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Original article

Characterization of a honey bee Toll related receptor gene *Am18w* and its potential involvement in antimicrobial immune defense[1]

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Abstract – Toll receptors are involved in intracellular signal transduction and initiation of insect antimicrobial immune responses. Here we report the isolation and characterization of a novel gene (*Am18w*) from honey bee *Apis mellifera,* which encodes for the Toll-like receptor and shares a striking 51.4% similarity with *Bombyx mori 18-wheeler*, 46.6% with *Drosophila Toll-7* receptor and 42.5% with *Drosophila 18-wheeler*. The sequence analysis of the deduced 18W protein revealed a conserved Toll-Interleukin–Resistance signaling domain (TIR) characteristic of signal transducing receptors found in Toll and mammalian interleukin 1 (IL-1) receptors. The expression profiles of the honey bee Toll-like receptor gene *Am18w* were examined in all developmental stages, before and after immune challenge. Here we demonstrate that injection of a dsRNA probe into honey bee larvae successfully disrupts the endogenous mRNA of the target gene and allows us to examine the effects of *Am18w* gene silencing (RNAi) on the expression level of antimicrobial peptides.

Toll receptor / gene silencing / antimicrobial peptides / immune response / honey bee / *Apis mellifera*

1. INTRODUCTION

Host defense against various microorganisms involves different recognition and signaling pathways. These signaling pathways regulate expression of antimicrobial peptides that are active against Gram-positive and Gram-negative bacteria, as well as fungi (Anderson, 2000; Tauszig et al., 2000; Hoffmann and Reichhart, 2002; Rutschmann et al., 2002). A large family of Toll-related receptor genes has been identified from a number of insects including Toll, (Toll-3 to -9), *18-wheeler, Trex, Tollo and Tehao* (Eldon et al., 1994; Williams et al., 1997; Luo and Zheng, 2000; Luna et al., 2002; Bilak et al., 2003). Toll-related receptors are a group of evolutionarily conserved proteins with diverse biological functions. In addition to their developmental role, they play a crucial role in insect humoral immune responses by transducing signals to downstream cellular components, which subsequently lead to induction of the antimicrobial molecules (Khush and Lemaitre, 2000; Kimbrell and Beutler, 2001). Toll receptors are characterized by the presence of repeated copies of the leucine-rich motif within receptor extracellular domain (LRR) and the Toll-Interleukin–Resistance signaling domain (TIR). LRRs are 20–29 amino acids (aa) residue sequence motifs, which were found in many proteins that participate in protein-protein interactions and had different functions and cellular locations. The TIR domain is an intracellular signaling domain, characteristic of signal transducing receptors found in MyD88 adaptor protein, interleukin 1 and Toll receptors.

Here, we report identification and cloning of the honey bee Toll-related receptor gene *18-wheeler* (*Am18w)* and its possible involvement

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in antimicrobial immunity. In *Drosophila* 18-Wheeler, the receptor had a crucial role in humoral immune responses. The mutations in *18w* compromised induction of all antimicrobial peptide genes and mutant flies showed increased lethality in the face of bacterial challenge (Williams et al., 1997). In contrast, Ligoxygakis et al. (2002) showed that in *Drosophila 18w* was not involved in control of antimicrobial peptides and that reduction of antimicrobial peptides expression in $18w^{7-35}$*/Df (2R)* mutant larvae was due to a general developmental delay of the fat body maturation rather then impaired NF-kB signaling.

We examined the potential involvement of the 18W receptor in honey bee humoral immune responses by testing the effect of the A*m18w* gene silencing on the level of expression of the antimicrobial peptide genes using gene interference assay (RNAi). The antimicrobial peptides can be used as efficient markers to study specific signaling immune pathways, as their level of expression can serve as a signature profile of the up-stream intracellular reactions. In this study, the level of the antimicrobial peptides expression was monitored in honey bee larvae in response to challenge by gram-positive, gram-negative bacteria and fungi.

2. MATERIALS AND METHODS

2.1. Honey bee sample sources

European honey bee (*Apis mellifera* L.) larvae were obtained from locally maintained colonies. Identification of racial types was confirmed using mitochondrial DNA analysis (Arias and Sheppard, 1996). Jenter™ oviposition cages (Brushy Mountain Bee Farm, Moravian Falls, NC) were placed in the hives for a day prior to caging the queen bee. Queens were placed in Jenter™ cages for ~24 h before being released. Embryos were collected from the cages using a Chinese grafting tool and preserved in RNA*later*® (Ambion) for subsequent isolation of total RNA. Honey bee larvae were collected directly from brood combs of healthy colonies and classified by approximate age, based on larval weight (Root, 1983). Larvae used in challenge assays were placed in a FALCON® 6-Well Non-Tissue Culture treated plate (5 larvae per well) and placed in an incubator at RH 95% and 33 °C. The bottom of each well was lined with Spectra/Mesh (Spectrum Laboratories Inc. CAT# 146418) to facilitate transfer of larvae. Larvae were fed, as needed, a mixed diet as described in Vandenberg and Shimanuki (1987). Subsets of larval samples for all treatments and time points were preserved in RNA*later*® at –20 °C. Adult bees were collected as they emerged from the comb.

2.2. Immune challenge by septic wounding and injections

Sixty larvae 4.5–5 days old (mean weight 148 mg) were collected for immune challenge assays. The larvae were randomly separated into four groups: control (N = 6), *Paenibacillus larvae* subsp. *larvae* (American foulbrood, AFB) infected (N = 15), *Ascosphaera apis* (chalkbrood) infected (N = 15), and *E.coli* infected (N = 15). Experimental larvae were pricked with a minutien pin that was dipped into a preparation of concentrated bacterial or fungal spores. Septic wounding with *E. coli* was done by touching a colony of bacteria growing on LB plates with the pin and then pricking the larvae. Three immune-challenged larvae from each experimental group were then collected at 2, 6, 24, 48, and 72 h time points post-wounding and preserved in RNA*later*® until further use. Control, non-challenged larvae were used as a base line for gene expression. The experiment was then repeated just as before but instead of septic wounding using injections of 1.0 µL of gram-negative cells (9.8×10^5 cells/µL), gram-positive and fungal spores ($5–6 \times 10^6$ spores/µL) directly into the larval hemolymph. A subset of larval samples from each treatment was dissected and examined for presence or absence of microbial cells in the larval haemolymph.

2.3. Bacterial isolates

Scales and larval remains from honey bee comb cells with AFB infection (Morse, 1978) were collected in 10 mL of PBS pH 7.2, homogenized and crude filtrated. The filtrate was centrifuged for 5 min at 12,000 rpm, the supernatant discarded, and the spore pellet suspended in 100 µL of sterile PBS. *Paenibacillus larvae* is a gram-positive spore forming bacteria that is highly resistant to environmental conditions (Hornitzky and Wilson, 1989). Suspensions of *P. larvae* spores were thus heat treated for 30 min at 90 °C to eliminate non-spore forming bacteria. A small aliquot of bacterial spores was then tested for spore viability. The concentrated bacterial spore suspension was stored at 4 °C in PBS until used in the immune challenge assay.

2.4. Viability test and species identification of bacterial isolates

A small aliquot of the heat-treated bacterial spores was suspended in 100 µL of PBS-pH 7.2 and

Table I. List of primer sequences for the honey bee Toll-like receptor gene *Am18w* and the antimicrobial peptide genes.

Gene Name	Forward Primer	Reverse Primer
Am18w	(DO9F) 5'-CGTCTGGAGCAGCTGCTTGT-3'	(D09R) 5'-GTTCTCGCCCAGGTCGAGAGT-3'
Defensin	5'-GTTGAGGATGAATTCGAGCC-3'	5'- TTAACCGAAACGTTTGTC CC-3'
Abaecin	5'-GGTAGTGATATTTATCTTCGC-3'	5'-TTGAGGCCATTTAATTTTCGG-3'
Hymenoptaecin	5'-ATGGATTATATCCCGACTCG-3'	5'-TCTAAATCCACCATTTATTCC-3'
Apidaecin	5'- GTTGTTACCTTTGTAGTCGCG -3'	5'- CACGTGCTTCATATTCTTCAT -3'
Lysozyme	5'-GGAGGCGAGGATTCTGACTCA-3'	5'-TGTTGCATATCCCTCCGCTGTG-3'
Actin	5'-GAAATGGCAACTGCTGCATC-3'	5'-GAGATCCACATGTGTTGGAA-3'

plated on semi-selective J. agar medium, containing Nalidixic and Pipemidic acid (Alippi, 1995; Govan and Allsopp, 1999). Plates were incubated at 33 °C in air containing 7% CO_2. Colonies were characterized by shape, margins and microscopic examination of Gram-positive smears. Bacterial cultures were tested for the catalase reaction (Leboffe and Pierce, 1999). The DNA-based assay was also performed to corroborate identification of the bacterial types present (Djordjevic et al., 2002; Govan and Allsopp, 1999). Bacterial cells from culture plates were added to 30 µL of PCR reaction. PCR conditions consisted of a 94 °C (4 min) step; 30 cycles of 94 °C (1 min), 55 °C (1 min), and 72 °C (1 min) step. The primers selectively amplify a fragment of 973 bp unique to the *P. larvae* 16s rRNA gene sequence. PCR products were visualized by 0.8% agarose gel electrophoresis in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and ethidium bromide (0.2 µg/mL) staining. Bacterial isolates, collected from an apiary in Wisconsin (USA), were verified as *P. larvae* and used in all subsequent immune challenge assays.

2.5. Total RNA isolation and cDNA synthesis

Total RNA was isolated from embryonic, larval and adult tissue of European honey bees using TRIzol® Reagent (Life Technologies) following the manufacturer's protocol. RNA was resuspended in DEPC treated water and stored at –80 °C. Samples were treated with DNase using the DNA-*free* kit (Ambion). To test for residual DNA contamination, 1 µL of the total RNA sample was amplified using primers for the defensin gene fragment, which contains a 286 bp intron. Samples that tested positive were treated with DNase a second time. A 1.0% denaturing agarose/formaldehyde gel was used to test the integrity and quantity of the RNA. Quantification was verified using Eppendorff's BioPhotometer with samples that had been diluted 200 times in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and incubated for 10 min at 60 °C. The cDNA was constructed using SuperScript™ First-Strand Synthesis system for RT-PCR (Invitrogen) using Oligo (dT) primers and following the manufacturer's protocol.

2.6. Cloning of *Am18w* gene and antibacterial peptide gene fragments

Honey bee cDNA was amplified with gene specific primers. The primers for the honey bee Toll receptor were designed based on several overlapping sequences found in the NCBI Database (Apis_Mellifera_WGS_Trace gnl/ti/173868101; gnl/ti/173827152; gnl/ti/250639420; gnl/ti/165859773). Specific primers were also designed for the anti-bacterial peptide genes: defensin (Accession number U15955), abaecin (Accession number U15954), apidaecin (Accession numbers: X72575, X72576, X72577), hymenoptaecin (Accession number U15956), lysozyme (Apis_Mellifera_WGS_Trace gnl/ti/166283321) and the housekeeping gene actin (Accession number AB023025), which was used as an internal control (Tab. I).

Further, 1 µL of cDNA was added to a PCR reaction and amplified after initial incubation in a thermocycler (M.J. Research, Inc) at 94 °C (3 min) with the subsequent 35 cycles at 94 °C (1 min), 60 °C (1 min) and 72 °C (1 min) followed by a final extension at 72 °C for 20 minutes. The PCR amplification products were gel purified (Qiagen), cloned into the PCR vector of the TOPO-TA cloning kits (Invitrogen), and sequenced (SeqWright). Sequences were analyzed by computer software "Sequencher" (Gene Codes, Inc.) and "EditSeq" (DNASTAR, Inc.).

2.7. Construction of the dsRNA probes

Three double stranded RNA probes were made using the MEGAscript® RNAi Kit (Ambion). Probe 1 was designed within LRR, leucin-rich repeats region

(307 aa–384 aa) of the honey bee Toll-like receptor gene *Am18w*. It was made by amplifying plasmid DNA containing a cloned 234 bp fragment with the D09F/R primers that had T7 promoter sequence (5'-TAATACGACTCACTATAGG-3') added to the primers 5' end. The resulting template was then transcribed for 13 h. Probe 2 spans the trans-membrane region and part of the TIR, cytoplasmic domain (812 aa–1096 aa). It was made by amplifying plasmid DNA containing a cloned ~852 bp fragment of *Am18w* with the forward primer T7F2Seq 5'-TAATACGACTCACTATAGGCCACGTGTTC-ATGGGCG-3' and reverse primer T7B05R5'-TAATACGACTCACTATAGGCACAGCCTGAG-GCCGGCCGCG-3'. The resulting fragment was transcribed for six hours. Probe 3 is located within TIR domain and a segment of the 3' glutamine-rich motif (1159 aa–1269 aa) of *Am18w*. Plasmid DNA containing a cloned ~330 bp fragment of *Am18w* was amplified using the following primers: TollHB3'F5 5'-TAATACGACTCACTATAGGAGCTGATT-ATCGTCCAAGCG-3' and TollHB3'R6 5'-TAATACGACTCACTATAGGCGCGTGTTGCA-ACATTAAC-3'. The resulting template was then transcribed overnight. DNA and ssRNA were removed and the probes were purified following the manufacturer's protocol. The concentrations for the dsRNA probes were 766 ng/μL, 312 ng/μL, and 210 ng/μL for Probes 1, 2, and 3 respectively.

2.8. Gene silencing assay and RT-PCR analysis

The dsRNA probes (0.6–1.0 μL) were injected into 4.5–5 day old (mean wt. 148 mg) honey bee larvae (N = 54) laterally around the 7th and 8th abdominal segment using a Becton, Dickenson Ultra-Fine™ II system (CAT# 328438) fitted with a screw plunger. Larvae were fed and kept as described above. The honey bee larvae were collected 36 h post injections and preserved in RNA*later*® for subsequent RNA extraction and reverse transcription. Gene expression was analyzed using multiplex RT-PCR. Total RNA (3–4 μg) was reverse transcribed as described earlier in Section 2.5. The cDNA was then amplified in a PCR multiplex reaction using the primer set for each individual gene plus the primer set for the honey bee actin gene used as an internal control in all reactions. The multiplex PCR reaction mixture of 30 μL contained 1 μL of cDNA, 1X of PCR reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100), 0.25 μM of primer pairs, 2 mM $MgCl_2$, 0.2 mM dNTP's and 1.25 units of Taq DNA polymerase (Promega Co.). Negative control reactions had the same PCR mixture minus the cDNA template. Amplification was conducted in the M J Research DNA engine thermal cycler under the following conditions: initial denaturation at 94 °C (3 min) with the subsequent 35 cycles at 94 °C (40 s), 60 °C (40 s) and 72 °C (40 s) followed by a final extension at 72 °C for 5 minutes. All antibacterial peptides were analyzed under the same conditions with the exception of lysozyme, which ran for only 30 cycles instead of the 35 cycles. The cycle number was determined experimentally by running a series of reactions. Individual reactions ran for 20, 25, 30, and 35 cycles. Screening of the RNAi samples was done by amplifying a 325 bp fragment of *Am18w* located between Probe 1 and Probe 2, with the following primer set: forward 5'-ACAACAAGCT-TCGCACCGT-3' and reverse 5'-GAGCCT-GATCGCCTCCAA-3'. The PCR conditions were the same as described above. PCR products were visualized by 2.0% agarose gel electrophoresis in TAE buffer and ethidium bromide staining. Gels were documented and band intensities were analyzed using BioRad's Quantity One (Gel Doc 2000™) imaging system.

3. RESULTS

3.1. *Am18w* amino acid sequence analysis

Three overlapping cDNA clones that shared similarity with the insect Toll family of receptors were analyzed by the "Sequencher" (Gene Codes Inc.) and "EditSeq" (DNASTAR, Inc) computer software. The deduced 18W protein sequence was aligned with the Toll related receptor sequences found in the database (NCBI Accession numbers NP_476814, AF247765, BAB85498) and the multiple protein sequences (Fig. 1) were analyzed by the CLUSTALW method in the LASERGENE 02 package version 5.03 (DNASTAR, Madison, WI). The major feature of the deduced 18W protein sequence is the presence of the conserved intracellular signaling domain (TIR) characteristic of Toll/interleukin 1 receptor family (1067 aa–1204 aa) and the presence of multiple leucine-rich repeats present in many proteins that participate in protein-protein interactions (74–924 aa). The conserved domains TIR and LRR were identified by using the NCBI Conserved Domain Database with Reverse Position Specific BLAST. Honey bee Toll-related receptor 18W shares a striking 51.4% identity with *Bombyx mori* 18 Wheeler, 46.6% with *Drosophila* Toll-7 receptor and 42.5% with *Drosophila* 18 Wheeler.

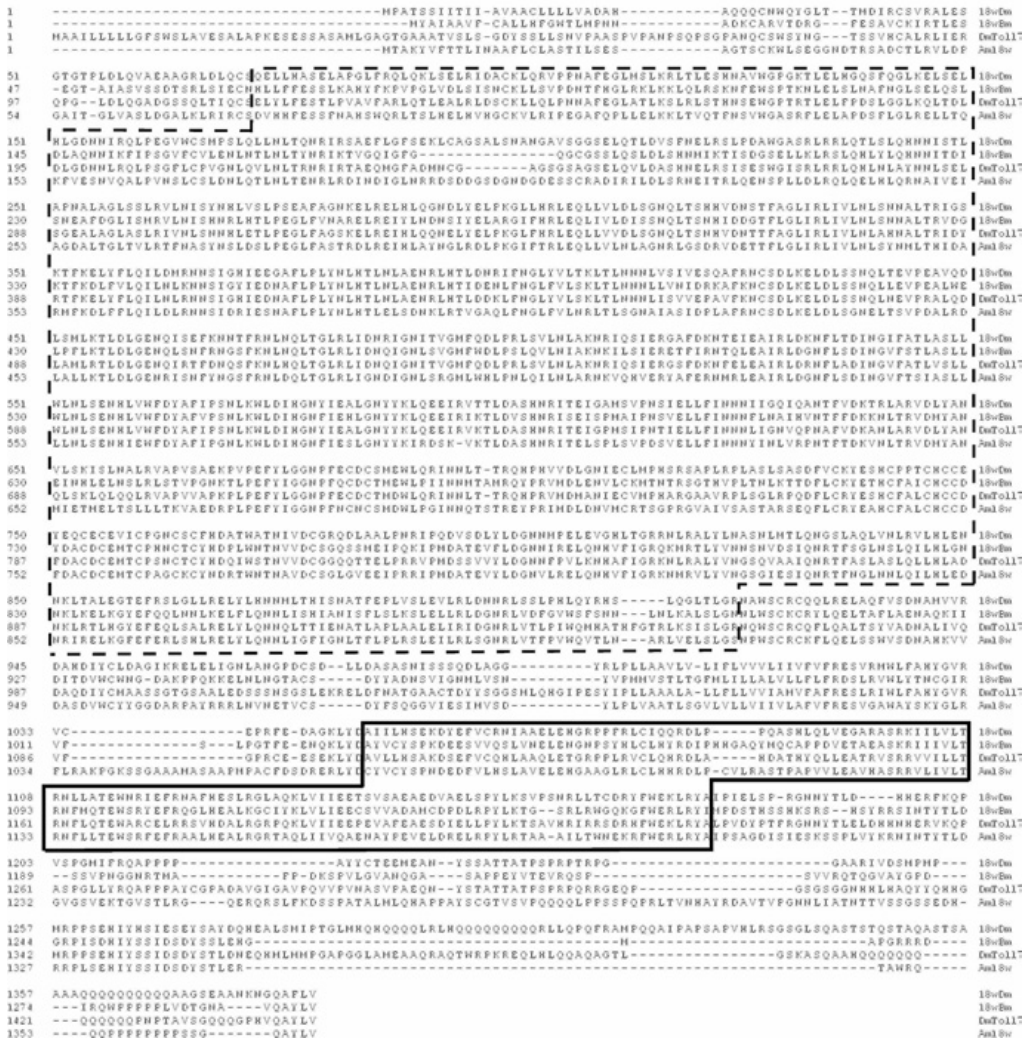

Figure 1. Multiple protein sequences alignment of the new *A. mellifera* Toll-like receptor 18W with selected members of the Toll family of receptors. Sequences were aligned by using the CLUSTALW method. NCBI Accession numbers: (18W *D. melanogaster* NP_476814), (Toll-7 *D. melanogaster* AF247765), (18W *B. mori* BAB85498). The leucine–rich repeats domain (LRR) and TIR domain are shown in dotted and solid-line boxes.

3.2. Developmental profile of the *Am18w* and antimicrobial peptides gene expression

Embryos (N = 475), ~2 day larvae (N = 12), ~5 day larvae (N = 12), brown eye pupae (N = 5), and emerging adults (N = 5) were analyzed for gene expression of *Am18w* receptor and antimicrobial peptides (Fig. 2). The *Am18w* receptor gene was highly expressed in honey bee embryos and pupa (Fig. 2A, Lanes 1 & 4, ~325 bp fragment). There is a dramatic decrease of the *Am18w* expression during the larval stages (Lanes 2 & 3). In adult bees, *Am18w* appears to be preferentially expressed in abdomen tissue (Fig. 2A, Lane 6). Initial attempts

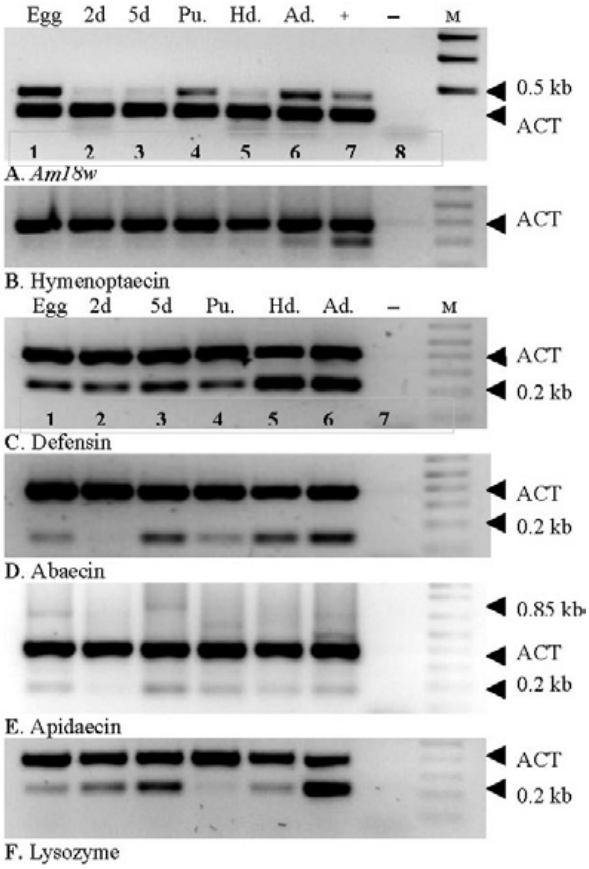

Figure 2. Expression of *Am18w* (A), Hymenoptaecin (B), Defensin (C), Abaecin (D), Apidaecin (E), and Lysozyme (F) in embryo, 2 day, 5 day, Pupae (Pu), Heads (Hd) and Abdomens (Ad) of adult honey bees (Lanes 1–6, respectively). Multiplex PCR reactions using a set of primers for actin (0.4 kb) and a set of the gene specific primers were visualized on 2.0% agarose gels stained with ethidium bromide. A nested forward primer for actin (ACT, 0.2 kb) was used when the size of the antimicrobial peptide was very similar to the (0.4 kb) actin band as in *Am18w* (A). Positive controls (+) for *Am18w* and Hymenoptaecin (Lane 7) are reactions with cDNA from larvae that were challenged by septic wounding with *E. coli*. Negative controls (Lane 8) for *Am18w* and Hymenoptaecin and lane 7 for Defensin, Abaecin, Apidaecin, and Lysozyme are PCR reactions without cDNA template.

to run Northern Blots to analyze *Am18w* expression were unsuccessful and the questions of size and number of transcripts will be addressed in future studies. Hymenoptaecin was expressed at levels too low to be detected with conventional PCR, with the exception of abdominal tissue of adult bees (Fig. 2B, Lane 6, ~299 bp fragment). Defensin was expressed at the same level from the embryonic stage to pupation (Fig. 2C, Lanes 1–4, ~227 bp fragment). However, there was a dramatic increase in the gene expression in adult tissues (Fig. 2C, Lanes 5–6). Abaecin was expressed at very low levels during embryogenesis, 2-day larvae and pupa stages. An increase in expression is seen in 5-day larvae and in the adults (Fig. 2D, Lanes 1, 2, and 4, ~150 bp fragment). Apidaecin expression was very low during all developmental stages (Fig. 2E, Lanes 1–6). We have consistently observed a number of PCR products (~700 bp, ~670 bp, 500 bp, and 181 bp) amplified from the pre-immune and immunized honey bee samples (Fig. 2E; Fig. 3E). Sequencing of these purified PCR products and TOPO-TA clones of these products revealed that all encoded for apidaecin polypeptides with a different number of Hb1b peptide repeats in the transcripts, which is consistent with the diversity of apidaecin transcripts in the database (NCBI Accession numbers: X72575, X72576, X72577). The number of such repeats is variable and was reflected in the observed diversity of amplicon lengths. We were able to amplify the entire diversity of the Apidaecin transcripts by designing primers within conserved regions of the pro-multipeptide; the forward primer was located within the polypeptide secretion signal and the reverse primer was located within the untranslated 3' UTR region including ATTTA "instability motif" (Casteels-Josson

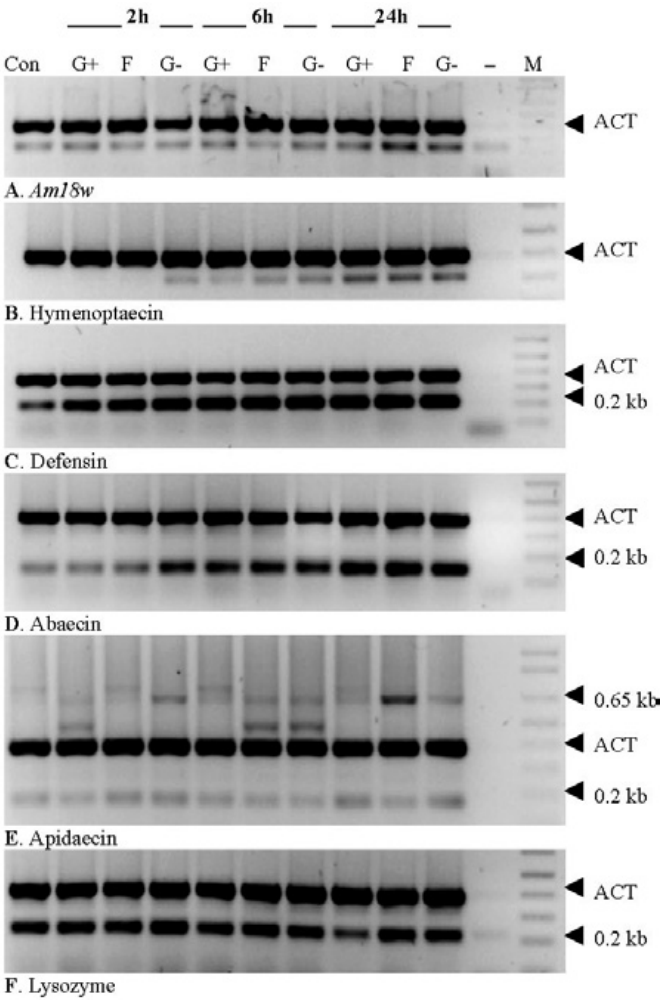

Figure 3. Septic wounding of ~5 day old European honey bee larvae with *Paenibacillus larvae* (G+), *Ascosphaera apis* (F) and *E. coli* (G-). Expression of *Am18w* (A), Hymenoptaecin (B), Defensin (C), Abaecin (D), Apidaecin (E), and Lysozyme (F) were examined at 2, 6, and 24 h time points post infection using multiplex PCR with the honey bee actin (ACT) gene fragment (0.4 kb). Controls (Con) are non-wounded larvae collected at the zero hour time point.

et al., 1994). We have classified the deduced peptide sequence based on the position of the 3/4/10/18 aa (N/R/Q/L) similarly to the classification by Casteels et al. (1994). The expression pattern of the lysozyme gene showed increased levels with each developmental stage from embryo to larvae (Fig. 2F, Lanes 1–3) and a sharp decline during pupation (Lane 4). In the adult bees, lysozyme expression was detected at a very high level in abdominal tissues (Fig. 2F, Lane 6).

3.3. Effect of septic wounding and injections on the level of *Am18w* gene expression and up-regulation of the antimicrobial peptides

We examined levels of *Am18w* gene expression in response to septic wounding of 5-day-old larvae challenged with gram-positive bacterial spores (*P. l. larvae*), fungal spores (*A. apis*), and gram-negative bacterial cells (*E. coli*). Up-regulation of the *Am18w* was delayed. A 3.1

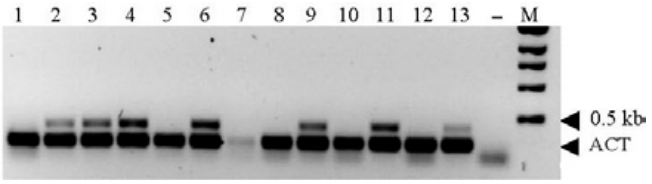

Figure 4. Silencing of *Am18w* using gene specific dsRNA. Gene expression was analyzed at 36 h post-injected with RT-PCR using multiplex reactions. *Am18w* was silenced in larval samples (Lanes 1, 5, 8, 10, 12). Partial silencing was noted in samples 2, 3, 9, and 13. Actin (ACT, lower band) was constitutively expressed in all samples with the exception of one reaction (Lane 7) where reverse transcription failed.

and 2.7-fold increase in gene expression occurred at 24 h post infection in response to a fungal (F) challenge and gram-negative bacteria (G-) respectively (Fig. 3A). Up-regulation was sustained up to the 48 h post infection time point in response to fungi and gram-negative bacteria, with the levels of gene expression returning to the pre-immune by 72 h (data not shown). A rapid induction of *Am18w* gene transcription was demonstrated by injection of 1.0 µL of gram-negative cells, gram-positive and fungal spores directly into the larval hemolymph. *Am18w* gene transcription reached its maximal 7.8 fold increase 2 h post-inoculation with gram-negative bacteria and 5 to 1.5 folds increase 6 h post inoculation with gram-positive bacteria and fungi respectively (data not shown).

Hymenoptaecin exhibited a delayed response similar to abaecin but at a far lower level of gene expression (Fig. 3B). The antimicrobial peptide defensin was rapidly induced by septic injections (Fig. 3C), showing strong anti-gram-positive, anti-gram-negative and anti-fungal activity. Abaecin expression was delayed in response to gram-positive bacteria and fungi compared to *E. coli,* with a gradual increase of gene expression by 6 h. At 24 h post infection, abaecin up-regulation was robust for all groups of microbes and comparable to that of the defensin (Fig. 3D). Apidaecin expression was delayed, most pronounced at 6 h post infection in response to gram-negative bacteria and fungi, with the gradual increase of the anti-fungal response by 24 h (Fig. 3E). Slightly varying levels of apidaecin expression were recorded by analyzing multiple sets of samples from the 24 h time point; in several samples up-regulation of the apidaecin was elevated and comparable to that of the 6 h time point (not shown). The bacteriolytic enzyme lysozyme was expressed at a high level, but not significantly up regulated by septic injections with any of the three microbial agents and comparable to the level of gene expression in pre-immune 5 day-old larvae (Fig. 3F, Con. Lane).

3.4. *Am18w* gene silencing (RNAi)

Probe 1 was used in the initial trial to target *Am18w* gene specific mRNA with no success. In the second trial we decided to target multiple regions of the gene; therefore we made two additional probes: Probe 2 and Probe 3. The three probes were mixed together in a 1:1:1 ratio and injected into 5-day-old larvae as described previously in this paper. Survival rate of larvae injected with the dsRNA probes was 67% 36 h post injection, compared to 88% for the control group injected with PBS. Total RNA was isolated from individual larval samples and three samples were discarded due to RNA degradation. Of the remaining samples, 42% exhibited successful disruption of *Am18w* gene expression (Fig. 4). The high specificity of the gene silencing was demonstrated by utilizing a multiplex PCR. Two sets of primers were used in the PCR reaction, *Am18w* gene-specific and actin gene-specific primers. The honey bee actin gene was constitutively expressed and detected in all samples except for Lane 7, where reverse transcription failed (Fig. 4, lower band). Expression of the honey bee Toll-like receptor was successfully disrupted after injection of the gene-specific dsRNA probe (Fig. 4, Lanes 1, 5, 8, 10, and 12, upper band). Partial silencing was recorded in samples 2, 3, 9 and 13. To validate that the gene had been silenced, the RT-PCR reaction was repeated the same as before with the exception that it cycled 40 times instead of 35. There was

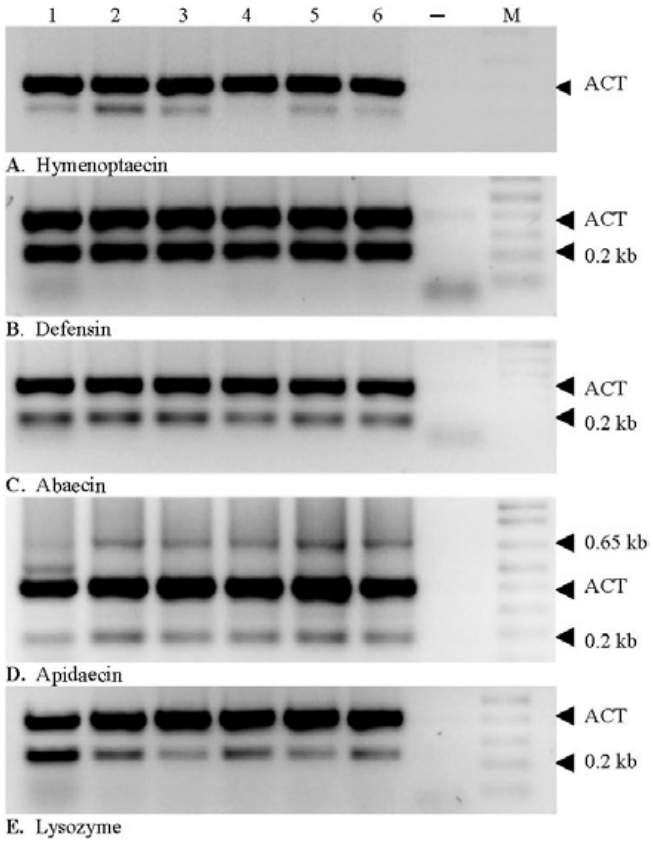

Figure 5. Expression of the antimicrobial peptide genes Hymenoptaecin (A), Defensin (B), Abaecin (C), Apidaecin (D), and Lysozyme (E) in honey bee larval samples that exhibited silencing of the Toll like receptor gene *Am18w* (Lanes 2–6) using multiplex PCR. Controls (Lane 1) are non-challenged ~5 day old larvae. Negative controls are PCR reactions without cDNA template.

indeed no amplification of *Am18w* at 40 cycles (data not shown). We investigated whether silencing of the receptor gene *Am18w* has any adverse effect on the development and survival rate of the honey bee larvae and found no significant differences between survival rates of the RNAi (injected with the dsRNA probe) and control larvae injected with buffer only. The mortality rate in both groups was high, considering difficulties associated with the rearing of the injected honey bee larvae. The number of animals that reached the adult stage in the RNAi group was minimal. To increase the survival rate of the RNAi group, we have begun a pilot study in which dsRNA probe is fed to the larvae. In preliminary results we have observed pupae with morphological defects in appendages (not shown).

3.5. Effect of *Am18w* gene silencing on expression of the antibacterial peptide genes

Samples that exhibited complete *Am18w* gene silencing (Fig. 4, Lanes 1, 5, 8, 10, and 12) were further analyzed to examine the effect, if any, this gene disruption has on the expression of antimicrobial peptides (Fig. 5). Silencing of the receptor gene appears to have no effect on the expression of any of the antimicrobial peptides. Although the level of lysozyme expression in the knock down samples was substantially down regulated (Fig. 5E), that was

consistent with the decline of the level of lysozyme expression during normal honey bee development (Fig. 2F, Lane 4).

4. DISCUSSION

4.1. *Am18w* sequence analysis and gene expression profile

We have identified and cloned the Toll-related receptor *18-wheeler* gene (*Am18w)* from honey bees. Analysis of the deduced protein sequence revealed that this gene has a striking similarity to the extended family of Toll receptors (51.4% to *Bombyx mori 18 wheeler*, 46.6% to *Drosophila Toll-7* and 42.5% to *Drosophila 18 wheeler*). Sequence analysis indicates that *Am18w* encodes a trans-membrane receptor protein with an extra-cellular moiety containing many leucine rich repeats (LRR) and cysteine motifs, and an intra-cytoplasmic (TIR) domain bearing homology to the cytoplasmic portion of the Toll/interleukin 1 receptors. The TIR domain presumably interacts with several intra-cytoplasmic partners upon induction of the Toll dependent signaling pathway (Hoffmann and Reichhart, 2002).

We examined expression of the *Am18w* gene by RT-PCR during normal honey bee development and in immune-challenged larvae. The *Am18w* gene expression profile in honey bees was consistent with that in *Drosophila* reported by Tauszig et al. (2000). Similar to *Drosophila Toll*, *18w*, Toll-6 and Toll-8, the honey bee *Am18w* was highly expressed in embryos and pupae, suggesting its developmental function. This Toll like receptor gene was also highly expressed in the abdominal tissue of the adult bees, suggesting its possible involvement in regulation of antimicrobial peptide genes expression (Rutschmann et al., 2002).

To determine whether the expression level of *Am18w* was affected by microbial challenge, we analyzed this gene expression in immune-challenged larvae and found delayed gene up-regulation when larvae were inoculated with a needle dipped in concentrated suspensions of spores or cells. In contrast, *Am18w* gene expression was induced faster and at a higher level when 1.0 µL of spore or cell suspensions were injected directly into the larval hemolymph.

4.2. Expression of the antibacterial peptide genes

Several distinct antimicrobial peptides have been identified in honey bees including defensin, abaecin, apidaecin, and hymenoptaecin (Kimbrell, 1991). Similar to observations reported by Rees et al. (1997), the entire bee antimicrobial peptide repertoire was induced by any of the microbial agents used in this study. In contrast, the bacteryolytic enzyme lysozyme was constitutively expressed in all larval stages before infection and the level of gene expression did not change significantly due to microbial challenge. The results presented here reflect the possibility that lysozyme is constitutively produced and stored in haemocyte granules. The microbial stimulation may induce release of the pre-existing lysozyme into the insect haemolymph as suggested by Lamberty et al. (2001). This may explain the increase in bacteryolytic activity of the haemolymph following infection as reported by Powning and Davidson (1973). Alternatively, according to Iketani and Morishima (1993), bacteria invading insect haemocoel have to be partially degraded to generate peptidoglycan fragments and subsequently induce production of antimicrobial peptides. Inoculations with intact microbial cells in this study may not be as effective as with processed peptidoglycans in modeling the actual mechanisms of induction.

4.3. Effect of *Am18w* receptor gene silencing on expression of the antimicrobial peptides

The RNA interference assay became instrumental for understanding of gene expression and function. In honey bees, this method was successfully implemented by using dsRNA embryo injections (Beye et al., 2002) and intra-abdominal injections of adult bees (Amdam et al., 2003). Here we demonstrated the use of dsRNA microinjections to down-regulate the Toll-related *Am18w* receptor gene expression in honey bee larvae and effect of that on the expression of antimicrobial peptides. Silencing of the *Am18w* had no apparent affect on the expression level of any of the antimicrobial peptide genes tested in this study. These results suggest that either the expression of the antimicrobial peptides is independent of *Am18w*

receptor signaling pathway similar to findings reported by Ligoxygakis et al. (2002) or the expression of these peptide genes is being compensated through alternative signaling pathways (Williams et al., 1997).

Future investigations will uncover other receptor(s) that control transcription of antimicrobial peptides in honey bees. We searched the honey bee database and identified four more Toll-related receptor genes in addition to *Am18w,* with high similarity to *Drosophila Toll-1*, *Toll-6*, *Toll-8* and *Anopheles Trex* genes. Our future investigations will provide more insights into the involvement of these receptor genes in the regulation of immune related molecules.

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Résumé – Caractérisation chez l'Abeille domestique d'un gène récepteur proche de Toll *Am18w* et de son rôle possible dans la défense immunitaire antimicrobienne. Les récepteurs de type Toll sont impliqués dans la transduction intracellulaire du signal et le début de la réponse immunitaire antimicrobienne chez les insectes. Ils se caractérisent par la présence de copies répétées d'un motif riche en leucine (LRR) à l'intérieur du domaine extracellulaire du récepteur et par une homologie de leur domaine de signalisation (TIR) avec le domaine cytoplasmique du récepteur Interleukin-1.
Nous présentons dans ce travail l'isolement et la caractérisation d'un nouveau gène (*Am18w*) de l'Abeille domestique, *Apis mellifera*, qui encode pour le récepteur de type Toll et partage une identité frappante à 51,4 % avec le 18-wheeler de *Bombyx mori*, à 46,6 % avec le récepteur Toll-7 de *Drosophila* et à 42,5 % avec le 18-wheeler de *Drosophila*. L'analyse de la séquence de la protéine 18W déduite a montré un domaine de signalisation intracellulaire conservé (TIR) caractéristique des récepteurs de transduction des signaux que l'on trouve dans la famille des récepteurs interleukin-1 (IL-1) et de type Toll des mammifères. Cette similitude d'un récepteur lié à Toll chez l'abeille avec les récepteurs de transduction des signaux suggère que le gène *Am18w* de l'abeille peut être impliqué dans les réponses immunitaires de l'hôte et dans l'activation des gènes de peptides antimicrobiens qui s'ensuit. Nous avons étudié le profil d'expression du gène récepteur de type Toll *Am18w* de l'abeille à tous les stades de développement, ainsi qu'avant et après une stimulation du système immunitaire. L'injection d'une sonde d'ARN à double brin (ARNds) dans les larves d'abeilles a réussi à provoquer la rupture de l'ADNm endogène du gène cible et nous a permis d'examiner les effets de l'inactivation du gène rendu silencieux sur l'expression des peptides antimicrobiens.

***Apis mellifera* / réponse immunitaire / récepteur Toll / inactivation de gène / peptide antimicrobien**

Zusammenfassung – Charakterisierung eines Toll-ähnlichen Rezeptorgens *Am18w* der Honigbiene und seine mögliche Rolle in der antimikrobialen Immunabwehr. Toll-Rezeptoren spielen eine wichtige Rolle in der intrazellulären Signalverarbeitung und in der Initiierung der antimikrobialen Immunantwort bei Insekten. Charakteristik ihrer Proteinsequenz sind wiederholte Kopien eines Leucin-reichen Motivs (LLR) innerhalb der extrazellulären Domäne des Rezeptors, sowie eine Homologie in ihrer Signaldomäne (TIR) mit der cytoplasmatischen Domäne des Interleukin-1 Rezeptors.
Die gegenwärtige Arbeit berichtet die Isolierung und Charakterisierung eines neuen Gens *(Am18w)* der Honigbiene, *Apis mellifera*, das für einen Toll-ähnlichen Rezeptor kodiert, und das eine hohe Ähnlichkeit von 51,4 % aufweist mit dem Gen *18-wheeler* des Seidenspinners, *Bombyx mori,* sowie mit den *Drosophila* Genen *Toll-7* Rezeptor (46,6 %) und *18-wheeler* (42,5 %). Die Analyse der Aminosäurensequenz des deduzierten 18W Proteins der Honigbiene zeigte eine konservierte (TIR) intrazelläre Signaldomäne als charakteristisches Merkmal des Signaltransduktionselements der Toll-Rezeptoren und der Interleukin-1 (IL-1) Rezeptoren der Säugetiere. Diese Ähnlichkeit eines Toll-ähnlichen Rezeptors der Honigbiene weist auf eine mögliche Rolle des *Am18w*-Gens in der Immunantwort der Honigbiene und der darauffolgenden Aktivierung von antimikrobialen Peptidgenen hin.
Wir untersuchten das Expressionsprofil des Toll-ähnlichen Rezeptorgens *Am18w* der Honigbiene in allen Entwicklungsstadien, sowie vor und nach einer Stimulierung des Immunsystems. Die Injektion einer doppelsträngigen RNA Probe (dsRNA) in Honigbienenlarven führte zu einer erfolgreichen Disruption der endogenen mRNA des Zielgens und ermöglichte es uns, die Effekte der Stillegung (RNAi) des *Am18w* Gens auf die Expression der antimikrobialen Peptide zu untersuchen.

Honigbiene / Toll Rezeptor / Immunantwort / Stillegung des Gens / antimikrobiale Peptide

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