

Full Length Research Paper

Cloning and characterization of the first actin gene in Chinese oak silkworm, *Antheraea pernyi*

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The first actin gene in Chinese oak silkworm, *Antheraea pernyi* (Apactin) was cloned and characterized. Remarkably, two types of the Apactin cDNA clones were isolated from the pupal cDNA library, which differed by the length and sequence of 5' untranslated region (UTR). The open reading frame (ORF) of the Apactin gene is 1131 bp and encodes a protein of 376 amino acids with a predicted molecular weight of 40.81 kDa and an isoelectric point of 5.30. The deduced amino acid sequence of the Apactin gene contains typical structural features of the actin genes. Sequence alignment and phylogenetic analysis showed that the Apactin gene was a cytoplasmic actin gene and had closest genetic relationship with *Antheraea yamamai* actin gene. The Apactin gene held the highest identity with the *Bombyx mori* actin A4 gene among the four known *B. mori* actin isoforms. RT-PCR analysis showed that the Apactin gene was constitutively expressed during four developmental stages and in all tested tissues. These results indicate that the Apactin gene presented here plays an essential role throughout the entire life cycle and can be used as a reference gene in the normalization of gene expression.

Key words: *Antheraea pernyi*, actin, cloning, expression pattern.

INTRODUCTION

Chinese oak silkworm, *Antheraea pernyi*, is an economically important insect of the Saturniidae family. It is known that the domesticated *A. pernyi* was originated in Shandong Province of China around the 16th century ago (Liu et al., 2010), and now it is commercially cultivated mainly in China, India, and Korea. This species feeds on the leaves of *Quercus* and produces coarse silk. In recent years, about 70,000,000 kg of cocoons (pupae) are produced each year in China, account for 90% of the world's production. In addition to being used for silk production, *A. pernyi* is also used as a source of insect food (larva, pupa, and moth) and an excellent natural bio-reactor for the production of recombinant proteins (Huang et al., 2002; Liu et al., 2008). Due to the economical

importance, we have constructed a full-length cDNA library from *A. pernyi* pupa to isolate and identify the functional genes (Li et al., 2009). Actin is a globular, roughly 42-kDa protein and participates in many important cellular processes, including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape (Pollard and Cooper, 1986; Cadoret et al., 1999). Actins are broadly categorized into two classes according to the N-terminal sequences as muscle or cytoplasmic actins (Hightower and Meagher, 1986; Herman, 1993). Moreover, the cytoplasmic actins are classical reference genes for gene expression level in northern blot analysis and semi-quantitative RT-PCR (Bunger et al., 2003; Kitade et al., 2008).

In this paper, we described the cloning and characterization of the first *A. pernyi* actin gene (Apactin). Remarkably, two types of the Apactin cDNA clones which

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differed by the length and sequence of 5' untranslated region were isolated, as found in the actin A4 gene of *Bombyx mori* (Mange et al., 1996). The expression analysis indicated that it can be used as a reference gene in the normalization of gene expression of the functional genes of *A. pernyi* in further studies.

MATERIALS AND METHODS

Experimental insects and tissues

Larvae of *A. pernyi* strain Shenhua No.1 used in this study were reared routinely. Eggs at day 5, fifth instar larvae, pupae and moths were frozen in liquid nitrogen and stored at -80°C for later use. The silkworm was dissected at 10th day of the fifth instar to obtain their blood, fat body, midgut, silk gland, body wall, Malpighian tubule, spermary, ovary, brain and muscle. The tissues were frozen in liquid nitrogen immediately and stored at -80°C

Mining of the Apactin gene and sequence analysis

A pupal cDNA library of *A. pernyi* was constructed in our laboratory to isolate and identify the functional genes (Li et al., 2009). Two EST sequences encoding *A. pernyi* actin genes (GenBank accession no. GH334889 and GH334891, respectively) were first isolated. Staden Package was used to assemble and edit the sequences generated to obtain a consensus sequence. Similar sequences were searched by blast program at <http://www.ncbi.nlm.nih.gov/blast/>. DNASTAR software was used to identify open reading frame and deduce amino acid sequence. The isoelectric point and molecular weight of the deduced amino acid sequences were predicted at http://www.expasy.org/tools/pi_tool.html. The deduced amino acid sequence was submitted to predict protein signal peptide with SignallP server on-line tools, <http://www.cbs.dtu.dk/services/SignalP/>. Conserved domains prediction was performed at <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>

Phylogenetic analysis

The cDNA sequences and deduced amino acid sequences of the actin genes were aligned with Clustal X software (Thompson et al., 1997). Eight actin genes of Bombycoidea and 19 actin genes of Lepidoptera were included. Phylogenetic tree was constructed with the aligned sequences by the neighbor-joining algorithms (Saitou and Nei, 1987) in the MEGA4 program (Tamura et al., 2007), 500 bootstrap replicates were performed for the phylogenetic analysis.

Total RNA extraction and first strand cDNA synthesis

Four different developmental stages (egg, larva, pupa and moth) and ten kinds of tissues of the fifth instar larvae (blood, fat body, midgut, silk gland, body wall, Malpighian tubule, spermary, ovary, brain and muscle) stored at -80°C were used to extract total RNA with RNAPrep pure Tissue Kit (TIANGEN Biotech Co. Ltd., Beijing) according to the manufacturer's instructions. DNase I was used to remove the contaminating genomic DNA. The purity and quantity of extracted RNA was quantified by the ratio of OD₂₆₀/OD₂₈₀ by ultraviolet spectrometer. Using 2 µg of total RNA per sample, first strand cDNA was generated with TIANScript cDNA Synthesize Kit (TIANGEN Biotech Co. Ltd., Beijing) following the manufacturer's

instructions.

RT-PCR analysis

The cDNA samples from the four developmental stages and ten tissues were amplified by PCR method using the gene-specific primer pair LYQ85 (5' CCAA GGCCA ACAGA GAGAA GA 3') and LYQ86 (5' CAAGA ATGAG GGCTG GAAGA GA 3'), which generated a 468 bp fragment of the Apactin gene. PCR amplification was carried out in a total reaction volume of 25 µl, containing normalized cDNA, 20 pmol of each primer, 2 mM MgCl₂, 0.25 mM dNTP, 1xbuffer and 2.5 units of Taq DNA polymerase (TIANGEN Biotech Co. Ltd., Beijing). PCRs were performed with the following cycles: initial denaturation at 95°C for 5 min; followed by 25 cycles of 1 min at 95°C, 30 s annealing at 55°C, 30 s extension at 72°C; and a final extension at 72°C for 10 min. The amplification products were separated on 1.0% agarose gels, purified, and directly sequenced using an ABI 3100 automated sequencer.

RESULTS AND DISCUSSION

Cloning and characteristics of the Apactin gene

The Apactin gene was isolated from a pupal cDNA library of *A. pernyi*. The cDNA sequence and deduced amino acid sequence is shown in Figure 1. The isolated cDNA was 1575 bp in length, including a 5' UTR of 97 bp, a 3' UTR of 347 bp, and an ORF of 1131 bp that encodes a polypeptide of 376 amino acids with an estimated molecular weight of 40.81 kDa and an estimated isoelectric point of 5.30. No typical AATAAA polyadenylation signal sequence was found in the 3' UTR, but a closely related motif, TATAAA, is present (Figure 1). Protein signal peptide prediction revealed no deduced signal peptide cleavage site in the N-terminal (Signal peptide probability: 0.000, Signal anchor probability: 0.000, Max cleavage site probability: 0.000 between position 20 and 21), meaning that it is a non-secretory protein as those found in other Bombycoidea insects. Conserved domains prediction revealed that it contains an ATP-Mg and ATP-Ca binding site (15, 155, 162, 215, 304, 338), a gelsolin binding site (145, 147, 168, 345, 347, 348, 351, 352, 353) and a profiling binding site (167, 168, 170, 172, 174, 288, 291, 353, 363, 374, 375), as observed in the other actin genes of Bombycoidea known so far. We therefore referred the cDNA as Apactin. The sequence data has been submitted to GenBank under accession no. GU073316.

Interestingly, another type of the Apactin cDNA clone was also discovered, which was different from the first one by the length and the 5' UTR sequence (Figure 2). The sequence data has also been submitted to GenBank under accession no. GU176616. The second cDNA had a 112 bp specific DNA at the 5' end, the nearly identical ORF of 1131 bp and the 3' UTR of 347 bp with the first one. This result suggested the existence of two alternative leader exons for the isolated Apactin gene, and the

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1                                     AGAAAGAAAC
11  CGGCCCGGCATACATTTCCCACGGTTTGTAAACCGCTGCGGATATTATCAATTTTACGTTATCTAAA
77  ITATCTACCGACTAATTCACAATGTCCGACGAGGAAAGTTGCTGCGTTGGTCGTAGACAAATGGCTCT
      M C D E E V A A L V V D N G S
143  GGTATGTGCAAGGCCCGTTTCGGGGCGATGATGCGGCCCGGTGCGCGTGTCCCAATCGATCGTAGGC
      G M C K A G F A G D D A P R A V F P S I V G
209  CGACCTCGCCACCAGGGTGTATTATGGCCGGCATGGGACAGAAAGGATTCCTACGTCCGGTGATGAGGCCA
      R P R H Q G V M A G M G Q K D S Y V G D E A
275  CAGAGCAAGAGAGGTATCCICACCTGAAGTACCCCATCGAACACGGIATCGTCCACCACTGGGAC
      Q S K R G I L T L K Y P I E H G I V T N W D
341  GACATGGAGAAAATCTGGCACCACACCTTCTACAATGAGGTGCGTGTAGCTCCCGAGGAGCACCCD
      D M E K I W M H T F Y N E L R V A P E E H P
407  GTCTGTCTCACCAGACTCCCTCAACCCAAAGGCCAACAGAGAGAGATGACACAGATCATGTTC
      V L L T E A P L N P K A N R E K M I Q I M F
473  GAGACGTTCAACACTCCCGCCATGACGTCCGGATCCAGGCTGTGCTCTCCTTGTACCGCATCCGGT
      E T F N I P A M Y V A I Q A V L S L Y A S G
539  CGTACTACCGGTATGCTGCTGGACTCCGGCGATGGTGTCTCCACACGGTGGCCATCTACGAAGGA
      R T I G I V L D S G D G V S H T V F I Y E G
605  TACGGCTGCGCGCACGCCATCCCTGCTGACTTGGACTTGGCCGGTCCGCGATCTCACAGACTACCTGATG
      Y A L P H A I L R L D L A G R D L I D Y L M
671  AAGATCTCACAGAGCGCGTTACTGCTTACCACCACCGCCGAGCGTGAATCGTTCGGCAGTC
      K I L T E R G Y S F T T T A E R E I V R D I
737  AAGGAGAAACTCTGCTACGTCCGCTCGACTTCGAGCAGGAGATGGCCACCGCCCGCTCCAGCAGC
      K E K L C Y V A L D F E Q E M A T A A S S S
803  TCCCTCGAGAAGTCTTACGAACCTCCCGACGGTCAAGTCAATCACAATCGGGAACGAAAGGTTCCGT
      S L E K S Y E L P D G Q V I I I G N E R F R
869  TGCCCCAGGGCTCTCTTCCAGCCCTCATTCTTGGGATGGAAGCTTGTGGCATCCACAGACTACCGACT
      C P E A L F Q P S F L G M E A C G I H E T T
935  TACAACCTCCATCATGAAGTCCGACTCGACTCCGTAAGACCTGTAGCCCAACAGATTTGTC
      Y N S I M K C D V D I R K D L Y A N T V L S
1001  GGTGGTACCACCATGTACCCCGGTATCCGGGACCGTATGCAGAAGGAATCACAGCCCTCGCTCCA
      G G T T M Y P G I A D R M Q K E I T A L A P
1067  TCTACGATGAAGATCAAAATCATTCGCCACACAGAGGAGTACTCTGTATGGATCGGTGGATCG
      S T M K I K I I A P P E R K Y S V W I G S
1133  ATCCTCGCGTCCCTCTCTACCTTCCAGCAGATGTGGATCTCGAAACAGGAGTACGACGAGTCTGGC
      I L A S L S T F Q Q M W I S K Q E Y D E S G
1199  CCCTCAATCGTGCACAGGAAGTCTTCTAAGCGCAGTGGCTCCCGCTGCACCCAGCAGCCCGCGGT
      P S I V H R K C F
1265  CCGGTCCGTCACCCAGCCCGCTGCTCAACTCAGGCTTTGTAATCTCGATTACTTTAATTGTACGTA
1331  AGATATTATTGTAATTAATCGTTAATAGTAGCGGGCGGAGTGAGCGATGCGCCCGCTGCGCACCT
1397  CTACCCCTGCGCTCGTACTGCACCTCGCCAAAGGATTTTGTGTAATCTCATGAATGATGATTTGTATGT
1463  GCTGAAACTGATAAGACTACTTTCTTACTAGTCAATTTATAAATTATAAAAATAAOGAAAATACA
1529  AAAAAAATCACAAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
    
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Figure 1. The full-length cDNA sequences and the deduced amino acid of the *Antheraea pernyi* actin gene (GU073316). The nucleotides are numbered on the left- side of the sequences. An asterisk (*) indicates the translation stop codon.

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Apactin-1  -----AGAAAGAAACCGGCCGGCATACATTTCCCACGGTTTGTAAACCGCTGCG-GA
Apactin-2  ACATTCACGAAGTGAGCTCGAGTTGTA-GCGTTGTGCAGCATTACTGAG-GCTGTGCAA
          * ** ** * * * * * * * * * * * * * * * * * * * * * * * * * *
Apactin-1  TATTA--TCAATTTTACGTTATCTA-AATTATCTACG----ACTAATTCACA
Apactin-2  TAGCAAGTTAATTTATTTATTTAATAGAACTATTTAATTTAATACTAATTCACA
          ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
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Figure 2. Sequence alignment of the 5' UTR of the Apactin-1 (GU073316) and Apactin-2 (GU176616). Identical nucleotide sites are indicated by the asterisk.

two cDNA clones might be arose from different mRNAs by alternative splicing, as found in the actin A4 gene of *B. mori* (Mange et al., 1996).

Homologous alignment and phylogenetic analysis

Besides the Apactin gene presented here, there were eight actin genes of Bombycoidea, including four *B. mori* actin gene isoforms in GenBank database to date. Multiple sequence alignment of these actin genes indicated that they were highly conserved with sequence

identity ranging from 83.2 to 96.5% at nucleotide level, 95.5 to 99.7% at amino acid level. However, the nucleotide sequences of the 5'- and 3'-UTR of the Apactin gene were significantly different from those of the other actin isoforms. Blast analysis showed that the Apactin gene held 96.5% identity at nucleotide level, and 99.7% identity at amino acid level, respectively, with the *A. yamamai* actin gene, which was partially sequenced with GenBank accession no. AB305643. *B. mori* is an important model insect of the silk-producing insect, and four actin genes have been isolated and characterized (Mange et al., 1996). To further subtype the Apactin gene,

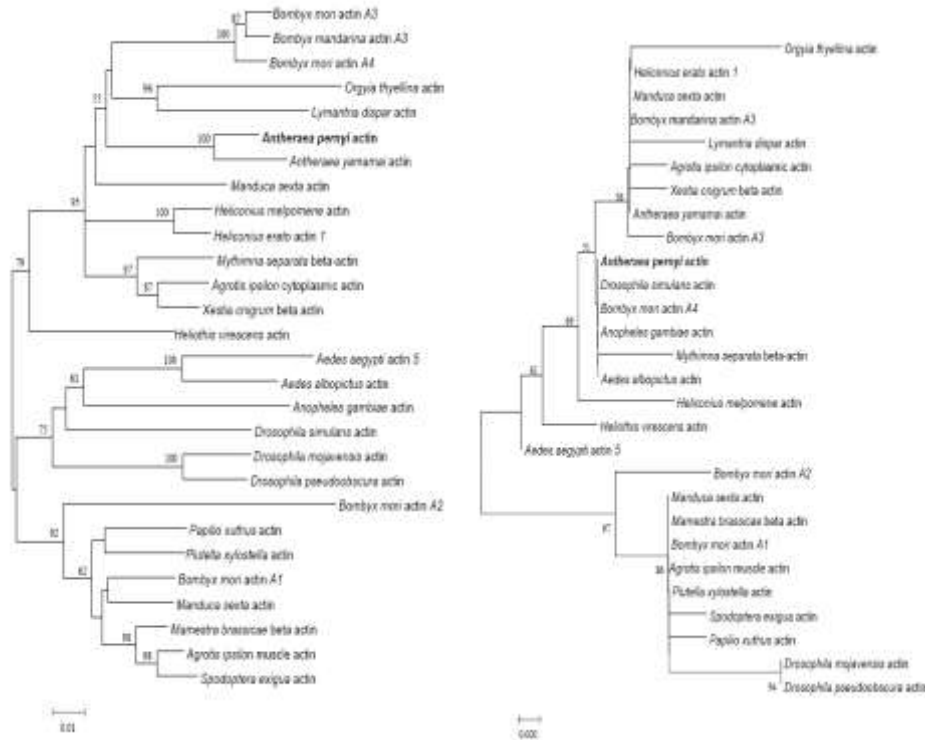


Figure 3. Neighbor-joining trees based on the nucleotide (A) and amino acid (B) sequences of Lepidoptera actin genes including the Apactin gene. The bootstrap values more than 50% were shown on the branch. Actin gene sequences data were obtained from GenBank database: *Antheraea yamamai* (AB305643); *Bombyx mori* A1 (NM_001126252), A2 (NM_001126253), A3 (NM_001126254), A4 (NM_001126255); *Bombyx mandarina* (EU780706); *Manduca sexta* (AJ519536; L13764); *Agrotis ipsilon* (muscle, EU601172; cytoplasmic, EU601173); *Heliothis virescens* (AF368030); *Xestia cnigrum* beta (EU035315); *Orgyia thyellina* (AB283042); *Heliconius melpomene* (EF211961); *Spodoptera exigua* (AY507963); *Papilio xuthus* (AB264725); *Mamestra brassicae* beta (EU035314); *Lymantria dispar* (AF182715); *Mythimna separata* beta (GQ856238); *Drosophila simulans* (XM_002106211); *Heliconius erato* 1 (EF207985); *Aedes aegypti* 5 (DQ074455); *Aedes albopictus* (DQ657949); *Anopheles gambiae* (U02930); *Drosophila mojavensis* (XM_002004219); *Drosophila pseudoobscura* (XM_002138481); *Plutella xylostella* (AB282645).

we compared the Apactin gene with the four known *B. mori* actin genes. The isolated Apactin gene shared 87.9, 83.1, 89.7, 90.4% identity at nucleotide level, and 95.5, 95.5, 99.2, 99.7% at amino acid level, with the *B. mori* actin A1, A2, A3, and A4, respectively. The results indicated that the Apactin gene is closely related to the *B. mori* actin A4 gene, which was confirmed by phylogenetic analysis (Figure 3). The sequence alignment of 3' UTR showed 89% sequence identity between the Apactin gene and the *B. mori* actin A4 gene, further confirming they were closely related. Moreover, as there were at least four actin isoforms in *B. mori*, we suggested that four actin genes might also be existed in *A. pernyi*. Two neighbor-joining trees inferred from the nucleotide and deduced amino acid sequences of the Apactin gene and 27 Lepidoptera actin genes were constructed to examine the relationships among them (Figure 3). In the trees, most samples were clustered clearly into two major

distinct clades: cytoplasmic actin and muscle actin, as previously reported (Hightower and Meagher., 1986; Mange et al., 1996). The Apactin gene was clustered into the cytoplasmic clade in both trees, indicating it is a cytoplasmic gene. The deduced amino acid sequences of actin genes were often used in studying phylogenetic relationships (Sun et al., 2007; Wang et al., 2007). In this study, the Apactin gene was closest to the *A. yamama* actin gene with 100% bootstrap in the tree based on the nucleotide sequences; however, it had closest genetic relationship with the actin A4 gene of *B. mori* with only 42% bootstrap in the tree based on the deduced amino acid sequences (Figure 3). Comparing the two trees constructed in this study, we found that the tree based on the nucleotide sequences could provide more information than that of the amino acid sequences. The genetic relationships of the actin genes were clear with higher than 60% bootstrap in the tree based on the nucleotide

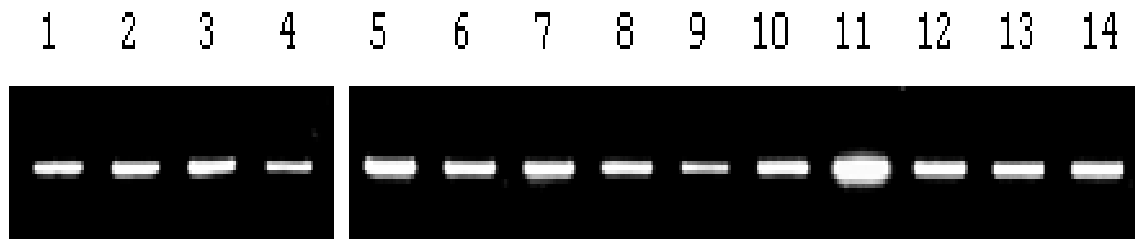


Figure 4. The expression patterns of the Apactin gene in different developmental stages and different tissues of the fifth instar larvae. The expression patterns were analyzed by RT-PCR, which was amplified after 28 cycles with specific primer pairs for the ApActin gene. Lanes: 1, eggs at day 5; 2, fifth instar larvae; 3, pupae; 4, moths; 5, blood; 6, fat body; 7, midgut; 8, silk gland; 9, body wall; 10, malpighian tubules; 11, spermary; 12, ovary; 13, brain; 14, musc.

sequences, but obscure with lower than 50% bootstrap in the tree based on the amino acid sequences. Therefore, we preferred to construct the phylogenetic tree based on the nucleotide sequences of the actin gene. This view was also found in previously reports (Xia et al., 2005; Wang et al., 2008).

Expression profiles of the Apactin gene

It is known that only the constitutively expressed gene can be used as reference gene. Most cytoplasmic actin genes are constitutively expressed (Lee, 2000), while some are not (Kim et al., 2009). In our study, expression of the Apactin gene in different life stages and tissues was assayed by RT-PCR analysis. The results revealed that the Apactin gene was constitutively expressed in all four life stages and in all ten tested tissues (Figure 4), indicating that the Apactin gene plays an essential role throughout the entire life cycle of *A. pernyi*, and can be used as a reference gene in the normalization of gene expression in further studies.

In conclusion, we cloned and characterized the first cytoplasmic actin gene from Chinese oak silkworm, *A. pernyi*. We found two types of Apactin cDNA clones which differ by the length and sequence of 5' UTR, as observed in *B. mori* A4 gene. Sequence alignment and phylogenetic analysis indicated that the isolated Apactin gene holds the highest identity with the *B. mori* actin A4 among the four known *B. mori* actin genes. RT-PCR analysis revealed that the Apactin gene is continuously and stably expressed during four developmental stages (egg, larva, pupa, and moth) and present in all tested tissues (blood, midgut, silk gland, Malpighian tubules, spermary, ovary, brain, muscle, fat body and body wall). This result confirms its reliability as a reference gene in the normalization of gene expression analysis.

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