamorphic molts, which lead to pupation and eclosion. Generally, molting consists of two phases, the lethargus, or inactive phase, and ecysis, or shedding of old cuticle. A cascade of physiological processes occurs during molting, including separation of the old cuticle from the underlying epidermis (apolysis), secretion of a new cuticle beneath the old, and finally shedding of the old exoskeleton [1]. Subsequent metamorphosis, the transformation of larva to pupa to adult, includes metamorphic molting. During this process, more complicated physiological processes occur, including histolysis of larval tissues, remodeling and formation of adult tissues, in addition to a molting cascade similar to the larval molt. Apoptotic and autophagic programmed cell death pathways are involved in tissue histolysis and remodeling during metamorphosis [2,3]. Insect larval molting and metamorphosis are governed by
ecdysteroids (20-hydroxyecdysone, 20E) and juvenile hormone (JH), with 20E orchestrating the molting process and JH determining the nature of the molt [4]. In the presence of JH, 20E directs larval molting. Otherwise, 20E directs metamorphosis. Twenty hydroxyecdysone (20E) and JH levels increase during the late stages of the final (wandering) instar in *Manduca* larvae, before pupal ec dysis, and then decrease at the pupal ec dysis. 20E levels peak midway through the pupal stage, but JH does not [4]. Increasing evidence indicates that other hormones and receptors may contribute to the complex developmental pathways associated with metamorphosis [5]. However, the endocrine circuits that regulate molting in response to environmental and physiologic cues are not well understood. Moreover, little is known about the molecular mechanisms regulating release and *de novo* production of the new exoskeleton [6]. The molecular mechanisms underlying metamorphosis are also enigmatic, although some key regulatory genes have been identified, such as *Broad complex, E74B* and *E93* [2]. In addition, very few genes downstream of Broad complex, *E74B* and *E93* are identified. Therefore, the molecular mechanisms that lead to larval molt and metamorphosis are poorly understood. Nevertheless, much remains to be learned regarding the molecular regulatory processes governing larval molting and metamorphosis.

Tremendous efforts have been made to identify larval molting and metamorphosis genes. Many genes have been shown to be involved in molting or metamorphosis, such as the transcription factors *EcR, USP, HR3, Broad C* [4], and the programmed cell death pathway genes [7]. The powerful technique of DNA microarray has extended the single-gene approach to the genome level by allowing simultaneous comparison of transcript levels of thousands of genes [8]. Several microarray and functional genomic studies have led to the identification of genes that exhibit a change in expression level at the onset of metamorphosis. For example, microarray technology was used to investigate gene expression changes in response to 20E, and dependent on *EcR*, at the onset of metamorphosis in *Drosophila* [9]. Tissue-specific gene expression and ecdysone-regulated genomic networks have also been examined by microarray in *Drosophila* [10]. Another study found that about one-third of all *Drosophila* genes undergo changes in expression by examining whole insects at different developmental timepoints [11].

Identification of genes differentially expressed during larval molting

cDNAs from five separate tissues from molting 5th instar larvae (5th-HCS, with head capsule slippage, HCS) were used as the tester and cDNAs from feeding 6th instar larvae (6th-48 h) were used as the driver in five separate rounds of SSH, resulting in 200 cDNAs with molecular masses greater than 300–500 bp. Sequencing revealed 27 putative genes with similarity to annotated genes in Genbank by BLAST search, and 8 novel ESTs (Table 1). Very high frequency genes included the ribosome proteins genes rpl27, S7, S2 and L23. Other high frequency genes were *CHK1* checkpoint homolog, *carboxypeptidase A2* (carbA2) and *hmg176*. However, most genes appeared at a lower frequency of 1 or 2 times (Fig. 1A).

Next, we grouped our genes according to predicted function, based on similarity to known genes in the National Center of Bioinformatics (NCBI) database, to produce seven main groups: ribosome proteins, rp); hydrolases, immune-related genes, regulators, enzyme, other genes, and unknown ESTs. Among those genes, eight encoded
Table 1: Identification of putative genes from SSH of tester cDNA from molting 5th instar larvae and driver cDNA from feeding 6th instar larvae

| Classification               | No | Tissue Sources          | Similar genes in GenBank                      | E-value | Functions of the genes                                                                                                           |
|------------------------------|----|-------------------------|-----------------------------------------------|---------|--------------------------------------------------------------------------------------------------------------------------------|
| Ribosome proteins            | 1  | head midgut epidermis   | ribosomal protein L27 [S. frugiperda]         | 1e-55   | participates in both 50 S subunit assembly and the peptidyl transferase reaction                                             |
|                              | 2  | head                    | ribosomal protein S7 [S. frugiperda]          | 7e-23   | initiating assembly of the head of the 30S subunit                                                                             |
|                              | 3  | fat body midgut         | ribosomal protein S2 [B. mori]                | 2e-40   | maintaining balance between the small and large ribosomal subunits                                                           |
|                              | 4  | midgut                  | ribosomal protein L17/23 [Apis mellifera]     | 8e-17   | regulator of the p53-MDM2 feedback regulation                                                                               |
|                              | 5  | midgut                  | ribosomal protein L38 [Plutella xylostella]   | 2e-17   | protein of 60 S ribosomal subunit                                                                                             |
|                              | 6  | haemocyte               | ribosomal protein S24 [B. mori]               | 1e-21   | protein of 40 S ribosomal subunit                                                                                            |
|                              | 7  | head                    | ribosomal protein L22 [S. frugiperda]         | 2e-29   | large ribosomal subunit                                                                                                       |
|                              | 8  | midgut                  | ribosomal protein L13 [S. frugiperda]         | 3e-43   | 60 S ribosomal subunit protein                                                                                               |
| Hydrolases                   | 9  | fat body                | molting fluid carboxypeptidase A [B. mori]-1  | 3e-31   | pupal ecdysis, recycling of the amino acids                                                                                |
|                              | 10 | haemocyte               | cathpsinogen Y like protease [Glossina morisia] | 1e-25   | larval molting and cuticle and eggshell remodeling                                                                         |
|                              | 11 | midgut                  | trypsinogen Y precursor [Gadus morhua]         | 2e-06   | hydrolase                                                                                                                   |
|                              | 12 | epidermis               | molting fluid carboxypeptidase A [B. mori]-2  | 1e-54   | melting-related                                                                                                             |
|                              | 13 | epidermis               | plasminogen activator, tissue [Mus muscula]   | 7e-07   | hydrolase                                                                                                                   |
|                              | 14 | midgut                  | lipase-I [B. mandarina]                       | 2e-23   | lipid digestion                                                                                                             |
|                              | 15 | fatbody                 | chitinase [H. armigera] (BLAST by nucleotide acids) | 0.42   | hydrolase                                                                                                                   |
| Immune-related Genes         | 16 | haemocyte               | lipopolysaccharide binding protein [B. mori]  | 6e-26   | immune                                                                                                                       |
|                              | 17 | fat body                | immune reactive putative protease inhibitor PrInh6 [Glossinamoritsans moritan] | 2e-09   | immune                                                                                                                       |
| Regulators                   | 18 | fat body haemocyte       | CHK1 checkpoint homolog [Xenopus tropicalis]  | 2e-07   | DNA damage and replication checkpoints                                                                                       |
|                              | 19 | midgut                  | CG13323-PA [D. melanogaster] (similar to hmg176) | 6e-05   | -                                                                                                                            |
|                              | 20 | epidermis               | thymosin beta homologue [Ciona intestinalis]  | 0.55    | actin-remodeling, wound healing, angiogenesis                                                                             |
| enzyme                       | 21 | head                    | Na-K-ATPase [Anopheles gambiae]                | 2e-09   | enzyme                                                                                                                       |
| Others                       | 22 | midgut                  | TNF receptor homolog 3 [Rattus norvegicus]    | 3       | cellular proliferation, differentiation and programmed cell death                                                          |
|                              | 23 | epidermis               | Titin-like protein [B. mori]                  | 8e-15   | roles in chromosomes and muscles                                                                                             |
|                              | 24 | midgut                  | Acp4a binding protein [B. mori]               | 4e-06   | intracellular acyl-CoA transporters                                                                                         |
|                              | 25 | fat body                | hypothetical protein Llacc01000286 [Lactococcus lactis subsp.cremoris SK11] | 1e-24   | -                                                                                                                            |
|                              | 26 | head                    | CG32972-PB, isoform B [D.melanogaster]         | 0.011   | -                                                                                                                            |
|                              | 27 | fat body                | 16S rRNA [S. frugiperda] (BLAST by nucleotide acids) | 7e-17   | -                                                                                                                            |
| Unknown ESTs                 | 28-35 | epidermis             | Unknown proteins                              | -       | -                                                                                                                            |
Genes identified in 5th instar molting larvae

**Figure 1**
Genes identified in 5th instar molting larvae. A) Gene frequency. B) Gene transcription composition (number, percentage).
ribosome proteins (22% of the genes), and seven encoded hydrolases, including carbA1 and c arbA2, cathesis L (cathL), trypsinogen Y, tissue plasminogen activator (tPA), lipase, and a chitinase-like gene (20% of the genes). The other encoded lipopolysaccharide binding protein (lbp), immune reactive putative protease inhibitor (irpp-inhibitor), CHK1 checkpoint homolog, and other putative genes, respectively (Fig. 1B).

Identification of genes differentially expressed during metamorphic molting

Two hundreds cDNA fragments were sequenced from SSHs between tester cDNAs of metamorphically committed larvae (6th-72, 96 and 120 h) and driver cDNAs of feeding 5th instar larvae (5th-24 h). By BLAST search, 31 putative genes with similarity to annotated Genbank genes, and 16 unknown ESTs were identified (Table 2). The most frequently appearing genes were ribosome protein encoding genes, including rpL27, S23 and S7. Another frequently occurring gene was the basic juvenile hormone-suppressible protein (BJHSP), which was not detected in 5th molting or 5th feeding larvae by SSH. Other genes identified were specifically detected only once or twice in this SSH (Fig. 2A).

These genes were classified into eight groups, as described above: ribosome proteins, hydrolases, immune-related genes, regulators, enzymes, transporters, others and unknown ESTs. A large number of unknown ESTs were common to tissues from the metamorphic molt (Fig. 2B). In addition, some important genes such as BJHSP, DEAD-box RNA helicases, basic leucine zipper (bzip), and guanine nucleotide binding protein gamma subunit (G-protein-γ) were detected at these stage.

Identification of genes differentially expressed in feeding 5th instar larvae

SSHs between feeding 5th instar larvae tester and metamorphically committed larvae (6th-72, 96 and 120 h) driver were performed to identify cDNAs enriched in feeding 5th instar larvae. In total, 230 cDNA fragments were sequenced following PCR. Of these, 23 putative genes and 3 unknown ESTs were identified (Table 3). Ribosome protein encoding genes were the most common, especially rpL27 that appeared very higher frequency, whereas other ribosome proteins had lower frequencies. Another high frequency gene was the gene encoding retinoblastoma-binding protein 6 isoform 2-like, which was not identified in molting 5th instar or metamorphically committed larvae by SSH. Other lower frequency genes were specifically detected in feeding 5th instar larvae (Fig. 3A).

Genes were classified into 6 groups, according to similarity, including ribosome proteins, hydrolases, regulators, enzymes, other genes, and unknown ESTs (Fig. 3B). Interestingly, three high frequency hydrolases genes were all vitellogenic-like carboxypeptidase, although their sequences were quite divergent. These three genes were detected in epidermis of feeding 5th instar larvae. The genes encoding ecdysteroid-regulated protein and a cytochrome P450 like-TBP-like gene were also detected from feeding 5th instar larvae. In addition, many genes with unknown function were detected.

Analysis of SSH efficacy by dot blot hybridization

To analyze the efficacy of SSHs, dot blot hybridization was performed using 70 cDNA fragments that were randomly chosen from the set of differentially expressed genes resulting from SSH between molting 5th instar epidermis tester and feeding 6th instar epidermis driver. Blots were hybridized with probes made from cDNA of molting 5th instar larval epidermis. Nearly all of the 70 dots stained positively. In contrast, when blots were hybridized with probes made from cDNA of feeding 6th larval epidermis, only 3 dots were obviously positively stained. This result indicates that 95% of the cDNAs detected by SSH were differentially expressed during larval molting, while 5% of the cDNAs may not be molting-specific (Fig. 4).

Northern blot analysis of gene expression

Two genes identified by SSH were chosen for analysis by Northern blot in different tissues at various developmental stages. One of these was hmg176, which was identified in molting 5th instar midguts by SSH between molting 5th instar larvae tester and feeding 6th instar driver. The other gene examined was cathL, identified in molting 5th instar hemocytes by the same SSH. The results indicated that hmg176 was highly expressed in the midgut during larval molting, but was not detected in 6th-48 h larvae. Similarly, cathL was obviously upregulated in hemocytes of molting larvae (Fig. 5). These data suggest that some of the ESTs identified by SSHs do represent stage-specific genes.

Semi-quantitative RT-PCR analysis of larval molting or metamorphosis genes

To further analyze larval molting and metamorphosis genes and to examine gene expression patterns in different tissues, such as head, midgut, fat body, epidermis, and hemocytes during development, 22 putatively differentially expressed genes were randomly chosen for semi-quantitative RT-PCR analysis. These included 18 genes from SSHs between molting 5th instar larva testers and feeding 6th instar larva drivers, 3 genes from SSHs between metamorphically committed larva testers and feeding 5th instar drivers, and 1 gene from SSHs between feeding 5th instar testers and metamorphically committed larva drivers. The beta-actin gene was used as a positive control.
| Classification        | No | Tissue sources | Similar genes in GenBank | E-value | Functions of the genes                      |
|-----------------------|----|----------------|--------------------------|---------|---------------------------------------------|
| **Ribosome proteins** |    |                |                          |         |                                             |
| 1 epidermis           |    | ribosome protein S23 [D.melanogaster] | 4e-06 | see Table 1 |                                             |
| 2 epidermis midgut    |    | ribosome protein L27 [S. frugiperda] | 4e-54 | see Table 1 |                                             |
| 3 fat body head       |    | ribosome protein S7 [B.mori] | 7e-24 | see Table 1 |                                             |
| 4 epidermis midgut    |    | ribosome protein L13 [Helicoverpa zeo] | 2e-24 | see Table 1 |                                             |
| 5 head                |    | ribosome protein L9 [B. mori] | 2e-26 | compose the large ribosomal subunit |                                             |
| 6 head                |    | ribosome protein L11 [B. mori] | 5e-22 | large ribosomal subunit protein |                                             |
| 7 head                |    | ribosomal protein L41 [S. frugiperda] (BLAST by nucleotide acids) | 1e-23 | mitochondrial ribosomal protein |                                             |
| **Hydrolases**        |    |                |                          |         |                                             |
| 8 fat body            |    | DEAD box RNA helicase [C. fumiferana] | 1e-25 | disrupt RNA-protein interactions |                                             |
| 9 midgut             |    | ribonuclease [Ceratitis capitata] | 8e-31 | degradation of RNA |                                             |
| **Immune-related proteins** |    |                |                          |         |                                             |
| 10 midgut            |    | immune-inducible protein [B. mori] | 3e-11 | immune-response |                                             |
| 11 midgut            |    | lysozyme [Heliothis wiescens] | 1e-17 | immune |                                             |
| **Regulator**        |    |                |                          |         |                                             |
| 12 epidermis          |    | basic juvenile hormone-suppressible protein 2 precursor (BjHSP2) [S. litura] | 6e-08 | metamorphosis related |                                             |
| 13 epidermis          |    | promoting protein [B. mori] | 3e-12 | promoting in vitro replication of nucleopolyhedrovirus |                                             |
| 14 head              |    | basic leucine zipper and W2 domains 2 [R. norvegicus] | 0.009 | transcription factor controlling molting and metamorphosis |                                             |
| 15 fat body          |    | guanine nucleotide binding protein gamma subunit-like protein [P. xylostella] | 4e-22 | Signal transduction |                                             |
| 16 midgut            |    | conserved hypothetical protein [Lactococcus lactis] | 5e-19 | - |                                             |
| **Enzyme**           |    |                |                          |         |                                             |
| 17 fat body          |    | ATP synthase subunit 6 [Pthionandria atrilineata] | 0.15 | ATP synthase |                                             |
| 18 midgut           |    | luciferase [Luciola lateralis] | 8e-13 | Fatty acyl-CoA synthetase |                                             |
| 19 head              |    | IMP dehydrogenase/GMP reductase [Desulfuromonas acetoxidans DSM 684] | 0.31 | re-utilization of free intracellular bases and purine nucleosides |                                             |
| **Transporter**      |    |                |                          |         |                                             |
| 20 epidermis         |    | cation channel family protein [Tetrahymena thermophila SB210] | 6.1 | Ca2+-permeable ion channel |                                             |
| 21 midgut           |    | nuclear transport factor 2 [Aedes aegypti] | 3e-24 | targeting proteins into the nucleus |                                             |
| **Others**           |    |                |                          |         |                                             |
| 22 midgut           |    | phenoloxidase inhibitor protein [Anopheles gambiæ] | 0.006 | enzyme inhibitor |                                             |
| 23 epidermis         |    | apolipophorin-III precursor [Agris convolvuli] | 1e-12 | lipid transfer and cell death |                                             |
| 24 midgut           |    | CG12120-PA [Tribolium castaneum] | 2e-09 | - |                                             |
| 25 epidermis         |    | hexamerine [H. armigera] | 2e-04 | storage protein during metamorphosis |                                             |
| 26 midgut           |    | cobatoxin long form B [S. frugiperda] | 8e-05 | - |                                             |
| 27 midgut           |    | sensory neuron membrane protein 2 [M. sexta] | 4e-05 | - |                                             |
| 28 head              |    | 60 S ribosome subunit biogenesis protein [Theileria annulata] | 9.7 | - |                                             |
| 29 midgut           |    | ENSANGP00000017373 [Apis mellifera] | 3e-10 | - |                                             |
| 30 head              |    | CG12926-PA [T. castaneum] | 3e-19 | - |                                             |
| 31 midgut           |    | deoxyribodipyrimidine photolyase [Marinobacter aquaeolei VT8] | 6.1 | - |                                             |
| **Unknown ESTs**     |    | midgut epidermis fat body head |               |         |                                             |
Figure 2
Genes identified in metamorphically committed larvae. A) Gene frequency. B) Gene transcription composition (number, percentage).
Table 3: Identification of putative genes from SSH of tester cDNA from feeding 5th instar larvae and driver cDNA of metamorphically committed larvae

| Classification | No. | Tissue sources | Similar genes in GenBank | E-value | Functions of the genes |
|----------------|-----|----------------|--------------------------|---------|------------------------|
| ribosome proteins | 1 | head midgut epidermis | ribosomal protein L27 [S. frugiperda] | 9e-55 | see Table 1 |
| | 2 | midgut | ribosomal protein L22 [S. frugiperda] | 9e-07 | see Table 1 |
| | 3 | midgut | ribosomal protein S29 [S. frugiperda] | 5e-29 | apoptotic inducer |
| | 4 | head | ribosomal protein L13 [H. zea] | 5e-43 | see Table 1 |
| | 5 | midgut | ribosomal protein L28 [S. frugiperda] | 6e-38 | ribosome assembly |
| | 6 | midgut | ribosomal protein S7 [Plutella xylostella] | 7e-25 | see Table 1 |
| | 7 | midgut | ribosomal protein S10 [Lonomia obliqua] | 1e-33 | mitochondrial import proteins |
| hydrolases | 8 | epidermis | carboxypeptidase, vitellogenic-like-1 [T. castaneum] | 1e-29 | inflammatory protease cascade |
| | 9 | epidermis | carboxypeptidase, vitellogenic-like-2 [T. castaneum] | 2e-16 | inflammatory protease cascade |
| | 10 | epidermis | carboxypeptidase, vitellogenic-like-3 [D. rerio] | 6e-04 | inflammatory protease cascade |
| regulators | 11 | epidermis | apoptosis inhibitor [Bos taurus] | 1e-16 | candidate of molting-related protein |
| | 12 | midgut | ecdysteroid-regulated protein [Litopenaeus vannamei] | 5e-19 | molting-related protein |
| | 13 | head | retinoblastoma-binding protein 6 isoform 2 [D. rerio] | 0.87 | candidate of molting-related protein |
| enzyme | 14 | head fat body | glutathione S-transferase [C. fumiferana] | 9e-09 | detoxification of electrophilic compounds |
| | 15 | midgut | cytochrome P450 like_TBP [Nicotiana tabacum] | 1e-10 | - |
| others | 16 | epidermis | ENSANGP00000014082 [A. gambiae] | 1e-17 | - |
| | 17 | epidermis | CG8031-PA [D. melanogaster] | 1e-35 | - |
| | 18 | midgut | CG13607-PA [T. castaneum] | 1e-18 | - |
| | 19 | midgut | CG13922-PA [T. castaneum] | 4e-05 | - |
| | 20 | midgut | fascin 3 CG5803-PA-isofrom A [D. melanogaster] | 8.0 | - |
| | 21 | midgut | CG10733-PAisoform [T. castaneum] | 8e-06 | - |
| | 22 | fat body | keratin associated protein 7 [Pan troglodytes] | 0.98 | - |
| | 23 | epidermis | sensory box histidine kinase/response regulator | 6.2 | - |
| Unknown ESTs | 24–26 | midgut fat body epidermis | unknown proteins | - | - |

Of these genes, 8 genes were differentially detectable during larval molting. Some genes were known larval molting genes, such as carbA1, irpp-inhibitor, hmg176, carbA2 and a tumor necrosis factor receptor (TNFR)-like gene. Interestingly, these genes exhibited distinct tissue-specific expression patterns as well. For instance, carbA1 was mainly detected in molting larval fat bodies, epidermis and hemocytes, the irpp-inhibitor was mainly detected in molting larval fat bodies and epidermis, hmg176 was mainly expressed in molting larval midguts, carbA2 was mainly detected in molting larval epidermis; and the TNFR-like gene was mainly detected in molting larval heads, midguts and fat bodies. An unknown EST was detectable at higher levels in all 5 tissues of molting larvae than in feeding larvae. CathL was detected at higher levels in molting larval hemocytes than feeding larval hemocytes, consistent with Northern blot analysis. The tPA gene was detected at higher levels in molting larval fat bodies compared to feeding larval fat bodies.

Some genes exhibited differential expression in different tissues during larval molting. For example, the Na-K-ATPase-like gene was detected at higher levels in the epidermis, but not in the head, midgut, or hemocytes of molting larvae. Titin appeared to be expressed at higher levels in the midgut, but not in other tissues, of molting larvae. Trypsinogen Y precursor and lipase only exhibited increased expression in molting larval fat bodies compared with other detected tissues.

Some genes did not show an obvious relationship to larval molting. For example, lbp was not differentially detected between larval molting and feeding by semi-quantitative RT-PCR, although it was detected specifically in hemocytes. Expression of the thymosin beta homologue-like gene, rpl27 and S7 were detected at almost constant levels in molting and feeding larval tissues. Rpl23 and the hypothetical protein gene (llacc) appeared higher expression during feeding in some tissues (Fig. 6A).
Figure 3
Genes identified in 5th instar feeding larvae. A) Gene frequency. B) Gene composition (number, percentage).
Three genes were differentially detected by semi-quantitative RT-PCR in a tissue-specific manner during metamorphosis. These were the genes encoding the gamma subunit of guanine nucleotide binding protein (G-protein-γ), nuclear transport factor-2 (NTF-2) and rpl11. The G-protein-γ was detected at higher levels in the epidermis, midgut and fat body of the metamorphically committed larvae in 6th-72 or 6th-96 h compared with the tissues from feeding 5th larvae. The NTF-2 was also detected at higher levels in the midgut of metamorphically committed larvae. The rpl11 was detected at higher levels during metamorphosis and in all 4 examined tissues compared with the tissues from feeding 5th larvae. In contrast, the ecdysteroid-regulated gene was upregulated in the tissues from feeding larvae, which is consistent with the fact that it was detected in feeding 5th instar larvae by SSH (Fig. 6B).

**Discussion**

We identified 100 ESTs, including 73 putative genes that were similar to genes in Genbank, and 27 unknown ESTs by SSH of larval tissues. Most similar genes had E-values below 0.01, while less similar genes had E-values greater than 0.01 (Tables 123). Eleven of the putative genes were differentially detectable during larval molting or metamorphosis via semi-quantitative RT-PCR, including regulatory genes expressed during molt and metamorphosis such as NTF-2, G-proteins, and the downstream genes that may play a direct role in the molting and metamorphosis such as carbA2 and cathL. Expression levels of these genes clearly correlated with larval molting or metamorphosis in a largely tissue-specific or developmental stage-specific manner by semi-quantitative RT-PCR analysis. Importantly some of these genes have been reported to play a
Figure 6
Semi-quantitative RT-PCR analysis of gene expression in tissues from different developmental stages. Panel A) Gene expression during larval molting and feeding. M = molting 5th instar larvae (5th-HCS), F = feeding 6th instar larvae (6th-48 h). Panel B) gene expression during metamorphosis. 5th-24 = 5th instar larvae 24 h after ecdysis, 6th-72 = 72 h after ecdysis (wandering-0 d, metamorphically committed larvae); 6th-96 h, 96 h after ecdysis (wandering-1 d, metamorphically committed larvae).
role in larval molting or metamorphosis. For examples, carbA2 has been reported to be a molting gene in Bombyx, and is expressed during molting in the epithelial tissues [16]. The CarbA2 protein has been suggested to degrade old cuticle during molting and to contribute to recycling of the amino acids in this tissue [16]. CathL, which encodes a cysteine proteinase, is involved in larval molting and cuticle and eggshell remodeling in Brugia pahangi [17]. Other genes identified in this study have not been previously known to play a role in molting or metamorphosis. Their predicted function, based on similarity, however, supports the notion that these genes participate in regulation of larval molting or metamorphosis. For instance, nuclear transport factor-2 (NTF-2) targets proteins to the nucleus in yeasts and mammalian cells in culture [18]. The gamma subunit of guanine nucleotide binding protein (G-protein-γ) is an essential component of one of the most prevalent signaling systems in mammalian cells. The fact that NTF-2 and G-protein-γ are upregulated during metamorphosis suggests that they participate in metamorphosis.

Some genes identified in this study, based on functions of similar genes, probably participate in larval molting or metamorphosis, although they have not yet been demonstrated to be upregulated in larval molting or metamorphosis in this study. For instance, the basic-leucine zipper gene, controls molting and metamorphosis and is likely to be involved in bZIP signaling pathways in Drosophila [19]. The basic juvenile hormone-suppressible protein 2 (BjHSP2), a juvenile hormone-sensitive protein, expresses and accumulates in the last instar larvae of Trichoplusia ni [20]. The DEAD box RNA helicase and leucine-rich repeat genes are important in molting C. elegans [6]. The Chk1 checkpoint protein was reported to monitor the state of DNA and can delay or arrest the cell cycle at multiple points [21]. The ribonuclease selectively attacks malignant cells, triggering an apoptotic response and inhibiting protein synthesis [22]. These putative genes represent attractive targets for further study into their functions in larval molting and metamorphosis.

Ribosomal proteins have been reported to participate in various cellular processes besides protein biosynthesis. They not only act as components of the translation apparatus, but also regulate cell proliferation and apoptosis [23]. For example, rlPL11 associates with and inhibits the transcriptional activity of peryosome proliferator-activated receptor-alpha [24]. Rpl23 regulates p53-DMDM2 feedback and the HDMD2-p53 pathway [25,26]. Mitochondrial ribosomal protein L41 plays an important role in p53-induced mitochondrion-dependent apoptosis by enhancing p53 stability and contributing to p53-induced apoptosis [27,28]. Rps29 induces apoptosis in H520 cells [29,30]. Rps10 participates in both transcription and translation [31]. We detected these ribosome proteins, and they appear to be highly conserved. Among these, rlPL11 was differentially expressed during metamorphosis. Whether or not other ribosome proteins participate in larval molting and metamorphosis need further study.

Interestingly, several putative immune-related genes were identified in molting larvae in this study. For instance, a lysozyme was identified in metamorphically committed larvae. Interestingly, a lysozyme has also been reported to be upregulated during larval metamorphosis in M. sexta, and has been proposed to provide protection from bacterial infection [32]. Other immune-related genes including the immune inducible protein and IRPP-inhibitor might play a similar role. These observations provide evidence that some immune-related genes are differentially expressed during larval molting or metamorphosis.

We also detected an ecdysteroid-regulated protein-like gene encoding an ML domain (MD-2-related lipid-recognition domain) in feeding 5th instar larvae. Single ML domain proteins are predicted to form a beta-rich fold containing multiple strands and to mediate diverse biological functions through interaction with specific lipids [33]. The function of this gene in larval development is unknown. In addition, three kinds of carboxypeptidase vitellogenic-like genes (cpvl) were detected in the epidermis of feeding 5th instar larvae. Human cpvl is upregulated during the maturation of monocytes (MO) to macrophages, and is involved in antigen processing, the secretory pathway and/or in actin remodeling and lamelipodium formation [34,35]. Because these genes were detected in feeding 5th instar feeding larvae, they may play important roles in larval growth and development.

We examined the efficacy of SSH by dot blot, Northern blot and RT-PCR. Dot blot analysis of cDNAs from the epidermis of molting 5th instar tester and feeding 6th instar driver suggested the efficacy was 95%. Northern blot analysis indicated that some were differentially expressed during larval molting. However, RT-PCR analysis suggested that only 11 of the 22 examined genes were differentially expressed during larval molting. Therefore, the efficacy of SSH was not as high as indicated by the dot blot analysis. This discrepancy might be due to limitation in sensitivity of the dot blot.

Intriguingly, several known genes critical for larval molting and metamorphosis in other species were not identified in this study. One possible explanation might be because that transcripts of these genes, most of them are transcription factors, are extremely rare, or they are
expressed for a very short time. Another possible explanation is that too few cDNAs were sequenced.

Finally, certain genes, including *rpl27*, *S7*, and *L13* were detected at higher levels in all SSHs. This might be due to incomplete subtraction of the extremely highly expressed housekeeping genes or to excessive cycles of PCR amplification. Sampling mRNA at correct developmental stages of larval molting and metamorphosis, increasing the amount of driver cDNA used, reducing the number of PCR cycles, and sequencing more cDNAs might improve the efficacy of SSH in future studies.

Of the 11 genes upregulated during larval molting or metamorphosis, 8 were differentially detected during larval molting (HCS), including *carbA1*, *irpp-inhibitor*, *hmg176*, *carbA2* and a *tumor necrosis factor receptor* (*TNFR*)-like gene, and 3 were differentially detected from metamorphically committed larvae, including the *gamma subunit of guanine nucleotide binding protein* (G-protein-γ), *nuclear transport factor-2* (*NTF-2*) and *rpl11*. According to studies in *Manduca*, 20E levels peak during larval molting several hours before HCS and then decrease to lower levels while JH levels peak at larval ecysis. 20E and JH again peak together at pupal commitment stage (wandering stages). After pupation, 20E peak again during pupal development [4]. *Helicoverpa armigera* belongs to Lepidoptera: Noctuidae and has similar 20E titer to *M. sexta*, which reaches peak levels during 5th and 6th larval molts and decreases to lower level during ecysis [36]. The 11 differentially expressed genes detected in this study are likely regulated by the puff of 20E, which present us new gene candidates for further study the functions and regulatory expression of these genes during larval molting or metamorphosis.

**Conclusion**

From the 100 ESTs identified from SSH of various tissues of *H. armigera*, we identified 11 that are differentially upregulated during larval molting or metamorphosis. In addition, we present several other candidates for further studies of larval molting or metamorphosis. These studies will extend our knowledge the regulatory mechanisms of holometabolous insect larval molting and the metamorphosis cascade.

**Methods**

**Insects**

The cotton bollworms, *Helicoverpa armigera* (Lepidoptera: Noctuidae), were reared on food composed mainly of wheat, soybean, vitamins, and inorganic salts, and maintained in a light:dark cycle of 14:10 h at 27°C. Developmental stages were defined as follows. Fifth-0 h larvae: immediately after ecysis with white head capsule (5th-0 h, WH). 5th instar larvae normally eat for 36~40 h and then head capsule slippage (HCS) occurs. HCS lasts for about 12 h, during which larvae considered 5th instar molting larvae (5th-molting, or 5th-HCS). Next, larvae shed their cuticle in a few minutes and enter the 6th instar (6th-0 h, WH). Sixth instar larvae eat for 48 h, then purge the gut and turn red, and begin wandering at 72 h (6th-72 h, W-0 d). Larvae continue to grow until 96 h (6th-96 h, W-1 d), then contract their legs and stop wandering at 120 h (6th-120 h, PP), and pupation begins at 140 h (P-0 h/d). Pupae are designated P-1 d, P-2 d, and so on. Pupae develop for 9~10 d, depending on sex, until eclosion. According to studies in *Manduca*, 20E levels peak several hours before HCS and then begin to decrease. JH levels peak at larval ecysis. 20E and JH again peak together at wandering and prepupal stages [4]. We prepared all samples for this study according to these developmental markers in individuals of equal body size.

**Isolation of mRNAs from tissues and synthesis of cDNA**

Messenger RNA was isolated from epidermis, midguts, fat bodies, hemocytes, and heads at four developmental stages using Micro mRNA Isolation kit (Amersham, Uppsala, Sweden) following the manufacturer's protocol. Developmental stages included the 5th to 6th instar transition, the feeding 6th instar stage (6th-48h), metamorphically committed larvae (6th-72, 96, and 120 h), and feeding 5th instar larvae (5th-24 h). First strand cDNAs were synthesized using SMART subtract cDNA construction kit from Clontech (BD Bioscience Clontech, Mountain View, USA) from 2 μg of mRNA with the following primers: 5′ SMART CDS Primer II A Oligonucleotide and 3′ SMART CDS Primer II A (5’-AAGCAGTGATATAACGCGGCGAGATACGCGGG-3’, 5’-AAGCAGTGATATAACGCAGTACT(30)N-1N-3-3’, N -1 = ACG; N = ATCG). Second strands were synthesized from first strand cDNAs using PCR Primer II (5’-AAGCAGTGATATAACGCGGAGTACGCGGG-3’).

**Suppression Subtractive Hybridization (SSH)**

Three kinds of SSH were performed, including tester cDNAs from five tissues from molting 5th instar larvae and driver cDNAs from five tissues from feeding 6th instar larvae, tester cDNAs from five tissues from metamorphically committed larvae and driver cDNAs from five tissues from feeding 5th instar, and tester cDNAs from five tissues of feeding 5th instar larvae and driver cDNAs from five tissues from metamorphically committed larvae. cDNAs from testers and drivers were first cut into fragments by RsaI digestion. Each cDNA from the testers was then separated into two portions and adapters 1 and 2R were each added to one of the two parts.

5’-CTATACCGACTCACATATAGGCTCGAGCCGCACCAGGGAGTTGAGTACGCGGG-3’ Adapter 1

(page number not for citation purposes)
3′-GGCCCGTCCA-5′
5′-CTAATACGACTCATATAGGGCAGCGTGTCGCG-GCCGAGGT-3′, to amplify differentially expressed
5′-GCCGCCCGGGCAGGT-3′/5′-AGCGTGGTCGCG-3′ and nested PCR primers 1/primer 2R (5′-TCGAGCG-
with PCR primer 1 (5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCG-
View, USA). PCR amplification was then performed twice
in a ratio of tester:driver 1:30 for the first hybridization,
and followed a second hybridization by adding 1 μl driver
mixture (1 μl hybridization buffer, 1 μl driver and 2 μl water) to the first hybridization solution according to the
Clontech PCR-Select cDNA SUBTRACTION kit protocol (BD Biosciences (BD Bioscience Clontech, Mountain
Make PCR products over 300 bp obtained by SSH were cloned
Escherichia coli DH5α. Insert sizes were screened by PCR and were
sequenced. A similarity search using each DNA fragment
as a query was performed using the Basic Local Alignment
Search Tool (BLASTX) [37] to identify the genes.

Cloning and identification of DNA fragments from SSH
PCR products were purified and cloned into the pGEM T-
Easy vector (Promega Company (Biosciences, Madison,
WI, United States) and transformed into Escherichia coli
DH5α. Insert sizes were screened by PCR and were
sequenced. A similarity search using each DNA fragment
as a query was performed using the Basic Local Alignment
Search Tool (BLASTX) [37] to identify the genes.

Preparation of digoxigenin-labeled anti-sense RNA probes
Target genes were inserted into the pGEM-T Easy plasmid.
Recombinant plasmids were linearized, depending on the
insertion direction of the target gene, and were used as
templates for in vitro transcription of anti-sense dig-
labelled probe using an RNA Labeling kit was used to syn-
thetize the probe and the procedures were performed
according to the manufacturer’s suggestions (Boehringer
Mannheim, Mannheim, Germany). The length of the tar-
gen genes was about 500 bp.

Preparation of digoxigenin-labeled DNA probes
Complementary DNAs (cDNA) synthesized from mRNAs
isolated from 5th and 6th instar epidermis were used as
templates for synthesizing dig-labeled DNA probes. The
probes were synthesized by random primed PCR using
the Dig High Prime DNA Labeling and Detection Starter
Kit (Boehringer Mannheim, Mannheim, Germany).

Dot blot hybridization
PCR products over 300 bp obtained by SSH were cloned
into pGEM T-Easy plasmid and transformed into DH5α.
Plasmids were amplified by PCR and the PCR products
were quantified by agarose gel electrophoresis to choose
candidates for dot blot hybridization. After denaturing for
10 min at 100°C, chilling on ice, and 2 μl of each PCR
product was then dotted onto a positively-charged nylon
membrane. After cross linking for 10 min under ultravo-
et light, blots were prehybridized, hybridized, washed,
and developed color. Prehybridization and hybridization
were performed at 40°C. The probe was denatured at
100°C for 5 min before use. The wash conditions were: 2 ×
SSC + 0.1% SDS for 2 × 5 min at room temperature, fol-
lowed by 0.1 × SSC + 0.1% SDS for 2 × 15 min at 68°C.
Color development was achieved using the NBT/BCIP
(nitroblue tetrazolium chloride/5-bromo-4-chloro-3-
indolyl phosphate) solution (45 μl NBT 50 mg/ml in 70% v/v dimethylformamide, plus 35 μl BCIP 50 mg/ml in 100% dimethylformamide).

Northern blot
Approximately 10 μg of total RNA was denatured and
electrophoresed in a 5% formaldehyde-containing agar-
ose gel. After electrophoresis, the RNA was transferred
onto a nylon membrane. Target mRNA was prehybridized
for 2 h and hybridized with dig-labeled antisense RNA
probe (100 ng/ml final concentration) in Northern
hybridization buffer, at 68°C overnight. After the strin-
gency wash at 68°C 2 × SSC + 0.1% SDS for 2 × 5 min at
room temperature, followed by 0.1 × SSC + 0.1% SDS for
2 × 15 min at 68°C, anti-dig-Phosphatase AP antibody
(1:10000 diluted in buffer 2, 100 mM maleic acid, 150
mM NaCl; pH 7.5, 1% blocking reagent) was used to
detect the probe at 37°C. NBT and BCIP were used to vis-
ualize the signals as description above. All steps were car-
ried out as recommended by the manufacturer (Roche).

Semi-quantitative reverse transcription-PCR (Semi-
quantitative RT-PCR)
Total RNA was isolated from epidermis, midguts, fat bod-
ies, hemocytes and heads of molting 5th instar and feed-
ing 6th instar larvae. Complementary DNAs (cDNA) were
then synthesized. The beta-actin gene was used as a cDNA
quantity control. PCR conditions were as follows: 94°C
for 2.5 min, and then 23 cycles of 94°C for 45 s, 53°C for
1 min and 72°C for 1 min, followed by a single cycle at
72°C for 10 min. The primers used are listed in Table 4.

Authors’ contributions
Du-Juan Dong constructed the SSH library and analyzed
the DNA sequences. Hong-Juan He constructed the SSH
library and analyzed the DNA sequences. Lian-Qin Chai
carried out RT-PCR and Northern blot. Xiao-Juan Jiang
constructed the SSH library and analyzed the DNA se-
quences. Lian-Qin Chai constructed the SSH library and did Dot blot hybridiza-
tion and Northern blot. Jin-Xing Wang participated in its
design and coordination. Xiao-Fan Zhao participated in
the design of the study and drafted the manuscript. All
authors read and approved the final manuscript.
Table 4: List of primers for semi-quantitative RT-PCR

| Number | Genes                                      | Primers                                                      |
|--------|--------------------------------------------|--------------------------------------------------------------|
| 1      | Carboxypeptidase A1                        | CarbA1F2 5'-gcttcttcttcttctttct-3' CarbA1RT 5'-actcttccacagttct-3' |
| 2      | Cathepsin L                                | CathFL1 5'-tgatcttcagctcttc-3' CathLR2 5'-tagatcttcagctcttc-3' |
| 3      | Lipopolysaccharide binding protein          | LipO2F2 5'-actccttcagctcttc-3' LipO2R2 5'-ctccttcagctcttc-3' |
| 4      | Immune reactive putative protease inhibitor | InhibFL2 5'-ttgatcttcagctcttc-3' InhibLR2 5'-tagatcttcagctcttc-3' |
| 5      | Lipase                                      | LipaseF2 5'-ctgatcttcagctcttc-3' LipaseR2 5'-tagatcttcagctcttc-3' |
| 6      | Trypsinogen Y precursor                    | TrypFL2 5'-tcgatcttcagctcttc-3' TrypLR2 5'-actcttccacagttct-3' |
| 7      | Conserved hypothetical protein [Lactococcus lactis] | LactFL2 5'-tctgatcttcagctcttc-3' LactLR2 5'-actcttccacagttct-3' |
| 8      | Titin-like protein                          | TitinFL1 5'-gcttcttcttcttctttct-3' TitinLR2 5'-actcttccacagttct-3' |
| 9      | Na-K-ATPase                                 | ATMaseF2 5'-actcttccacagttct-3' ATMaseR2 5'-actcttccacagttct-3' |
| 10     | HMG176                                      | HMG176F 5'-actcttccacagttct-3' HMG176R 5'-actcttccacagttct-3' |
| 11     | Carboxypeptidase A2                        | CarbA2F2 5'-ctccttcagctcttc-3' CarbA25-P 5'-aaagccttaacggccagtgtc-3' |
| 12     | Tissue plasminogen activator               | tPAF2 5'-tggatcttcagctcttc-3' tPAF3 5'-tagatcttcagctcttc-3' |
| 13     | Thymosin beta homologue                    | ThymoFL1 5'-ggaacccaaaccggttca-3' ThymoLR1 5'-atgatcttcagctcttc-3' |
| 14     | TNF receptor homolog 3                     | TNFRII 5'-aagctttttgcttcatc-3' TNFRI 5'-actcttcagctcttc-3' |
| 15     | Unknown EST                                 | UNRF1 5'-aagctttttgcttcatc-3' UNR1 5'-actcttcagctcttc-3' |
| 16     | Ribosome protein S23                       | L11F 5'-cagcttcagctcttc-3' L11R 5'-ggctttttgcttcatc-3' |
| 17     | Ribosome protein L27                       | L27F 5'-aagctttttgcttcatc-3' L27R 5'-actcttcagctcttc-3' |
| 18     | Ribosome protein S7                        | L7F 5'-aagctttttgcttcatc-3' L7R 5'-actcttcagctcttc-3' |
| 19     | Ribosome protein S7                        | L7F 5'-aagctttttgcttcatc-3' L7R 5'-actcttcagctcttc-3' |
| 20     | G-protein-γ                                | G-protein-γ 5'-aagctttttgcttcatc-3' G-protein-γ 5'-actcttcagctcttc-3' |
| 21     | NTF-2                                      | NTF2 5'-atgatcttcagctcttc-3' NTF2R 5'-ttgatcttcagctcttc-3' |
| 22     | Ecdysteroid-regulated gene                 | EcdyF5 5'-cagcttcagctcttc-3' EcdyR5 5'-actcttcagctcttc-3' |
| 23     | β-actin                                     | ActinF5 5'-aagctttttgcttcatc-3' ActinR5 5'-actcttcagctcttc-3' |

Accession numbers

Accession numbers of the putative genes and unknown expression sequence tags (ESTs) in this study are: [GenBank: DQ875214–DQ875283 and EF332468–EF332497].

Acknowledgements

This work was supported by grants from the Natural Science Foundation of China (No: 30330070; 30670265), the Special Fund for the Major State Basic Research Project (2006CB102001), and the Natural Science Foundation of Shandong Province (No.Z2003D04).

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