Insect Molecular Biology (2008) **17**(4), 395–404

Identification and expression of the *achaete-scute* complex in the silkworm, *Bombyx mori*

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Abstract

Recently, the study of *achaete-scute* (*AS-C*) homologues has contributed enormously to understanding of gene duplication and function evolution, particularly in Diptera. We identified four *AS-C* homologue genes in the silkworm, *Bombyx mori*, referred to as *BmASH*, *BmASH2*, *BmASH3*, and *Bmase*. The complex displayed tandem array structure in the genome. Analysis of spatial expression profiles showed that they all were expressed in obviously higher levels in wing disc than in other tissues, suggesting that they might play important roles in the development of the wing. Furthermore, we found that their expression profiles in the wing discs were mostly correlated with the development of the scales, especially the *BmASH* gene. RNA interference results further indicated that *BmASH* was necessary for scale formation in silkworm wing.

Keywords: *achaete-scute* (*AS-C*) homologue, *Bombyx mori*, genomic organization, expression pattern, RNA interference.

Introduction

The *achaete-scute* (*AS-C*) complex encodes transcriptional regulators of the basic-helix-loop-helix (bHLH) protein family (Villares & Cabrera, 1987; Alonso & Cabrera, 1988; Ghysen & Dambly-Chaudière, 1988; Gonzalez *et al*., 1989). Studies of *AS-C* genes in several species have shown that they are a highly conserved family that plays important roles in the development of neural cells in both vertebrates and invertebrates (Chan & Jan, 1999; Ledent & Vervoort, 2001). Expression of *AS-C* genes provides cells with neural potential (Galant *et al*., 1998; Wulbeck & Simpson, 2000, 2002; Skaer *et al*., 2002).

In *Drosophila*, the *AS-C* complex consists of four genes, the adjacent *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) proneural genes, and the neural precursor gene *asense* (*ase*). They scatter over about 100 kb at the distal tip of the X-chromosome in *Drosophila* genome. They are required for development of the central (CNS) and peripheral nervous systems (PNS) (Ghysen & Dambly-Chaudière, 1988). In the adult Drosophila PNS, the *AS-C* proneural genes promote external sensory organ precursor (SOP) formation. The SOPs arise from small clusters of cells co-expressing *ac* and *sc* that prefigure the sites of each of the future bristles (Romani *et al*., 1989; Cubas *et al*., 1991). Loss of the activity of these genes results in flies devoid of bristles (Garcia-Bellido & Santamaria, 1978). Once the neural precursors formed, the expression of proneural gene extinguished rapidly, *ase* began to express in all neural precursors and appear to promote the division and differentiation of these cells (Dominguez & Compazano, 1993; Jarman *et al*., 1993).

To date, homologues of *AS-C* genes have been identified in various invertebrates and vertebrates. They all have a conserved bHLH domain, and the functional roles of these genes in nervous system development appear to be well conserved during evolution (Cabrera *et al*., 1987; Johnson *et al*., 1990; Jasoni *et al*., 1994; Galant *et al*., 1998; Wulbeck & Simpson, 2000, 2002; Wheeler *et al*., 2003; Hayakawa *et al*., 2004; Wheeler & Skeath, 2005). The analysis of the insect *AS-C* homologues showed that the number of *AS-C* genes varies among species, with two in *Tribolium*, three in both of *Ceratitis* and *Calliphora* and four in *Drosophila*. It seemed that the number of *AS-C* genes increased during insect evolution, particularly in the Diptera lineage (Skaer *et al*., 2002). In Drosophila, the four genes were thought to have arisen from a single ancestral gene by three independent duplication events. Furthermore, most of insects include both the proneural genes and the neural precursor gene *ase*.

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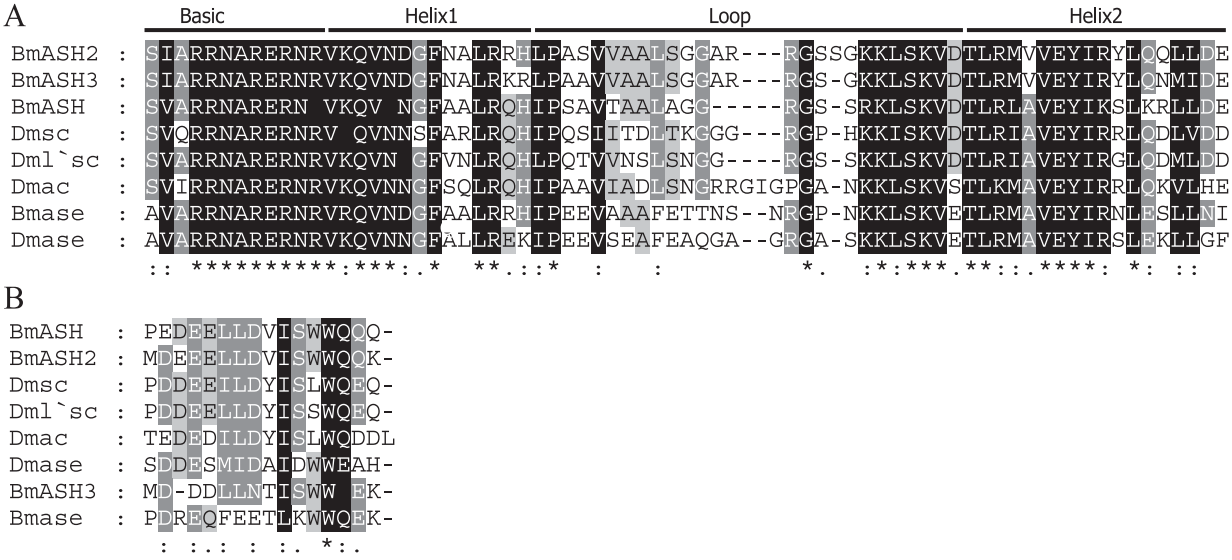

Figure 1. Alignments of *AS-C* homologue sequences from the silkworm and *Drosophila*. (A) Comparison of the amino acid sequences from the bHLH domains of the silkworm *AS-C* genes (*BmASH*, *BmASH2*, *BmASH3*, *Bmase*) with those from *Drosophila* (*Dmac*, *Dmsc*, *Dml'sc*, *Dmase*). Most changes are found within the loop. (B) Alignment of the C-terminal motif of silkworm AS-C proteins with the C-terminal motif of *Drosophila AS-C* genes. Amino acids conserved in five or more proteins are dark and gray shaded asterisks indicate residues conserved in all AS-C bHLH domains.

In Lepidoptera, only one *AS-C* homologue, *JcASH1*, has been found in the buckeye butterfly, *Junonia coenia*. Expression studies showed that *JcASH1* was expressed in embryonic proneural clusters, in putative sensory mother cells of the larval wing discs and also expressed in pupal wings. It appears to perform multiple functions throughout butterfly development, and essential to wing scale formation (Galant *et al*., 1998). In silkworm, an *AS-C* homolog, *BmASH*, was used to detect if the gene was abnormal in the scaleless wing mutant (Zhou *et al*., 2006). The question therefore arises: Are some other *AS-C* genes present in the Lepidoptera insects? In order to address this issue, we focused our study on the domesticated silkworm, *Bombyx mori*, one of the well-known model systems for Lepidoptera (Willis *et al*., 1995). The recent completion of the genome sequence of *B. mori* (The International Silkworm Genome Sequencing Consortium, 2007) provides a unique opportunity to analyze the *AS-C* complex in the silkworm. Here, we investigated the genomic organization, expression profiles and functions of *AS-C* complex in the silkworm. Our results provided important information for future studies on their functions and their spatio-temporal expressions during development.

Results

Four AS-C *genes in the silkworm*

AS-C genes encode related transcriptional regulators of the bHLH protein family (Villares & Cabrera, 1987). So far, homologues of *AS-C* genes have been identified in several species. Although the number of *AS-C* genes varies among species, their bHLH motifs are highly conserved and can be distinguished from other bHLH genes. To identify how many *AS-C* genes existing in the silkworm, we used the bHLH motif sequences from known *AS-C* genes as queries to search the 9× coverage silkworm genome sequence (The International Silkworm Genome Sequencing Consortium, 2007) with the TBLASTN program. The genome sequence could be accessed on the website http://silkworm.swu.edu.cn/silkdb/genome/. The results revealed that four sequences contained the conserved bHLH domain with similarities from 70.5% to 74.2%. We used their predicted amino-acid sequences as queries to search the NCBI, and found four matched sequences previously deposited in the Genebank (NP_001037416.1, ABR20839.1, ABR20840.1, and ABR20841.1). They were named as *BmASH*, *BmASH2*, *BmASH3*, *Bmase*, and their encoded proteins contain 193, 239, 241 and 404 amino acids, respectively.

The alignment of the bHLH domains of the silkworm and *Drosophila AS-C* genes showed that they were highly conserved. Conservation within the loop was weaker than that in the carboxy-terminal region of the loop (Fig. 1A). The similarity outside the bHLH region was much lower. Furthermore, BmASH, BmASH2, and BmASH3 were more closely related to *Drosophila* proneural l'sc than others with the similarity ranged from 70.5% to 71.4%. They also showed high similarities to *Drosophila* l'sc within C-terminal motif (data not shown) that had been used to distinguish insect proneural AS-C proteins from insect ase proteins (Fig. 1B) (Wheeler *et al*., 2003, 2005). On the other hand, the Bmase showed 74.2% amino acid similarity with *Drosophila* ase, and it had lower similarities (ranging from 57.1 to

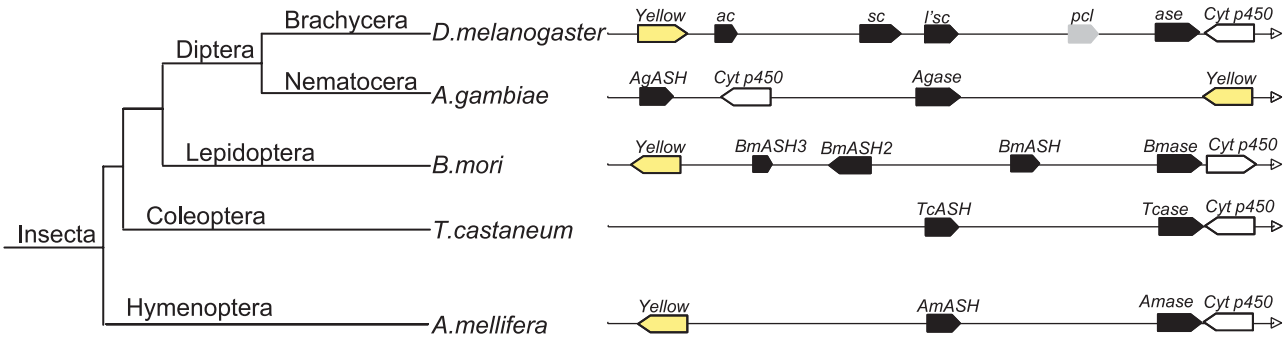

Figure 2. Comparison of genomic structures of the silkworm, *Drosophila*, *Anopheles*, *Tribolium* and *Apis AS-C* complexes. The *Drosophila AS-C* complex contains three proneural genes *ac*, *sc* and *l'sc* and one *ase* gene located 48 kb downstream of *l'sc*, a *pcl* gene (gray) located on the middle of *l'sc* and *ase* in *Drosophila*. The complex spans about 100 kb. The Anopheles *AS-C* complex contains *AgASH* and *Agase*, they lie 22 kb apart. The silkworm *AS-C* complex contains three proneural genes, *BmASH*, *BmASH2* and *BmASH3*, and one *ase* gene, *Bmase*. The complex scattered over ~300 kb. The *Tribolium AS-C* complex contains one proneural gene, *TcASH* and one *ase* homolog, *Tcase*, which reside 55 kb apart. The only two *AS-C genes*, *AmASH* and *Amase* of *Apis* reside 43 kb. *AS-C* genes are shown in black, *yellow* genes in yellow, and *cyt p450* in black. Arrows denote the direction of transcription.

58.5%) with other *AS-C* proteins within the bHLH domain. The above data suggested that *BmASH*, *BmASH2*, and *BmASH3* were proneural genes and *Bmase* was an *ase* homolog.

Genomic structure of the silkworm AS-C *complex*

In the silkworm, the four *AS-C* genes *BmASH*, *BmASH2*, *BmASH3*, and *Bmase* were found to cluster on the same scaffold scattered over ~300 kb (Fig. 2). The scaffold is mapped on chromosome 13 by the markers of simple sequence repeat and single nucleotide polymorphism (http://silkworm.swu.edu.cn/silkdb/genome/). There is no any other predicted gene within the silkworm *AS-C* complex. In addition, a *yellow* gene and a *cyt P450* locate at the two ends of the complex (Fig. 2). The *yellow* gene is about 8 kb upstream of *BmASH3*, and the *cyt P450* is about 3 kb downstream of *Bmase*. *BmASH3* has higher homology with *BmASH2* than with *BmASH*, and shares the lowest homology with *Bmase*. This order is consistent with the order of locations of the four genes in the silkworm genome, similar to those in *Drosophila* (Campuzano *et al*., 1985). The distances between the each of two adjacent genes are about 46 kb, 118 kb, and 115 kb, respectively. Three of four genes are transcribed in the same orientation whereas *BmASH2* is transcribed in opposite direction (Fig. 2). It is interested to note that all of four *AS-C* genes have no intron in the ORFs.

Evolutionary relationship of AS-C *homologues*

The highly conserved residues within the bHLH region and the carboxyl terminus were used to reconstruct phylogenetic tree with the NJ method to determine the relationships between the silkworm and previously identified AS-C proteins. The phylogenetic analysis revealed that all the insects AS-C proteins formed proneural and ase clades, while the AS-C proteins of human and mouse (vertebrates) and the two spider (chelicerate) homologues formed a cluster and diverged first (Fig. 3). In the proneural clade, the homologs of the three genes in Dipteran always grouped together, while the tree placed the *Anopheles gambiae* and *Aedes aegypti ASH (AS-C)* homologus genes that were closest to *Drosophila l'sc* in the most basal position within the proneural clade in Dipteran. *An. gambiae* and *Ae. aegypti* are the basal species of Diptera belonging to the Nematocera (McAlpine *et al*., 1981).

However, within the higher Diptera, the proneural proteins formed three major clusters ac, sc, l'sc, with strong support as bootstrap values of 98, 100 and 80%, respectively. The ac group and sc group clustered together, and then they clustered with the l'sc group. A previous study suggested that the *ac* and *sc* genes seemed to have a common ancestor because of their high similarities (Skaer *et al*., 2002). The branching pattern of Diptera on the phylogenetic tree (Fig. 3) was entirely consistent with a tree defined by morphological comparison (McAlpine *et al*., 1981). However, outside Diptera group in proneural clade, BmASH2 clustered with butterfly JcASH1, and then clustered with BmASH3. Furthermore, BmASH was closely related to AmASH and TcASH rather than BmASH2 and BmASH3. The branching pattern of the ase clade was basically consistent with that of proneural clade. The ases of silkworm in Lepidoptera, *Apis mellifera* in hymenoptera, and *Tribolium castaneum* in coleoptera were also located on the most basal position within the clade. For each clade, the relationships among individual AS-C proteins were generally congruent with the phylogenetic relationships of species established by morphological traits.

Spatial expression profiles of silkworm AS-C *genes*

To investigate the spatial expression patterns of the *AS-C* genes, we used semi-quantitative RT-PCR to analyze total RNA samples of 12 silkworm tissues on the sixth day of the

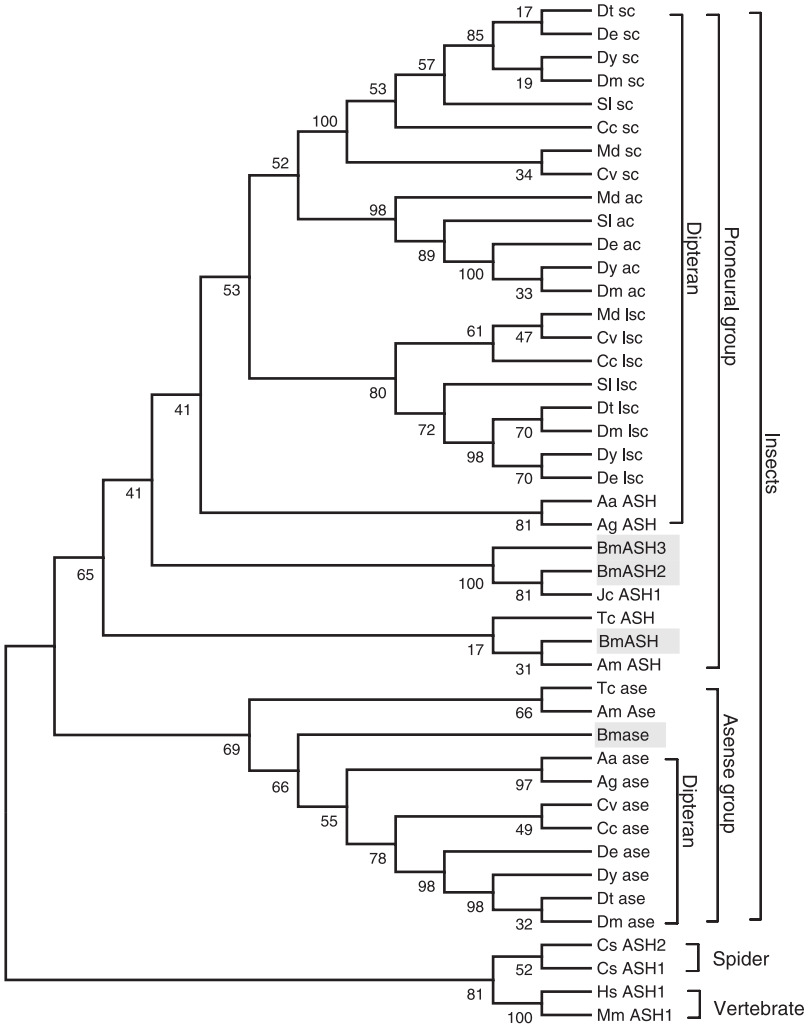

Figure 3. A phylogenetic tree of the *AS-C* homologues. NJ tree reconstructed based on the bHLH domais and the conserved C-terminal domains using MEGA v3.0. For simplicity, branch lengths of the tree are not proportional to distances between sequences, and the values given at each node correspond to the bootstrap values (1000 repetitions). The abbreviations were as follows: *ase*, *asense*; *l'sc*, *lethal of scute*; *sc*, *scute*; *ac*, *achaete*; *ASH*, *AS-C* Homologue; *Dm*, *Drosophila melanogaster*; *De*, *Drosophila erecta*, *Dt*, *Drosophila teissieri*, *Dy*, *Drosophila yakuba*; *Cc*, *Ceratitis capitata*; *Cv*, *Calliphora vicina*; *Md*; *Musca domestica*, *Sl*, *Scaptodrosophila lebanonensis*, *Aa*, *Aedes aegypti*, *Ag*, *Anopheles gambiae*, *Am*, *Apis mellifera*, *Bm*, *Bombyx mori*, *Tc*, *Tribolium castaneum*, *Jc*, *Junonia coenia*, *Cs*, *Cupiennius salei*, *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*. The *AS-C* genes we focused are colored with gray.

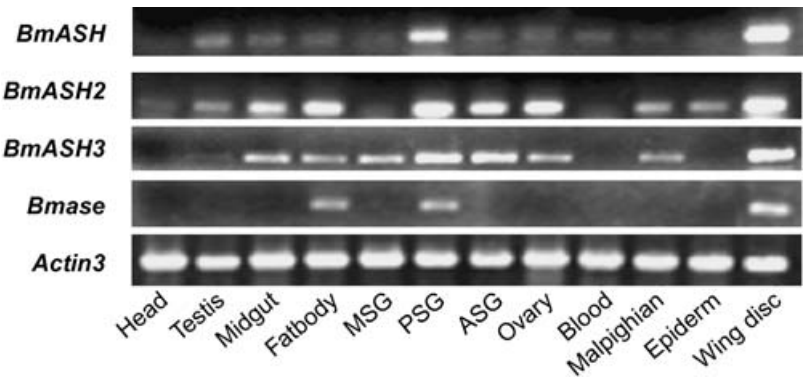

Figure 4. The expression profiles of four *AS-C* genes in various tissues of the silkworm. They were analyzed by semi-quantitative RT-PCR. *Actin3* gene, as internal control. ASG, MSG and PSG are short for the anterior, middle and posterior silk glands respectively.

5th instar larva, including the ovary, testis, head, epiderm, midgut, malpighian tubule, fat body, hemocyte, wing disc, anterior (ASG), middle (MSG), and posterior (PSG) silk glands. The results indicated that the silkworm *AS-C* genes were distinctly expressed in different tissues (Fig 4). The PSG and wing disc were the only two tissues expressed all of the four genes. Furthermore, their expression levels in wing disc were obviously higher than in other tissues. Interestingly, *BmASH* appeared to be more tissue-specific expression, its expression was very low or nearly undetectable in other tissues except PSG and wing disc. However, *Bmase* could also be detected in fat body beside PSG and wing disc. *BmASH2* and *BmASH3* were more widely expressed than *BmASH* and *Bmase*. These results

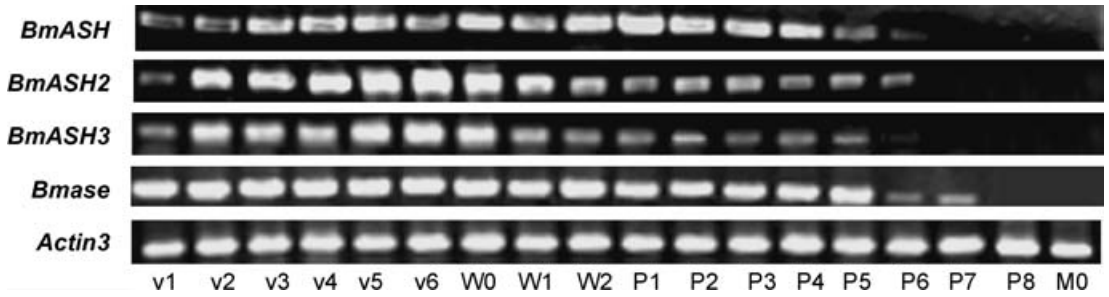

Figure 5. The expression profiles of four *AS-C* genes in the silkworm during wing development. *Actin3* gene, as internal control, was also analyzed under the same experimental conditions. V1–V6 represent days of the 5th instar larva, W0–W2 represent days after the beginning of wandering, P1–P8 represent days after pupation. M0 represent the day at beginning of moth.

suggested that the silkworm *AS-C* genes might play different roles in different tissues, and that they all may play very important roles in PSG and wing disc. Alternatively, the analysis might partially reflect their spatial expression since only one stage was used in our analysis.

The expression profiles of four AS-C *genes in the silkworm during wing development*

The highest expressions of the silkworm *AS-C* genes were detected in the wing disc. So we examined the expression profiles of the four *AS-C* genes during wing development in the silkworm by semiquantitative RT-PCR. The total RNA samples from silkworm wing discs or pupal and adult wings after every day from the 5th instar larva to the first day adult were extracted (Fig. 5). This period is selected because it is important for wing maturation and wing will experience enormous changes on morphology during the metamorphosis.

The results indicated that the four *AS-C* genes were all expressed at high abundance in wing disc, but their expression profiles were slightly different. As shown in Fig. 5, the four genes all began to be expressed from the first day of the fifth instar larvae and continued up to the end of the pupal stage. The expression profiles of *BmASH2* and *BmASH3* were almost the same. They had a low expression level during the beginning of the 5th instar larvae and reached the peak at the end of larvae, just before the wandering, then followed by a gradual reduction. No expression was detected for *BmASH2* and *BmASH3* on the seventh and sixth day after pupation, respectively. The expression peak of the *BmASH* appeared at the onset of pupation, gradually decreased thereafter, and ended on the seventh day after pupation, which was similar to the observation in a previous study (Zhou *et al.*, 2006). However, *Bmase* maintained high expression levels from the first day of the 5th instar larvae to the fifth day after pupation, and decreased largely on the sixth and seventh days after pupation. No expression was detected on the eighth day of pupal stage. Generally, the highest expressions of the four genes appeared at the beginning of wandering or pupal stages, which were two important transitional periods of wing development.

BmASH *RNAi phenotypes*

RNA interference (RNAi) is the process of sequence-specific post-transcriptional gene silencing triggered by double stranded RNAs (dsRNAs) homologous to the target genes. RNAi was used to evaluate the function of one silkworm *AS-C* genes, *BmASH*, during early scale precursor cell differentiation in the wing. Based on the gene expression profile of *BmASH*, we deemed the first day after the silkworm spinning (W1) as the best time for dsRNAs injection, meanwhile, we also performed the injection with *BmASH* dsRNAs on the first day of 5th instar (V1), but the result was not significant (data not shown). Phenotypes of the injected individuals were examined during the moth stage.

To examine *BmASH* expression level in the wings injected with the dsRNA was significantly reduced or not, we performed real-time RT-PCR analyses of *BmASH* expression for mRNAs from the wings injected by *BmASH* dsRNA, DEPC ddH_2O, and the non-injected ones on the first day of pupae. As shown as Fig 6, we found that gene silencing

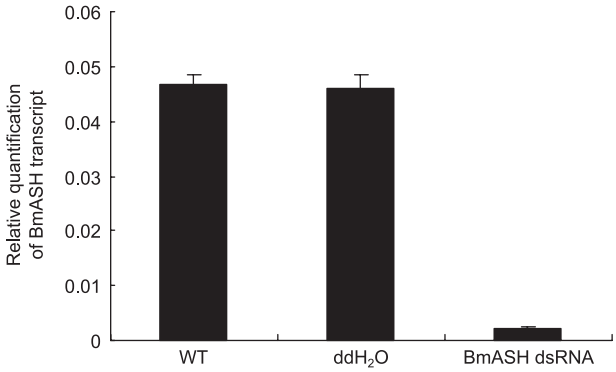

Figure 6. *BmASH* expression was detected using real-time RT-PCR analysis after *BmASH* dsRNA treatment. Relative quantification of *BmASH* of the samples treated with *BmASH* dsRNA is obviously lower than that of in the unmanipulated (*WT*) and treated with DEPC ddH_2O silkworms taken as the control on the first day of the pupal stage. Expression levels were normalized to the expression of the silkworm *BmActin3* gene.

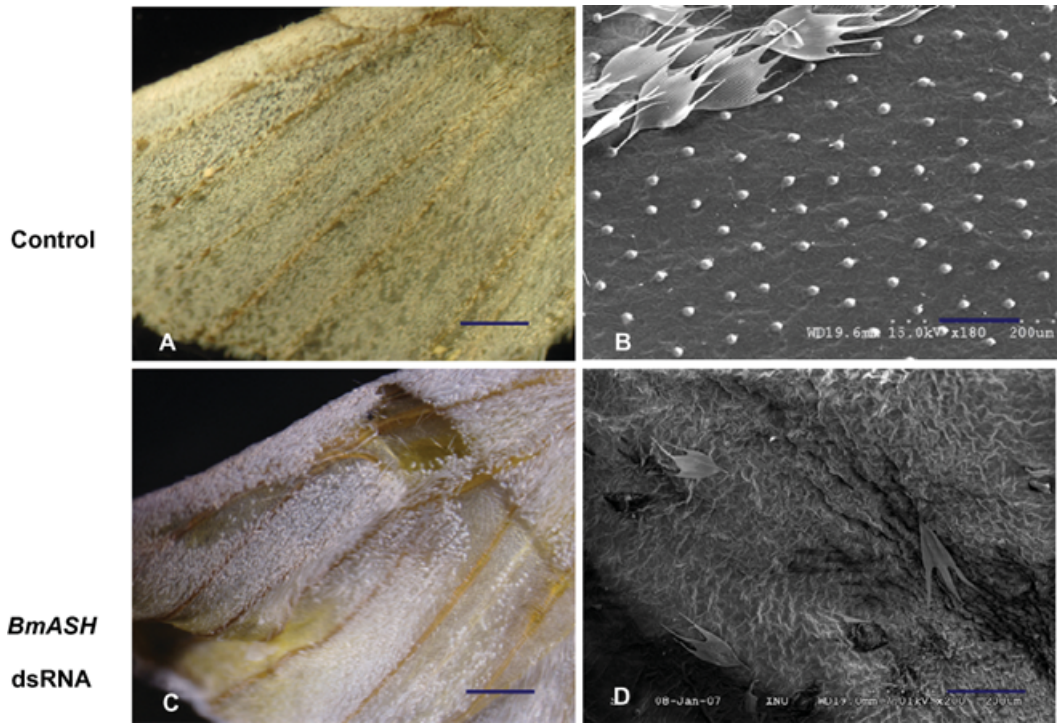

Figure 7. Silkworm *BmASH* RNAi phenotypes: Phenotypic changes in scales formation induced by *BmASH* dsRNA (C, D) injection with the ddH_2O (A, B) as the control. A and C are the phenomenon of the wings amplified by stereoscope. B and D are the partly wing of A and C under the scanning electron microscopy, most scales of control (B) were washed off before observation, sockets (white dots) are arranged compactly, while the wings injected by *BmASH* dsRNA (D) appear in their natural state, without washing. There are quite fewer scales and sockets in *BmASH* dsRNA injected wing than in control. Bars 1 mm in (A) and (C), 100 μm in (B) and (D).

with the injection of *BmASH* dsRNA does not fully inhibit the expression of the target gene, however, the *BmASH* mRNA was significantly reduced after RNAi treatment compared with the controls. The loss of *BmASH* expression in these experiments is unlikely to arise from non-specific targeting of *BmASH2*, *BmASH3* or *Bmase*, because *BmASH* share 22.2–44.3% nucleotide sequence similarity with others in the ORF, and 52.3–60.0% in the conserved bHLH region.

In our experiment, 92% and 96% of total individuals treated by *BmASH* dsRNA and DEPC ddH_2O survived to eclosion, respectively. Of the 92 adults treated by the *BmASH* dsRNA, 21 (22.8%) individuals showed unsuccessful scale development, the remaining individuals had no abnormal phenotype. No abnormal wing phenotype was observed in the adults treated by the DEPC ddH_2O and in the non-injected adults as controls. On the abnormal wings, only a part uncovered with scales on the surface, the remaining area had the normal phenotype (Fig. 7). To determine that the phenomenon was not caused by the physical damage during the molting, we examined the socket arrangement on the part surface of the wings without scales on the surface by scanning electron microscopy. In addition, we used the normal wings that be carefully washed off most of the scales as a control. The results showed that there were many sockets visible in the normal wings, in contrast, only few sockets and scales were present in the area of uncovered scales on the wings treated by *BmASH* dsRNA. The surface of these areas had more wrinkles than the normal (Fig. 7D). These phenotypes were never observed in silkworm adults injected with a control DEPC ddH_2O, suggesting that they were specific to *BmASH* dsRNA injection. Only a small proportion of injected individuals showed discernible phenotype, presumably due to incomplete inactivation of the gene or function complementation within the complex. Our results indicated that *BmASH* had obvious effects on the development of scales on the silkworm wing.

Discussion

Evolution of AS-C *genes in insects*

Our results suggested that there are four *AS-C* genes in the silkworm. Among them, *BmASH*, *BmASH2*, and *BmASH3* did not show one-to-one relationship with the three proneural genes *ac*, *sc*, and *l'sc* in *Drosophila* based on amino acid sequence similarities. However, they showed higher similarities with l'sc protein than others. *Bmase* was more closely related to *Drosophila ase* than other *AS-C* genes. Our analysis suggested that *BmASH*, *BmASH2*, and

BmASH3 were proneural genes and *Bmase* was an *ase* homolog.

Drosophila has an *AS-C* complex with four genes. The genome structure of the complex has been described in details (Campuzano *et al*., 1985). Although *AS-C* genes have been identified in several other insect species, a little is known about the genomic organization of *AS-C* genes outside the Diptera. In this study, we analyzed the genomic organization of *AS-C* complex in the silkworm and compared it with the *Drosophila*, *Anopheles*, *Tribolium* and *Apis AS-C* complexes (Fig. 2). The four *AS-C* genes in the silkworm exist in a complex scattered over ~300 kb on chromosome 13. They are organized in tandem arrays in the genome. In *Drosophila*, the four *AS-C* gene complex spans ~100 kb region at the distal tip of the X-chromosome, while the *Anopheles AS-C* complex contains *AgASH* and *Agase* which are 22 kb apart (Skaer *et al*., 2002). The *Tribolium AS-C* complex contains one proneural *AS-C* gene, *TcASH* and one *ase* homolog, *Tcase*, which reside 55 kb apart from each other. The two *Apis AS-C* genes, *AmASH* and *Amase*, lie about 43 kb apart. We found that the *AS-C* complex spanned larger genome region in silkworm than in other insects, probably this is due to the fact that there are larger intergenic regions in the silkworm genome (Xia *et al*., 2004). In addition, the silkworm genome size (432 Mb) (The International Silkworm Genome Sequencing Consortium, 2007) is considerably larger than those of *Drosophila melanogaster* (180 Mb) (Adams *et al*., 2000), *Anopheles gambiae* (278 Mb) (Holt *et al*., 2002), *T. castaneum* (200 Mb) (Alvarez-Fuster *et al*., 1991) and *Ap. mellifera* (238 Mb) (The Honeybee Genome Sequencing Consortium, 2006).

In a comparison of *AS-C* genome regions among insects, we also found a conserved syntenic structure that a *yellow* gene and a *cyt P450* reside at two ends of the *AS-C* complex (Fig. 2). *Yellow* gene lies upstream of the complex and the *cyt P450* resides the downstream in most insects. In the case of *Anopheles*, it has a different structure: the *cyt P450* resides between the *AS-C* genes and the *yellow* gene lies downstream of *Agase*. Moreover, in these insects, *AS-C* complex showed the same arrangement pattern that the *ase* lied downstream of the proneural genes. In *Drosophila*, *Anopheles*, *Tribolium*, and *Apis*, all of the *AS-C* genes were transcribed in the same orientation. In the silkworm, however, the *BmASH2* was transcribed in the different orientation from other *AS-C* genes, suggesting that an inversion might have occurred in the corresponding region. In general, the structure comparison suggests that the organization of the subregion containing *AS-C* complex and its neighboring genes is a general feature of most insects.

Based on the previous reports about the *AS-C* complex in *Drosophila*, *Tribolium* and *Anopheles*, only silkworm and *Drosophila* have four genes in the *AS-C* complex (Alonso & Cabrera, 1988; Wulbeck & Simpson, 2002; Wheeler *et al*., 2003). With respect to homologies among the four *AS-C* genes in *Drosophila*, *ac* is more closely related to *sc*, and then to *l'sc*, and distant from *ase*. The order of the four genes in the genome is *ac*, *sc*, *l'sc*, and *ase* (Campuzano *et al*., 1985). For the four *AS-C* genes in the silkworm, they show the similar order to those in *Drosophila* (Fig. 2). Moreover, there is no other predicted gene among the silkworm *AS-C* complex genes. In contrast, an unrelated gene *pepsinogen-like (pcl)* inserted between *l'sc* and *ase* in *Drosophila*. Probably, the insertion of the *pcl* gene in *Drosophila AS-C* complex occurred recently, because there is no similar insertion happened in other insects.

Previous studies suggested that there is an increase in gene copy number of *AS-C* genes in the Diptera lineage during insect evolution (Skaer *et al*., 2002). Phylogenetic analysis showed that the ancestral *AS-C* gene have independently divided into *proneural*-like and *ase*-like genes in the insect groups. If so, one would expect *ase* to be present in *J. coenia*. Within the three proneural gene in silkworm, *BmASH2* and *BmASH3* seem to be close relatives each other based on the amino acid identity. This might suggest that *BmASH* is more ancient. Notably, *JcASH1*, the single *AS-C* homologue found in the butterfly *J. coenia* was clustered closely with *BmASH2* but not with the more ancient gene *BmASH* in our phylogenetic tree. This observation indicates that there might be other two proneural genes to be found in *J. coenia*, or because the single *ASH* can fulfill the complex roles (Galant *et al*., 1998). In summary, our data also indicated that the number of genes in the complex also expanded in Lepidoptera or at least in the silkworm.

A conserved role for AS-C *homolog in the development of the silkworm wing*

Morphological, cell biological, developmental and molecular evidence indicated that Lepidopteran scales and insect sensory bristles are homologous structures (reviewed in Galant *et al*., 1998; Reed, 2004). It has been demonstrated that, in *Drosophila*, the *AS-C* genes exhibit significant abilities to promote the differentiation and formation of sensory bristles (Cubas *et al*., 1991; Brand *et al*., 1993). The *AS-C* homolog in the butterfly, *J. coenia*, *JcASH1*, expressed both in the wing disc of the fifth instar and the wing blade during pupation, and is essential for wing scale formation (Galant *et al*., 1998). In this study, we investigated gene expression of the four *AS-C* genes in silkworm using semi-quantitative RT-PCR. The results showed that the four genes were all expressed at high abundance in the wing disc, indicating that they may play important roles in the wing formation.

In *Drosophila*, the *ac* and *sc* genes share cis-regulatory sequences, expression studies showed that they are expressed in identical patterns (Skeath & Carroll, 1991; Gomez-Skarmeta *et al*., 1995). In silkworm, the *BmASH2*

and *BmASH3* were also coexpressed as that in *Drosophila*. However, the *Bmase* showed a consistently high expression at the beginning of the 5th instar larvae, earlier than the other *AS-C* genes. It is different from the expression of *ase* in *Drosophila*, in which it expressed after the proneural genes *ac* and *sc*. The result indicated that there might be some slight function distinctions between silkworm *ase* and *Drosophila ase* during the wing development. In butterfly, the expression of *JcASH1* was detected the following 24 h after pupate, the critical period when the first scale precursors are formed (Galant *et al*., 1998). These results strongly suggested that the expression profiles of the four *AS-C* homologes in wing discs were mostly correlated with the development of the scales in silkworm.

The peak levels of the *BmASH* appeared at the onset of pupation, which was the time of the first scale precursors formed in butterfly. To examine whether the *BmASH* is essential for the wing scales formation in the silkworm, we used RNAi to reduce the expression of *BmASH* in wing disc at the late stage of the larvae and then assayed scale precursor formation molecularly by *BmASH* expression after injection and by morphological examination. We observed the correlation between reduce of *BmASH* expression and the absence of scales (Fig. 6). It demonstrated that *BmASH* was essential for the scales differentiation and formation in silkworm. Based on the expression patterns of the other three silkworm *AS-C* genes during the wing development and previous references on the roles of *AS-C* genes in *Drosophila* and butterfly, we supposed that the *BmASH2*, *BmASH3* and *Bmase* might be also involved in the process of the development and differentiation of the scales in silkworm.

Our data indicated that the silkworm *AS-C* complex expanded compared with other insects, resulting in the four *AS-C* genes that arose from duplication events. Ohno (1970) suggested that gene duplication could enable one copy to acquire new functions while the other performs the original function. In this study, through RT-PCR analysis, the expressions of the *AS-C* genes could also be detected in the non-neural tissues in the silkworm, indicating that the *AS-C* genes might have other functions in the silkworm besides their roles in the scales formation. This is consistent with the hypothesis that descendant of the gene duplication may obtain additional roles compared with their ancestor. It is concluded that the data presented in this study provide some new insights into evolution and functions of the *AS*-C genes in the silkworm.

Materials and methods

Insects

B. mori larvae of the N4 strain (maintained in our laboratory) were reared at 25 °C under a photoperiod of 12 h of light and 12 h of dark for the experiments. Under these conditions, most fifth instar larvae began wandering in the late photophase of day 6–6.5 and became pupae 3 days after they began to wander.

Alignment and phylogenetic analysis

Complete AS-C protein sequences of vertebrates (*Mus musculus*, *Homo sapiens*), insects (*J. coenia*, *B. mori*, *T. castaneum*, *Ap. mellifera*, *Ae. aegypti*, *An. gambiae*, *Calliphora vicina*, *Ceratitis capitata*, *Musca domestica*, *Scaptodrosophila lebanonensis* and *D. melanogaster*) and *Cupiennius salei* were downloaded from GenBank (http://www.ncbi.nih.gov/GenBank/). In order to compare equivalent regions, the bHLH domains were retrieved using their secondary structure prediction from SMART. As many as 71 amino acids of AS-C protein sequences spanning the bHLH domain and nearly 17 amino acids of the conserved carboxyl terminus were aligned using Clustal X (Thompson *et al*., 1997). Phylogenetic tree was reconstructed using MEGA v3.0 (Kumar *et al*., 2004) with the neighbor-joining (NJ) method and 1000 bootstrap samples.

Expression-profile analysis of silkworm AS-C *genes by RT-PCR*

To investigate the spatial expression pattern of the *AS-C* genes, we used 12 silkworm tissues on the sixth day of 5th instar larva, including the ovary, testis, head, epiderm, midgut, malpighian tubule, fat body, hemocyte, wing disc, anterior (ASG), middle (MSG), and posterior (PSG) silk glands. To show expression of the silkworm *AS-C* genes in wings at different developmental stages, silkworm wing discs or pupal and adult wings were dissected out at given stages. The tissues were stored immediately in RNASafer™ (Omega Bio-Tek; Norcross, GA, USA) for RNA extraction. Total RNA was extracted using E.Z.N.A.™ MicroElute™ Total RNA Kit (Omega) according to the manufacturer's protocol. Two micrograms of total RNA was used to synthesize first-strand cDNA using M-MLV Reverse Transcripts e (Promega, Madison, MI, USA) as the protocol described.

RT-PCR was performed for 25 cycles. The primers designed for *BmASH* (GenBank accession no. NP_001037416.1) were 5′-ATG CCG ATG GCG GCG ATT CAC-3′ (sense) and 5′-TCA CTG TTG TTG CCA CCA C-3′ (anti-sense), for the *BmASH2* gene (GenBank accession no. ABR20839.1) were 5′-ACA CGA CAC TCC ACC GAA-3′ (sense), and 5′-AGA CAC ACC CGA AGA ACA-3′ (anti-sense), for *BmASH3* (GenBank accession no. ABR20840.1) were 5′-GCG TTG AGG AAA AGA CTG-3′ (sense) and 5′-CTT GCC ACC ACG AGA TAG-3′ (anti-sense), for *Bmase* (GenBank accession no. ABR20841.1) were 5′-GCG TCA GTC AAC AAG GC-3′ (sense) and 5′-TTT TCG GGA AGA ATA AGA T-3′ (anti-sense), and for *Bm-actin A3* (GenBank accession no. X04507) gene were 5′-AAC ACC CCG TCC GCT CAC TG-3′ (sense) and 5′-GGG CGA ACT GTG ATT TCC T-3′ (anti-sense), yielded 582, 469, 391, 989 and 637

bp-length bands, respectively. The amplification of *Bm-actin A3* cDNA sequence was taken as the inner control (Mounier & Prudhomme, 1986), and the quantitative RT-PCR was repeated three times.

Construction of dsRNA and Injection

To evaluate the functional role of *BmASH* in silkworm wing during early scale precursor cell differentiation, we produce dsRNA of *BmASH* for RNA interference by ligating the 582 bp fragment including the ORF of it into the double T7 vector pL4440 double T7 script II (Timmons *et al*., 2001). Double stranded RNA was produced from this clone using T7 RNA polymerase (Promega), the plasmid DNA was then removed by using DNaseI from the RNase-free kit (Promega), the RNA transcripts were precipitated by ethanol precipitation, quality and quantity of the dsRNA were checked by electrophoresis on an agarose gel and spectrophotometer, respectively. About 50 ng of dsRNAs (or DEPC ddH_2O for negative controls) were injected into per wing disc of silkworm on the first day after spinning using a IM300 microinjector (Narishige, Tokyo, Japan). Approximately 200 silkworms at this stage were treated with *BmASH* dsRNA. The injected silkworms were reared under standard conditions. Half of the injected silkworm at the first day of the pupa stage after injection and non-injected silkworms were used for the gene expression analysis. The remains were reared under standard conditions and developed to adulthood.

Detection of BmASH *expression after dsRNA injection by real-time RT-PCR*

Pupal wings were dissected from the sampled treated with *BmASH* dsRNA, with DEPC ddH_2O and from non-injected silkworms. Total RNA was extracted with E.Z.N.A.™ MicroElute™ Total RNA Kit (Omega) and subjected to cDNA synthesis using oligo(dT) primers and a M-MLV Reverse Transcriptase (Takara, Tokyo, Japan) according to the manufacturer's instructions. The resultant cDNAs were examined by real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Forster City, CA, USA) with a SYBR Premix EX Taq_ kit (Takara) according to the manufacturer's instructions. The expression of *BmASH* was examined, PCR primers used in the real-time PCR were as follows: for detection of the *BmASH*: 5′-GCC CCA TCA TTC GTG TCT-3′ (sense) and 5′-GCT CCA TCG GGT CTT CCT-3′ (anti-sense), for detection of *Bm-actin A3* (GenBank accession no. X04507) gene as a control was 5′-CAT GAA GAT CCT CAC CGA GCG-3′ (sense) and 5′-CGT AGC ACA GCT TCT CCT TGA TA-3′ (anti-sense).

Analysis of RNAi phenotypes by SEM

The wings of the adults were observed with stereoscope and the socket cells were examined with the scanning electron microscopy (SEM). A number of non-injected silkworms, together with some ones treated by *BmASH* dsRNA and DEPC ddH_2O were examined. Most scales of the controls were washed off before observation by SEM. The wings were cut in the appropriate size and mounted onto SEM 'stubs' with adhesive tape and then sputter coated with 30 nm gold. The samples were visualized on a scanning electron microscope and the images were captured.

Acknowledgements

This work was supported by the National Basic Reaserch Program of China (No. 2005CB121000), Hi-Tech Research and Development (863) Program of China (No. 2006AA10A117), Technological Innovative Foundation Project for postgraduates of Southwest University (No. 2006052).

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