



## The immune signaling pathways of *Manduca sexta*

Xiaolong Cao <sup>a</sup>, Yan He <sup>a</sup>, Yingxia Hu <sup>a</sup>, Yang Wang <sup>a</sup>, Yun-Ru Chen <sup>b</sup>, Bart Bryant <sup>c</sup>, Rollie J. Clem <sup>c</sup>, Lawrence M. Schwartz <sup>d</sup>, Gary Blissard <sup>b</sup>, Haobo Jiang <sup>a,\*</sup>

<sup>a</sup> Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA

<sup>b</sup> Boyce Thompson Institute, Cornell University, Ithaca, NY 14853, USA

<sup>c</sup> Division of Biology, Kansas State University, Manhattan, KS 66506, USA

<sup>d</sup> Department of Biology, University of Massachusetts, Amherst, MA 01003, USA



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### ABSTRACT

Signal transduction pathways and their coordination are critically important for proper functioning of animal immune systems. Our knowledge of the constituents of the intracellular signaling network in insects mainly comes from genetic analyses in *Drosophila melanogaster*. To facilitate future studies of similar systems in the tobacco hornworm and other lepidopteran insects, we have identified and examined the homologous genes in the genome of *Manduca sexta*. Based on 1:1 orthologous relationships in most cases, we hypothesize that the Toll, Imd, MAPK-JNK-p38 and JAK-STAT pathways are intact and operative in this species, as are most of the regulatory mechanisms. Similarly, cellular processes such as autophagy, apoptosis and RNA interference probably function in similar ways, because their mediators and modulators are mostly conserved in this lepidopteran species. We have annotated a total of 186 genes encoding 199 proteins, studied their domain structures and evolution, and examined their mRNA levels in tissues at different life stages. Such information provides a genomic perspective of the intricate signaling system in a non-drosophilid insect.

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**Abbreviations:** Atg, autophagy-related protein; aPKC, atypical protein kinase C; CF, control fat body; CH, control hemocytes; IF, induced fat body; IH, induced hemocytes; Deaf, deformed epidermal autoregulatory factor; Dnr1, defense repressor-1; Dsp1, Dorsal switch protein-1; ECSIT, evolutionarily conserved intermediate in Toll pathways; FPKM, fragments per kilobase of exon per million fragments mapped; GPRK, G protein-coupled receptor kinase; IAP, inhibitor of apoptosis; Imd, immunodeficiency; IKK, I $\kappa$ B kinase; JNK, Jun N-terminal kinase; Jra, Jun-related antigen; MAPK, mitogen-activated protein kinase; MASK, multiple ankyrin repeats single KH domain; ML, MD2-like; MLK, mixed-lineage kinase; NF $\kappa$ B and I $\kappa$ B, nuclear factor- $\kappa$ B and its inhibitor; NTF, nuclear transport factor; PIAS, protein inhibitor of activated STAT; PIRK, poor Imd response upon knock-in; PVF, platelet-derived and vascular endothelial growth factor; PVR, PDGF/VEGF receptor; STAT, signal transducer and activator of transcription; PG and PGRP, peptidoglycan and peptidoglycan recognition protein; SUMO, small ubiquitin-like modifier; TAK, transforming growth factor  $\beta$  activated kinase; TAMP, Toll activation mediating protein; TIR, Toll/interleukin-1 receptor; TRAF, tumor necrosis factor receptor-associated factor.

\* Corresponding author. Tel.: +1 405 744 9400; fax: +1 405 744 6039.

E-mail address: [haobo.jiang@okstate.edu](mailto:haobo.jiang@okstate.edu) (H. Jiang).

### 1. Introduction

Insects fight against invading pathogens and parasites via their innate immune system (Gillespie et al., 1997; Lemaitre and Hoffmann, 2007). Like other physiological processes, insect immune responses involve sensors, effectors, and signal transducers, linking pathogen recognition with cellular and humoral responses. Some of the responses occur in minutes while others involve transcriptional activation of genes that are not highly expressed under normal conditions, and thus may provide responses in hours to days. In the latter case, a relay system must exist to transduce the extracellular signals of wounding or invasion into the nuclei of cells, where transcriptional regulation occurs. If pathogens are sensed by receptors (e.g. PGRP-LC) on the cell surface, responses are more direct than if recognition occurs in hemolymph. In the latter scenario, receptors (e.g. PGRP-SA) in hemolymph bind to the pathogens and initiate extracellular signal transduction to generate active cytokines. The cytokines then interact with their receptors on the cell surface to induce cellular responses including phagocytosis, encapsulation, apoptosis, autophagy, and synthesis of immune effectors (Strand, 2008; Jiang et al., 2010). Consequently, the intracellular signal

transduction network is essential for mediating immune responses in insects.

Receptor-mediated Toll, Imd, MAPK-JNK-p38, JAK-STAT and other pathways are widely conserved in metazoans, functioning as regulators and mediators of humoral and cellular immune responses (Buchon et al., 2014). Extensive studies in *Drosophila melanogaster* have revealed many details of the signal transduction network. The Toll pathway was discovered in the screens that identified mutations in genes affecting the establishment of dorsoventral axis and later found to regulate the expression of immunity-related genes through Dorsal and Dif, transcription activators of the Rel family (Valanne et al., 2011). Gram-positive bacteria and fungi trigger this pathway via an extracellular serine protease cascade that activates the cytokine Spätzle through limited proteolysis. This activated cytokine binds to the Toll receptor, leading to antimicrobial peptide synthesis and differentiation of certain hemocytes into lamellocytes. These lamellocytes are capable of encapsulating and killing parasites such as parasitoid wasps (Sorrentino et al., 2004). In the case of Gram-negative bacteria, DAP-type peptidoglycans (PGs) elicit the Imd pathway via transmembrane PGRP-LC and intracellular signal mediators (Kaneko et al., 2006; Rämet et al., 2002). Activated Relish, another Rel factor, then migrates into the nucleus to turn on a set of immunity-related genes overlapping with that induced by Dorsal and Dif (Imler and Hoffmann, 2001; Mellroth et al., 2005). Cytokines, growth factors, or stress signals stimulate the MAPK-JNK-p38 pathway to regulate apoptosis, Imd pathway, and cell differentiation (Ragab et al., 2011; Chen et al., 2010; Dong et al., 2002). The JAK-STAT pathway, RNA interference, autophagy and other defense mechanisms are involved in antiviral responses (Kisseleva et al., 2002; Baeg et al., 2005; Kingsolver et al., 2013). Based on the available information, these pathways are mostly conserved among insects but differences do exist. For instance, the honeybee *Apis mellifera* has considerably fewer immunity-related genes (Evans et al., 2006). *A. mellifera* has five Toll genes compared with the nine found in *D. melanogaster*. The pea aphid *Acythosiphon pisum* lacks the entire Imd pathway (Gerardo et al., 2010). With such plasticity observed among the few genomes available in the Insecta, it is therefore critically important to examine and characterize the immune signaling components in different major orders of insects.

Lepidoptera comprises about 160,000 described species of moths and butterflies in 126 families and 46 superfamilies (Kristensen et al., 2007). Larvae of many lepidopterans are serious agricultural pests but they are susceptible and can be controlled by biological agents such as entomopathogens (e.g. viruses, bacteria, fungi) and parasitoid wasps. Studies of lepidopteran immune systems and the associated signaling pathways are extremely important for developing effective biological control methods. *Manduca sexta* and *Bombyx mori* have been used as powerful biochemical models to explore various aspects of innate immunity (Jiang et al., 2010). Immunity-related genes in the silkworm were previously compared with those in *D. melanogaster*, *Anopheles gambiae* and *A. mellifera* (Tanaka et al., 2008) and analyses of the *M. sexta* hemocyte and fat body transcriptomes revealed a set of 232 genes encoding proteins for pathogen recognition, signal transduction, microbe killing (Gunaratna and Jiang, 2013), and modulation of mRNA levels in response to an immune challenge (Zhang et al., 2011). Recently, the *M. sexta* genome assembly became available along with 52 RNA-Seq datasets of tissues at various life stages (X et al., 2015). To better understand immune signal transduction in this undomesticated pest species, we have annotated genes for the putative pathway members, studied their expression patterns, and proposed a signal transduction network based on 1:1 orthology. The results represent working models for future studies on *M. sexta* and other lepidopteran pests.

## 2. Materials and methods

### 2.1. Gene identification, sequence improvement, and feature prediction

*Manduca* Genome Assembly 1.0 and gene models in *Manduca* Official Gene Sets (OGS) 1.0 and 2.0 and Cufflinks Assembly 1.0 (X et al., 2015) were downloaded from *Manduca* Base (<ftp://ftp.bioinformatics.ksu.edu/pub/Manduca/>). Protein sequences of the putative signal transducers from *M. sexta* (Gunaratna and Jiang, 2013) and other insects were used as queries to search Cufflinks 1.0, OGS 1.0 and OGS 2.0 using the TBLASTN algorithm ([http://darwin.biochem.okstate.edu/blast/blast\\_links.html](http://darwin.biochem.okstate.edu/blast/blast_links.html)). Hits with aligned regions longer than 30 residues and identity over 40% were retained for retrieving corresponding cDNA sequences. Errors resulting from problematic regions (e.g. NNN ...) in the genome assembly were manually corrected after BLASTN search of *Manduca* Oases and Trinity Assemblies 3.0 of the RNA-Seq data (Cao and Jiang, 2015). The two genome-independent RNA-Seq assemblies were developed to cross gaps between genome scaffolds or contigs and detect errors in the gene models. In some complex cases, all exons of a gene were examined based on the GT-AG rule and sequence alignment to identify the splicing junctions. Correct open reading frames in the improved sequences were identified using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and validated by BLASTP search against GenBank (<http://www.ncbi.nlm.nih.gov/>) or Uniprot (<http://www.uniprot.org/>). Signal peptides were predicted using SignalP4.1 (Petersen et al., 2011). Conserved domain structures were identified using SMART ([http://smart.embl-heidelberg.de/smart/set\\_mode.cgi](http://smart.embl-heidelberg.de/smart/set_mode.cgi)) and InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>).

### 2.2. Sequence alignment and phylogenetic analysis

Multiple sequence alignments of immune signal transducers from *M. sexta* and other insects were performed using MUSCLE, a module of MEGA 6.0 (Tamura et al., 2013) at the following settings: refining alignment, gap opening penalty (−2.9), gap extension penalty (0), hydrophobicity multiplier (1.2), maximal iterations (100), UPGMB clustering (for iterations 1 and 2) and maximum diagonal length (24). The aligned sequences were used to construct neighbor-joining trees by MEGA 6.0 with bootstrap method for the phylogeny test (1000 replications, Poisson model, uniform rates, and complete deletion of gaps or missing data).

### 2.3. Gene expression profiling

Coding DNA sequences from the improved gene models were retrieved and employed as templates for mapping reads in the 52 *M. sexta* RNA-Seq datasets, representing mRNA samples from whole insects, organs or tissues at various developmental stages. Illumina reads (*M. sexta* genome and transcriptome project; <http://www.ncbi.nlm.nih.gov/bioproject/PRJNA81039>) were trimmed to 50 bp and mapped to the coding regions using Bowtie (0.12.8) (Langmead et al., 2009). Numbers of the mapped reads were used to calculate FPKM (fragments per kilobase of exon per million fragments mapped) values using RSEM (Li and Dewey, 2011). Hierarchical clustering of the  $\log_2(\text{FPKM} + 1)$  values was performed using MultiExperiment Viewer (v4.9) (<http://www.tm4.org/mev.html>) with the Pearson correlation-based metric and average linkage clustering method. To study transcript level changes after immune challenge, the entire CDS set was used to search for corresponding contigs in the CIFIH09 database ([http://darwin.biochem.okstate.edu/blast/blast\\_links.html](http://darwin.biochem.okstate.edu/blast/blast_links.html)) (Zhang et al., 2011) by TBLASTN. The numbers of CF, CH, IF, and IH reads (C for control, I for induced after

injection of bacteria, F for fat body, H for hemocytes) assembled into these contigs were retrieved for normalization and calculation of IF/CF and IH/CH ratios. When a polypeptide sequence corresponded to two or more contigs, sums of the normalized read numbers were used to calculate its relative mRNA abundances in fat body and hemocytes (Gunaratna and Jiang, 2013).

### 3. Results and discussion

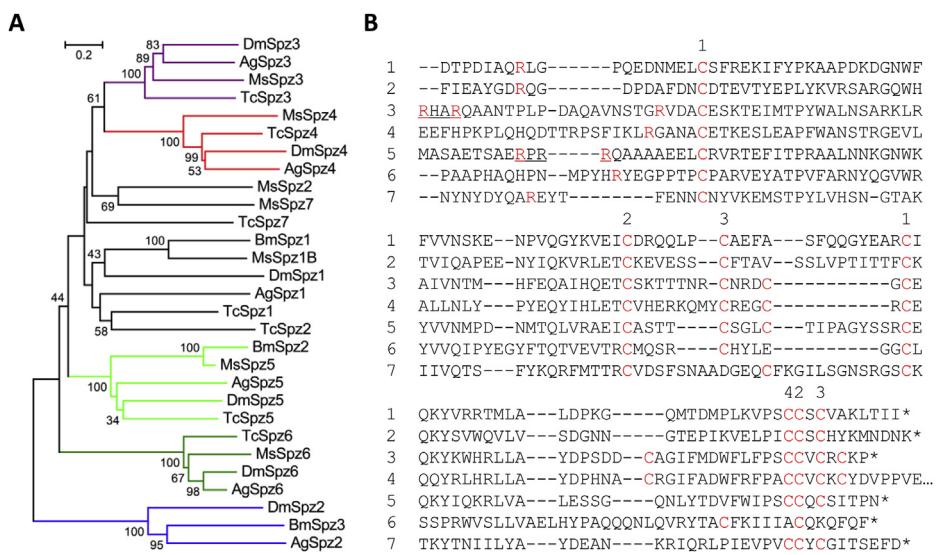
#### 3.1. Spätzle-1–7, cytokines with distinct structures, functions, and expression patterns

There are seven genes encoding Spätzle-like proteins in *M. sexta* (Table S1), which differs from the number present in *Tribolium castaneum* (9), *D. melanogaster* (6), and *A. gambiae* (6), *B. mori* (3) and *A. mellifera* (2) (Tanaka et al., 2008). The *M. sexta* proteins contain a signal peptide, a 50 to 360-residue segment with 0–4 low complexity regions, and a cystine-knot cytokine domain (Fig. 1). For Spätzle-1, cleavage between QR and LG results in a dimer of the C-terminal fragment that induces antimicrobial peptide synthesis (An et al., 2010), presumably via a Toll receptor. While Spätzle-2–7 may be activated by trypsin-like serine proteases, Spätzle-3 and 5 can also be processed by furin-like enzymes next to their recognition sequences, RHAR and RPRR, respectively. The C-terminal fragments of Spätzle-3–6 contain an even number of Cys residues, which might allow them to possibly dimerize via additional disulfide bonds. Molecular modeling suggests that Spätzle-1–5 and 7 adopt a similar fold with three pairs of antiparallel  $\beta$ -strands stabilized by 3 or 4 intrachain disulfide bonds (data not shown). Phylogenetic analysis of the entire proteins indicates that Spätzle-3–6 each forms a tight group with their orthologs from the other insects (Fig. 1A), suggestive of conserved functions. From parallel studies (Cao et al., 2015; Rao et al., 2015; He et al., 2015), we have noticed that the mRNA levels of many immunity-related genes in fat body and midgut greatly increase at the onset of wandering stage and peak on day 1 of the pupal stage. This infection-independent gene up-regulation during metamorphosis also occurs in other lepidopterans such as *Galleria mellonella* (Altincicek and Vilcinskas, 2008). Spätzle-1, 2 and 7 transcripts display this pattern with the

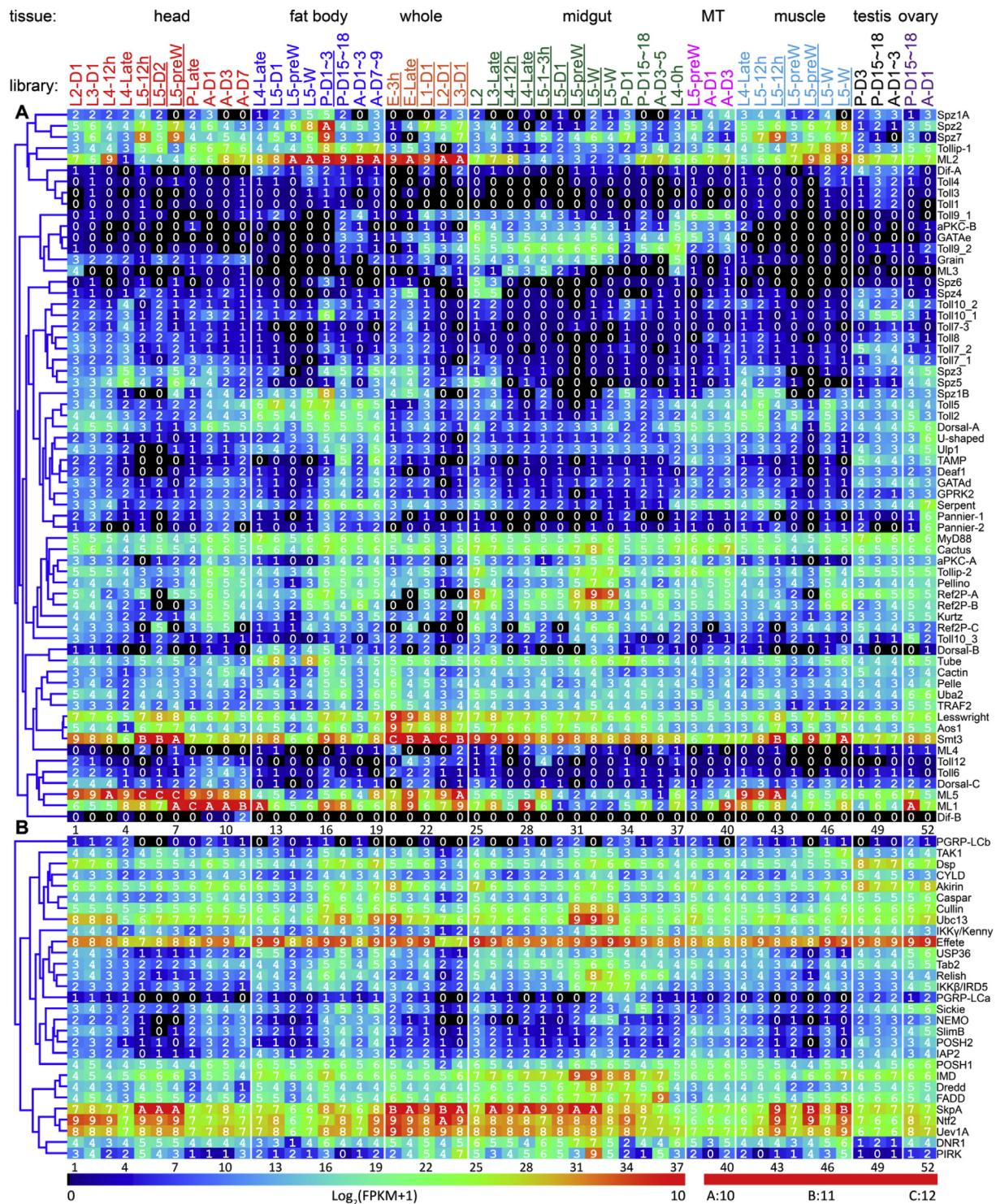
highest FPKM values of 224, 760 and 564, respectively (Fig. 2A). These three genes are induced upon immune challenge, whereas Spätzle-3–6 mRNAs were detected only at very low levels (Gunaratna and Jiang, 2013; Zhang et al., 2011, Table 1). Spätzle-1B mRNA level is low in ovary, higher in eggs and down-regulated after hatching. In contrast, Spätzle-2 mRNA levels are high in ovary, lower in eggs, and become higher in 1st instar larvae. The expression patterns of Spätzle-3 and 5 are similar to each other. Spätzle-4 and 6 are almost exclusively produced in the midgut of 2nd and 3rd instar larvae. The detection of Spätzle-2, 3, 5 and 7 mRNAs in head is interesting, since *Drosophila* Toll6, Toll7 and Toll8 act as receptors of neurotrophins (*Drosophila* Spätzle-2, 3 and 5) (McIlroy et al., 2013; Ballard et al., 2014).

#### 3.2. Structure, expression, and evolution of Toll receptors

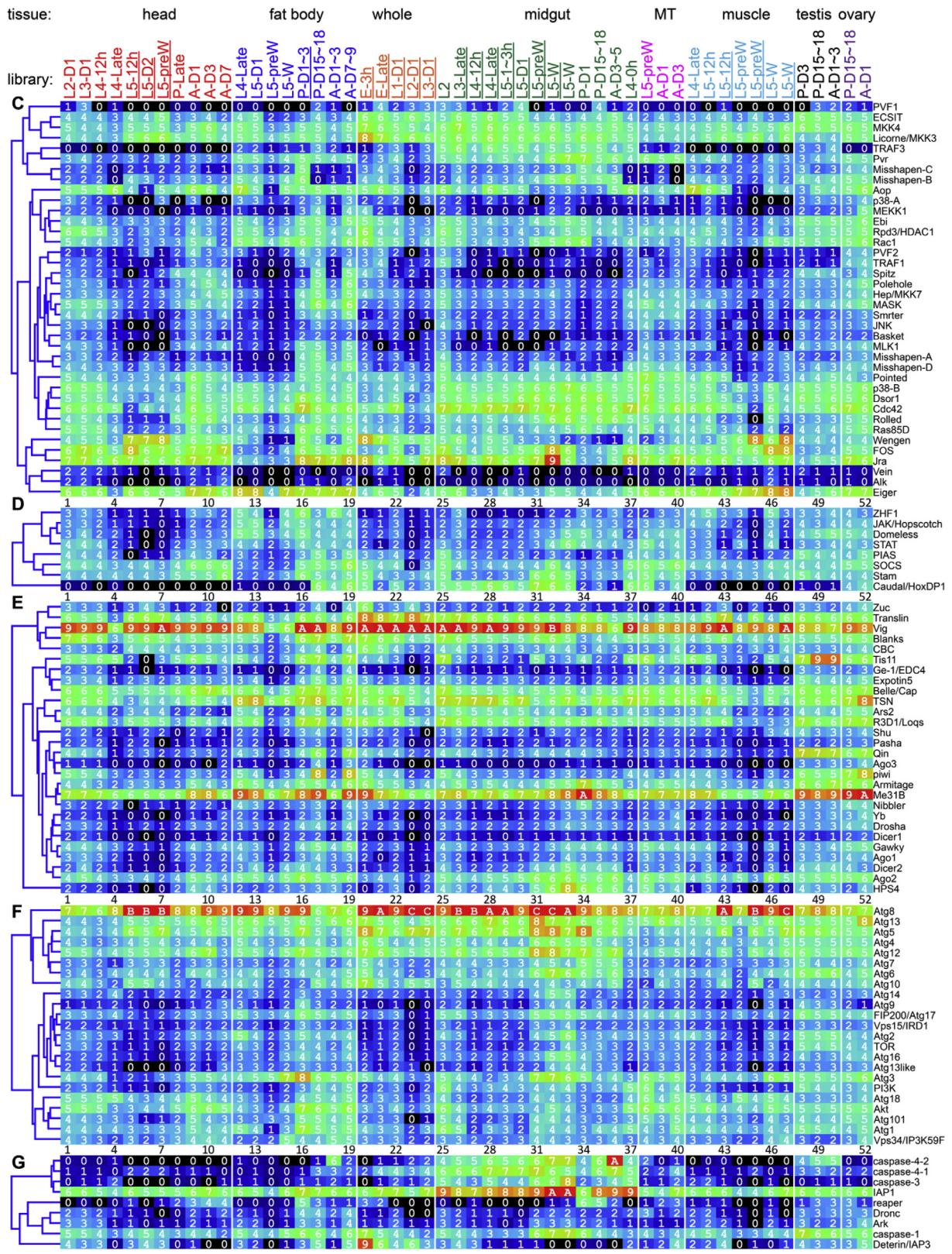
Toll receptors are a group of transmembrane proteins with extracellular Leu-rich repeats (LRRs) and a cytoplasmic Toll/interleukin-2 receptor (TIR) homology domain (Fig. 3A). We have identified sixteen such genes and named them Toll1–6, 7\_1–3, 8, 9\_1, 9\_2, 10\_1–3 and 12 (Table S1). These names are based on and mostly consistent with their orthologs in *B. mori* (Tanaka et al., 2008). Toll1 is reported as an immune-inducible gene that is predominantly expressed in hemocytes (Ao et al., 2008b; Gunaratna and Jiang, 2013) (Table 1). Along with Toll2–5 and *B. mori* Toll3\_1–3, *M. sexta* Toll1 is grouped with *D. melanogaster* Toll1, 3–5, *A. gambiae* Toll1A, 1B, 5A and 5B, and *T. castaneum* Toll1–4 (Fig. 3B). Nonetheless, *M. sexta* Toll1, 3 and 4 have only 4 to 5 LRRs (Fig. 3B), instead of the 12 LRRs and 2 Cys-rich C-terminal domains that are present in *Drosophila* Toll1. The mRNA levels of Toll1, 3 and 4 are very low in the 52 libraries (Fig. 2A). Hence, the putative roles as Spätzle-1 or 2 receptors need validation. In contrast, Toll2 and 5 transcripts are highly abundant in fat body and their profiles of expression are closely similar to those of Dorsal, Serpent and Spätzle-1B. Interestingly, *Manduca* Dorsal and Serpent may interact with each other to activate moricin gene transcription (Rao et al., 2011). Toll2 and 5 containing a Cys-rich C-terminal domain, are more similar in domain structure to *Drosophila* Toll1. Based on this and other evidence, we suggest *M. sexta* Toll2 and 5 are better



**Fig. 1.** Phylogenetic relationships of Spätzles in *M. sexta*, *B. mori*, *T. castaneum*, and *D. melanogaster*. (A) Tree. Based on the sequence alignment of 29 full-length Spätzles, a tree was generated with branches shown in colors representing closely related groups. (B) Aligned sequences of the cystine-knot cytokine domains in *M. sexta* Spätzles-1 through 7. Cys residues are indicated in a red font. Some Cys residues may form intra- (1–1, 2–2, 3–3) and inter- (4) chain disulfide bonds. Proteolytic activation sites, known for Spätzle-1, are predicted to be next to the Arg (red) in Spätzle-2 through 6. The putative processing site (RXXR) is underlined in Spätzle-3 and 5.



**Fig. 2.** Transcript profiles of the putative signaling protein genes in the 52 tissue samples. The mRNA levels, as represented by  $\log_2(\text{FPKM} + 1)$  values, are shown in the gradient heat map from blue (0) to red ( $\geq 10$ ). The values of 0–0.49, 0.50–1.49, 1.50–2.49 ... 8.50–9.49, 9.50–10.49, 10.50–11.49, and 11.50–12.49 are labeled 0, 1, 2 ... 9, A, B and C, respectively. The cDNA libraries are constructed from the following tissues and stages: head [2nd (instar) L (larvae), d1 (day 1); 3rd L, d1; 4th L, d0.5; 4th L, late; 5th L, d2; 5th L, pre-W (pre-wandering); P (pupae), late; A (adults), d1; A, d3; A, d7], fat body (4th L, late; 5th L, d1; 5th L, pre-W; 5th L, W; P, d1-3; P, d15-18; A, d1-3; A, d7-9), whole animals [E (embryos), 3h; E, late; 1st L; 2nd L; 3rd L], midgut (2nd L; 3rd L; 4th L, 12h; 4th L, late; 5th L, 1–3h; 5th L, 24h; 5th L, pre-W; 5th L, W; P, d1-3; P, d15-18; A, d3-5; 4th L, 0h), Malpighian tubules (MT) (5th L, pre-W; A, d1; A, d3), muscle (4th L, late; 5th L, 12h; 5th L, pre-W; 5th L, W; P, d1-3; P, d15-18; A, d1-3), and ovary (P, d15-18; A, d1). Some libraries (underlined) are from single-end sequencing; the others are from paired-end sequencing. Note that some synonymous libraries exhibit different FPKMs due to method differences. Panel A, Toll; B, Imd with JNK branch; C, MAPK-JNK-p38; D, JAK-STAT; E, pi- si- and mi-RNA pathways, F, autophagy; G, apoptosis.



**Fig. 2.** (continued).

candidates than Toll1 as receptors of Spätzle-1, 2 and 7. In *D. melanogaster*, Toll6, 7 and 8 (i.e. Tollo) are involved in neurotrophism (McIlroy et al., 2013; Ballard et al., 2014) and recent studies suggest that Toll7 may also be a pattern recognition receptor for vesicular

stomatitis virus, activating cellular autophagy of the virus (Nakamoto et al., 2012). Their orthologous genes (Toll6, 7\_1–3 and 8) are expressed in heads at levels higher than other tissues (Fig. 2A) and may play similar roles in *M. sexta*. The *M. sexta* Toll9\_1

**Table 1**Relative mRNA abundances of the intracellular signaling pathway members in induced (I) and control (C) fat body (F) and hemocytes from the larvae of *M. sexta*.

Name	IF/CF	IH/CH	Name	IF/CF	IH/CH	Name	IF/CF	IH/CH	Name	IF/CF	IH/CH
Spätzle1*	1.4	3.9	Dredd*	1.8	1.2	MLK1*	1	2.1	R3D1/Loqs	0.8	1.3
Spätzle2	1.5	2.2	Relish*	5.2	1.5	MKK4*	0.8	0.5	Dicer1	0	0.7
Spätzle7	4.1	3.6	NTF2*	1.2	1.7	JNK*	1.8	1.6	Ago1	1	2.6
Toll1*	2.5	6.2	TAK1*	0.5	3.6	Basket	1.3	2.7	Drosha	1.3	1.1
Toll2	0.7	0.8	Tab2*	2.5	1	ECSIT*	1	1.7	Pasha	-	1.5
Toll3	2.5	6.2	IKK $\beta$ *	0.5	0.2	MEKK1*	0	0.6	Expotin5	4.1	1.7
Toll4	2.5	6.4	IKK $\gamma$ *	2.5	1.2	MKK3*	1.4	1.1	Nibbler	0.7	1.4
Toll5	2.9	0.9	NEMO	1	1.2	p38*	2.2	1	Gawky	0.9	0.9
ML1	2.2	0.6	Dnr1	2.2	0.5	Aop*	3.1	1.2	Me31B	1	0.8
ML2	1.8	0.8	Sickie*	1	33.2	FOS*	2	1.9	Ge-1	0.5	1
MyD88*	1.5	1.5	Caspar*	3.1	1.6	Jra*	1.6	0.9	Atg1	2.8	0.9
Tube*	8.4	0.8	IAP2*	0.3	0.9	Ebi	1	1.4	Atg13	0.4	1.3
Pelle*	5.1	2.2	Bendless*	1.2	2.1	Smrter	1.8	1.2	Atg101	1.3	0.6
Pellino*	2.3	1.3	Uev1A*	1.4	1.1	Rpd3/HDAC1	0.2	1.2	Atg17	0.6	0.7
Cactus*	9.2	1.8	Effete	0.8	1.1	Domeless*	2.4	0.8	Vps34	0.5	1.2
Dorsal*	1.3	1.2	USP36	0.5	1.4	Stam*	2	1.1	Vps15	0.5	1.6
Tollip-1*	1	-	POSH1*	1.7	0.9	JAK/Hopscotch*	1	0.5	Atg6	0.5	1.2
Tollip-2*	0.8	1.1	POSH2	0.8	1.3	STAT*	0.4	0.7	Atg18	3.6	0.8
Ref2P*	1.5	1	CYLD	3.6	0.8	PIAS*	1.6	1	Atg12	0.5	3
aPKC*	-	1	SkpA	0.5	1.7	SOCS*	2.5	0.8	Atg7	0.5	0.5
GPRK2	0.1	0.7	Cullin	0.9	1.3	ZHF1	0.7	1	Atg5	0.5	1
Cactin	0.5	2.5	SlimB	1.5	0.7	Piwi	0.4	0.8	Atg4	0.2	1.1
Aos1*	0.7	1.7	Akirin	9.2	1.7	Armitage	1.4	1	Atg8	3.1	0.6
Uba2*	2.5	1.5	Dsp	2	1.4	Yb	0.5	0.2	Atg3	0.3	1
Lesswright*	4.6	1.2	Eiger*	0.8	8	Shu	0.5	5.9	Atg2	1.2	0.2
Ulp1	5.6	0.8	PVR*	1	1.4	Qin	0.7	0.9	Atg9	1.5	0.9
Kurtz	1	0.7	Ras85D*	0.7	1.7	Dicer2	1.3	1.5	Akt	1	1
Smt3*	1.6	1.6	Rac1*	2.5	1.3	Ago2	0.8	1	TOR	0.2	0.8
Deaf1	-	0.9	Cdc42*	1.3	1.4	Vig	0.9	1.1	PI3K	1.5	0.9
Serpent*	0.4	0.9	MASK*	1.2	1.4	TSN	0.6	1.2	IAP1	1.6	1.1
Pannier-1	0.5	2.4	Polehole	0.5	1	Ars2	3.1	1.7	Deterin	-	0.7
Pannier-2	1	2.4	Dsor1*	0.5	1.1	CBC	-	3.1	Dronc	0.5	1.2
GATAe	0.5	2.4	Rolled	1.9	1.1	Belle/Cap	1.2	1.5	Ark	2	0.6
U-shaped	0.3	1.1	Pointed	0.3	0.9	Blanks	2.5	1.1	Caspase-1	1.5	1.4
Imd*	2.7	1	Misshapen*	1.6	0.8	Translin	0.5	0.9			
FADD*	0.6	1.3	Hep/MKK7*	1.5	1.2	Tis11	1.3	0.9			

As described in Section 2.3, the transcriptome data of larval fat body and hemocytes before and after the immune challenge (Zhang et al., 2011) were processed again according to Gunaratna and Jiang (2013), based on the BLAST search using 196 complete coding sequences as queries. The ones with no hit in the Cifh library are omitted from the table. Note that, due to the increase in query sizes and contig hits, the reported relative abundances (\*) (i.e. IF/IH and CF/CH) (Gunaratna and Jiang, 2013) may be different for certain genes. “-”: C and I = 0.

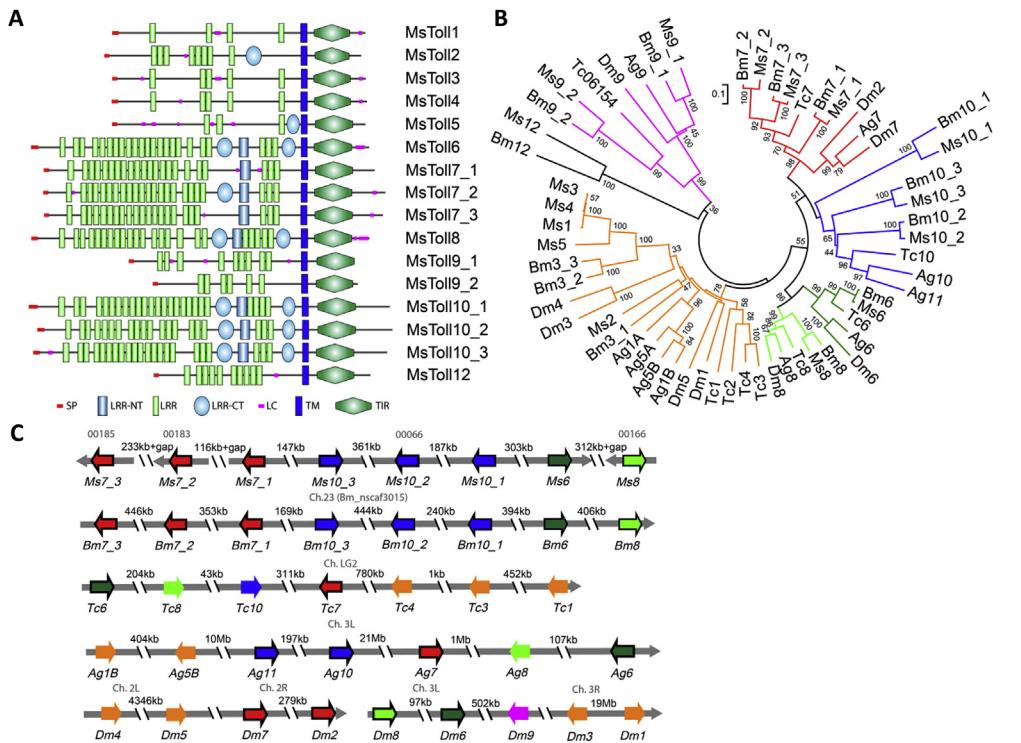
mRNA levels are high in Malpighian tubules of pre-wandering larvae and adults, as well as in midgut of feeding larvae. Human myeloid differentiation factor-2 (MD2) forms a complex with Toll-like receptor-4 to recognize lipopolysaccharide and lead to inflammation and cytokine production. A MD2-like protein (ML1) from *A. gambiae* specifically regulates the resistance to *Plasmodium falciparum* (Dong et al., 2006). We have identified five MD2-like proteins (MLs) in *M. sexta*, which contain a signal peptide and may increase binding specificity of the Toll receptors (Ao et al., 2008a).

Despite the fact that the coding regions being 2.2–4.0 kb in length, half of the 16 genes (Toll6, 7\_1–3, 8, 10\_1–3) only contain a single exon (Fig. 3C). They correspond 1:1 with their orthologous genes on chromosome 23 in *B. mori*. *M. sexta* Toll7\_1, 10\_3, 10\_2, 10\_1 and 6 on Scaffold (S) 00066 have the same orientations as those in the silkworm, flanked by Toll7\_3 (S00185), 7\_2 (S00183), and 8 (S00166) (Fig. 3C). When we compared the orthologous genes in *A. gambiae*, *D. melanogaster* and *T. castaneum*, similar gene orders were found. These orthologous genes include: Toll7\_3 to 1, 10\_3 to 1, 6 and 8 in the lepidopterans; Toll11&10, 7, 8 and 6 in the mosquito; Toll2&7, 8 and 6 in the fruit fly; Toll6, 8, 10, and 7 in the beetle. The underlined genes result from lineage-specific gene duplications. Except for AgToll8, TcToll8 and TcToll10 (with 5, 2 and 2 exons, respectively), the remaining genes are intronless. In comparison, MsToll1–5 have 7 or 8 exons, BmToll3\_1–3 have 7, 5 and 8 exons, DmToll1, 3–5 have 2 or 4 exons, AgToll1A, 1B, 5A and 5B have 3 exons, and TcToll1–5 have 3 or 4 exons. Together, these

observations reveal a dramatic evolutionary history of this ancient family of genes along the lineages of holometabolous insects.

### 3.3. Intracellular members of the Toll pathway and their regulation

We have identified 1:1 orthologs for most of the intracellular pathway members and modulators known so far. These include MyD88, Tube, Pelle, Pellino, Cactus, G protein-coupled receptor kinase-2 (GPRK2), Tollip-1&2, Cactin, Aos, Uba2, Smt3, Lesswright, and deformed epidermal autoregulatory factor-1 (Deaf1) (Fig. 4A, Table S1) in *Drosophila*. In the current model, activated Toll receptor associates with its adaptor MyD88 via their TIR domains. MyD88, Tube and Pelle (a kinase-like protein) form a complex via their death domains to phosphorylate Cactus. Pellino, with a RING E3 ubiquitin ligase domain, may ubiquitinate Pelle to enhance the Toll signaling. Unlike its ortholog in the fruit fly, the C-terminal Ser/Thr protein kinase domain of *M. sexta* Tube is predicted to be active catalytically and thus, actively involved in the pathway activation. The phosphorylation of Cactus by Pelle and perhaps Tube, causes it to dissociate from Dorsal or Dif become polyubiquitinated and degraded by the proteasome. Dorsal and Dif appear to be the products of a lineage-specific gene duplication (data not shown). GPRK2 may interact with Cactus to enhance signaling. Atypical protein kinase C (aPKC), together with its partners Ref2P and TRAF2 (TNF-receptor-associated factor-2), may interact with Pelle and directly activate Dorsal/Dif (Avila et al., 2002). Free, active Dorsal/Dif translocates into the nucleus to activate target gene



**Fig. 3.** Domain structures (A), phylogenetic relationships (B), and gene orders (C) of Tolls in *M. sexta*. (A) Signal peptide (SP), Leu-rich repeat (LRR), amino- and carboxyl-terminal (NT & CT) LRRs, low complexity (LC) region, transmembrane (TM) segment, and TIR (Toll/interleukin-1 receptor) domain are shown in different colors and shapes as indicated. (B) Amino acid sequences of the 58 full-length Toll proteins from *M. sexta*, *B. mori*, *T. castaneum*, *A. gambiae*, and *D. melanogaster* are aligned to generate the tree with its branches in different colors for closely related groups. (C) Orientations and orders of the Toll genes in the five insects are schematically shown as arrows in the same colors as in panel B. Arrows for the single exon genes are in black frame.

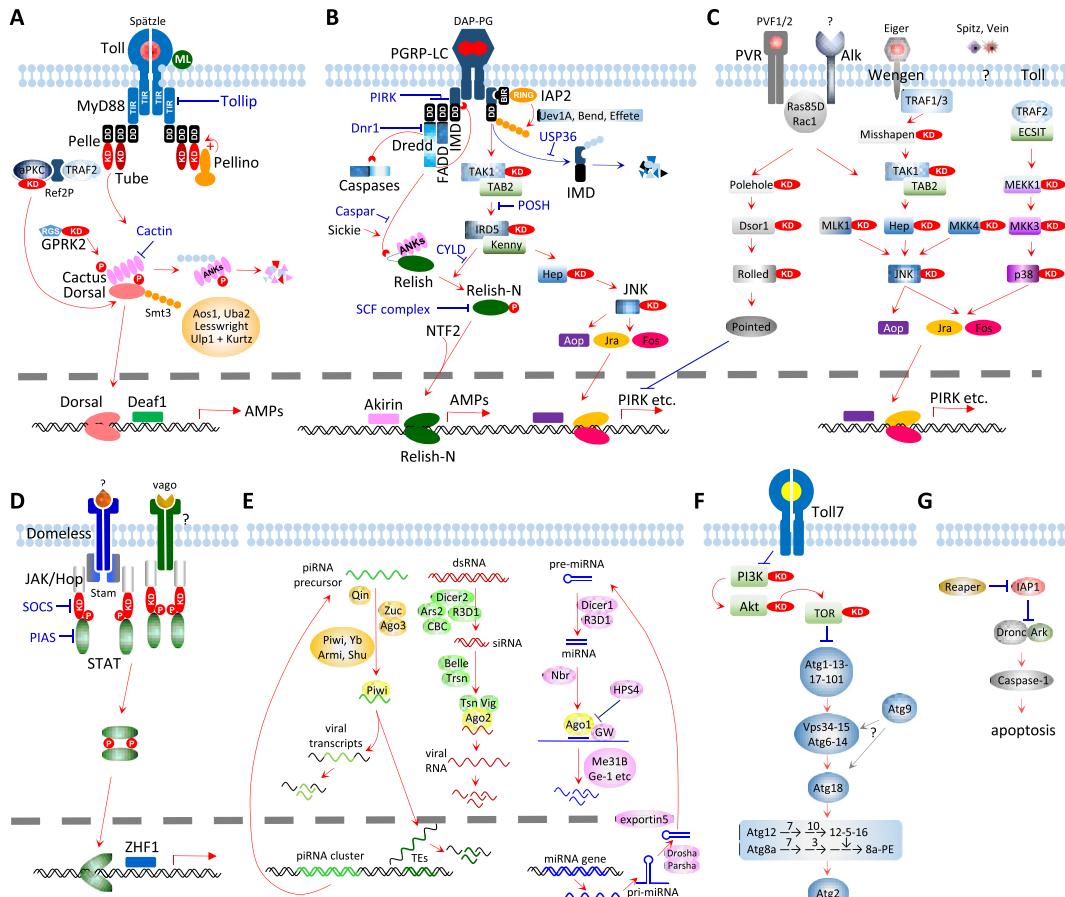
transcription along with *Deaf1* and other transcription factors (e.g. U-shaped and Toll activation mediating protein, TAMP). This pathway is likely regulated at other steps. For instance, Tollipps may associate with the Toll receptor and suppress the kinase activity of Pelle (Zhang and Ghosh, 2002). In *D. melanogaster*, Cactin may bind Cactus to block its function and cause embryonic ventralization (Lin et al., 2000). Conjugation of Dorsal/Dif by Smt3, a small ubiquitin-like modifier (SUMO), may potentiate function of Dorsal/Dif (Bhaskar et al., 2002). Aos1 and Uba2 may form a dimer which acts as an E1 SUMO-activating enzyme (Paddibhatla et al., 2010). The Lesswright homolog of Ubc9, an E2 SUMO-conjugating enzyme, negatively impacts the pathway (Chiu et al., 2005). The E3 SUMO ligase, Ulp1 peptidase and its helper Kurtz, reduces SUMO conjugation and response level of Dorsal/Dif-induced genes (Anjum et al., 2013).

Three Dorsal and two Dif variants are generated via alternative splicing (Table S1). The major Dorsal A is widely produced in tissues whereas B- and C-forms are preferentially expressed in fat body and head, respectively (Fig. 2A). Dif mRNA levels are lower compared to Dorsal. Like *MyD88*, *Aos1* and *Smt3*, *Manduca* Tube, Pelle, Pellino, Lesswright, Uba2, Ref2Ps, aPKC-A, TRAF2, Cactus, Dorsal-A, ML2, cactin, Tollip-1 and 2 are widely expressed in all the tissues examined. However, mRNA levels of the latter genes (Tube through Tollip-2) increase considerably in fat body during the wandering stage and reach peaks in pupae at day 1. As well, most of these genes are induced by 24 h following an immune challenge (Table 1).

#### 3.4. The Imd pathway, JNK branch, and their regulation

The Imd pathway, considered specific for Gram-negative bacteria, regulates the transcription of a set of immunity-related genes

that overlaps with that controlled by the Toll pathway (Kleino and Silverman, 2014). This pathway is also branched to JNK and apoptosis (Fig. 4B). We have identified 1:1 orthologs for nearly all of the pathway components (Table S1) and, therefore, propose that the *M. sexta* Imd pathway is triggered by DAP-PG, a component of the cell wall in most Gram-negative bacteria as well as Gram-positive *Bacillus* and *Listeria* species. Since there is no PGRP-LE ortholog in the moth (Zhang et al., 2015), membrane-bound PGRP-LC and LCb may work together to detect them. The longer splicing variant LCa contains two transmembrane domains, raising the possibility that it detects intracellular bacteria. Upon DAP-PG binding, a cytosolic portion of these variants may interact with the adaptor Imd and then FADD through their death domains. FADD recruits Dredd, the mammalian caspase-8 homolog, which cleaves Imd and Relish (Ertürk-Hasdemir et al., 2009) or a pro-caspase that leads to apoptosis. Cleaved Imd is susceptible to ubiquitination by IAP2 (inhibitor of apoptosis-2, an E3 ubiquitin ligase), Uev1A, Ubc13/Bendless and Ubc5/Effete (E2 ubiquitin-conjugating enzymes) (Paquette et al., 2010). Following ubiquitination, Imd likely recruits transforming growth factor β-activated kinase-1 (TAK1) and its binding protein TAB2 (Aggarwal, 2003). The dimer of TAB2 and TAK1 may then phosphorylate both Kenny/IKKβ and IRD5/IKKγ/NEMO in a complex, and JNK and Basket through MKK4 or MKK7/hemipterous (Hep) (Geuking et al., 2009). JNK may activate Aop and the AP-1 complex of *Jra/Jun* and *Fos/Kayak* to regulate downstream genes (e.g. PIRK). The IKK complex may phosphorylate the cleaved Relish to cause chain separation. While the C-terminal ankrin repeats and death-like domain are destined to be degraded, the N-terminal fragment (Relish-N), assisted by nuclear transport factor 2 (NTF2), could translocate into the nucleus and activate expression of immunity-related genes (e.g. antimicrobial peptides) via its Rel homology domain.



**Fig. 4.** Putative signaling pathways and regulators for antimicrobial immune responses in *M. sexta*. Panels **A**, Toll; **B**, Imd with JNK branch; **C**, MAPK-JNK-p38; **D**, JAK-STAT; **E**, pi- si- and mi-RNA pathways, **F**, autophagy; **G**, apoptosis. Panels A through G are described in the text.

Additional regulatory mechanisms are known for the Imd pathway in *Drosophila* (Kleino and Silverman, 2014). PIRK interferes with the association of Imd, FADD, and Dredd (Kleino et al., 2008). Dnr1 (defense repressor-1) inhibits the caspase Dredd while Sickie and Caspar have opposite effects on Dredd-induced activation of Relish (Foley and O'Farrell, 2004). USP36 deubiquitinates Imd for its degradation and, thus, represses Imd signaling (Thevenon et al., 2009). Another deubiquitinase, CYLD (for Cylindromatosis), modulates the IKK complex to control Relish phosphorylation (Tsichritzis et al., 2007). POSH controls the complex of TAK1 and TAB2; an SCF complex of Skp1, Cullin and F-box protein regulates the phosphorylated Relish-N; Akirin and Relish-N co-regulate some target genes of the Imd pathway (Tsuda et al., 2005; Cardozo and Pagano, 2004; Bonnay et al., 2014).

Most genes in the putative Imd pathway are widely expressed in different tissues at various life stages (Fig. 2B). The mRNA levels of Imd, FADD, Dredd, Relish, and many other genes are considerably higher in midgut than in fat body. This is consistent with the finding that local immune response of epithelial cells is Imd pathway-dependent, as the Imd pathway is fast and can be activated within minutes following a challenge (Kleino and Silverman, 2014; Paquette et al., 2010). While mRNA levels of a few genes are higher at 24 h after the immune challenge, others are similar to or even lower than the control levels (Table 1). This contrasts drastically with most of the Toll pathway genes, whose induced expression in fat body and hemocytes lasts longer than 24 h. Consistent with their immune inducibility, most Imd pathway members are highly expressed in fat body from the pre-wandering larval stage to the

early pupal stage (Fig. 2B). Their up-regulation in midgut is less pronounced and varies among the Imd pathway members during the same period, perhaps due to gut purging.

### 3.5. MAPK-JNK-p38 pathways

MAPK pathways are responsive to growth factors, cytokines and stress signals, and thereby regulate cell proliferation, differentiation, inflammation, and death. In *Drosophila*, components of these pathways activate MAPKs (Rolled, JNK and p38), down-regulate the Imd pathway, and stimulate hemocyte proliferation and lamellocyte formation (Fig. 4C) (Ragab et al., 2011; Dong et al., 2002; Lee and Ferrandon, 2011). We have identified homologs of two platelet-derived and vascular endothelial growth factors (PVFs), a PDGF/VEGF receptor (PVR), two small GTPases (Ras85D and Rac1), three kinases (Polehole, Dsor1 and Rolled), and a transcription factor (Pointed) that induces PIRK (poor Imd response upon knock-in) production. By interfering with Imd-FADD-Dredd association, PIRK, a small protein with no known domain structure, may inhibit Imd signaling. JNK may be activated through an Imd branch (Fig. 4B) and perhaps also by MLK1, MKK4, PVR or Alk (PVR and Alk are receptors with a Ser/Thr kinase domain). We have also found putative members of the cytokine-triggered MAPK pathway, namely Eiger, Wengen, TRAF1, and Misshapen that may recruit and sequentially activate TAK1-TAB2 dimer, MKK7/hemipterous, and JNK (Liu et al., 1999; Geuking et al., 2009). A protein called ECSIT (evolutionarily conserved intermediate in Toll pathways) is linked to the Toll receptor through TRAF2, and may activate a kinase

cascade of MEKK1, MKK3, and p38 to induce the formation of the AP-1 complex (Kopp et al., 1999). In addition, Spitz and Vein may induce MAPK signaling in the presence of reactive oxygen species but their receptors are unknown in *Manuda*.

Certain members of the putative MAPK-JNK-p38 pathways (i.e. Eiger, Rac1, MASK, Rolled, JNK, p38, Aop, Jra, Fos) in *M. sexta* are transcriptionally activated in larval fat body or hemocytes after an immune challenge (Gunaratna and Jiang, 2013) (Table 1). In addition to these, PVF2, PVR, Wengen, Ras85D, Cdc42, Dsor1, Misshapen, MLK and Pointed show mRNA level increases in fat body from pre-wandering to early pupal stage (Fig. 2C). Transcript levels for most of these genes in midgut are similar to or higher than those in fat body. Levels of PVR, Rac1, Misshapen-B&C, p38B, Ras85D, Jra and Fos mRNAs reach peak levels during pupation. Expression of the pathway members in head, muscles, Malpighian tubules, testis, and ovary clearly indicates that roles of the MAPK-JNK-p38 pathways are beyond immunity.

### 3.6. JAK-STAT pathway and other antiviral mechanisms

#### 3.6.1. JAK-STAT pathway and its regulation

The JAK-STAT pathway is involved in antiviral immune responses in insects (Dostert et al., 2005; Kingsolver et al., 2013). In *Drosophila*, an extracellular protein, Unpaired3, binds to Domeless, causes receptor dimerization, and recruits STAM and Hopscotch/JAK, which in turn phosphorylates itself and then STAT (Fig. 4D). We did not find an Unpaired3 ortholog in *M. sexta* or *T. castaneum* (Zou et al., 2007). However, the *M. sexta* ortholog of Vago may bind to an unknown receptor to activate JAK and STAT in a way similar to the unknown ligand of Domeless. After phosphorylation, the STAT dimer translocates into the nucleus to induce antiviral gene expression. SOCS (a JAK inhibitor) and PIAS (protein inhibitor of activated STAT) may down-regulate the pathway. Except for the ligand, orthologs of all the pathway components are present in *M. sexta* (Table S1). Domeless and SOCS mRNA levels increased 2.6-fold in larval fat body at 24 h after the injection of a mixture of bacteria (Gunaratna and Jiang, 2013) (Table 1). We also found that their mRNA levels became more abundant in fat body and midgut between wandering larval and early pupal stages (Fig. 2D). Similar increases were observed for other members of the predicted pathway, including JAK, STAT and STAM.

#### 3.6.2. RNA interference (RNAi) pathways

RNA interference plays important roles in limiting viral infection in insects (Kingsolver et al., 2013; Fablet, 2014). There are three RNAi pathways (Fig. 4E): 1) small interfering RNAs (siRNAs) are generated from double-stranded RNA (dsRNA) of viruses and siRNAs degrade or inhibit viral RNA and thereby disrupt the viral infection cycle; 2) microRNAs (miRNAs) are produced from cellular gene transcripts and typically function to control the translation or half-life of their target transcripts, including those regulating immune responses; 3) Piwi-interacting RNAs (piRNAs) provide epigenetic control of transposable elements and viral transcripts in germ-line cells in order to prevent genome disruption. The siRNA pathway is mostly responsible for antiviral activity in insects. Viral RNAs may form double stranded RNAs due to innate secondary structures or via replication intermediate, and these dsRNAs are recognized and cleaved by Dicer-2 to generate siRNAs, which are then loaded into RNA-induced silencing complexes consisting of Argonaute-2 and other proteins. Unwinding of the duplex occurs along with guide strand selection. After target RNA recognition by the guide RNA, the targeted viral RNA is degraded by Argonaute-2. We have identified 28 putative pathway members suggesting that these pathways are functional in *M. sexta* (Fig. 4E, Table S1). Since R2D2 is not found in *M. sexta*, we suggest that R3D1 (an ortholog of

*Drosophila* Loquacious) acts as a Dicer-1 partner in the miRNA pathway, as well as a Dicer-2 partner in the siRNA pathway. Unlike *Drosophila*, which has distinct Piwi and Aubergine genes, lepidopteran insects have a single PIWI-clade protein that we refer to as Aub/Piwi. Transcript levels for members of the siRNA pathway are relatively higher than those for either piRNA or miRNA pathways (Fig. 2E), consistent with its greater role in antiviral immunity (Kingsolver et al., 2013). Expression profiles of these pathways do not exhibit fat body- and midgut-specific up-regulation from wandering to early pupal stage, except for Dicer-2 and Argonaute-2. The Argonaute-2 mRNA levels increased moderately in induced fat body and hemocytes (Table 1). Although transcript abundances for piRNA pathway components vary, they are almost always higher in testis and ovary than the other tissues, consistent with their roles in the germline cells.

#### 3.6.3. Autophagy

Autophagy is a cellular process in which dysfunctional or unnecessary cellular materials or components are selectively targeted, then separated from the cytoplasm in double membrane vesicles (autophagosomes), and ultimately degraded by lysosomes (Mulakkal et al., 2014). Some pathogens may also be targeted to autophagosomes. Autophagy recycles the cellular materials and maintains cellular homeostasis under a variety of conditions. It is implicated in cellular responses to stress by nutrient-restriction, developmental changes involving tissue reorganization during metamorphosis, and certain pathological processes. The signaling of autophagy is mediated through the phosphoinositide 3-kinase (PI3K)-Akt pathway (Fig. 4F), which phosphorylates TOR to suppress autophagy. Autophagy itself involves about 20 components conserved throughout eukaryotes from yeast to mammals. In *Drosophila*, autophagy is induced upon infection by some viruses, intracellular bacteria (e.g. *Listeria monocytogenes*), and other pathogens (Yano et al., 2008; Kingsolver et al., 2013), suggesting that in addition to other cellular functions, it may also serve as an ancient cellular immune response. We have identified orthologs of all known autophagy pathway members (Fig. 4F, Table S1) and examined their expression profiles (Fig. 2F). As components of a ubiquitination complex, Atg3, 4, 5, 7, 8, 10, 12 and 16 are highly expressed in all the tissue samples used for RNA-Seq analyses. The mRNA levels of these autophagy pathway genes are generally higher in midgut than in fat body, testis and ovary. Since there is no major increase in mRNA levels in the pupal stage, autophagy may be partly supported by pre-existing proteins. Transcript levels of Atg2 through 6, 8, 9, and 16 are up-regulated in fat body and midgut from wandering larvae and young pupae, and decrease in the later stages. These changes may correlate with cellular reorganization in cells undergoing metamorphosis. In contrast, the PI3K, Akt, TOR, Vps34, Atg1, 7, 10, 12, 13, 17, 18, and 101 mRNA levels remain high from pupal to adult stage. Based on our current data (Fig. 2F), expression of autophagy-related genes appears to be a development-regulated process. There is no strong correlation with their immune inducibility, perhaps due to the fact we did not use viruses or intracellular bacteria to challenge the larvae.

#### 3.6.4. Apoptosis

Apoptosis, the best characterized mechanism of programmed cell death, is a part of normal developmental processes such as tissue modeling and homeostasis, but apoptosis can also participate in pathological processes including cancer and defense against pathogens (Opferman and Korsmeyer, 2003). In *Drosophila*, the initiator caspase Dronc and an adaptor protein (Ark) form a large protein complex (apoptosome) in response to intrinsic signals (Hay and Guo, 2006). It is not clear how the other *Drosophila* initiator caspases, Dredd and Strica, are activated. Once Dronc is activated, it

cleaves and activates effector caspases such as Drice and Dcp1 to cleave other protein substrates that lead to the downstream events of programmed cell death. Negative regulators of caspases (e.g. IAPs, Dnr1) control the pathway by inhibiting the activation of initiator caspases through either direct binding or by ubiquitination-induced degradation (Orme and Meier, 2009). Likewise, IAP antagonists (e.g. Reaper, Hid, Grim and Sickle) inactivate IAPs and, thus induce apoptosis. We have identified 12 members of the core apoptosis pathway in *M. sexta*, including Reaper, IAP1, IAP2, Deterin/IAP3, Dnr1, Ark, Dredd/caspase-6, Dronc/caspase-5, caspase-1, -3, and -4 (Fig. 4G) (Courtade et al., 2011). While Dnr1, Dredd, and IAP2 are likely involved in the balance between the Imd and apoptosis pathways, the other proteins may be devoted to programmed cell death. Reaper, an indirect pathway activator, is produced in the embryo, pupal fat body and midgut, as well as adult head, Malpighian tubules, testis and ovary (Fig. 2G), suggesting a possible role of apoptosis in tissue remodeling. The IAP3 mRNA, which is related to Survivin, a mitotic spindle-associated protein, is strikingly high and may perhaps regulate embryonic development. With a similar expression profile, IAP1 may block caspase-3 and -4 in cells of midgut, fat body, and other tissues. The high transcript abundances in midgut of feeding and wandering larvae, pupae and adults could indicate that the tissue is poised to undergo or carefully regulate active programmed cell death and regeneration. In addition, the caspase-1 and IAP1 mRNA peaks in fat body and midgut from wandering to early pupal stage correlate with their immune inducibility (Table 1).

### 3.7. Concluding remarks

Our search of the *M. sexta* genome has yielded 187 genes encoding 198 putative members of the immunity-related signal transduction pathways, namely Toll, Imd, MAPK-JNK-p38, JAK-STAT, piRNA, siRNA, miRNA, autophagy and apoptosis. Analysis of the expression profiles reveals differences among the proposed pathways (e.g. Toll, Imd, and MAPK-JNK-p38) and among some of the components (e.g. Spätzles, Tolls). These results suggest that the intracellular signaling system is functional in this undomesticated insect, and thus pave the way for understanding and potentially modulating similar pathways in pest lepidopteran species. The proposed signaling network needs experimental validation using biochemical, molecular and cellular biological methods.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2015.03.006>.

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