Novel Characteristics of Immune Responsive Protein IRP30 in the Bumble Bee Bombus lantschouensis (Hymenoptera: Apidae)

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Abstract

Immune responsive protein 30 (IRP30) is a Hymenoptera-specific protein first identified from honey bee hemolymph in response to bacterial infection. However, its function remains elusive. Here, we cloned the full-length IRP30 gene and clarified its expression pattern in the bumble bee Bombus lantschouensis (Vogt). The full-length IRP30 gene measures 1443 bp and contains two exons and one intron. The length of the cDNA is 1082 bp, including a 36-bp 5' UTR and a 218-bp 3' UTR, and it encodes a putative protein of 275 amino acids. As expected, the sequence of the B. lantschouensis IRP30 protein was clustered with the bumble bee group, which appeared as a single clade next to honey bees. The family shared similar conserved protein domains. Moreover, bumble bee IRP30 belongs to a recently diverged clade that has four leucine-rich repeat (LRR) conserved domains. IRP30 is highly expressed in the worker caste, during pupal developmental stages, and in the head and thorax tissues. Interestingly, its expression increases 20- to 90-fold when female bumble bees (B. lantschouensis) and honey bees (Apis mellifera L.) begin laying eggs. Overall, based on the expression of IRP30 during development and egg laying in female bumble bees, this protein not only responds to immune challenge but also may play an important role in metamorphosis and reproduction.

Key words: Bumble bee, Bombus lantschouensis, immune responsive protein 30, leucine-rich repeat, expression pattern
different tissues. In honey bee, the fat body was the main tissue for IRP30 expression, especially in winter bees (Albert et al. 2011). In summary, although previous studies have demonstrated that the expression of IRP30 in hymenopteran insects is affected by microbial stimulation and environmental factors, the characteristics and biological function of IRP30 remain puzzling.

As an endemic bumble bee, Bombus lantschouensis (Vogt) is one of the most important pollinators in North China (An et al. 2014, Zhang et al. 2018). Due to its excellent pollination performance and potential for factory reproduction, this species has been selected and artificially reared to provide pollination services for greenhouse crops. This species has become a bumble bee of choice to replace the imported European bumble bee B. terrestris (L.), and its genetics have been explored (Zhou et al. 2015, Huang and An 2018). IRP30 was identified as a significantly differentially expressed protein by comparative hemolymph proteomics of egg-laying versus non-egg-laying B. lantschouensis queens in our laboratory (unpublished data). Therefore, we focused on whether IRP30 protein is involved in female reproduction.

In this study, we report the structure of the IRP30 gene of B. lantschouensis. Its phylogenetic relationships and conserved domains were predicted. Moreover, the expression levels of IRP30 mRNA were explored in different castes, developmental stages and adult tissues. Finally, the relationship of IRP30 with reproductive status was demonstrated by comparing egg-laying and non-egg-laying females from bumble bee (B. lantschouensis) and honey bee (Apis mellifera L.).

Materials and Methods

Bees and Sample Collection

Six colonies of B. lantschouensis were obtained from the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China. They were reared under constant darkness in a room with a temperature of 29 ± 0.5°C and a relative humidity of 60 ± 5%. Fifty-percent sucrose solution and fresh pollen pellets were supplied every other day. Six colonies of A. mellifera were maintained in the yard of the institute campus.

The Expression Profile of IRP30 in Bumble Bee

To investigate the expression profile of IRP30, different samples were collected as follows:

(a) Workers in different developmental stages were collected according to Dong et al.’s (2017) description, including eggs within 24 h of laying, fifth-instar larvae (Lar5), tenth-instar larvae (Lar10), white-eyed pupae with unpigmented cuticles (Pw), pink-eyed pupae with unpigmented cuticles (Pp), brown-eyed pupae with unpigmented cuticles (Pb), brown-eyed pupae with lightly pigmented cuticles (Plb), brown-eyed pupae with darkly pigmented cuticles (Pbd), and pharate adults (Pha). Eighteen eggs were collected and grouped into biological replicates of six eggs each. For the other developmental stages of workers, three individuals per stage were collected and treated as separate biological replicates.

(b) To obtain the different tissues, callow workers (those that had emerged in the past 24 h) were collected and marked. These workers were introduced into queenright colonies. Fifteen 7-d-old workers were collected and used for dissection. Using a stereomicroscope (Olympus, Japan), we dissected the bees on ice and collected antenna (AN), head (HD), thorax (TH), leg (LG), midgut (MG), ovary (OV), fat body (FB), and venom gland (VG) specimens. Tissues were pooled into three biological replicates of five bees each.

(c) Seven-day-old queens, workers, and drones were collected to explore the expression characteristics in different castes. The head, thorax, and abdomen were separated on ice for RNA extraction. Each caste had three biological replicates.

All samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

The Expression of IRP30 in Female Bumble Bee Hemolymph

To collect hemolymph from female bumble bees for Western blotting, queens and workers of different reproductive statuses were anesthetized by chilling on ice. Haemolymph was collected by gently puncturing the abdominal membrane between sternum segments 5 and 6 with a glass capillary. The hemolymph was immediately transferred into Eppendorf tubes and stored at −80°C. Three biological replicates were collected for each sample.

The Expression of IRP30 in Female Bumble Bee and Honey Bee

To characterize the expression profile of IRP30 under different egg-laying statuses, ovaries were collected from female bumble bees. To obtain egg-laying worker bumble bees, we reared queenless microcolonies with three callow workers each in small rearing boxes. The egg-laying workers were sacrificed on the seventh day. Non-egg-laying 7-d-old workers were collected from queenright colonies. The egg-laying status of workers was confirmed according to their ovarian developmental conditions as described by Duchateau et al. (Duchateau and Velthuis 1989). Egg-laying and non-egg-laying queens were selected among queens that had recently awakened from diapause. The egg-laying status of the queens was defined based on their ovarian development.

To verify the differences in IRP30 expression in female honey bees of different egg-laying statuses, sterile and reproductive workers were obtained according to Grozinger et al.’s (2007) approach. Non-egg-laying virgin queens were collected on the 15th day after emergence. The egg-laying queens were selected from the newly mated and laying queens.

The abdomen of each bee was cut and the gut was removed. Three biological replicates, each consisting of one bee, were collected for each egg-laying status. The abdomen without gut samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA and DNA Extraction

Total RNA was extracted from the different samples using the TRIzol reagent (Invitrogen, United States) following the manufacturer’s instructions. Genomic DNA was isolated from the thoraces of bumble bee workers using Wizard Genomic DNA Purification Kits (Promega Inc., United States). The quality of the RNA and DNA was assessed using gel electrophoresis and a Micro-Volume UV-VIS spectrophotometer (NanoDrop 2000, United States).

Rapid Amplification of cDNA Ends

One microgram of total RNA was used as template, and 3’ and 5’ rapid amplification of cDNA ends (RACE) cDNAs were synthesized with a Smart RACE cDNA Amplification Kit (Clontech, United States) according to the manufacturer’s instructions. Specific
primers were designed from the retrieved IRP30 gene sequence of *B. terrestris* (GenBank accession number: JN181870.1) (Table 1). Then, 3′ and 5′ RACE were performed to obtain the full-length IRP30 gene using the universal primers provided in the kit as well as gene-specific primers (Table 1). The PCR conditions were as follows: initial denaturation at 94°C for 3 min; five cycles (94°C for 30 s and 72°C for 3 min), five cycles (94°C for 30 s, 70°C for 30 s, and 72°C for 3 min), and 27 cycles (94°C for 30 s, 68°C for 30 s, and 72°C for 3 min); and a final extension at 72°C for 10 min. The amplified PCR products were checked by gel electrophoresis and purified using a gel extraction kit (TaKaRa, Japan) according to the manufacturer’s protocol. We cloned the PCR products into pMD19-T vectors. To ensure that the full cDNA was obtained, we selected thirty clones for sequencing using an ABI 3730XL sequencer (Applied Biosystems, United States). Sequences were analyzed with BioEdit version 7.2.1. Genomic DNA was used to amplify the intron and exon sequences with gene-specific primers (Table 1).

**Sequences and Phylogenetic Analysis**

The nucleotide sequences were verified, merged and aligned using BioEdit (version 7.2.1) (Hall 1999). Open reading frame (ORF) detection was performed using the NCBI online tools (http://www.ncbi.nlm.nih.gov/orf/orf.html). Gene structure was displayed with GSDS (version 2.0) (Hu et al. 2015). The point values (pl) and molecular weight (Mw) were calculated using ExPaSy tools (http://WebExPaSy.org/compute pl/). The presence of a signal peptide in the IRP30 gene in Hymenoptera were identified through a BLAST search in the non-redundant (nr) protein database. The conserved domains in IRP30 were determined with the Conserved Domain Database software (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The phylogenetic analysis was performed using MEGA X (Kumar et al. 2018). The best protein model selected based on AIC values was the general reversible mitochondrial + frequency model. The maximum likelihood method with 1,000 bootstrap replicates was adopted (Felsenstein 1985, Adachi and Hasegawa 1996, Kumar et al. 2018). The conserved domains in IRP30 were determined with the Conserved Domain Database software (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

**Real-Time Quantitative Polymerase Chain Reaction Analysis**

The expression profiles of the IRP30 gene in different castes and tissues of bumble bees and in female bumble bees and honey bees of different reproductive statuses were examined by real-time quantitative polymerase chain reaction (qPCR). Each sample included three biological replicates. One microgram of total RNA from each sample was used as template. First-strand cDNA was generated using the PrimeScript RT–PCR Kit (TaKara, Dalian, China), and real-time qPCR was performed using the SYBR Premix Ex Taq II kit (TaKara, Dalian, China). The IRP30F and IRP30R primers (Table 1) were designed and synthesized for the real-time qPCR experiment. The primers for the endogenous control gene, β-actin, were from Dong et al. (2019). The amplification was performed as follows: 95°C for 40 s followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Three replicates were performed for each sample. The relative expression levels of the IRP30 gene were estimated using the 2−ΔΔCt method (Livak and Schmittgen 2001). The expression differences of IRP30 between different developmental stages and different tissues were determined using one-way analysis of variance (ANOVA) with Tukey’s post hoc test. The expression differences of IRP30 in different castes and tissues were determined using two-way ANOVA with Bonferroni post hoc tests. The expression differences of IRP30 between egg-laying and non-egg-laying status in workers and queens were determined using Student’s t-test. Data analysis was performed using R (version 3.1.1).

**Western Blotting**

A polyclonal IRP30 antibody was generated using prokaryotic expression and mouse immunization according to a previously described method (Dong et al. 2017). The expression primers used to amplify the full-length CDS are listed in Table 1. The IRP30 antibody was kept at −80°C until required. Twenty-microgram samples of total protein from the haemolymph of non-egg-laying and egg-laying female bumble bees were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto a methanol-activated PVDF membrane (Millipore Immobilon FL, United States) and incubated with antibody using an Eu-Labeled Anti-Mouse ScanLater Western Blot Assay Kit (MD, United States) according to the manufacturer’s protocol. The blots were scanned on a SpectraMax i3 Multi-Mode Detection Platform (MD, United States) using a ScanLater Western Blot (WB) Detection Cartridge. Experiments were repeated in triplicate. Total gray value analyses of each band were performed digitally using ImageJ (version 1.5.2) (http://rsweb.nih.gov/ij/). β-Actin (Transgen, China) was used as an internal reference protein to normalize protein expression. The statistical significance (*P < 0.05* or *P < 0.01*) between egg-laying and non-egg-laying status was assessed using Student’s *t*-test. Data were analyzed using R (version 3.1.1) and reported as the mean ± SE.

**Table 1. Primers used for cloning, prokaryotic expression and transcription analysis of the IRP30 gene**

| Primer name | Sequence (5′–3′) | Purpose |
|-------------|-----------------|---------|
| IRP30F      | GGATATCGAGCCCGAGATGCTTTCAGAGGTC    | 3′-RACE  |
| IRP30R      | CCTGACGAAAACCGATATGGTTGCTCC       | 5′-RACE  |
| IRP302F     | CGATGACCTGTTGATTTGGGGACAACTATA    | 3′-RACE  |
| IRP302R     | TGTATGCTGCCCATAATCAGGTTATCG       | 5′-RACE  |
| IRP30EF     | CGGAATTCTATAGAGGCGTTCGAGAGCAGCC  | Expression/Gene |
| IRP30ER     | GCGGCCGCTTTACATTTTGCTTTATCCCAGGTCGC | Expression/Gene |
| IRP30F      | GTCTTATAGGTTGTTCTCC               | qPCR     |
| IRP30R      | GTGCCTCATATCCTCAATC               | qPCR     |
| β-actinF    | CGACTACCTCATGAAGATT               | qPCR     |
| β-actinR    | CGACGTAACAAAGGTCTTC               | qPCR     |

The italicized bases are added enzyme sites.
Results

Molecular Cloning of the IRP30 Gene

The RACE results showed that the full-length cDNA of IRP30 was 1082 bp (including a 36-bp 5′-UTR and a 218-bp 3′-UTR) (GenBank accession no. MN027563) and contained a full ORF of 825 bp that encoded a putative protein of 275 amino acids. The transcript had a start codon (ATG), stop codon (TAA), and a non-classical putative polyadenylation signal (TATAAA) (Fig. 1A). The IRP30 protein had a calculated molecular mass of 30.7 kDa and a theoretical isoelectric point of 6.82. The signal peptide cleavage site determined by SignalP 4.1 showed that the cleavage site of the protein product from IRP30 was between positions 19 and 20 aa. Conserved domain analysis showed four leucine-rich repeat (LRR) conserved domains. The LRR region was located between 35 and 180 aa and contained three LRR_8 superfamily domains located at 73–132 aa, 97–156 aa, and 121–180 aa (Fig. 1C). Genomic amplification results showed that the length of the IRP30 gene was 1443 bp (GenBank accession no. MN027562) and that it contained two exons and one intron (Fig. 1B).

Phylogenetic and Conserved Domain Analysis of IRP30

To understand the phylogenetic relationships of IRP30 in Hymenoptera, a total of 18 homologous sequences were retrieved from GenBank. Phylogenetic tree analysis indicated that the IRP30 proteins of the Apidae, Formicidae, and Vespidae families formed a monophyletic group with a high posterior probability (Fig. 2). Among the Apidae family, the proteins were divided into two groups, Apis and Bombus, which had diverged recently. IRP30 of B. lantschouensis was clustered with the Bombus clade at the top of the tree.

The conserved domain results showed three types of LRR domains in the LRR superfamily region, except in the outgroup species Nasonia vitripennis. The LRR types were LRR_4 (PF12799), LRR_8 (PF13855), and LRR_9 (PF14580), of which LRR_8 had the highest occurrence frequency. Furthermore, the same LRR repeat combination occurred throughout the Bombus clade, including three LRR_8 domains.

Expression Profile of IRP30 in Different Developmental Stages

Real-time PCR showed that IRP30 was expressed at different levels in each developmental stage (Fig. 3, F_{1,16} = 11.057; P < 0.001). The expression levels of IRP30 increased gradually from the fifth-day larval stage, reached a peak in the brown-eyed pupae with unpigmented cuticle stage (Pb), and then began to decline until the pharate adult stage (Pha). The expression levels of IRP30 were significantly higher in the brown-eyed pupae with unpigmented cuticle (Pb) and brown-eyed pupae with lightly pigmented cuticle (Pbl) stages compared with other stages (P < 0.01). It can be demonstrated that IRP30 plays an important role in the developmental stages in bumble bee workers, especially in the brown-eyed pupae with unpigmented cuticle (Pbl) stages.

Expression Profile of IRP30 in Different Tissues and Castes

Real-time PCR showed that the expression levels of IRP30 differed in eight tissues in workers and that the relative expression was significantly higher in the head and thorax than in the other tissues (Fig. 4, F_{7,16} = 3.248; P = 0.024). Furthermore, IRP30 relative expression was analyzed across different tissues. The results indicated that the expression of IRP30 showed significant differences not only in different tissues but also in different castes. The expression levels of IRP30 were higher in the thorax than in the head or abdomen in all three castes of bumble bees. The expression levels of IRP30 were highest in the thorax of workers, followed by queens and then drones (F_{8,18} = 118.420; P < 0.001). The results indicated that the expression of IRP30 showed significant differences not only in different tissues but also in different castes. The expression levels of IRP30 were higher in the thorax than in the head or abdomen in all three castes of bumble bees. The expression levels of IRP30 were highest in the thorax of workers, followed by queens and then drones (F_{8,18} = 118.420; P < 0.001).

Protein Expression Profile of IRP30 in Female Bumble Bee Haemolymph

To directly assess the expression levels of IRP30 protein in egg-laying versus non-egg-laying workers and queens, we performed a...
conventional western blot analysis using prepared antibodies. The results showed that IRP30 protein was expressed in the haemolymph of female bumble bees (*B. lantschouensis*) (Fig. 6A). There was a significant difference in the expression of IRP30 protein between egg-laying and non-egg-laying female bees. The ratio of IRP30 to β-actin band density indicated that the expression level of IRP30 protein was significantly upregulated in egg-laying workers compared with non-egg-laying workers (Fig. 6B, Worker: $t(16) = -10.020, P < 0.001$). Similar results were observed in queens with different reproductive statuses (Fig. 6B, Queen: $t(16) = -16.975, P < 0.001$). These changes in IRP30 protein levels confirmed that IRP30 could play an important role in egg-laying bumble bee females.
of the abdomens of female bumble bees (B. lantschouensis) with egg-laying and non-egg-laying status, while using female honey bees (A. mellifera) to further verify the change in IRP30 in different reproductive statuses. The results indicated that IRP30 mRNA expression levels were significantly increased in egg-laying female bumble bees compared with non-egg-laying females (P < 0.01, Student’s t-test). As shown in Fig. 7A (Worker: t (4) = −6.296, P = 0.003; Queen: t (4) = −4.700, P = 0.009), IRP30 mRNA was upregulated greater than 90-fold in egg-laying workers compared to non-egg-laying workers, and a similar trend was observed in bumble bee queens with different reproductive statuses. Surprisingly, IRP30 transcripts increased greater than 20- and 30-fold in egg-laying female honey bees compared with non-egg-laying honey bees, similar to bumble bees (Fig. 7B, Worker: t (4) = −5.068, P = 0.007; Queen: t (4) = −8.134, P = 0.001). These results indicated that IRP30 expression is positively correlated with changes in reproductive status in both bumble bees and honey bees and in both queens and workers. These results further confirmed that IRP30 may play an important role in female reproduction.

Discussion

IRP30 has been conserved during evolution in social hymenopteran insects and is involved in response to challenge by external stimuli, especially bacterial infection (Randolt et al. 2008, Albert et al. 2011, Gatschenberger et al. 2013). Despite previous studies focused on the changes in different social insects’ IRP30 in various environments, very little information is available about the precise expression pattern and biological function of this protein. In this study, we clarified the structural features and expression patterns of the IRP30 gene in the bumble bee B. lantschouensis for the first time and presented an interesting and novel finding that IRP30 expression levels were related to egg laying in female bumble bees and honey bees. Therefore, in addition to its involvement in immune response, IRP30 may play an important role in the reproductive process in bees.

According to the phylogenetic tree and domain structure, IRP30 was highly conserved throughout its evolution among genera. The IRP30 gene has been found only in hymenopteran arthropods, and the critical residues in the LRR domains are conserved (Albert et al. 2011). We found that the LRR domains were more highly conserved in the Bombus clade, while different types and numbers of LRR conserved domains were present in the Apis genus. In addition, we observed that compared with other social hymenopteran insects, the N. vitripennis homologue of IRP30 lacked one section of the LRR conserved domain (marked with yellow in Fig. 2). This result may be caused by different numbers of introns and does not share a common origin (Albert et al. 2011). Therefore, N. vitripennis IRP30 is highly divergent from the others.

Immune response protein 30 is an LRR-containing protein. One representative LRR section is present in IRP30 of bumble bees; it includes three LRR8 motifs that confirm its important function. This protein, LRRs are short sequence motifs that have a specific conserved eleven-residue signature, LxxLxLxxNCxL, composed of a β-strand and adjacent loop regions, whereas the remaining parts of the repeats are variable (Kobe and Deisenhofer 1994, Kobe and Kajava 2001). It has been demonstrated that LRRs provide a structural framework for the formation of protein–protein interactions (Gay et al. 1991, Enkhbayar et al. 2004) and that the main functions of LRR protein family members are in the immune response (Zhu et al. 2010) and signal transduction (Takeemura et al. 2020). LRR proteins (LRM1, APL1, and LRRD7) in Anopheles hemocytes generally form a complex that can interact with pathogenic surface molecules.
the head, thorax and fat body showed higher expression levels than the seasons and bacterial challenge (Albert et al. 2011). In this study, high amounts of mRNA. Expression may also change with IRP30 in honey bees. Compared to the other tissues, fat body expressed IRP30 in bumble bees was similar to that of metamorphosis. However, a previous study showed that IRP30 does not appear to function in identifying bacteria. It does not interact with bacterial cell wall components or any components of bee haemolymph (Albert et al. 2011). Therefore, whether IRP30 is involved in signal transduction or another mechanism in the immune response requires further exploration.

In this study, we examined the expression pattern of IRP30 in bumble bees. First, we observed that IRP30 was continuously expressed in different developmental stages of worker bumble bees from egg to adult (Fig. 3), especially in the brown-eyed pupae with unpigmented cuticle and brown-eyed pupae with lightly pigmented cuticle stages, indicating that IRP30 may play an important role in the metamorphosis developmental stages of bumble bee pupae. The function of IRP30 may be associated with its LRR sequence motifs. Previous research has shown that Toll-like receptors, a class of LRR-containing proteins, are expressed at high levels during embryogenesis and moulting in Drosophila (Tauszig et al. 2000, Bell et al. 2003). We speculate that IRP30 may perform developmental functions similar to those of Toll-related receptors in the developmental stages of metamorphosis. However, a previous study showed that IRP30 is not produced in honey bee larvae, even after immune challenge, and has only minimal expression in the early pupal stages (Albert et al. 2011). These expression differences may be caused by different immune pathways in fruit fly and honey bee. Moreover, we found that IRP30 was expressed in multiple tissues in adult bumble bees, but its expression varied considerably among the different tissues (Fig. 4). The expression trend of IRP30 in bumble bees was similar to that in honey bees. Compared to the other tissues, fat body showed high amounts of IRP30 mRNA. Expression may also change with the seasons and bacterial challenge (Albert et al. 2011). In this study, the head, thorax and fat body showed higher expression levels than the other tissues in uninfected bumble bees with no signs of disease. These results suggested that IRP30 was expressed constitutively and had a significant tissue bias in bumble bees.

The most interesting result of this study is that IRP30 had a female bias in bumble bees. Furthermore, its expression varied with changing reproductive status in females. The expression level of IRP30 was increased significantly in egg-laying female bees compared with non-egg-laying female bees. This phenomenon may be associated with female immunity. Cardoen et al. (2012) found that the viral load in the brains of infertile workers was higher than in fertile workers, indicating that infertile workers increase their immunity when they start to lay eggs. We speculate that the high expression of IRP30 in egg-laying female bees may be related to immunological competence. The queens and workers need to improve their immunity to ensure the successful completion of reproduction. Nevertheless, the function of IRP30 in bumble bees still remains to be validated.

**Conclusions**

This study successfully identified the IRP30 gene of the bumble bee B. lantschowensis, and the expression pattern of IRP30 in bumble bees was recorded for the first time. IRP30 had a significant bias with respect to castes and tissues. Due to its high expression in pupal developmental stages, IRP30 plays an important role in the growth and development process in workers. The last but most interesting novel finding was that IRP30 expression levels were related to egg-laying status in female bumble bees and honey bees. Therefore, the existing IRP30 not only responds to immune challenge but also participates in reproductive function. These results provide new insights into the expression patterns and biological functions of IRP30 in hymenopteran insects.
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