Cloning, expression pattern and functional characterization of fused, an important kinase of the Hedgehog signalling pathway from *Locusta migratoria* (Orthoptera: Acridoidea)

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**ABSTRACT**

*Locusta migratoria* is one of the global destructive migratory agricultural pests. The species serves as a model insect for the study of morphology, physiology, developmental biology, and neurosciences and so on. In recent years, there has been growing interest in the research of the cell and molecular biology of *L. migratoria*, and a lot of genes of signalling pathways have been cloned. The Hedgehog (Hh) signalling pathway is essential for cellular proliferation and differentiation during embryonic development. Fused (Fu) is a conserved component of the Hh signalling pathway, and it plays a central role in embryonic development. The Fu gene was cloned from the grasshopper *L. migratoria*. The gene encodes a 717 amino acid protein. Then, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression pattern of the *Lm*-Fu gene at different development stages and tissues. The result showed that the gene was upregulated at the embryonic period and second instar, and the expression level was the highest in the head at the different tissues. Western blotting was used to assess the abundance of the *Lm*-Fu protein, which was expressed at different development stages. The embryogenesis of *L. migratoria* was examined by immunohistochemical staining, and the *Lm*-Fu protein was detected in the head, prothorax and legs. The results showed that the *Lm*-Fu gene plays an important role during embryonic development.

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**Introduction**

Hedgehog (Hh) signalling plays a highly conserved role in the embryonic development and postnatal life of multicellular organisms ranging from invertebrates to vertebrates. During embryonic development, Hh signalling contributes to cell differentiation, proliferation and survival [1–10], and in postnatal life, in the maintenance of tissue homeostasis [11–13]. In the human body, activation of the Hh pathway results in tumorigenesis, malignancy, and metastasis. This is one of the most widely studied signalling pathways [14,15]. According to research reports, the Hh signalling pathway regulates the formation of many organs during the embryonic stage and determines the direction of cell differentiation; it plays a vital and universal role in cell growth and morphology in the region of *Drosophila* wing buds [16–20]. Hh is an important signalling pathway regulating the development of wings, legs and primordia; it is also involved in the regulation of other biological processes, such as the migration of germ cells, the development of the lobes, the gonads and the intestines [21–27].

Most of what is known about the Hh signalling cascade comes from studies of *Drosophila*, where the pathway was originally identified [28]. The Hh signalling pathway is driven by the ligand Hh and transduces signals through binding to its receptor, a 12-transmembrane protein Patched (Ptc). The binding of Hh to Ptc inhibits its repression of Smoothened (Smo), a GPCR-like seven-transmembrane protein [29–36]. Activated Smo functions as the Hh signal transducer to recruit an intracellular protein complex containing the kinesin-like protein Cos2 and the kinase Fused (Fu), leading to Fu phosphorylation and activation [37–45]. In *Drosophila*, this phosphorylation is sequential and triggered by several kinases that include protein kinase A(PKA;PKA-C1-FlyBase), the

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serine-threonine kinase Fused (Fu) and G protein-coupled receptor kinase 2 (Gprk2), which promote the conversion of Smo to an open conformation [46–56]. The Ser/Thr kinase Fused (Fu), a known downstream effector of Smo, is part of the Hh signalling pathway, which is one of the most conserved components and may play a central role [57]. The wing imaginal disc model in *Drosophila* has revealed that phosphorylation at the Hh signalling pathway is induced by the kinase Fused (Fu); in turn, activation of Smo induces Fu to act on its downstream targets [29]. It has been reported that the activity of Fu kinase activates the anterior compartment cells of the wing disc and induces the expression of related genes, thereby affecting the development of the wing. In *Drosophila*, a biochemical study established that Fu plays a vital role in the embryonic development [58,59].

Fu has been cloned from *Drosophila melanogaster* (NM_058151.5) and *Bombyx mori* (XM_004923160.3). However, the structure and function of the Fu protein remains unclear in *Locusta migratoria*, which has long served as a model organism for many aspects of research, such as insect morphology, behaviour and physiology [60–63]. This insect is also one of the most destructive agricultural pests. In China, outbreaks of *L. migratoria* have been increasing in recent decades, which are associated with locust's long-distance migration and resistance to pesticides [64, 65]. Studying the physiological characteristics of grasshoppers is important for the control of *L. migratoria*. In this paper, the cDNA of the *Lm-Fu* gene was obtained from *L.migratoria*. The expression levels of *Lm-Fu* at different developmental stages and adult tissues were investigated. In addition, the protein expression and localization of Fu were analyzed by Western blotting and immunohistochemistry. Our aim was to understand the function of *Lm-Fu* in *L.migratoria*.

### Materials and methods

#### Animals and reagents

Eggs of *Lmigratoria* were purchased from Junan, Shandong Province. The *L. migratoria* eggs were held in a constant temperature incubator at 30 °C and 75% relative humidity with an L12:D12 photoperiod. When the eggs hatched, wheat seedlings were given as food. Nymphal instars were identified by the development of wings. Appropriate amounts of *L.migratoria* from each instar were taken and rapidly frozen in liquid nitrogen and then stored at −80 °C. All the restriction enzymes, T₄ DNA ligases and Taq DNA polymerase were from TaKaRa Biotechnology (Dalian, China).

#### Cloning of the Fu gene

Total RNA was extracted from adult *L.migratoria* using RNAiso Plus (TaKaRa, Dalian, China), and then was transcribed into cDNA. Based on the transcriptomic database from our laboratory of *Lmigratoria*, specific primers (Fu-F, Fu-R, Table 1) were synthesized by Genewiz (China). The amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 1 min), and followed by a 10 min of extension at 72 °C. The PCR amplification products were detected using 1.0% agarose gel by electrophoresis. The objective bands were purified by a DNA gel extraction kit (Takara, Dalian, China), and then sequenced (TSINGKE, China).

#### Bioinformatics analysis

The sequence of *Lm-Fu* was analyzed using BLAST program at the National Center for Biotechnology Information (NCBI, US National Institute of Health) website (http://www.ncbi.nlm.nih.gov/BLAST). SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP) and TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were used to predict the signal peptide of the *Lm-Fu* amino acid sequences and transmembrane region. ClustalX2.0 and DNAman were used to carry out multiple alignment analysis. Then, a phylogenetic tree of the insect system was constructed using the neighbour-joining (NJ) method and MEGA6.0 software.

#### Quantitative real-time polymerase chain reaction (PCR)

RNA samples of *L.migratoria* were extracted from different development instars of nymphae (the first, second, third, fourth and last fifth instars) and different tissues of adult females and males (prothorax, abdomen, legs, wing, head, midgut). Total RNA was extracted by the Trizol purification method. After that,
the extracted mRNA was reverse transcribed into cDNA using a PrimeScript® RT Reagent Kit for Perfect Real Time (Takara, Dalian, China). According to the full-length DNA of Lm-Fu, the qRT-PCR primer pairs (qFu-F, qFu-R, Table 1) were designed in Primer Premier 5.0 (qFu-F, qFu-R, Table 1). The normalization control gene was the β-actin housekeeping gene (β-actinF, β-actinR, Table 1) Real-time qPCR was performed in LightCycler 96 System (Roche, Potsdam, Germany). Each 10 μL PCR reaction mixture contained: 5 μL SYBR Premix Ex Taq (Roche, Potsdam, Germany), 0.4 μL of each primer (10 mmol/L), 1 μL cDNA solutions, and 3.2 μL sterile distilled water. PCR conditions were: an initial denaturation at 95°C for 30 s; followed by 35 cycles of 95°C for 10 s, 53°C for 30 s and 72°C for 15 s. Each experiment was repeated at least three times. Finally, we used the delta-delta Ct method to calculate the relative quantitative expression [66].

Prokaryotic expression

After obtaining the complete sequence of the Lm-Fu gene, primers exFu-F and exFu-R (Table 1) with restriction enzyme sites (KpnI and EcoRI) were designed. The PCR product and pET-30a were ligated with the restriction enzymes. After the two fragments were ligated, the recombinant pET-30a-Fu-plasmid was transferred into E. coli DH5α via the heat shock method. The insertion fragment was sequenced (Geneviz company; Suzhou, China) to ensure that there were no errors in the sequence and then was transferred into E. coli BL21 (DE3). When the optical density (OD) at 600 nm of the transformed cells increased to 0.6–0.8, IPTG was added for induction. Sonication was performed in an ice bath to disintegrate the cells. The fractions were collected and tested by SDS-PAGE on a 12% gel. Finally, the protein was purified and sent to BGI (Shenzhen, China) for antibody preparation.

Western blot

Total proteins from different instars of Lmigratoria were extracted and analyzed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to a nitrocellulose (NC) membrane. To block non-specific protein binding, the membrane was incubated with skimmed milk for 2 hours; then the NC membrane was incubated with Rabbit anti-Fu polyclonal antibodies (1:100) at 4°C overnight. After washing with TBST (TBS/0.1%Tween 20), the NC membrane was incubated with secondary antibody of goat anti-rabbit IgG-HRP (ProteinTech, China) (1:10,000) at room temperature for 2 h. Finally, colour development was performed using an ECL chromogenic kit (Sangon, Shanghai, China). β-actin was used as an internal reference.

Immunohistochemistry

Different periods eggs of Lmigratoria were collected, soaked with 10% NaClO for 15 minutes, then the embryos were dissected manually in 1× PBS (phosphate buffered saline) by cutting off the anterior pole and squeezing the embryo and yolk out of the egg shell. Subsequently, the eggs were cleaned form yolk and fixed on ice for 30 min in PEM-FA fixative. The embryos were rehydrated stepwise into PBS/0.1% Triton X-100 (PBT), and blocked in PBT/1% BSA for 2 h at room temperature. The treatment embryos were incubated with Rabbit anti-Fu polyclonal antibodies (1:50) at 4°C overnight. The control embryos were incubated with negative serum. After washing 3 times in PBST, the samples were incubated with secondary antibody of goat anti-rabbit IgG-HRP (1:10000) at room temperature for 1 h, after further washing with PBST. After incubating for 12 minutes using the DAB kit (Sangon, Shanghai, China), the samples were observed.

Data analysis

Data are mean values with standard deviation (±SD) of three independent replicates. Student’s t test and Fish’s LSD multiple comparison test were used for assessment of significant differences among the developmental stages and different tissues.

Results and discussion

Cloning and sequence analysis of the Lm-Fu gene

A 4783-bp cDNA of the Lm-Fu gene was obtained from the grasshopper L. migratoria by RT-PCR, and the gene was named Lm-Fu (GenBank Accession No. MH704853). The sequence contained an open reading frame of 2154 bp, and was predicted to encode 717 amino acids. The theoretical amino acid sequence of Lm-Fu was analyzed and predicted by the CD search program of NCBI. This result is consistent with the report for Drosophila [67] (Figure 1). The results of the multiple alignments showed a high degree of homology to the Lm-Fu amino acid sequence. Moreover, Standard protein BLAST (blastp) also confirmed that
the Lm-Fu amino acid sequence and the amino acid sequence of Neodopiron lecontei shared 50% homology (Figure 2). The analysis of the phylogenetic tree constructed by the NJ method showed that all sequences from the same group were clustered together. The homology between the Lm-Fu protein of L. migratoria and the Hymenoptera protein situated them more closely, and the confidence level reached 93%. The mammals were the outgroup of the phylogenetic tree (Figure 3).

Expression analysis of Lm-Fu at different stages and different tissues

qRT-PCR was used to analyze the expression levels of the Lm-Fu gene at different developmental stages and in different tissues. The results showed that the Lm-Fu gene was expressed in the different tissues and developmental stages. The expression levels of the Lm-Fu gene in the second instar were higher than those in other instars. At the second instar, the wing primordium of the L. migratoria begins to develop. Fu protein plays a role in the formation of wing imaginal discs. Fused kinase activity is required at the A/P boundary in the anterior compartment of the wing disc [58]. The fu mutant wings present alterations in the region of the wing blade between veins 3 and 4 and thus are affected in anterior and posterior compartments on both sides of the A/P boundary in D. melanogaster [58]. In the fly wing imaginal disc, the protein kinase Fused (FU) plays a key role in the responses to high levels of Hh [58]. In our study, the transcripts of the Lm-Fu gene were at lower levels in the third and fourth instar. Among them, the expression of Lm-Fu reached the lowest levels in the fourth instar (Figure 4).

In order to better study the function of Lm-Fu in embryonic development, we observed and analyzed the expression levels of Lm-Fu in different periods of embryogenesis. The relative expression level was higher during the embryonic period, which is in agreement with the important role that the Ser/Thr kinase Fused (Fu) has in the Hh signalling pathway during the embryonic development [68,69]. The expression level was the highest on the 8th day of the embryonic period. The reason could be that the Lm-Fu gene promotes development in the nervous system. The nervous system development begins on the 8th day of the embryonic period. In the developing Drosophila eye, Hh controls the initiation and propagation of the morphogenetic furrow, leading to photoreceptor differentiation. Hh signalling is important during Drosophila nervous system development [70]. Fused is involved in the development of various organs; for example, it is expressed in the central nervous system during development [71,72]. In our study, the expression level of the Lm-Fu gene reached the lowest on the 14th day of the embryonic period (Figure 5).

As shown in Figure 6, the Lm-Fu gene was expressed at different degrees in different tissues of male and female adults. In all tissues, the expression was higher in males than in females. The reason could be the effect of estrogen on the Lm-Fu gene [73]; however, the mechanism is not yet clear. The expression level of the Lm-Fu gene in different tissues of the female was low. The Lm-Fu gene had the lowest expression level in the male and female midgut, and it reached higher levels in the male and female head.

SDS-PAGE analysis of Lm-Fu and Western blot

The partial sequences were selected for prokaryotic expression. The recombinant cells were disintegrated by sonication. The results of SDS-PAGE showed that the target protein was expressed in inclusion bodies, and a protein band of 48 kD was obtained. After purification and concentration, the target protein of relative purity was obtained (Figure 7A).

We detected the expression of Lm-Fu by western blot. Experiments were carried out by selecting different days of L. migratoria from each instar nymphs. As shown in Figure 7B, Lm-Fu had the highest expression in the second instar, and relatively low expression in the third and fourth instars, reaching the lowest level at the fourth instar. The western blot results were consistent with qRT-PCR.

Immunohistochemistry analysis of Lm-Fu prorein

Chin et al. [74] divided the embryonic development of L. migratoria into 18 stages. In different stages,
embryos express different forms. Immunohistochemistry was used to detect the location of *Lm*-Fu protein of *L. migratoria* in different developmental stages of the embryo (Figure 8). During the E-6 period, compared to the control group (Figure 8, E6A1), *Lm*-Fu was expressed in the head,
but the expression was relatively small (Figure 8, E6A2). At E-8 period, there was a groove in the middle of the hind leg bud to divide the hind leg into the femur and tibia. It could be observed that Lm-Fu was expressed in the upper mandible and the antennae, but the effect was not obvious (Figure 8, E8A2). At E-10 period, Lm-Fu was expressed in the head, as well as in the fore leg and middle leg, and the effect was obvious (Figure 8, E10A2). At E-12 period, a red-brown crescent-shaped compound eye began to appear. There was transposition in the fore leg, middle leg and hind leg, and Lm-Fu was detected in the head and legs (Figure 8, E12A2). At E-14 period, the segmentation of the legs was clearly visible and the expression of Lm-Fu was increased (Figure 8, E14A2). At E-16 period, the herringbone pattern on the femur leg was clearly visible, the black crescent-shaped femur appeared at the tarsus, and Lm-Fu was distributed in the head, prothorax and legs (Figure 8, E16A2). The immunohistochemistry results showed that Lm-Fu prorein was located in the head appendages and legs segments in the embryo development, which is consistent with the previous report that Fu prorein exhibits constitutive, non-specific expression [70]. There are research reports that the Hh signalling pathway plays a critical role in determining proper embryonic patterning and cell fate determination in the central nervous system [71]. Since the Ser/Thr kinase Fused (Fu) is a positive regulator of the Hh signalling pathway during the embryonic development [68,69], we hypothesize that Lm-Fu prorein plays an important role in the development of the embryo, especially in the early development of the embryonic head appendages and legs segments.

Figure 3. A phylogenetic NJ tree of Lm-Fu with other homologues.

Figure 4. Expression analysis of the Lm-Fu gene at different developmental stages of L. migratoria. 1: embryonic period; 2: first instar; 3: second instar; 4: third instar; 5: forth instar; 6: fifth instar. Data are means with standard deviation (± SD) of triplicate experiments. Asterisks indicate significant differences compared to the control: *p < .05; **p < .01.

Figure 5. Expression analysis of the Lm-Fu gene at different developmental stages in the embryonic period of L. migratoria. Data are means ± SD of triplicate experiments. Asterisks indicate significant differences compared to the control: *p < .05; **p < .01.
Figure 6. Expression analysis of the *Lm-Fu* gene in different tissues of *L. migratoria*. Data are means ± SD of triplicate experiments. Asterisks indicate significant differences compared to the control: *p* < .05; **p** < .01.

Figure 7. SDS-PAGE (A) and Western blot (B) analysis of *Lm-Fu*. Note: (A) SDS-PAGE analysis: M, protein Marker (TransGen Biotech, Beijing); Lane 1, recombinant product of pET-30a-*Lm-Fu* without inducer; Lane 2, recombinant product with inducer; Lane 3, supernatant of cell lysate; Lane 4, precipitate fraction; Lane 5, purified recombinant protein. (B) Protein expression analysis of *Lm-Fu* at different stages.

Figure 8. Immunohistochemistry of *L. migratoria* in different developmental stages. A1, control group; A2, experimental group. E6, sixth stage of embryonic development; E8, eighth stage of embryonic development; E10, tenth stage of embryonic development; E12, twelfth stage of embryonic development; E14, fourteenth stage of embryonic development; E16, sixteenth stage of embryonic development.
Conclusions

In this study, the sequence of Lm-Fu from L. migratoria was cloned, and sequence alignment was performed. The analysis of the expression patterns of the Lm-Fu gene by qRT-PCR showed that the Lm-Fu gene was expressed in different developmental stages and different tissues at different levels. The expression of the Lm-Fu gene was the highest in the second instar. The expression of the Lm-Fu gene in males was higher than that in females. The results of qRT-PCR and immunohistochemistry showed that Lm-Fu played an important role in embryonic development. The research on the Lm-Fu gene of L. migratoria established a theoretical foundation for exploring new approaches to the control of locust plagues.

Disclosure statement

No potential conflict of interest was reported by the authors.

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