JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Molecular Cloning and Characterization of Two 12 kDa FK506-Binding Protein Genes in the Chinese Oak Silkworm, *Antheraea pernyi*

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ABSTRACT: Two 12 kDa FK506-binding protein (FKBP12) genes were isolated and characterized from Chinese oak silkworm *Antheraea pernyi*, an important agricultural and edible insect, designated *ApFKBP12 A* and *B*, respectively. Both *ApFKBP12 A* and *B* contained 108 amino acids with 82% sequence identity. Phylogenetic analysis showed that FKBP12 B sequences of *A. pernyi*, *Bombyx mori*, and *Danaus plexippus* were clearly separated from FKBP12 A sequences of these three species, suggesting that insect *FKBP12 A* and *B* may have been evolving independently. RT-PCR analyses revealed that two *ApFKBP12* genes were expressed during the four developmental stages and in all tested tissues, and that the mRNA expression level of the *ApFKBP12 A* gene was significantly higher than that of the *ApFKBP12 B* gene. After heat shock treatment, expressions of the two *FKBP12* genes were up-regulated, but at different time points. The results suggested that each paralogue of the *FKBP12* genes may play a distinct functional role in the development of *A. pernyi*.

KEYWORDS: *Antheraea pernyi, 12 kDa FK506-binding protein, evolution, expression pattern*

INTRODUCTION

The FK506-binding proteins (FKBPs) belong to the superfamily of the PPIases (peptidylprolyl cis−trans isomerases), which catalyze cis−trans conversions around Xaa−Pro bonds.[1,2] PPIases catalyze the reaction of the peptide bond preceding proline in the proteins.[3−7] During the past decades, a large number of FKBPs from vertebrates, invertebrates, plants, fungi, and bacteria have been isolated. FKBPs range in mass from 12 to 135 kDa and participate in diverse cellular functions.[6] It has been shown that FKBP family proteins play important functional roles in the T-cell activation when they combine with their ligands.[8,9]

The 12 kDa FKBPs, known as FKBP12, are the most comprehensively studied proteins of the family, partially because of their clinical relevance as T-cell suppressors in mammalian models.[8,10] In mammalian systems, two *FKBP12* genes have been identified and designated *FKBP12 A* and *FKBP12 B*, respectively. When the FK506 emerges in the cells, *FKBP12 A* inhibits the phosphatase activity of calcineurin, an activator of nuclear factor of activated T-cells (NF-AT).[1,10] Each *FKBP12* isoform generates the production of a specific IL (interleukin). *FKBP12 A* regulates expression of IL-2 and IL-5, whereas *FKBP12 B* is related to IL-2, but it has no effect on the expression of IL-5.[8]

In insect systems, several proteins that associate with *FKBP12* have been identified, including calmodulin and calcineurin homologues in the silkworm *Bombyx mori*, mTOR and ryanodine receptors in *Drosophila melanogaster*, and two calmodulin-like proteins in the tobacco hornworm *Manduca sexta*. It has been suggested that similar FKBP-mediated pathways may exist in insects.[11] Subsequently, in insects, two *FKBP12* genes have been available only from *B. mori*.[11,12] However, knowledge on insect *FKBP12* remains extremely limited.

The Chinese oak silkworm, *Antheraea pernyi* (Lepidoptera: Saturniidae), is one of the most well-known wild silkmoths used for silk production.[13] Recently, it is mainly used as a source of insect food (its larva, pupa, moths) in China.[14] The protein of silkworm pupae has been thought to be a newly available source of high-quality protein that contains all of the amino acids needed by the human body.[15] With the development of biotechnology, more and more attention has been paid to isolation of the functional genes related to growth and development of this species.[16] In this study, we have isolated and identified two *FKBP12* genes from the *A. pernyi* pupal cDNA library by expressed sequences tag (EST) sequencing. Then, we compared the deduced protein sequence of the two genes with related FKBP12s from other organisms and determined their phylogenetic relationships. In addition, we comparatively examined the expression patterns at various developmental stages and in different tissues. Lastly, we examined the effects of heat shock on relative mRNA expression levels of the two targeted genes. The results presented here would provide essential information for a better understanding of the evolution and functional diversity of insect FKBP12s.

Received: February 8, 2013
Revised: April 21, 2013
Accepted: April 25, 2013
Published: April 25, 2013

dx.doi.org/10.1021/jf4006092 | *J. Agric. Food Chem.* 2013, 61, 4599−4605

MATERIALS AND METHODS

Insects and Tissues. The larvae of *A. pernyi* strain Shenhuang No. 1 used in this study were reared routinely on oak trees, *Quercus liaotungensis*. A number of tissues were dissected from the larvae at day 10 of the Fifth-instar, including hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes (male), ovaries (female), brain, and muscle. They were frozen in liquid nitrogen immediately and stored at −80 °C. Eggs at day 5, fifth-instar larvae, pupae, and moths were also sampled. To examine the effect of heat shock on the mRNA expression of two *FKBP12* genes, *A. pernyi* eggs in embryonic reversal stage (at day 6) were treated in a water bath at 50 °C for 20 s and then incubated at 25 °C. Control eggs were incubated at 25 °C throughout development. After 1 and 3 h of treatment, eggs were frozen in liquid nitrogen and then stored at −80 °C until RNA isolation.

Cloning of Two ApFKBP12 Genes and Sequence Analysis. Using a pupal SMART full-length cDNA library of *A. pernyi* constructed in our laboratory,[16] two EST encoding *ApFKBP12* homologues were isolated by random EST sequencing. Therefore, the cDNA clone was used to complete the full-length cDNA sequences of the *ApFKBP12* gene. The ORF Finder tool of NCBI was used to identify the open reading frame (ORF) at the Web site http://www.ncbi.nlm.nih.gov/gorf/gorf.html. The Blast search was performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The isoelectric point (p*I*) and molecular weight (MW) of the deduced amino acid sequences were predicted using the Compute pI/MW Tool at the Expert Protein Analysis System (ExPAsy) site (http://web.expasy.org/compute_pi/). The deduced amino acid sequence was submitted to predict protein signal peptide with the SignalP server online tool (http://www.cbs.dtu.dk/services/SignalP/). Transmembrance protein topological structure was analyzed with the TMHMM server online tool (http://www.cbs.dtu.dk/services/TMHMM/).

RNA Isolation and First-Strand cDNA Synthesis. Total RNA was extracted with the samples from *A. pernyi*, using an RNAprep Pure Tissue Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. DNAase I was used to remove the genomic DNA. The quality and quantity of this extracted RNA were determined by the ratio of OD_{260}/OD_{280} with an ultraviolet spectrophotometer. The integrity of the RNA was analyzed by 1.2% (w/v) agarose gel electrophoresis. The first-strand cDNA was generated with 2 μg of total RNA per sample by using a TIANScript RT Kit (TIANGEN Biotech) following the manufacturer's instructions. Then the first-strand cDNA was stored at −20 °C for later use.

Quantitative RT-PCR Analysis. The primers used are listed in Table 1. A gene encoding eukaryotic translation initiation factor 4A (eIF-4A; GenBank accession no. KC481238) was used as the internal control.[17] The RT-PCR method was first used to determine the expression patterns of two *ApFKBP12* genes. The PCR reactions were performed on a Bio-Rad S1000 thermal cycler (Bio-Rad Laboratories, Inc., USA). The PCR amplification was carried out in a total reaction volume of 25 μL containing 0.5 μL of cDNA sample, 10 pM of each primer, 2 mM $MgCl_2$, 2 mM of dNTP, 10× buffer, and 1 U Taq DNA polymerase (TIANGEN Biotech). The cycling parameters were an initial denaturation at 95 °C for 3 min, followed by 27 cycles, each comprising denaturation at 95 °C for 30 s, annealing temperature of 56 °C for 30 s, and extension temperature of 72 °C for 30 s. The amplification products were analyzed by 1.5% (w/v) agarose gel electrophoresis. We used total RNA as templates in negative control of the RT-PCR reactions to assess the contamination of DNA. The RT-PCR products were purified from the gel and sequenced to ensure the specificity.

Then, we used real-time quantitative RT-PCR (qRT-PCR) to determine the mRNA expression levels of two *ApFKBP12* genes. Primers for qRT-PCR were designed using Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA). The qRT-PCR was carried out using a Roche Light Cycler 480 (Hoffmann-La Roche Ltd., Switzerland). Each reaction in a final volume of 10 μL contained 4.5 μL of 2.5× RealMaster Mix/20× SYBR solution (TIANGEN Biotech), 0.5 μL cDNA sample, and 10 pM gene-specific primer. The cycling parameters were an initial denaturation at 95 °C for 2 min, followed by 40 cycles, each comprising denaturation at 95 °C for 15 s, annealing temperature of 60 °C for 30 s, and extension temperature of 68 °C for 30 s, followed by a stage of 60−95 °C to determine melting curves of the amplified products. The relative changes for each gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression for qRT-PCR experiments.[18] Three parallel measurements for each cDNA sample from independent RNA isolation were detected, and three replicates of each cDNA sample were performed for qRT-PCR analysis. A two-tailed Student's test was used to determine the statistical difference between the groups, and $P < 0.01$ was considered to be significant.

Phylogenetic Analysis. The amino acid sequence alignments were performed by Clustal X software.[19] A phylogenetic tree was constructed by MEGA 5.0 using the maximum parsimony (MP) method and the bootstrap test performed with 1000 replications to test the statistical significance of the nodes.[20]

Table 1. Primers for RT-PCRs Used in This Study

gene	primer pairs and sequences (5′−3′)	PCR product (bp)
RT-PCR		
FKBP12 A	F: TCCTA AGCCT GGACA AACTG	168
	R: CGCTC ACCCA CTGAC ATTTT	
FKBP12 B	F: ATGGG CGTAG ATGTA GAAAC	256
	R: TAGAG CCATA AGCAA AGTCG	
eIF-4A	F: TCCAT CGCTC AGGCT GTTAT	340
	R: GTGCT CGTCT GTCAC TTTCA	
qRT-PCR		
FKBP12 A	F: ATTCT GTCCG TGTTC AAC	181
	R: AAGTG CCTGT GTAGT GTA	
FKBP12 B	F: TGGCT AAGAT GTCTG TAGG	82
	R: ACTCC TGGAT GTCCT CTA	
eIF-4A	F: TCCTC TCGTG TGCTT ATC	128
	R: CCACC TCTTC CGATT CTAT	

RESULTS

Sequence Analysis of Two *A. pernyi FKBP12* Genes. In our present study, two *A. pernyi FKBP12* genes were isolated and identified from the *A. pernyi* pupal cDNA library.[16] The cDNA sequence and deduced amino acid sequence of the *ApFKBP12 A* gene are shown in Figure 1A. The obtained 516 bp cDNA sequence contains a 5′ untranslated region (UTR) of 100 bp, a partial 3′ UTR of 89 bp, and an ORF of 327 bp that encodes a polypeptide of 108 amino acids. The deduced amino acid sequence has a predicted molecular weight of 11.79 kDa and an isoelectric point of 7.84. Blastp analysis revealed that the predicted protein sequence of this cDNA shared 97% identity with that of FK506-binding protein of *B. mori* (ABF51286).

The cDNA sequence and deduced amino acid sequence of the *ApFKBP12 B* gene are shown in Figure 1B. The obtained 467 bp cDNA sequence contains a 5′ UTR of 91 bp, a partial 3′ UTR of 48 bp, and an ORF of 327 bp encoding a polypeptide of 108 amino acids with a predicted molecular weight of 11.68 kDa and an isoelectric point of 7.88. The cDNA sequence differed by the sequence of 5′ and 3′ UTR compared with that of the *ApFKBP12 A* gene. Blastp analysis revealed that the predicted protein sequence of this cDNA shared 96% identity with that of FK506-binding protein of *B. mori* (ABF51512).

Signal peptide prediction showed that both were non-secretory proteins. Analyses of transmembrane protein topological structure revealed that they contained no

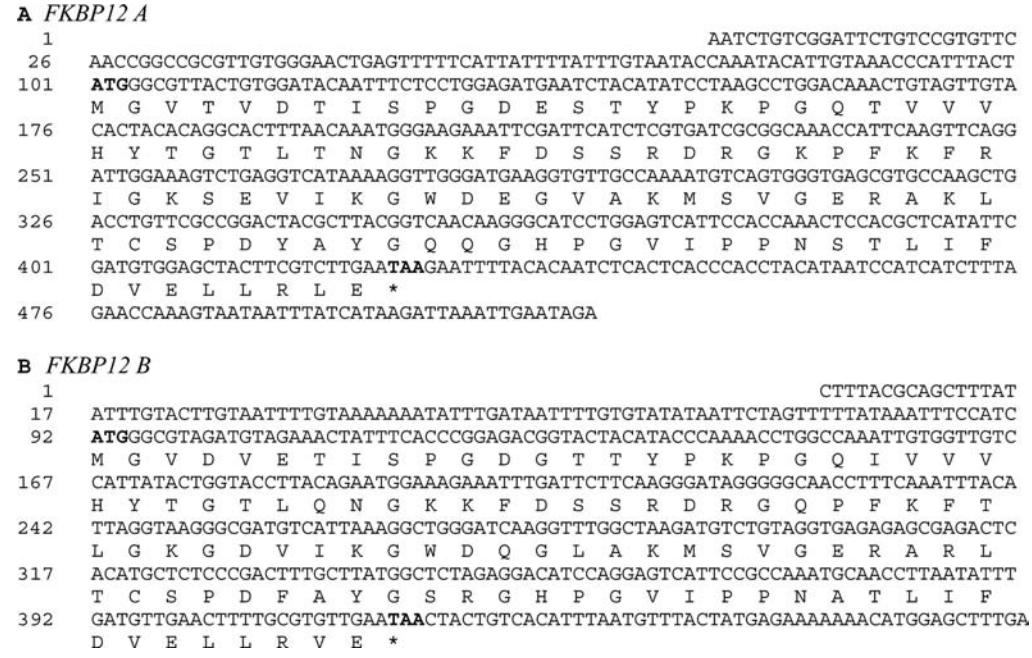

Figure 1. Complete nucleotide and deduced amino acid sequence of the *A. pernyi FKBP12 A* and *B* genes. The amino acid residues are represented by one-letter symbols. The initiation codon ATG is bolded, and the termination codon TAA is bolded and marked with an asterisk.

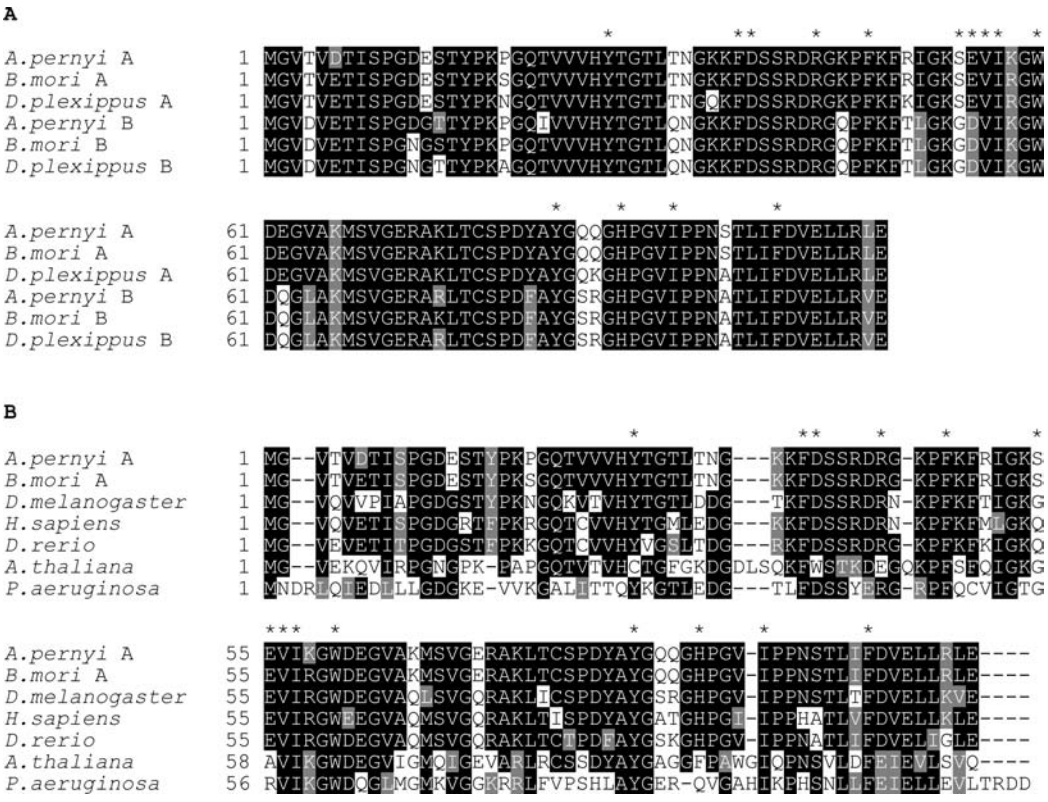

Figure 2. Sequence comparisons of FKBP12s. (A) Sequence alignment of FKBP12 A and B from *A. pernyi*, *B. mori*, and *D. plexippus*. Three FKBP12 A show 91−97% sequence identities to each other, and three FKBP12 B show 96% sequence identities. However, three FKBP12 A exhibit only 78−84% sequence identities to three FKBP12 B from the same species. (B) Sequence alignment of FKBP12 A from *A. pernyi* and other organisms. *A. pernyi* FKBP12 A shows 97% sequence identity to *B. mori* (ABF51286), 77% to *Drosophila melanogaster* (NP_523792), 75% to *Homo sapiens* (NP_000792), 78% to *Danio rerio* (NP_956239), and 51% to *Arabidopsis thaliana* (AED97876), 35% to *Pseudomonas aeruginosa* (EGB71364). The ∗ shows the position of the critical residues necessary for PPIase activity and FK506 binding.[11,24].

membrane helices. The residue compositions between ApFKBP12 A and B were similar (data not shown). The conserved domain prediction showed that both of the two ApFKBP12 belonged to the FKBP_C superfamily, known as FKBP-type peptidyl-prolyl cis−trans isomerase. Therefore, we referred to the two proteins as ApFKBP12 A and B,

dx.doi.org/10.1021/jf4006092 | J. Agric. Food Chem. 2013, 61, 4599−4605

respectively. The cDNA sequences of the *ApFKBP12 A* and *B* genes have been deposited in GenBank under accession nos. KC481239 and KC481240, respectively.

Homologous Alignment. In mammalian species, the presence of two FKBP12 isoforms has been evidenced.[11] In our study, the *ApFKBP12 A* and *B* genes were used to search against the GenBank database available; we then found that two FKBP12 isoforms have been available only from two lepidopteran insect species, including *B. mori* (ABF51286 and ABF51512, respectively) and *Danaus plexippus* (EHJ65443 and EHJ74432, respectively).[21] In insects, by search in the available database including ButterflyBase[22] and WildSilkbase,[23] many complete sequences of the *FKBP12 A* genes are available; however, no other sequences of the *FKBP12 B* genes were retrieved.

The amino acid sequences of six FKBP12s from *A. pernyi*, *B. mori*, and *D. plexippus* were compared (Figure 2A and Table 2).

Table 2. Protein Sequence Identities between the Two FKBP12 Isoforms from *A. pernyi*, *B. mori*, and *D. plexippus*

	A. pernyi A	*B. mori* A	*D. plexippus* A	*A. pernyi* B	*B. mori* B
B. mori A	97				
D. plexippus A	91	93			
A. pernyi B	82	81	79		
B. mori B	84	83	81	96	
D. plexippus B	80	80	78	96	96

As shown in Table 2, three FKBP12 A showed 91−97% sequence identities to each other, and three FKBP12 B showed 96% sequence identities; however, three FKBP12 A exhibited only 78−84% sequence identities to three FKBP12 B. This result showed that FKBP12 A is more similar to their orthologues from different species than to paralogues, FKBP12 Bs, from the same species.

In addition to three FKBP12 B amino acid sequences from *A. pernyi*, *B. mori*, and *D. plexippus*, we also used another 37 FKBP12 sequences from different organisms to calculate the sequence identity. All of these FKBP12 sequences were retrieved from the NCBI database, including vertebrate (10), invertebrate (16), plants (5), fungi (3), and bacteria (3). As shown in Figure 2B, these sequences are aligned with various FKBP12s from insects (*A. pernyi*, *B. mori*, and *D. melanogaster*), vertebrates (*Homo sapiens* and *Danio rerio*), plants (*Arabidopsis thaliana*), and bacteria (*Pseudomonas aeruginosa*). The alignment of these amino acid sequences revealed that *A. pernyi* FKBP12 A had the highest identity (97%) to *B. mori* (ABF51286) and the lowest identity (34%) to *Shewanella putrefaciens*. FKBP12 A of *A. pernyi* revealed 62−97% identity to invertebrates, 73−78% identity to vertebrates, 49−54% identity to plants, 55−62% identity to fungi, and 34−43% identity to bacteria.

Phylogenetic Analysis. A total of 40 representative FKBP12 sequences from different organisms, including *A. pernyi*, were used to reconstruct their phylogenetic relationships. Bacteria FKBP12 sequence from *P. aeruginosa* was used as outgroup. A maximum parsimony tree was constructed using amino acid sequences and a Poisson-corrected distance (Figure 3). The obtained phylogenetic tree followed classical evolutionary trends. In the phylogenetic tree of FKBP12s, the used FKBP12 sequences were well divided into five groups, corresponding to vertebrates, invertebrates (insects and nematodes), plants, fungi, and bacteria, respectively. It was seen that FKBP12 A and B sequences from *A. pernyi*, *B. mori*, and *D. plexippus* were separated into two different clades, respectively, as observed for two FKBP12 isoforms in mammalian species.[11]

Expression Patterns. In the first step, the RT-PCR approach was employed to detect the expression patterns of two *ApFKBP12* genes at different developmental stages and in different tissues of the fifth-instar larvae (Figure 4A). By sequencing, we confirmed that the positive RT-PCR products were amplified from the *FKBP12* gene sequence. Two *ApFKBP12* genes were found to be expressed throughout all four developmental stages, including egg, larva, pupa, and moth. Also, two *ApFKBP12* genes were found to be present in all tested tissues of the fifth-instar larvae, including hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes, ovaries, brain, and muscle.

qRT-PCR was further used to determine the expression levels of two *ApFKBP12* genes (Figure 4B). During the four developmental stages, the expression levels of the *ApFKBP12 A* gene were significantly higher than those of the *ApFKBP12 B* gene. Moreover, the relative expression levels of the two *ApFKBP12* genes at the stage of moth were higher than that of the other three stages including larva, egg, and pupa. In all tested tissues, it was obvious that the relative expression levels of the *ApFKBP12 B* gene were also significantly lower than those of the *ApFKBP12 A* gene. For the *ApFKBP12 A* gene, the relative expression level was highest in fat body and lowest in silk glands. For the *ApFKBP12 B* gene, the relative expression level was highest in integument and lowest in silk glands.

As shown in Figure 5, the relative expression level of the *ApFKBP12 A* gene was significantly up-regulated ($P < 0.01$ and fold change = 2.6) at 1 h after heat shock treatment. In contrast, the *ApFKBP12 B* gene showed a significantly up-regulated expression at 3 h after treatment ($P < 0.01$ and fold change = 1.8).

DISCUSSION

In past years, more attention has been paid to the biology of FKBP12, and a large number of FKBP12s from vertebrates, invertebrates, plants, fungi, and bacteria have been identified. Until now, there are about 20 complete insect genome sequences available. However, few complete insect FKBP12 B sequences are available; the majority of sequences are from FKBP12 A. By searching in the available EST databases including ButterflyBase[22] and WildSilkbase,[23] no other partial FKBP12 B sequences were retrieved. As a result, only three complete sequences of FKBP12 B from *A. pernyi*, *B. mori*, and *D. plexippus* are available. For the *B. mori FKBP12 A* and *B* genes, both cDNA and DNA sequences are available. For the *D. plexippus FKBP12 A* and *B* genes, only DNA sequences are available. In this study, we evidenced the cDNA sequences of the *A. pernyi FKBP12 A* and *B* genes. We found that both the *ApFKBP12 A* and *B* genes encoded 108 amino acids, but only with 82% protein sequence identity. Moreover, we comparatively examined the expression patterns of the *A. pernyi FKBP12 A* and *B* genes during different developmental stages and in various tissues. To our knowledge, this is the first study to comparatively examine the expression patterns of insect *FKBP12 A* and *B* genes. The results presented here would provide basic information of the two genes for further functional analyses.

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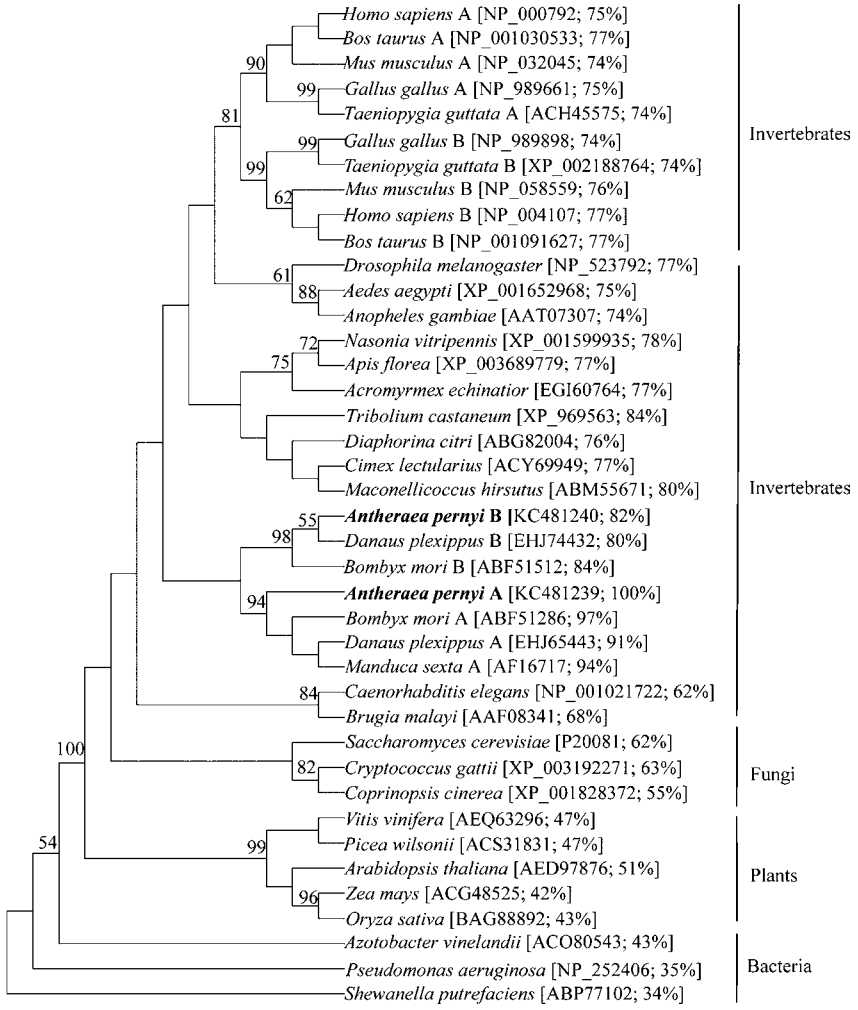

Figure 3. Phylognetic tree based on the amino acid sequence comparisons of FKBP12s from various organisms including *A. pernyi*. The topology was tested using bootstrap analyses (1000 replicates). Numbers at nodes are bootstrap *P* values. Public database accession numbers of FKBP12s and their identities to *A. pernyi* FKBP A are shown in square brackets. The maximum parsimony tree was constructed by MEGA version 5.0 based on a Poisson-corrected distance.

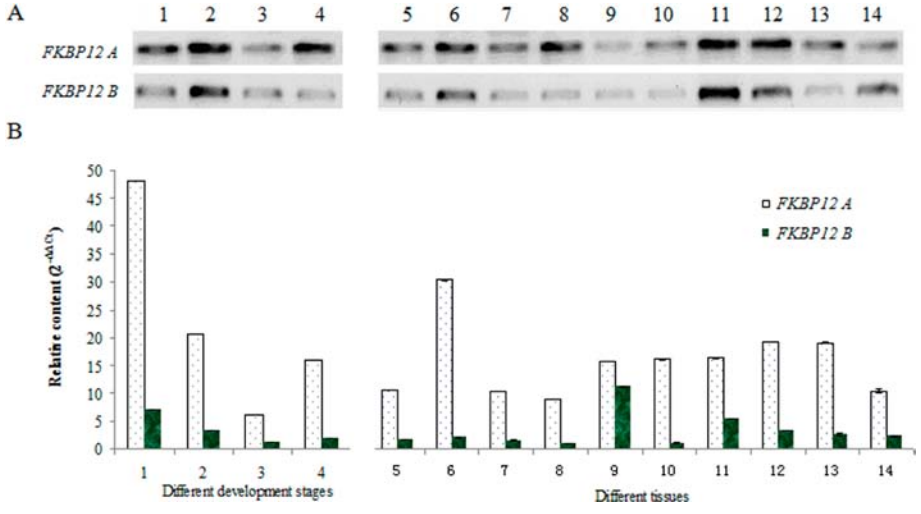

Figure 4. Expression of the *A. pernyi FKBP A* and *B* genes during the four developmental stages and in various tissues of the fifth-instar larvae: (A) expression patterns analyzed by RT-PCR; (B) expression levels analyzed by real-time quantitative RT-PCR. Horizontal line 1−4 represents the four developmental stages of moths, pupae, eggs at day 5, and fifth-instar larvae, respectively; and line 5−14 represents 10 tissues in the fifth-instar larvae of hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes, ovaries, brain, and muscle, respectively.

dx.doi.org/10.1021/jf4006092 | J. Agric. Food Chem. 2013, 61, 4599−4605

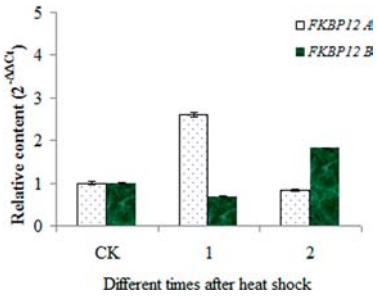

Figure 5. Expression changes in response to temperature stress. Horizontal line 1−2 represents eggs at 1 and 3 h after heat shock, respectively. CK represents the control.

Protein sequence comparison of the six lepidopteran *FKBP12* genes from *A. pernyi*, *B. mori*, and *D. plexippus* revealed that the identity degrees within FKBP12 A (91−97%) or B (96%) from different species were obviously larger than between them from the same species (about 80%). This result suggested that insect *FKBP12 A* and *B* may be evolving independently. Furthermore, the phylogenetic analysis provided insight into the relationship of two insect FKBP12s within the insect lineage. In the phylogenetic tree, the used six lepidopteran FKBP12s clustered together, with the A and B types segregating from each other. The fact further validates the hypothesis that insect *FKBP12 A* and *B* may have been evolving independently, possibly due to their highly specific roles in regulation of calcium release and/or T-cell activation, as observed in mammalian species.[11]

It is of note that eukaryote FKBP12s maintain >50% amino sequence identity. This implies that FKBP12s are highly conserved throughout the evolution of eukaryote organisms. Fourteen critical residues necessary for PPIase activity and FK506 binding have been identified.[24,25] As shown in Figure 2A, only 2 of 14 critical residues changed between FKBP12 A and B, positioned at 54 and 55. It has been suggested that there may be a functional constraint imposed on this protein which limits the degree of change as a result of amino acid divergence.[11] Does the alteration of the two critical residues lead to the functional difference between *FKBP12 A* and *B* of lepidopteran species? To address this question, more studies need to be performed.

Large-scale EST resource and extensive microarray information for *B. mori*, a major lepidopteran insect model for research, are available at GenBank and SilkDB.[26] By searching at GenBank and SilkDB, we can get EST and DNA evidence for the *B. mori FKBP12 A* gene, whereas we get only one EST evidence for the *B. mori FKBP12 B* gene. The in silico gene expression analysis based on the EST resources at the site http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi (Bmo.1161) showed that ESTs for the *B. mori FKBP12 A* gene are observed at the stages of egg, larva, and pupa and in the tissues including eye, maxilla, and prothoracic glands. The genome-wide microarray data at SilkDB also indicated that the *B. mori FKBP12 A* gene (sw13189) is highly expressed in the tissues including hemolymph, head, Malpighian tubules, spermaries, ovaries, fat body, integument, and silk glands. The expression patterns during the developmental stages and in larval tissues of two *B. mori FKBP12* genes have been investigated by RT-PCR technique.[12] Our results showed that two *A. pernyi FKBP12* genes are expressed during all four developmental stages and in all tissues tested, which agrees well with the expression patterns of the two *B. mori FKBP12* genes.

Our results revealed a distinct expression pattern of the two *ApFKBP12* genes. It has been reported that, in mammalian systems, each *FKBP12* isoform can generate the production of a specific IL. *FKBP12 A* regulates expression of IL-2 and IL-5, whereas *FKBP12 B* is related to IL-2, but it has no effect on the expression of IL-5.[8] In the present study, our results show that both *A. pernyi FKBP12* genes are expressed during the four developmental stages and in all tissues tested, which suggest that each of the two *ApFKBP12* genes may play an important role in the development of *A. pernyi*. However, during the four developmental stages and in all tissues, the relative expression levels of the *ApFKBP12 B* gene are significantly lower than those of the *ApFKBP12 A* gene. Furthermore, after heat shock treatment, the *ApFKBP12 A* gene reaches a higher expression level at 1 h, whereas the *ApFKBP12 B* gene reaches a higher expression level at 3 h. This observation suggests that the two genes are involved in temperature stress, and the *ApFKBP12 A* gene functions earlier than the *ApFKBP12 B* gene. Taken together, our results suggest that the two isoforms of the *FKBP12* genes may play distinct functional roles in the development of *A. pernyi* that remain to be elucidated.

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Author Contributions

‖M.C. and M.-M.C. contributed equally to this work.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 31072082), the China Agriculture Research System (CARS-22), the Program for Liaoning Excellent Talents in University (2012060), and the Project for Tianzhushan Scholar of Shenyang Agricultural University.

Notes

The authors declare no competing financial interest.

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