Molecular Cloning and Characterization of a *Bombyx mori* Gene Encoding the Transcription Factor Atonal

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The *atonal* genes are an evolutionarily conserved group of genes encoding regulatory basic helix-loop-helix (bHLH) transcription factors. These transcription factors have a critical antioncogenic function in the retina, and are necessary for cell fate determination through the regulation of the cell signal pathway. In this study, the *atonal* gene was cloned from *Bombyx mori*, and the transcription factor was named BmAtonal. Sequence analysis showed that the BmAtonal protein shares extensive homology with other invertebrate Atonal proteins with the bHLH motif. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses revealed that BmAtonal was expressed in all developmental stages of *B. mori* and various larval tissues. The BmAtonal protein was expressed in *Escherichia coli*, and polyclonal antibodies were raised against the purified protein. By immunofluorescence, the BmAtonal protein was localized to both the nucleus and cytoplasm of BmN cells. After knocking out nuclear localization signals (NLS), the BmAtonal protein was only detected in the cytoplasm. In addition, using the *B. mori* nuclear polyhedrosis virus (BmNPV) baculovirus expression system, the recombinant BmAtonal protein was successfully expressed in the *B. mori* cell line BmN. This work lays the foundation for exploring the biological functions of the BmAtonal protein, such as identifying its potential binding partners and understanding the molecular control of the formation of sensory organs.

Key words: Atonal, *Bombyx mori*, Bioinformatics, Sub-Cellular Location

Introduction

The basic helix-loop-helix (bHLH) proteins form a large superfamily of transcription factors that play important roles in a variety of regulatory processes in organisms. Since the first characterization of the murine bHLH transcription factors E12 and E47, a predictive motif for the bHLH domain was developed (Atchley *et al.*, 1999; Murre *et al.*, 1989). The bHLH domain is approximately 60 amino acids in length and comprises a DNA-binding basic region of 15 amino acids followed by two α-helices separated by a variable loop region (Ferré-D'Amaré *et al.*, 1993).

Members of the bHLH family have been identified in several organisms including fruit fly, mouse, and human. At present, 52 bHLH members have been identified in the silkworm (*Bombyx mori*). Phylogenetic analysis showed that they belong to 39 bHLH families and 6 groups, A to F (Wang *et al.*, 2007). Group A and B proteins bind to hexameric DNA sequences called E-boxes (CANNTG). Group A includes the original E-box-binding protein E12/E47 (Seipel *et al.*, 2004), and the Atonal protein belongs to group A.

The *atonal* gene, which encodes a member of the Atonal family of transcription factors, was the first reported to be active in the *Drosophila* peripheral nervous system (Jarman *et al.*, 1993). Subsequently, the cloning of *atonal* homologues was reported for several organisms such as red flour beetle, puffer fish, chicken, mouse, and human (Ben-Arie *et al.*, 1996). However, so far no report has been published on the *atonal* gene of *Bombyx mori*.

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In *Drosophila*, like in all metazoans, the proneural gene *atonal* plays critical roles during neurogenesis. The Atonal protein family promotes the formation of neurogeneic cells and is necessary for the formation of several specialized sensory organs mediating visual, olfactory, and mechano-sensation, respectively, in the *Drosophila* peripheral nervous system (PNS). The *atonal* gene is also dedicated to the formation of chordotonal organs and photoreceptor neurons, and flies that lack the Atonal protein are ataxic and blind (Ben-Arie *et al.*, 1996). The Atonal protein is necessary for cell fate specification and differentiation, and affects the fates of neighbouring cells through the regulation of cell signaling pathways. In the fly retina, it has been shown that the *atonal* transcription factor has a critical antioncogenic function (Hastie *et al.*, 2009; Witt *et al.*, 2010). Moreover, selective *atonal* gene delivery can also improve the balance function in a mouse model of vestibular disease (Schlecker *et al.*, 2011). Therefore, investigation of Atonal family members may bring new insights into the molecular control of the formation of sensory organs.

The silkworm is an important model insect for the Lepidoptera, which represents the second largest order of insects with ~200,000 species (Nardi, 1995). With the completion of the Silkworm Genome Project (Xia *et al.*, 2004), *B. mori* has become a useful tool in insect genomics and genetic studies. At present, while many bHLH family members have been identified in a multitude of organisms, but experimental aspects of genes encoding bHLH transcription factors in *B. mori* have rarely been reported (Markova *et al.*, 2003).

In this paper, the silkworm *atonal* gene was cloned and then the protein BmAtonal was expressed in *Escherichia coli*. Polyclonal antibodies were raised against the heterologously expressed protein and used to determine the sub-cellular localization of BmAtonal. The expression profiles of the silkworm *atonal* gene in various tissues were determined. Moreover, the BmAtonal protein was overexpressed in the BmN cell line and identified by Western blot analysis. This work will help to successfully study the biological functions of BmAtonal.

Materials and Methods

Materials

Bombyx mori strain C108 (standard strain of silkworm) and the *B. mori* cell line BmN derived from the ovary of the silkworm (Grace, 1967) were maintained in our laboratory. The larvae were reared with fresh mulberry leaves at (25 ± 2) °C under a 12-h light/12-h dark photoperiod. The *B. mori* cell line BmN was cultured at 27 °C in TC-100 insect medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Fbs) (Gibco-BRL, Gaithersburg, MD, USA).

All primers were synthesized by Generay Company (Shanghai, China). RNase-free DNase I, r-Taq polymerase, LA-Taq polymerase, restriction enzymes, T4 DNA ligase, and the DNA subcloning vector pMD18-T were purchased from TaKaRa (Dalian, China). Cellfectin® reagent was purchased from Invitrogen (Carlsbad, CA, USA). Chemicals were purchased from Sigma-Aldrich or a domestic provider in China unless stated otherwise.

Bioinformatic analysis

The full length of the *B. mori atonal* gene was obtained by searching the silkworm EST database with the sequence encoding the BmAtonal bHLH motif. In order to elucidate the genomic organization, the cDNA sequence was blasted to the contigs of the *B. mori* genome in the GenBank. SIM4 (http://pbil.univ-lyon1.fr/sim4.php) was used to align the cDNA sequence with the genomic sequences to detect potential introns. The ExPASy translate tool (http://au.expasy.org/tools/dna.html) was used to deduce the amino acid sequence corresponding to the cDNA. Multiple sequences alignment was performed with the Clustal W program (Thompson *et al.*, 1994), and edited with Genedoc. A phylogenetic tree was constructed by the neighbour-joining method using the MEGA 4.1 program (Saitou and Nei, 1987; Tamura *et al.*, 2007). The chromosomal location of the gene and the analysis of the open reading frame (ORF) were carried out online at the silkworm cDNA database (http://silkworm.genomics.org.cn/).

RNA extraction and transcriptional analysis of BmAtonal

The larvae at different developmental stages and various tissues (hemolymph, testis, ovary, epidermis, silk gland, and ganglion) of the fifth instar larvae were dissected, immediately frozen in liquid N_2, and stored at −80 °C for later use. Total RNA was extracted from the frozen samples with the RNeasy mini kit (Qiagen, Shanghai, China), treated for 20 min at 37 °C with RNase-free DNase I (TaKaRa), purified

with phenol/chloroform, precipitated with ethanol, and finally dissolved in diethyl pyrocarbonate (DEPC)-treated double distilled water (ddH_2O). cDNA were generated from these RNA using M-MLV RTase (Promega, Beijing, China) and an oligo-dT primer following the manufacturer's instructions.

To determine the tissue distribution of BmAtonal transcripts, the larval tissues mentioned above were analysed by reverse transcription-polymerase chain reaction (RT-PCR). PCR was then carried out using BmAtonal-specific primers: 5′-CCCTCGAG-TTACTGCAGGAGCTCGTAAAG-3′ and 5′-CG-GGATCCATGTTAGAGTACGCTACAGAAGAAT-3′, in which the underlined characters are restriction sites of Xho I and BamH I, respectively. A 284-bp fragment of the *B. mori* cytoplasmic actin gene A3 was amplified in parallel with each RNA sample using the primers: Bm-actinA3-F (5′-GCGCG-GCTACTCGTTCACTACC-3′) and Bm-actinA3-R (5′-GGATGTCCACGTCGCACTTCA-3′) as an internal control for adjustment of template RNA quantity. The PCR reaction was carried out for 30 amplification cycles (94 °C/45 s, 52 °C/45 s, and 72 °C/40 s) in a Gene Amp 2400 System thermocycler (Perkin-Elmer, Waltham, MA, USA). Ten μl of each PCR product were electrophoresed on an 1% agarose gel and visualized by ethidium bromide staining.

Protein expression in E. coli

The purified PCR product was ligated into the pMD18-T vector (TaKaRa) using T4 DNA ligase (TaKaRa). The vector was transformed into *E. coli* (TG1 strain), and the positive clones were isolated and sequenced. Then the purified PCR fragment was subcloned into the pGEX-4T-2 prokaryotic expression vector and transformed into *E. coli* strain BL21 cells. The transformants harboring the recombinant plasmid were confirmed by restriction enzyme (BamH I and Xho I) analysis.

To express the recombinant protein, a positive clone was cultured in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) overnight at 37 °C with shaking. This overnight culture was then transferred to fresh LB medium containing ampicillin of the same concentration. The cultures were grown at 37 °C to an optical density of 0.6 at 600 nm (OD600), then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration, 0.03 – 0.8 mM during optimization), and further cultured for another 5 h at 37 °C. Cells were harvested by centrifugation (4500 × *g*, 4 °C, 15 min), proteins separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the Mini-Protein system (Bio-Rad, Hercules, CA, USA) and visualized by staining with Coomassie Brilliant Blue R250.

Preparation of specific polyclonal antibodies

Bands of the recombinant BmAtonal protein were excised manually from the gel with a sterile scalpel and ground in liquid nitrogen. The purified protein was used to raise polyclonal antibodies in New Zealand white rabbits. The antiserum was prepared using standard techniques. The BmAtonal protein (about 2 mg) in complete Freud's adjuvant was injected subcutaneously to immunize a rabbit, followed by two booster injections of antigen in incomplete Freud's adjuvant. After the last injection, blood samples were taken from the marginal vein of the rabbit ear. After the specificity of the antibody had been verified by Western blot analysis, the rabbit was exsanguinated by tremulous pulse. The blood was centrifuged, and the serum was collected for testing in an immunoassay.

Immunofluorescence microscopy

For immunoblotting analysis, six-well tissue culture plates were seeded with $2 \cdot 10^5$ BmN cells and grown to 80% confluence. The cells were washed with phosphate-buffered saline (PBS) and fixed with 2 ml of 4% paraformaldehyde in PBS for 20 min. Then cells were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and further incubated in blocking buffer (1% skimmed milk powder in PBS) for 1 h. After washing four times with cold PBS, cells were incubated with anti-BmAtonal polyclonal antiserum (1:1000) as primary antibody for 2 h at 37 °C. Cells were washed three times with PBS and then incubated with the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:3000) (Qualex, Durham, NC, USA), for 1 h at 37 °C. Cells were then washed three times with PBS. After staining nuclear DNA with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min, cells were examined with a Leica confocal laser scanning microscope (Zeiss, Heidenheim, Germany). In negative controls, preimmune serum replaced the antiserum.

Preparation of nuclear and cytoplasmic fractions was performed by a nuclear and cytoplasmic protein extraction kit (Beyotime, Nanjing, China). Total cellular protein was also extracted by radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM

Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40]. Equal volumes of the protein fractions were resolved in 12% denaturing SDS-PAGE, the proteins transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Shanghai, China) and incubated with the anti-BmAtonal polyclonal antibody as primary antibody (1:1000). A goat anti-rabbit antibody conjugated to horseradish peroxidase was used to detect the specific band by using diaminobenzidine (DAB) as peroxidase substrate.

Expression of the atonal gene in BmN cells and Western blot analysis

BmAtonal was expressed in the bac-to-bac/*B. mori* nuclear polyhedrosis virus (BmNPV) baculovirus expression system (Miao *et al.*, 2005). In brief, the atonal ORF was inserted into pFastBacHTc at the BamH I/Xho I sites and transformed into *E. coli* DH10 Bac/BmNPV. Through inside transposition in bacteria, the *atonal* gene was transferred to a baculovirus shuttle vector (bacmid) within a mini-att Tn7 target site. The recombinant bacmid-positive baculovirus was obtained by blue/white selection and then transfected into BmN cells, which were incubated in a humidified incubator at 27 °C. The recombinant P1 viral solution was collected 120 h post infection and stored at 4 °C, protected from light. The P1 viral stock was further used to infect BmN cells to generate the high-titer P2 stock that was used for expression of the recombinant baculovirus. BmN cells ($2 \cdot 10^6$ cells/flask) were infected with recombinant virus for expression of the Atonal protein and collected after 48 and 72 h, respectively.

Western blot analysis was performed as described previously (Towbin *et al.*, 1979). After 15% SDS-PAGE, the gel was immersed in cold transfer buffer (0.025 M Tris, 0.19 M glycine, 20% methanol), and the proteins were transferred to a PVDF membrane. The membrane was subsequently blocked in 3% skimmed milk powder in PBS Tween-20 (PBST) medium overnight at 4 °C, and immunoblotted with the anti-BmAtonal polyclonal antiserum diluted 1:1000 for 1.5 h at room temperature. After washing 5 times with PBST medium, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG diluted 1:2000 for 1.5 h at room temperature. After washing with PBS for 5 times, the bands were visualized using DAB or the enhanced chemoluminescence (ECL) method (Thermo, Shanghai, China).

Atonal mutant constructs and plasmid transfection

The *atonal* mutant construct was obtained by overlap extension PCR and missed the basic nuclear localization signal (NLS) (KRRGRATSAAVLRRRR), which was predicted by the algorithm on the website https://www.predictprotein.org/. According to the methods of Ho (1989) and Senanayake and Brian (1995), using the above prepared cDNA as template, the 5′ terminal fragment was amplified with primer A (5′-CCGCTCGAGCTGCAGGAGCTCGTAAAG-3′) and primer B (5′-TAGAAGGTTCTGGATTGGCCGCTAACGC-3′), which were used in the first round of PCR. Similarly, the 3′ terminal fragment was amplified with primers B and C (5′-CGGGATCCATGTTAGAGTACGCTACAGAAGAAT-3′), which were used in the second round of PCR. The amplified products of the first and second reaction, respectively, were electrophoretically resolved on an 1% agarose gel and recovered by gel puncture with a micropipet tip for a third round of PCR using primers A and C. The DNA fragment obtained in the third round of PCR was ligated into the transient expression vector pFastBacHTb-IE1p-EGFP which was cut with BamH I and Xho I. Then the ligation product (pFastBacHTb-IE1p-EGFP-atonal) was transformed into cells of *E. coli* strain TG1. The resulting clone was confirmed by DNA sequencing.

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gccgaaacttacgggcatcgcttagtttactcggaaaaggatattttctctaatgacgtc
atgttagagtacgctacagaagaatgttatcttacgtggcccagatctcccgattcggga
 M  L  E  Y  A  T  E  E  C  Y  L  T  W  P  R  S  P  D  S  G
aggtctagtttggagccgacgcccctcgatcgatggaagcatcagccatgactctacgcac
 R  S  S  L  E  P  T  P  S  I  D  G  S  I  S  H  D  S  T  H
atcgcctaccgtggcctgtccagggacatggttctagaagacagcgcggaagacaacgac
 I  A  Y  R  G  L  S  R  D  M  V  L  E  D  S  A  E  D  N  D
ttactagaaggttctggaaagaggagagggagagcaaccagcgctgctgtcttgaggaga
 L  L  E  G  S  G  K  R  R  G  R  A  T  S  A  A  V  L  R  R
cgtcggttggccgctaacgcgagagaaagacggaggatgcacaatttgaacaaagcattt
 R  R  L  A  A  N  A  R  E  R  R  R  M  H  N  L  N  K  A  F
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 D  R  L  R  G  H  L  P  S  L  G  A  D  R  Q  L  S  K  Y  E
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 T  L  Q  M  A  Q  T  Y  I  A  A  L  Y  E  L  L  Q  *
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acagaatcgcttttgtagactataaaaatgtatgtttctgagtgtgtgttacatgtaata
cgcttattttgactaagttaaatattgaaaaaaaaaaaaaaaaa
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the BmAtonal protein. The amino acid sequence is represented by a single capital letter below the nucleotide sequence. The initiation and stop codons are boxed. The bHLH motif is underlined.

Six-well tissue culture plates were seeded with $2 \cdot 10^5$ BmN cells per well and grown to 80% confluence at 37 °C. Cells were transfected with the construct pFastBacHTb-IE1p-EGFP-atonal using the Lipofectamine 2000 reagent (Invitrogen) at a ratio of 1 μg DNA per 8 μl per well. As a negative control, a second set of BmN cells was transfected with the plasmid pFastBacHTb-IE1p-EGFP. At 24 and 72 h post transfection, cells were observed with a confocal laser scanning microscope (Zeiss).

Results

Bioinformatic analysis of BmAtonal

Using the tblastn program, we found only a single silkworm EST fragment with the BmAtonal bHLH motif, but its coding sequence was complete, since it contained the transcription starting site and the poly(A) tail signal. According to the silkworm *atonal* gene structure, the ORF began with the initiation codon

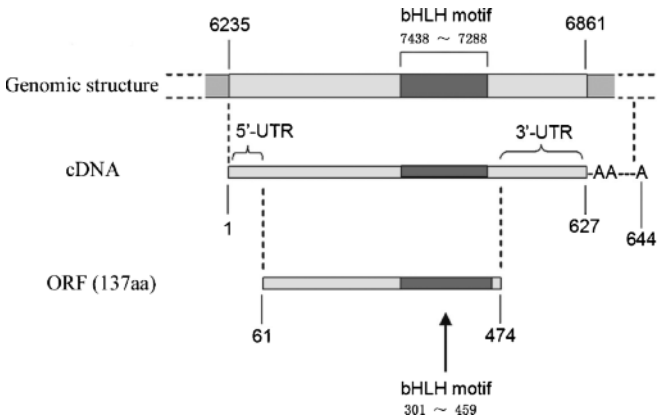

Fig. 2. Structure diagram of the silkworm *atonal* gene, the corresponding cDNA, and protein. The predicted cDNA length is 644 bp, with its coding region from nucleotides no. 6235 to no. 6861 on ctg15472 in the *Bombyx mori* genomic database. The cDNA includes a 5′-UTR of 60 nucleotides and a 3′-UTR of 153 nucleotides. Its ORF consists of 414 bp and codes for a protein of 137 amino acid residues. The bHLH motif is encoded by nucleotides no. 301 to no. 459 on the cDNA.

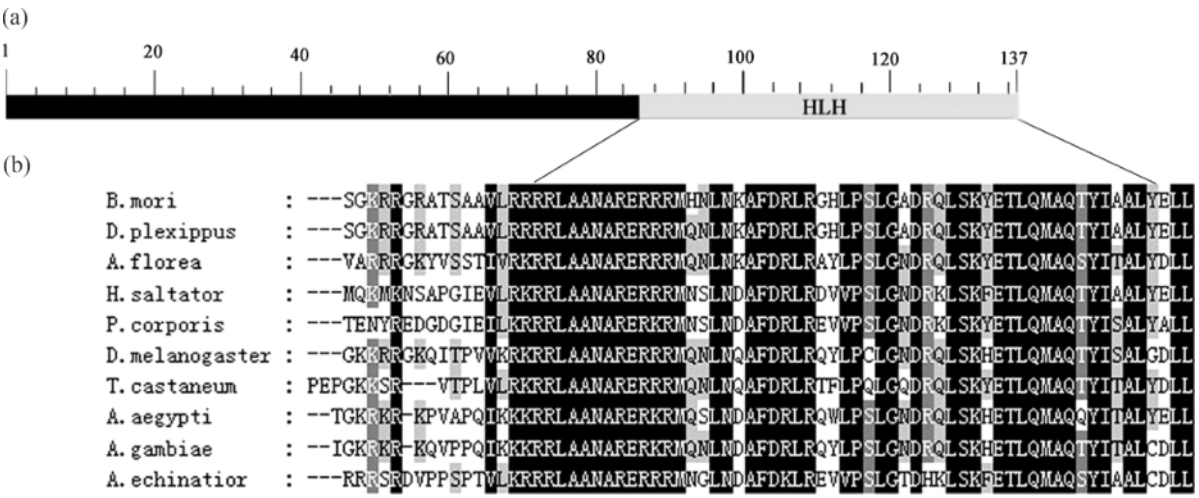

Fig. 3. Multiple alignment of the conserved domain of the Atonal protein of *Bombyx mori* and nine other insect species. (a) Conserved domain of BmAtonal. The bHLH motif extends from no. 81 to no. 133 of the amino acid sequence. (b) Multiple alignment of the conserved domain of Atonal proteins. The sources of sequences and their GenBank accession numbers are: *Danaus plexippus* (EHJ69641.1), *Apis florea* (XP_003696174.1), *Harpegnathos saltator* (EFN82082.1), *Pediculus humanus corporis* (XP_002425798.1), *Drosophila melanogaster* (CAL26767.1), *Tribolium castaneum* (XP_970709.1), *Aedes aegypti* (XP_001649519.1), *Anopheles gambiae* str. PEST (XP_321345.3), *Acromyrmex echinatior* (EGI59393.1).

ATG at position 61 bp, ending with TAG at 474 bp, and there was no intron in the coding region (Figs. 1 and 2). Multiple sequence alignment of the *atonal* transcription factor sequence of *B. mori* and nine other insect species revealed that they all contained the highly conserved bHLH motif (Fig. 3). The analysis of the phylogenetic tree of the BmAtonal sequences (Fig. 4) indicated the closest affinity between *B. mori* and *Danaus plexippus* (both Lepidoptera), and a distant relationship between *B. mori* and *Drosophila melanogaster* (Diptera). Of these eleven sequences, only the function of the Atonal protein of *Drosophila melanogaster* had been elucidated, so we speculate that the function of BmAtonal was similar to that of DmAtonal (*Drosophila melanogaster* Atonal). BmAtonal was located on chromosome 6 of the *B. mori* genome by using the silkworm cDNA database (http://silkworm.genomics.org.cn/).

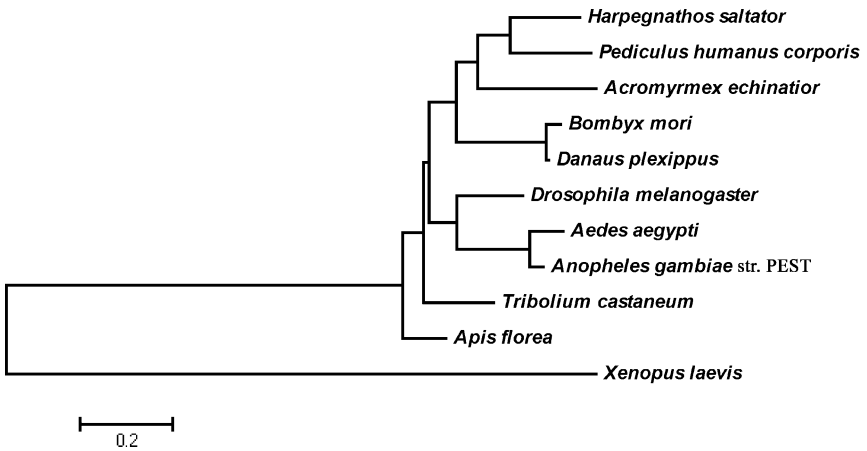

Fig. 4. Phylogenetic relationship of Atonal proteins between *Bombyx mori* and ten other species. A neighbour-joining tree is shown. For simplicity, branch lengths of the tree are not proportional to distances between sequences, and bootstrap values less than 50 are not shown. The Atonal sequence of *Xenopus laevis* (NP_001081213) was used as the outgroup and was defined as the tree root. For the sequences used in the phylogenetic tree analysis and their GenBank accession numbers see Fig. 3.

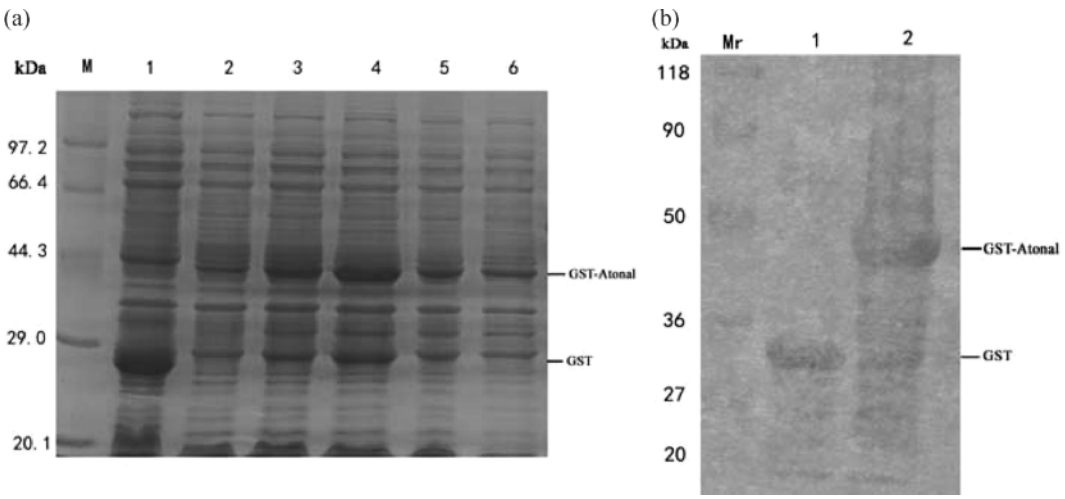

Fig. 5. SDS-PAGE stained with Coomassie Brilliant Blue of recombinant GST-BmAtonal. (a) Lane 1, lysate of host cells transformed with empty vector pGEX-4T-2; lanes 2 to 6, lysate of *E. coli* BL21 cells harboring pGEX-4T-2-BmAtonal induced with 0.03, 0.06, 0.25, 0.45, 0.80 mM IPTG. (b) Western blot analysis of recombinant GST-BmAtonal with GST antibody. Lane 1, protein extracts of transformed cells transformed with pGEX-4T-2; lane 2, protein extracts of cells transformed with the vector containing the GST-BmAtonal construct. The bands were visualized by staining for peroxidase with DAB as substrate.

Expression and identification of recombinant BmAtonal protein

The coding sequence of the silkworm *atonal* gene was subcloned into the pGEX-4T-2 prokaryotic expression vector and successfully expressed in *E. coli* (strain BL21) as a GST fusion protein. Expression of BmAtonal at 37 °C was optimally induced by 0.25 mM IPTG (Fig. 5a). The expression of the GST fusion BmAtonal protein was also confirmed by the anti-GST monoclonal antibody. The molecular mass of the GST-BmAtonal fusion protein was 41.5 kDa which was in accordance with the predicted mass (Fig. 5b, lane 2), while the molecular mass of GST was 26 kDa (Fig. 5b, lane 1). The purified protein was also used to immunize rabbits to produce a specific antiserum against BmAtonal.

Expressional analysis of BmAtonal at various developmental stages

The levels of the BmAtonal transcript at various developmental stages of the silkworm were examined by RT-PCR. BmAtonal mRNA was detectable throughout from the egg to the fifth instar larvae and the pupa (Fig. 6a). To investigate the expression profile of BmAtonal, the total RNA from fifth instar larvae was isolated from hemolymph, testis, ovary, epidermis, silk gland, and ganglion, and subjected to RT-PCR (Fig. 6b). The results showed that the *atonal* gene was expressed in all tissues.

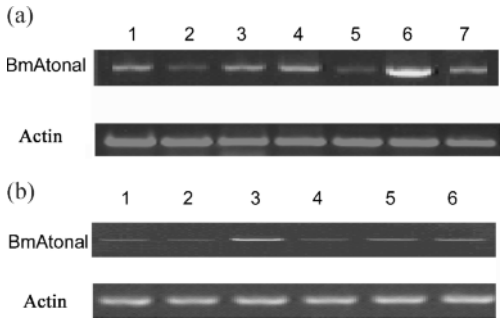

Fig. 6. (a) RT-PCR analysis of BmAtonal transcript at various developmental stages of *Bombyx mori*. Lane 1, egg; lanes 2 to 7, larvae of instar 1, 2, 3, 4, 5, and silkworm pupa, respectively. (b) BmAtonal transcript in various *Bomyx mori* tissues. Lanes 1 to 6, testis, ovary, ganglion, epidermis, blood, and silk gland, respectively. The Bm-actin A3 transcript was used as internal control.

Sub-cellular localization of BmAtonal and its mutant forms in BmN cells

Nucleo-cytoplasmic protein trafficking of bHLH proteins is an important regulatory checkpoint in the control of target gene expression (Franco *et al.*, 2011). Mutations in Atonal may affect their nuclear location, and therefore affect their binding to DNA. In order

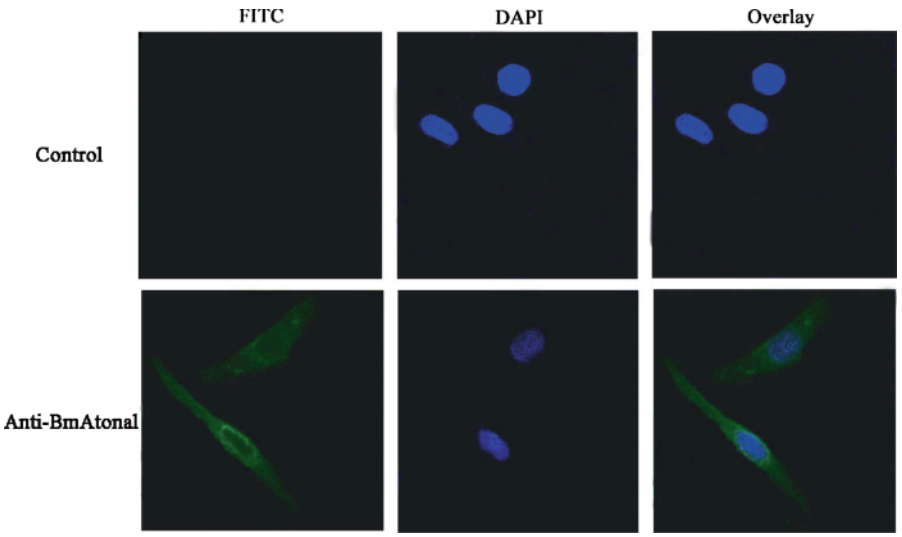

Fig. 7. Sub-cellular localization of BmAtonal in BmN cells. BmN cells were treated with anti-BmAtonal antibody, followed by treatment with FITC-conjugated goat anti-rabbit IgG, and DAPI (blue) was used to delineate the nuclei. From left to right: green fluorescence for FITC-treated BmAtonal, DAPI-treated nucleus, and the overlay images. For a control, preimmune serum was used as the primary antibody.

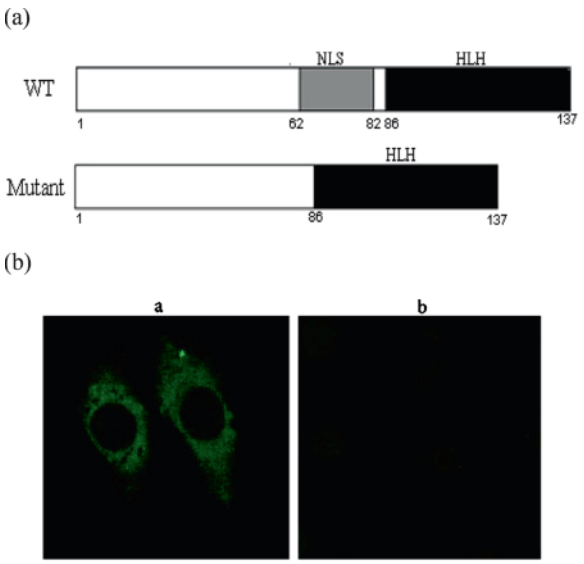

Fig. 8. Sub-cellular localization of BmAtonal mutant forms in BmN cells. (a) Diagram of the BmAtonal protein and its mutant form. The nuclear localization signal (NLS) is located from no. 62 to no. 82 in the amino acid sequence. The mutant form lacking NLS was obtained by overlap extension PCR. (b) Fluorescence microscopy of the mutant form of BmAtonal in BmN cells. a, GFP-Atonal fusion protein in cytoplasm of BmN cells; b, no fluorescent protein in BmN cells.

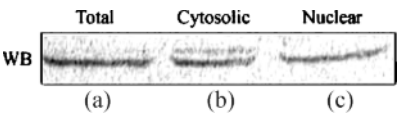

Fig. 9. Western blot analysis of nuclear and cytosolic BmN cells with anti-BmAtonal antibody. Lanes from left to right: (a) total BmN cell lysate, (b) cytosolic lysate, (c) nuclear lysate.

to address this issue, we detected the intracellular localization of BmAtonal by immunofluorescence using the anti-BmAtonal serum (Fig. 7). Wild-type (WT) BmAtonal was seen in both cytoplasm and nucleus, and predominantly in the cytoplasm in the perinuclear area. However, the mutant BmAtonal-GFP fusion protein that lacked the NLS (Fig. 8a) was only detected in the cytoplasmic fraction (Fig. 8b). Nuclear and cytoplasmic protein extracts were used for immunoblotting analysis (Fig. 9). Wild-type BmAtonal was detected both in cytoplasmic and nuclear fractions, consistent with the results of the immunofluorescence.

Discussion

Transcription factors with the bHLH motif play important roles in the control of cellular proliferation, tissue differentiation, and development in animals, plants, and fungi (Jan and Jan, 1993). One of the most structurally and functionally conserved group within this family is the Atonal protein (Hastie *et al.*, 2009). This protein can serve as a useful molecular marker for proneural maturation. The stepwise resolution of proneural clusters during the initiation of ommatidial differentiation in the developing eye disc can be observed (Dokucu *et al.*, 1996). So the Atonal protein is essential for the proper formation of the *Drosophila* eye (Melicharek *et al.*, 2008). Moreover, in the fly retina, *atonal* plays an important role in a very early step of the oncogenic process (Hastie *et al.*, 2009).

As far as we know, currently there is no report on the characterization and functional analysis of BmAtonal. Thus, our work presents the first report on the cloning and characterization of BmAtonal. We found that the *B. mori atonal* gene contained a 414-bp ORF encoding a peptide of 137 amino acid residues. There was a bHLH domain consisting of a basic region and a helix-loop-helix region, responsible for DNA binding and dimerization (Tanaka-Matakatsu and Du, 2008). Sequence alignment revealed that the bHLH domain of BmAtonal is highly homologous to the corresponding domains in the Atonal proteins of other species, indicating a highly conserved and similar role for these Atonal proteins. The Atonal protein was successfully expressed in *E. coli* for preparation of the polyclonal antibody. Furthermore, the Atonal protein was successfully overexpressed in the BmN cell line.

These results provide a substantial base for further elucidation of the BmAtonal function. RT-PCR analysis showed that *atonal* was expressed in all silkworm tissues and examined at all developmental stages. Analysis of the sub-cellular localization revealed that the wild-type Atonal protein was located in both cytoplasm and nucleus, predominately in the cytoplasm in the perinuclear area. Transcription factors are present in phosphorylated and non-phosphorylated forms. Phosphorylation regulates the activity of the transcription factors and controls their translocation from the cytoplasm to the nucleus. The *atonal* transcription factor may be affected by phosphorylation. The mutant BmAtonal that lacked the NLS was only located in the cytoplasm. The amino acid sequence of the NLS is enriched in lysine and arginine residues. As a bHLH transcription factor, the Atonal protein may be regulated at the level of its subcellular localization. In addition, *B. mori* provides a simple model system to study the function of the Atonal protein, because its complete genomic sequence has been established. This may help us to understand the molecular control of sensory organ formation.

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