A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae

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Abstract **We examined the role of carotenoid-binding protein (CBP) in yellow cocoon pigmentation. First, using yellow or white cocoon races, we investigated the linkage between the yellow pigmentation and CBP expression. CBP was expressed only in the silk gland of the yellow cocoon races, which utilize carotenoids for cocoon pigmentation. Furthermore, CBP expression in the silk glands of day 1–7 fifth instar larvae matched the period of carotenoid uptake into the silk gland. Finally, we gave double-stranded CBP RNA to *Bombyx mori* (*B. mori*) larvae to induce RNA interference. The significantly reduced expression of CBP in the silk gland of fifth instar larva was confirmed on day 4 and a decrease in yellow pigmentation was observed in the cocoon. We showed that CBP plays a key role in the yellow cocoon pigmentation caused by carotenoids.**

Keywords: Carotenoid-binding protein; StAR family; Cocoon pigmentation; Cocoon color; Lipophorin; Camouflage

1. Introduction

There are many varieties of the silkworm *Bombyx mori* (*B. mori*), and these produce brilliantly colored yellow, pink, golden-yellow, flesh, sasa (yellowish green) and green cocoons. The pigments in yellow, pink, golden-yellow and flesh cocoons are derived from carotenoids [1], while those of sasa and green are from flavonoids [2,3]. These pigments are absorbed from mulberry leaves, transferred from the midgut to the silk gland via the hemolymph, and are accumulated in the layers of the cocoon sericine [4]. Consequently, the cocoon color is determined by the ''features of pigment transport'' into the tissue or hemolymph.

Carotenoids transport in the hemolymph involves a lipoprotein called lipophorin, which serves as a reusable shuttle transferring carotenoids from one tissue to another [5,6]. Lipophorin transports carotenoids that interact with the lipid transfer particle into the hemolymph [7].

In *B. mori,* the loci of the genes involved in pigment transport have been determined [8].

Carotenoids transport involves three genes: the *Y*, *I*, and *C* genes. The *Y* gene colors the hemolymph yellow and may control the uptake of carotenoids from the midgut lumen into the midgut epithelium. The *I* gene is a suppressor gene that down-regulates the yellow coloring of the hemolymph and controls carotenoids transfer from the midgut epithelium into the hemolymph. The *C* gene colors the cocoon golden-yellow by controlling the uptake of carotenoids from the hemolymph to the silk gland. However, the *Y*, *I* and *C* genes products that are involved in cocoon pigmentation are not clear. Only larvae with the *Y*, *+I* (recessive allele of the *I* gene), and *C* genes make yellow cocoons.

A previous study described a link between the expression of CBP and the existence of the *Y* gene and suggested that CBP plays a crucial role in cocoon pigmentation with carotenoids [9].

An analysis of the deduced amino acid sequence of CBP revealed its homology to StAR protein. The C-terminus of CBP contains the StAR-related lipid transfer (START) domain [10,11]. Therefore, CBP is a novel member of the StAR protein family, but its physiological function in the pigmentation of the *B. mori* cocoon is unclear.

Lutein-binding protein (LBP) has also been identified from the *B. mori* midgut [12]. LBP is a second intracellular carotenoid transfer protein in *B. mori,* but it is also not clear whether LBP is involved in cocoon pigmentation.

In this paper, we examine the physiological roles of CBP and present evidence that CBP is a key protein in the yellow pigmentation of the cocoon.

2. Materials and methods

2.1. Materials

The protease inhibitor mixture was purchased from Roche Molecular Biochemicals. p123-T vector was purchased from Molecular Bio Technology Co. Ltd. RNase and the MEGAscript High Yield Transcription kit T7 and T3 were purchased from Ambion. Polyvinylidene difluoride (PVDF) membrane, horseradish peroxidase-conjugated anti-rabbit IgG goat serum, and the immunodetection kit were purchased from Bio-Rad. The T4 DNA ligation kit was purchased from TaKaRa.

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The QIAprep Miniprep Plasmid DNA Purification kit was purchased from Qiagen. The PCR products purification kit was purchased from Promega. The ECL Western blotting detection system was purchased from Amersham Pharmacia Biotech.

2.2. Insects

Bombyx mori of the N4 race was reared on artificial diet (containing mulberry powder, Yakult, Japan) at 25 °C on a 12-h light/12-h dark photoperiod. *B. mori* of the FL50×FL501 yellow cocoon race was reared on mulberry leaves. We studied various races of *B. mori* with different cocoon colors: c440 (pink), e21 (whitish sasa), N13 (yellow), N15 (white), N16 (golden-yellow), N17 (deep sasa), N21 (golden-yellow), N71 (white), and N72 (pastel yellow). These races were reared on fresh mulberry leaves.

2.3. Detection of CBP expression in the silk gland by immunoblotting

To show CBP expression in the silk gland during the fifth instar larval stage, silk glands were dissected between day 0 (shortly after the fourth ecdysis) and day 7 (one day before pupation).

To show the link between cocoon color and CBP expression, we dissected silk glands from day 4 fifth instar larvae of the following races: c 440, e21, N13, N15, N16, N17, N21, N71 and N72.

Each silk gland was weighed and homogenized in three volumes of PBS (containing protease inhibitor mixture), using a Polytron homogenizer, and was centrifuged at $10\,000 \times g$ for 15 min. The concentration was determined using the Bradford method [13].

For immunoblotting, 25 μg of protein in the supernatant was separated by SDS–PAGE and stained with Coomassie Brilliant Blue R250 or transferred to PVDF membrane using the method of Bjerrum and Schafer–Nielsen [14], and immunoblotted using rabbit anti-CBP antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase. The filters were developed using a horseradish peroxidase color-development kit.

2.4. Detection of APN-1 expression in brush border membrane vesicles (BBMV) in the midgut using immunoblotting

BBMV from the midgut of fifth instar larvae of *B. mori* were prepared according to the method described by Wolfersberger et al. [15]. BBMV proteins were separated using SDS–PAGE, transferred to a PVDF membrane using the method of Bjerrum and Schafer-Nielsen [14], and immunoblotted using rabbit anti-APN-1 antibody, and goat anti-rabbit IgG conjugated with horseradish peroxidase. The filters were developed using the ECL Western blotting detection system.

2.5. Construction of dsRNAs

To produce the CBP for RNA interference, a 308-bp PCR fragment of CBP was amplified using CBP cDNA[1] as template and the primers 5′-ATGGCCGACTCTACGTCGAAAAGC G-3′ and 5′-TCCCCTG-ACGGTCCCTCGCTTCTCC-3′, and cloned into p123-T vector.

To produce aminopeptidase N-1 (APN-1) dsRNA for RNA interference, a 458-bp PCR fragment of APN-1 was amplified using APN-1 cDNA[2] as template and the primers 5′-TTCTTCTGGAATCG-CTACCTTCAGGAAGAT-3′ and 5′-TAGCCT GACTCGAAG-TAGTTCGTGAATTCT-3′, and cloned into p123-T vector.

The cloned fragments were amplified using M 13 forward and reverse primers. The PCR products were purified using a PCR purification kit and used as template to generate sense (sRNA) and antisense (asRNA) RNA using a T7 and T3 MEGA script kit. To generate dsRNA, equal amounts of sRNA and asRNA were mixed, heated at 95 °C for 3 min, and gradually cooled to 25 °C. Then, the solution was treated with RNase for 1 h at room temperature. The dsRNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in PBS. One microgram of dsRNA was analyzed using 1% agarose gel electrophoresis to ensure that the majority of the dsRNA existed as a single band of approximately 300 or 450 bp, respectively.

[1] The oligonucleotides employed in this study were designed on the basis of the CBP nucleotide sequence (GenBank/DDBJ SAKURA Data Bank Accession No. AB062740).

[2] The oligonucleotides employed in this study were designed on the basis of the APN-1 nucleotide sequence (GenBank/DDBJ SAKURA Data Bank Accession No. AF084257).

2.6. Injection of dsRNA, observation of cocoon pigmentation, and estimation

Thirty micrograms of dsRNA was injected intrahemocoelically into day 0 fifth instar larvae using micro-injector (1710 TLL, Hamilton). PBS was injected as a control. The silk glands or midgut of several insects were dissected after 4 days (day 4 fifth instar) for immunoblotting and the expression of CBP or APN-1 in each treatment was quantified using densitometry. Other insects were allowed to grow and pupate, and cocoon color was observed.

3. Results and discussion

3.1. CBP expression in silk glands producing yellow cocoons

Variations in cocoon coloration result from the proportions of lutein, β-carotene, α-carotene, and other xanthophylls in the cocoon [16]. Lutein is the most important determinant of cocoon color. For example, yellow (Fig. 1A, 3) and pastel yellow (Fig. 1A, 8) cocoons contain greater than 80% lutein, while golden-yellow (Fig. 1A, 5 and 7) cocoons contain 60% lutein (1). For these cocoon colors, CBP likely plays a key role in cocoon pigmentation because it binds lutein strongly [9].

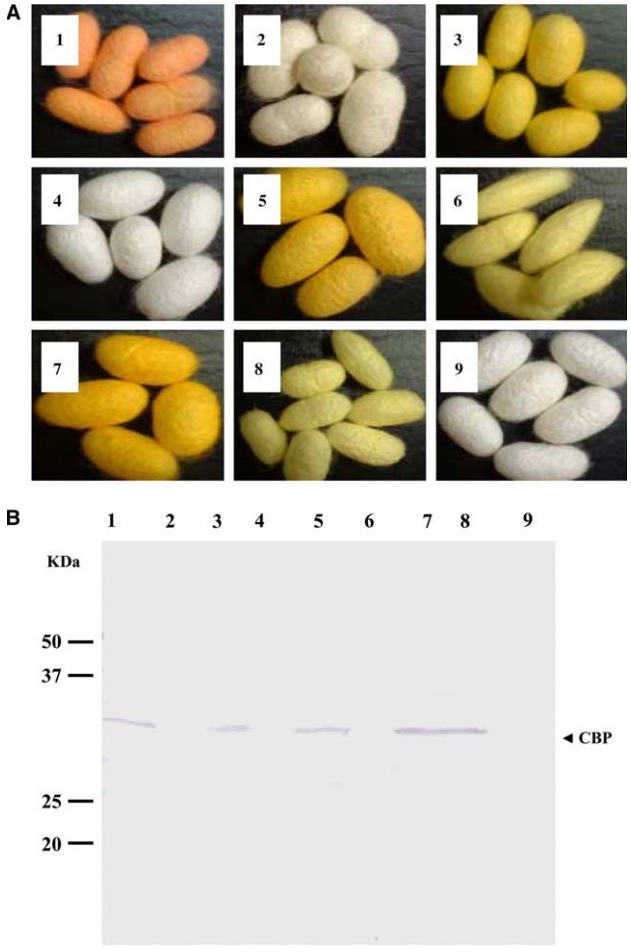

Fig. 1. Expression of CBP in races with yellow cocoons. A, Races with different cocoon colors. 1, c440 (pink cocoon); 2, e21(whitish sasa); 3, N13 (yellow); 4, N15(white); 5, N16 (golden-yellow); 6, N17 (deep sasa); 7, N21 (golden-yellow); 8, N72 (pastel yellow) and 9, N71 (white). B, Immunoblot analysis of CBP expression in the silk gland of races producing different cocoon colors. Silk gland was dissected from day 4 of the fifth instar larvae. Protein samples (25 μg) were separated by SDS–PAGE, transferred to PVDF membranes, and immunoblotted.

To determine the role of CBP in the coloration of carotenoid-containing cocoons, protein samples from the silk glands of larvae from seven yellow and two white cocoon races (Fig. 1A) were analyzed using immunoblotting with anti-CBP antibody (Fig. 1B).

CBP was found in the silk glands of the yellow cocoon races (Fig. 1A and B, 1, 3, 5, 7 and 8). In the cocoons from races N13, N16, N21 and N72, lutein is the main component of the yellow pigment [1]. Lutein contributed 88% of the carotenoids extracted from the purified CBP of race N4, which produces yellow cocoons [9]. Further, rCBP can bind lutein [9]. By contrast, CBP was not found in the silk gland of the races producing flavonoid-containing cocoons (e21 and N17) or white cocoons (N15 and N71) (Fig. 1A and B, 2, 4, 6, and 9). Therefore, CBP plays a role in cocoon pigmentation due to lutein.

CBP exists in the silk gland of c 440 (Fig. 1A and B, 1) in which the carotenoids are 90% β-carotene and 8% lutein [1]. β-Carotene is an important component of cocoon pigmentation and it forms a complex with CBP from the silk gland. CBP from the silk gland of race N4, which makes yellow cocoons, contains 9% β-carotene [9]. This implies that CBP plays a role in the transport of β-carotene in the silk gland and is involved in cocoon pigmentation by β-carotene.

Therefore, it was thought that CBP might play a role in the transport of several carotenoids in the silk gland or in other tissues of *B. mori*.

3.2. Linkage of CBP expression and silk gland pigmentation

In a yellow cocoon race of *B. mori*, the cells of the silk gland begin to take up most of the carotenoids from the hemolymph at the fifth instar stage [17,18]. Pigmentation changes in the silk gland were first observed at day 3 in fifth instar larvae (Fig. 2A, 4). The intensity of yellow, which presumably depended on the carotenoid uptake, gradually increased until day 6 (Fig. 2A, 7) and then plateaued (Fig. 2A, 8).

In order to examine the relationship between carotenoid uptake into the silk gland and CBP expression, we collected silk glands from fifth instar larvae to quantify the expression of CBP by immunoblotting. CBP was detected from days 1 to 7 in fifth instar larvae, and the expression peaked on day 4 (Fig. 2B). The expression of CBP was consistent with the increase in the density of yellow color in the silk gland.

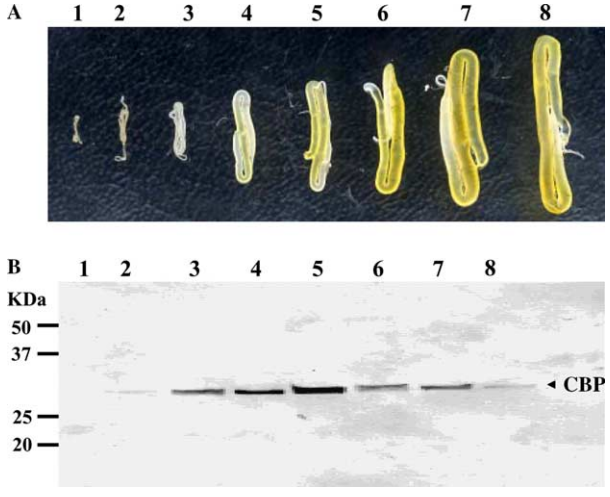

Fig. 2. Pigmentation and expression of CBP in the silk gland during the fifth instar larval stage. A, Pigmentation of the silk gland during the fifth instar larval stage. Each day from day 0 (shortly after the fourth ecdysis) to day 7 (one day before pupation), silk glands were dissected from fifth instar larvae. B, Immunoblot analysis of CBP expression in the silk gland during the fifth instar larval stage. Protein samples (25 μg) were separated by SDS–PAGE, and transferred to PVDF membranes, and immunoblotted; 1–8 are days 0–7, respectively.

3.3. CBP gene silencing by RNAi and its effect on yellow cocoon pigmentation

To evaluate the functional role of CBP in cocoon pigmentation, we used RNA interference by injecting CBP dsRNA to suppress the production of CBP in *B. mori*.

As shown in Fig. 3A, we synthesized sense and anti-sense ssRNA from the N-terminal region of CBP, which did not include the START domain, using a MEGAscript ssRNA synthesis kit. The ssRNA was annealed to generate dsRNA, and 30 μg/insect was injected into the hemolymph of day 0 fifth instar larvae using a micro-injector.

Four days after dsRNA injection, the silk glands were dissected from several insects, and the amount of CBP was estimated using immunoblotting. The band patterns on a polyacrylamide gel stained with Coomassie brilliant blue R250 were similar for the control and for larvae injected with 30 μg of dsRNA (Fig. 3B, 1, 2, 5, and 6). However, injection of 30 μg dsRNA resulted in an 80% reduction in CBP levels (Fig. 3B, 4 and 8) versus controls (Fig. 3B, 3 and 7) in almost every silk gland tested.

Furthermore, to evaluate whether this reduction in the CBP level was specific, we used aminopeptidase N-1 (APN-1) dsRNA. APN-1 is a glycoprotein from *B. mori* midgut that is suspected of being the *Bacillus thuringiensis* Cry1Aa toxin receptor [19]. Injection of 30 μg of APN-1 dsRNA resulted in equal CBP levels in controls and in almost every silk gland tested (Fig. 3C, 1 and 2). Conversely, the APN-1 levels were reduced to 15% of the control levels (Fig. 3C, 3 and 4). These results show that injecting CBP dsRNA specifically reduces the CBP level.

Other insects were allowed to grow and pupate, and the effect of dsRNA injection was measured by the reduction in cocoon color density. An injection of 30 μg of dsRNA resulted in marked reduction in the density of cocoon color as compared to controls (Fig. 3D). We also examined the effect of a 10-μg injection of dsRNA, which reduced cocoon color density less than that of a 30-μg injection (data not shown). Furthermore, injection of 30 μg APN-1 dsRNA did not affect the density of cocoon color (data not shown).

Even with an injection of 30 μg CBP dsRNA, CBP expression was not inhibited completely. It has been reported that gene silencing with the injection of dsRNA does not fully inhibit the expression of the target gene in insects [20,21]. Moreover, a massive amount of mRNA is produced in the silk glands of fifth instar larvae [22], which might cause incomplete inhibition of CBP expression.

It is also possible that other proteins affect cocoon pigmentation and function in the silk gland. Another carotenoid-binding protein has been identified in *B. mori*, an LBP that was purified from the *B. mori* midgut [12]. The purified LBP binds only lutein and is expressed in the midgut and silk gland throughout larval development. However, precise information on the role of LBP in carotenoid transfer and cocoon pigmentation is lacking.

Bombyx mori absorbs carotenoids from mulberry leaves. Of the dietary carotenoids contained in mulberry leaves, 70% are lutein, and the other components are β-carotene, α-carotene, and other xanthophylls [4]. In *B. mori,* lipophorin plays a role

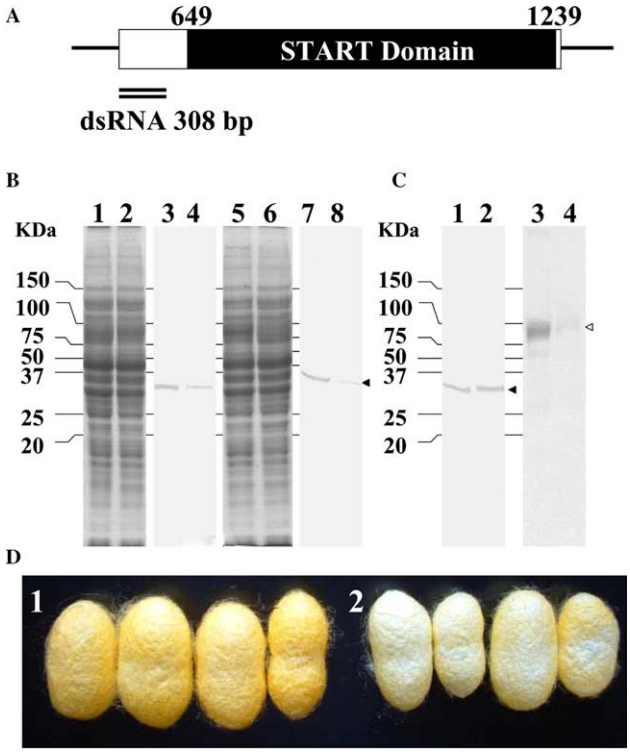

Fig. 3. Injecting CBP dsRNA to silence the CBP gene and its effect on the yellow cocoon pigmentation. A, Source of the dsRNA used for RNA interference of CBP. The structure of the silkworm CBP cDNA. The 308-bp dsRNA was prepared from the N-terminal region of CBP cDNA denoted by the double lines. B, Immunoblot analysis of CBP expression in the silk glands of larvae injected with CBP dsRNA; 30 μg of CBP dsRNA (2, 4, 6 and 8) or PBS (1, 3, 5 and 7) was injected into day 0 fifth instar larvae (shortly after the fourth ecdysis), and the silk glands were dissected on day 4. The proteins (25 μg) from each silk gland were separated by SDS–PAGE and stained with Coomassie brilliant blue R250 (1, 2, 5 and 6) or transferred to a PVDF membrane (3, 4, 7 and 8). CBP was detected using rabbit anti-CBP serum; 1–4 are representatives of the first trial; 5–8 are representatives of the second trial. C, Immunoblot analysis of CBP expression in the silk glands or APN-1 expression in the midgut of larvae injected with APN-1 dsRNA; 30 μg of APN-1 dsRNA (2 and 4) or PBS (1 and 3) was injected into day 0 fifth instar larvae (shortly after the fourth ecdysis), and the silk glands or midgut were dissected on day 4. The proteins (25 μg) were separated by SDS–PAGE and transferred to a PVDF membrane. CBP was detected using rabbit anti-CBP serum and APN-1 was detected using rabbit anti-APN-1 serum. D, Phenotypic changes in yellow cocoons induced by CBP dsRNA injection. The cocoon color was observed after cocoon formation. 1, Yellow control cocoon; 2, cocoons of fifth instar larvae injected with 30 μg of CBP dsRNA on day 0 were of a paler color. The closed arrowhead indicates the 33-kDa band of CBP and the open arrowhead indicates the broad 115-kDa band of the glycoprotein APN-1.

in the transport of absorbed carotenoids in the hemolymph. Purified lipophorin from the yellow-cocoon race N4 contained lutein (99%), β-carotene (0.6%), and α-carotene (0.2%) [8]. By contrast, purified CBP contained lutein (88%), β-carotene (9%), and α-carotene (3%), which is consistent with the carotenoid composition of lipophorin and mulberry leaves. Furthermore, the distribution of CBP in the midgut and silk gland has been examined using immunohistochemistry [9,23]. CBP was found in midgut and silk gland cells. These results suggest that carotenoids from mulberry leaves are absorbed by midgut cells, captured under the membrane of the microvilli, and transported through the cells by CBP. After transport in the hemolymph by lipophorin, the carotenoids are again received by CBP in silk gland cells, probably relying on the help of receptors such as the lipophorin receptor [24–27], and transferred through the silk gland cells by CBP, to accumulate in the layers of cocoon sericin, causing cocoon pigmentation.

In this study, we provided direct and indirect evidence that CBP is a protein that is involved in cocoon pigmentation by carotenoids in *B. mori*.

In future studies, we will investigate whether CBP alone regulates cocoon color.

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