Molecular cloning and characterization of High Mobility Group box (HMGB) gene from Beauveria bassiana-infected silkworm, Bombyx mori

H Chengxiang1,2, W Han1,2, L Dingding1,2, L Rulin1,2, G Xije1,2

1Sericultural Research Institute, Jiangsu University of Science and Technology, Zhenjiang 212018, China
2Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China

Abstract

The cDNA sequence of High Mobility Group box (HMGB) from Bombyx mori was cloned by rapid amplification of cDNA ends and submitted to GenBank (the accession number was JF969272). The full-length cDNA of BmHMGB included 3015 bp, with five exons and four introns. It contained a 313 bp 5’ UTR, a 2114 bp 3’ UTR with a polyadenylation signal sequence AATAAA and a poly(A) tail. It encodes a 195- amino acid polypeptide with a predicted molecular weight about 22991.9Da and a theoretical isoelectric point 10.00. Phylogenetic analysis revealed that BmHMGB is grouped in insect HMGB proteins. BmHMGB was mainly expressed in hemolymph, cuticles, midgut and fat body by RT-PCR. In these tissues, the relative expression of BmHMGB with Beauveria bassiana-infected silkworm was higher than the normal ones by fluorescent quantitative real-time PCR. Therefore, BmHMGB may play an important function in the response to B. bassiana infection of silkworms.

Key Words: Bombyx mori; Beauveria bassiana; High Mobility Group box protein; rapid amplification of cDNA ends; qRT-PCR

Introduction

High Mobility Group box (HMGB) proteins are one of the cytokines with diverse functions, exist at the intersection of foreign and endogenous molecules in all eukaryotes (Andersson and Tracey, 2011; Malarkey and Churchill, 2012; Choi et al., 2016). These proteins have one or multiple HMG-box, which is an unique and versatile DNA-binding domain. They can many substrates, such as different immunogenic nucleic acids, bended/lopped and unwinded DNA, damage associated molecular pattern (DAMP), Toll-like receptor (TLR) (Stros, 2010; Malarkey and Churchill, 2012). As DNA chaperones, they also influence many biological processes in chromatin from maintenance of genome integrity to transcription, such as DNA replication and repair, recombination, transcription, genomic stability, so they throw a critical vote in decisions of life and death (Gerlitz, 2009). HMGBs have another crucial role that they act as an endogenous immune signal to inform other cells that damage or invasion has occurred (Yang and Tracey, 2010). In the nucleic-acid-mediated innate immune responses, HMGBs act as an universal sentinels, that is all exogenous nucleic acids must bind firstly HMGBs, then be sent to specific pattern recognition receptors and activate the innate immune responses (Yanai et al., 2011). They also function as DAMPs, which can activate inflammatory and induce immune responses to protect against infection and promote healing after tissue damage in organisms (Manigrasso et al., 2014; Choi et al., 2016).

The silkworm, Bombyx mori, an important economical insects and model organisms of lepidoptera, only has the natural immune system. Beauveria bassiana is one of the major fungal pathogens for silkworms and can cause economic damages to the sericultural industry. During the silkworm defense pathogen-infected, immune recognition is the first step of the responses. In our previous studies, we found a different expressed gene BmHMGB with recognized function (Hou et al., 2014). To further study it, we first cloned the full-length cDNA of BmHMGB by rapid amplification of cDNA ends. Then the expression characteristics of BmHMGB was analyzed in different tissues and at different time in the same tissue after B. bassiana infected. These results provide some useful information for further study of the immune recognition mechanism of silkworm to fungi infection.
Materials and Methods

Silkworm and B. bassiana strain

In our study, the silkworm strain p50 and B. bassiana HN6 were provided by the Sericultural Research Institute of the Chinese Academy of Agricultural Sciences. The larvae of silkworm were reared with mulberry leaves at standard temperature and humidity until the newly exuviated fifth instar larvae for the experiments. HN6 were originally inoculated from the cadaver of the infected silkworm to potato dextrose agar (PDA), then isolated and cultured on PDA for about 10 days at 25 °C.

Inoculation of B. bassiana

Conidia of B. bassiana were scraped from PDA and diluted to 3×10^9 spores/ml using sterile distilled water (containing 0.01 % Tween-80). The newly exuviated larvae were immersed in the conidia solution for 15 s. The control larvae were immersed in sterile distilled water (containing 0.01 % Tween-80) for the same period. Then regulated temperature and humidity to 28 °C and 95 % RH, the larvae were reared in these conditions.

Collection tissues

The different tissues were collected from both the HN6-infected and control larvae at 8 h post-inoculation (hpi). First, hemolymph was collected, which was directly mixed with pre-joined Trizol reagent (Invitrogen) in an Eppendorf tube. Then the larvae were rapidly dissected to collect gonad, martensite, mid-gut, mid-silk gland, fat body and cuticles. These tissues from 10 - 15 larvae of fifth instar larvae were pooled as one sample. The tissues were quickly washed in diethylpyrocarbonate-treated PBS solution (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) and immediately frozen in liquid nitrogen before stored at -80 °C refrigeratory. The collected time was 8, 10, 12, 15, 18, 24 and 36 hpi, respectively. The larvae hemolymph of 36 hpi was used to cDNA cloning.

Cloning Full-length cDNA and sequencing of BmHMGB

Total RNA was extracted respectively from different tissues by using a RNipure- total RNA rapid extraction kit (Beijing Boling kewei Biotechnology) according to the manufacturer’s protocol. Then the degraded condition of rRNA and its concentration was determined separately. Subsequently the eligible rRNA from hemolymph of 36 hpi was used to synthesize the first-strand cDNA according to the protocol of PrimerScript™ RT reagent Kit with gDNA Eraser (Takara). Full-length cDNA of BmHMGB was synthesized using the first-strand cDNA as templates and SMART™ RACE cDNA Amplification Kit (Clontech). The specific primers for 5’ and 3’ RACE were designed according to the EST sequence revealed in our previous study. 3’-RACE-ready cDNA was prepared according to the Clontech Kit protocol. The specific primer P1 (5’-CGTTGACTGACATGAGAAAGCGAACGATTG-3’, 5’-TACCTTGCTGCTGTCATAGG-3’, 5’-GGTAGTACATGAGAAAGCGAACGATTG-3’) and P2 (5’-CAACTTGTGCTTTGATTGATGACTCAACG-3’) were separately used for amplification of 5’and 3’-end in the RACE reactions. The PCR amplification progress was: 95 °C for 1m; 28 cycles of 95 °C for 15 s, 68 °C for 3min; then 72 °C for 10 min. The PCR products were gel-purified and cloned into pMD19-T simple vector (Takara). The positive recombinants were selected by anti-Ampicillin after being transformed into Escherichia coli Top10, then PCR screening was done with M13 primers (F: 5’-TGGATAAACGACGGCCAGT-3’, R: 5’-CAGGAAACAGCTATGACC-3’) and then sequenced. The sequences of BmHMGB were assembled with the obtained fragments from RACE and analyzed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The amino acid sequence of BmHMGB was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and SMART program (http://smart.embl-heidelberg.de/). The work of multiple protein sequences and phylogenetic tree was employed by the Clustal W and neighbor-joining method in MAGE 6.06 software. Bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

RT-PCR

RT-PCR method was used to detect the expression condition of BmHMGB in different tissues of silkworm. 1μL (500 ng) rRNA, extracted from different tissues, was used as template to synthesize cDNA using the Prime Script™ RT Reagent Kit (Takara). The PCR reaction was performed to describe the expression in these tissues or not.

Quantitative real-time PCR (qRT-PCR) of BmHMGB

The expression level of BmHMGB in different tissues of silkworm was detected by qRT-PCR method. QRT-PCR was performed using 2 μl of diluted cDNA in each 20 μl reaction mixture according to the manufacturer’s instructions of the SYBR Premix Ex Taq™ (Takara). cDNA templates were synthesized from the hemocyte, cuticles, fat body and midgut of B. bassiana-infected and control larvae at 8, 10, 12, 15, 18, 24 and 36 hpi. Specific primers for BmHMGB (F: 5’-ACAGTCTGAACCTGTTGATCCA-3’, R: 5’-AATCAACAGTGCTCCCAA-3’) and β-actin (an endogenous control gene, F: 5’- AAATGCTCCGGATGTC3’, R: 5’-TGCTTCTGTGGCTCTG-3’) were designed by Primer Premier 5.0 software. Reactions were run in triplicate on a 7300 Sequence Detection System (Applied Biosystem). The thermal cycling parameters of qRT-PCR was: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 31 s; 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. Each tissue contained the control and affected sample at different times and its β-actin was run in the same plate. The relative expression levels of BmHMGB gene were normalized using the Ct values obtained from the β-actin amplification run in the same plate. The mean value ± SD and 2^-ΔΔCt method were used to analyze the relative transcript levels of each time point.
Fig. 1 The full-length nucleotide sequence information of BmHMGB. The initiation and stop codons are boxed, the trailing signal (AATAAA) is underlined. Black area is the HMGB domains. Green area is the coiled coil region. Red word is N-myristoylation site (No. 12-17).

Results

Analyse of the BmHMGB full length and structure

The full length cDNA of BmHMGB protein was obtained by RACE, then sequenced, spliced and uploaded to GenBank (Accession no. JF969272). It contained a 313 bp 5' untranslated region (UTR), a 2114 bp 3' UTR and a 588 bp open reading frame encoding a 195 amino acids protein with a predicted molecular weight about 22991.9 Da and a theoretical isoelectric point 10.00 by Protparam software (https://www.expasy.org) (Fig. 1). A putative polyadenylation signal AATAAA was detected in the 3'UTR 310 bp upstream from the poly(A) tail. At the No.106-178 amino acid sequences of the polypeptide chain, there was a HMG domain which is a DNA binding site domain and this protein belongs to the HMGB family. There was a coiled coil domain at the No. 41 - 96 amino acid sequences (Fig. 2). The sequence contained a N-glycosylation site (No. 12-17), three Protein kinase C phosphorylation sites (No. 120-122, 140-142, 151-153) and five Tyrosine kinase phosphorylation sites (No. 52-55, 75-78, 120-123, 149-152, 151-154) (https://blast.ncbi.nlm.nih.gov, http://smart.embl-heidelberg.de) (Fig. 1). Analysis of the amino acid sequence by TMpred revealed that, this protein had no remarkable transmembrane region.

Aligning the full cDNA of BmHMGB to the genome sequence of B. mori, it was contained on the Bm_scaf1_contig476, this contig has 44217 bp and the accession number is BABH01000476.1. It showed that BmHMGB cotained five exons and four introns and each exon-intron boundary conforms with “GT-AG” rule (http://www.expasy.org/).

Fig. 2 Domain architecture analysis of BmHMGB (Green area is coiled coil region)
The analysis of homologous sequences

The phylogenetic tree was constructed according to the amino acid sequences of HMGB homologous genes by MAGE 6.06 (Fig. 3). The results showed two separate clusters of vertebrate and invertebrate HMGBs, BmHMGB was grouped in insect HMGBs of the invertebrate group. In the invertebrate group, some insects which should live in water for some period, including Anopheles gambiae, Culex quinquefasciatus, Aedes aegypti which showed high identity (75 %, 69 %, 66 %) with BmHMGB.

Confirmation of infection

At approximately 48 hpi after B. bassiana spores infected the silkworm larvae, oily spots appeared on their body, which is one of the typical symptoms of the white muscardine disease. All of the infected larvae died in 60 hpi. The disease was further confirmed by the observation of hypha in hemolymph under a microscope and the appearance of the symptom of muscardine.

Expression of BmHMGB in different tissues

The test result of RT-PCR revealed that BmHMGB was expressed in hemolymph, cuticles, malpighian tubule, mid-gut, fat body, mid-silk gland, post-silk gland, and gonad. In contrast, it had lower transcript level in gonad and malpighian tubule. It mainly expressed in hemocyte, cuticles, fat body and midgut (Fig. 4).
The relative expression level of BmHMGB in normal tissues and B. bassiana-infected tissues (A, B, C, D are hemocyte, cuticles, fat body and midgut, separately. ◊ is infected tissues, × is normal tissues. 1-7 is different time points post-inoculation, namely 8, 10, 12, 15, 18, 24 and 36 h). The relative expression levels of BmHMGB gene at each time points were normalized using the Ct values obtained from the β-actin amplifications run in the same plate. All samples were tested in triplicate. The mean value±SD was used for the analysis of relative transcript levels for each time point using the ΔΔCt method. The infected and control individuals are blue and red, respectively.

Expression difference of BmHMGB in control and B. bassiana-infected tissues of silkworm

According to the result of RT-PCR, the tissue-specific expression of BmHMGB transcripts at different times was examined by qRT-PCR (Fig. 5). Between the normal and infected tissues, the expression variety of BmHMGB was significantly different. In the control tissues, there was almost no variety in different times. While in the infected ones, there was significant variation. The relative expression of BmHMGB in the infected tissues was higher than the control ones, especially with the extension of the infected time. For example, at 24 hpi, the relative expression levels in the fat body was 6.11-fold, in the cuticles was 8.34-fold, in the midgut was 10.31-fold and in the hemolymph was 21.71-fold.

Discussion

Beauveria is an important entomogenous fungi with a very broad host range, high pathogenicity and adaptability. Some Beauveria species have a wide use in the biological control of agricultural and forestry pests, but some threaten severely to silkworm and other economic insects. So far, the mechanism of the silkworm how to recognize fungi and initiate immune responses against B. bassiana infection is still poor. In our previous study, we screened and identified BmrHMGB, an up-regulating gene which encoding High Mobility Group Box protein (Hou et al., 2011, 2014).

HMGBs are one kind of multifunctional proteins, architectural chromatin nuclear proteins which have been implicated as a mediator or alarmin of inflammatory and autoimmune diseases in multicellular organisms (Voll et al., 2008). They act as an innate alarm and trigger in the initiation of host defenses or tissue repair, and they may be an integrators of signals that maintain the peace and stress, life and death (Bianchi and Celona, 2011). They participate the activation of TLRs and other cytosolic receptors in immune response (Tian et al., 2007; Kazama et al., 2008). In these activation progress, HMGBs act as an universal sentinels, that is all immunogenic nucleic acids of the exogenous microorganisms first be promiscuous sensed and binded by HMGBs, then be discriminative sensed by specific pattern recognition receptors and activate immune responses of host. HMGBs shuttle continuously between nucleus and cytoplasm to hunt the invading nucleic acid of virus (Bonaldi et al., 2003). They bind to nucleic acid without specific sequences, so it is very difficult to evade HMGBs surveillance for virus (Bianchi and Celona, 2010). If
HMGBs knockout, the activation of TLR3, TLR7 and TLR9 by their cognate nucleic acids will be severely impaired so the cells can’t sense the invasion of pathogens (Wang, 2009). Now they have attracted considerable attention due to they can be recognized by various cell surface receptors and their potent pro-inflammatory activities associated with diverse and major human diseases (Andersson and Tracey, 2011; Venereau et al., 2012; Yang et al., 2015; Eunjun et al., 2016).

HMGB proteins include single or multiple HMG boxes. B box is a domain of induced inflammatory response and can efficiently activate macrophages to release tumor necrosis factor and other cytokines, while box A antagonizes it (Li et al., 2003; Andersson and Tracey, 2011). When the host cells are exposed to pathogen-derived molecules, HMGBs can be actively secreted by immunological cells, or passively released by injured, apoptotic or necrotic cells (Andersson et al., 2000; Andrassy et al., 2008; Tsung et al., 2005). The passively released HMGB can induce an inflammatory response, promote tissue repair and angiogenesis (Yun et al., 2013).

The innate immunity of insect emerges as the defensive frontline that coordinates the interaction between host and pathogen. Insect can use apoptosis as another defense strategy, while pathogen synthesize anti-apoptotic proteins like p35 to prevent host cell death against its defense (Narayanan, 2004). So, it is important to understand the interaction between host and pathogen, the defensive mechanisms about host response infection and the anti-ective mechanisms of pathogen. These are important for better controlling pathogen spread, researching efficient utilization of entomopathogenic fungi and developing new drugs. In our study, the relative expression of BmHMGB in the Beauveria-infected tissues were higher than that of the control ones, especially in the hemocyte and midgut. Among of these tissues, there was a remarkable difference in the hemocyte. at 24 hi, the relative expression of the infection was approximately 21.71-fold of that in the normal one. We suppose that after the invasion of B. bassiana, the response of natural immunity of host was activated causing the immune cells to become active, infected and apoptotic cells passively released HMGBs which led the transcript level of BmHMGB in infected tissues was increased. This may be one of the reasons that led the dysfunction of host and eventually caused its death.

In summary, during Beauveria infect the silkworm, a series of physiological and pathological changes and immune responsive reactions take place in the host. As a corresponding result, the transcript levels of some related genes also change. According to our study, we suppose that BmHMGB may play an important role in the interaction of silkworm and Beauveria. Further functional experiments of BmHMGB should be taken.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (Grant No. 31672358) and the Natural Science Foundation of JiangSu Province (BK20161364).

Reference
Sohn E, Kim J, Kim CS, Lee YM, Kim JS. Extract of Polygonum cuspidatum attenuates Diabetic Retinopathy by Inhibiting the High-Mobility Group Box-1 (HMGB1) Signaling Pathway in Streptozotocin-Induced Diabetic Rats. Nutrients


