RESEARCH

Transcription Analysis of the Beta-Glucosidase Precursor in Wild-Type and *l-4i* Mutant *Bombyx mori* (Lepidoptera: Bombycidae)

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ABSTRACT. Lethal fourth-instar larvae (*l-4i*) mutant of *Bombyx mori*, a recently discovered novel mutant, die from energy depletion due to genetic mutation. Beta-glucosidase is a common digestive enzyme that hydrolyzes cellulose in the diet to provide energy. In this study, the mRNA expression profiles of *B. mori* beta-glucosidase precursor (*BmpreBG*) were characterized by reverse transcription polymerase chain reaction and quantitative real-time polymerase chain reaction. The transcription level of *BmpreBG* varied in different tissues and developmental stages, except in the pupa and moth, which are the no-diet period. Remarkably, the mRNA expression level of *BmpreBG* was sharply reduced in *l-4i* but not in the wild type, which suggested that the digestive function of the mutant was severely damaged. This was consistent with the *l-4i* phenotypic traits of not eating mulberries, lack of energy, and ultimate death. 5′-rapid amplification of cDNA ends showed, for the first time, that *BmpreBG* has a 160-bp 5′-untranslated region. These findings suggested that *B. mori* β-glucosidase precursor was involved in the death process of *l-4i* mutant larvae.

Key Words: β-glucosidase precursor, *BmpreBG*, lethal mutant

Glycoside hydrolases selectively catalyze the hydrolysis of glycosidic bonds in oligosaccharides, polysaccharides, and their conjugates (Liu et al. 2005). Among them, beta-glucosidase (BG, EC 3.2.1.21) is a common cellulose hydrolase in bacteria, fungi, protists, plants, and animals, which catalyzes the hydrolysis of β-glucosidic linkages of various oligosaccharides and glycosides to form glucose and shorter/debranched oligosaccharides (Zhang et al. 2009, Singhania et al. 2013). In insects, digestive β-glucosidases are vital for hydrolysis of di- and oligo-β-saccharides derived from hemicelluloses and cellulose in the diet. In addition, β-glucosidases play an important role in the interaction between insects and plants (Mattiacci et al. 1995, Marana et al. 2000).

Lethal mutant in the fourth-instar larva (*l-4i*) is a novel lethal mutant that is discovered during rearing of silkworm (*Bombyx mori*) strain P_{33}. Compared with the normal larva, the mutant grows and develops slowly, resulting in a smaller body shape and poor vitality after day 2 of third instar as well as the duration of the third instar is extended by about 2 d. The fourth newly exuviated larva barely take mulberry leaves and almost stop growth and development and begin to die on the 3rd to 4th day of the fourth instar (Fig. 1) (Kang et al. 2015).

The *l-4i* mutant that dies from energy depletion is attributed to the genetic mutation, so the *B. mori* β-glucosidase precursor (*BmpreBG*) which is associated with sugar metabolism aroused our interest. To verify the relationship between the *BmpreBG* and the phenotype of the *l-4i* mutant, the transcription profiles of *BmpreBG* were investigated by reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) in this study. Interestingly, qPCR analysis revealed that the mRNA expression level of *BmpreBG* decreased significantly in the midgut of *l-4i* mutant at three successive developmental stages, while such decreases were not observed in the wild type. Our results contribute to the comprehensive understanding of the underlying mechanism of *l-4i* mutation.

Materials and Methods

Materials. *B. mori* strain C108 (standard silkworm strain), the wild-type P_{33}, and *l-4i* mutant strain were supplied by the Sericultural Research Institute (Zhenjiang, China). The larvae were reared on fresh mulberry leaves at 25 ± 2°C under a photoperiod of 12:12 (L:D) h and 65 ± 5% relative humidity.

RNA Extraction and cDNA Synthesis. Larvae at different developmental stages as well as the egg, pupa, and moth, and various tissues (trachea, midgut, ovary, hemocytes, testis, fat body, malpighian tubule, epidermis, and silk gland) from day 3 in the fifth-instar larvae of silkworm strain C108, as well as the midguts of the wild-type and *l-4i* mutant larvae on day 2, molting of third instar and the fourth newly exuviated larva were dissected, frozen in liquid nitrogen immediately, and stored in RNA-free Eppendorf tubes at −80°C for later use. Total RNA was extracted from frozen samples using an RNeasy mini kit (Qiagen, Germany), treated with RNase-free DNaseI (Takara, China) for 20 min at 37°C, purified with phenol–chloroform, precipitated with ethanol, and finally dissolved in DEPC-treated ddH_2O. The cDNAs were synthesized using M-MLV RTase (Promega, USA) and an oligo-dT primer, according to the manufacturer's instructions.

Transcriptional Analysis of *BmpreBG* by RT-PCR. To determine the tissue and development specificity of *BmpreBG*, mRNA expression in the larvae, trachea, midgut, ovary, hemocytes, testis, fat body, malpighian tubule, epidermis, and silk gland of the day 3 in the fifth-instar larvae, as well as the egg, larvae from first to fifth instar, pupa, and moth of strain C108 were analyzed by RT-PCR, which was performed using the following primers: *BmpreBG*-F: 5′ATGCGCTTGATGCCGGA ATT 3′; *BmpreBG*-R: 5′GCGGATTTTCTTGGAGTACG 3′. A 284-bp fragment of *B. mori* cytoplasmic actin gene *A3*, corresponding to nucleotides 680–963 (GenBank accession no: X04507), was amplified in parallel with each RNA sample as an internal control for adjustment of template RNA quantity. Intensities of the bands were quantified with Gel-Pro Analyzer (version 4.5).

Quantitative Real-Time PCR. To compare the *BmpreBG* transcription levels in the midguts of the wild-type and *l-4i* mutant at the three developmental stages mentioned above, qPCR was carried out using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA). *BmpreBG* mRNA and Bm-actin *A3* mRNA were quantified

Fig. 1. Body size comparison of lethal mutation in the fourth-instar larva (*l-4i*) (right) and normal larva (left) of the normal variety at day 3 of the fourth instar of silkworm.

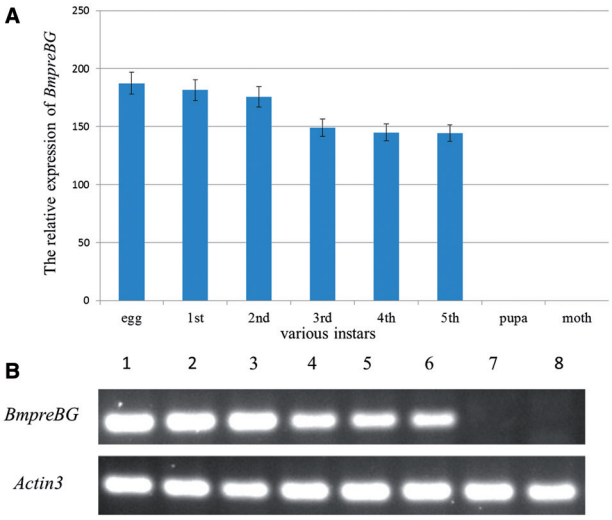

Fig. 2. RT-PCR analysis of *BmpreBG* at different developmental stages. The graph shown in (A) was obtained from the image in (B) by quantification using Gel-Pro Analyzer. (B) Lines 1–8 represent the egg, the first-, second-, third-, fourth-, and fifth-instar larvae, the pupa, and the moth, respectively. Bm-actin *A3* was used as the internal control to normalize the amount of template in the PCR reaction.

using 2 µl of the reverse transcription reaction (equivalent to 100 ng single-stranded cDNA) as a template in the qPCR. A 151-bp product for *BmpreBG* cDNA corresponding to nucleotides 1240–1391 was amplified using the following primers: forward 5′-ATGCGCTTGATGCCG GAATT-3′ and reverse 5′-GCGGATTTTCTTGGAGTACG-3′. qPCR was carried out in a 20-µl reaction mix using SYBR Green Supermix (Takara), according to the manufacturer's instructions. The thermal cycling profile consisted of initial denaturation at 94°C for 5 min; and 40 cycles at 94°C for 30 s, 58°C for 25 s, and 72°C for 35 s. All reactions were performed in triplicate, and the relative expression level was analyzed using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$, and CT refers to the cycle threshold (Livak and Schmittgen 2001).

Cloning of the Open Reading Frame and Putative Promoter, and 5′-Rapid Amplification of cDNA Ends in the Wild-Type and *l-4i* mutant. To compare the gene sequence between the wild-type and the mutant, we tried to get the open reading frame (ORF), putative promoter, and untranslated region (UTR) sequences. The *BmpreBG* specific primers, forward primer (5′-GGCTGATAGCTCGTCTGTTT-3′) and reverse primer (5′-TTCACGAGTCGTTGATGGTC-3′) were designed to amplify the ORF of the putative *BmpreBG* gene (SilkDB accession no. BGIBMGA005602-TA). The PCR reaction was carried out with 30 amplification cycles (94°C for 30 s, 58°C for 25 s and 72°C for 90 s) in an ABI2720 96-well thermocycler (Applied Biosystems). To obtain the 5′-UTR (d'Alencon et al. 2010) of the *BmpreBG* cDNA, a gene-specific primer for 5′-rapid amplification of cDNA ends (RACE, 5′-GGGGCTTTAGCCGTACTTCCATATCC-3′) was designed, following the SMART RACE cDNA Amplification Kit (Biosciences Clontech, USA). Specific PCR was performed using the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 60°C for 25 s and extension at 72°C for 1 min. The promoter sequence was predicted by the 5′-UTR and the BDGP tool (http://tools.genome.duke.edu/generegulation/McPromoter/). About 1 kb of sequence containing the predicted promoter was extracted using specific primers based on the genome sequence: forward primer: 5′-CAATCTGCTAACGCATT TATCTG-3′ and reverse primer: 5′-CCAGGCACCTTCAATTTGATA AG-3′. All PCR products were cloned into vector pMD18-T (Takara), transformed into *Escherichia coli* strain Top10 and sequenced.

Results

Developmental and Tissue-Specific Transcription Pattern of *BmpreBG*. The *BmpreBG* transcription levels at various developmental stages were examined by RT-PCR. The results indicated that mRNA was detectable from the egg to the fifth-instar larvae, except in the pupa and moth (Fig. 2). To confirm the expression of *BmpreBG* gene at transcriptional level, RT-PCR was performed using mRNA prepared from trachea, midgut, ovary, hemocytes, testis, fat body, malpighian tubule, epidermis, and silk gland, respectively. The results showed that the mRNA of *BmpreBG* gene could be detected in the most of tissues: the midgut had the highest level, whereas the epidermis had the lowest (Fig. 3).

Transcription Analysis of *BmpreBG* in the Midgut of the Wild-Type and the *l-4i* at Various Developmental Stages. The mutant larvae have smaller body sizes and slower growth rates after day 2 of the third instar; therefore, qPCR was conducted to analyze the mRNA expression pattern of the *BmpreBG* in the midgut of the wild-type and *l-4i* on day 2 and the molting of third instar and the fourth newly exuviated larva. The results showed that *BmpreBG* transcription was downregulated significantly in all the examined developmental stages of *l-4i*, while this trend was not observed in the wild type. Besides, the expression level of *BmpreBG* in the wild-type midgut at different stages was gluttonous stages > newly molted silkworm > newlymolting silkworm (Fig. 4).

Cloning and 5′-RACE. The *BmpreBG* cDNA contains a 1,482 bp ORF encoding a protein of 493 amino acids with a calculated molecular mass of 56.28 kDa. The ORF begins with the initiation codon ATG at 161 bp, ends with TGA at 1,640 bp; therefore, the 5′-UTR is 160 bp (Fig. 5). Amplification from genomic DNA produced a PCR product containing the putative promoter that was about 1 kb upstream the initiation codon (Fig. 6). By comparing these sequences between the wild-type and *l-4i* mutant, there was no sequence variation among the putative promoter, 5′-UTR and ORF.

Discussion

The cellulose digestive system of herbivorous insects has been intensely investigated. β-glucosidase is a digestive enzyme associated with cellulose degradation in termite species (Hirayama et al. 2010, Scharf et al. 2010, Uchima et al. 2011). In addition, endogenous

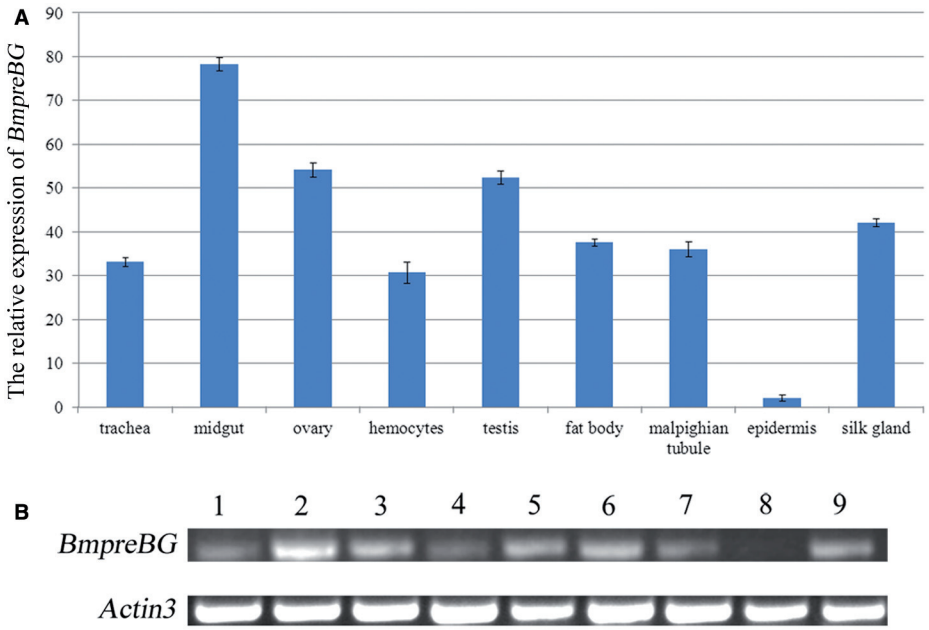

Fig. 3. Expression profile of *BmpreBG* in different tissues of *B. mori.* The graph shown in (A) was obtained from the image in (B) by quantification using Gel-Pro Analyzer. (B) Lines 1–9 represent trachea, midgut, ovary, hemocytes, testis, fat body, malpighian tubule, epidermis, and silk gland. Bm-actin *A3* was used as the internal control.

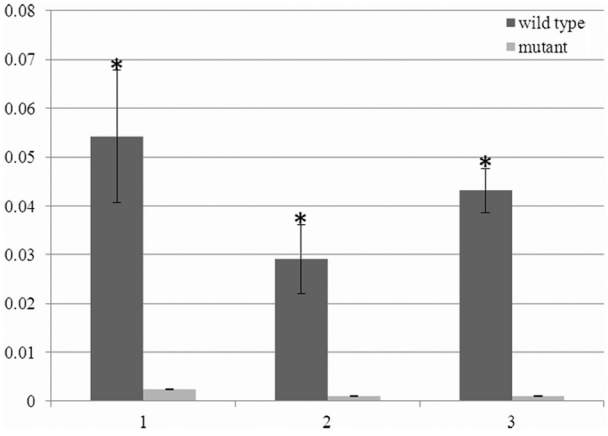

Fig. 4. Relative expression levels of *BmpreBG*, as determined by quantitative real-time PCR. Each real-time PCR analysis was repeated at least three times for each set of RNA samples. Each point represents the mean value ± SD. The relative amounts of *BmpreBG* were determined using the Bm-actin *A3* as a standard. *Significant difference ($P < 0.05$) compared with the mutant.

β-glucosidases have been purified from many orders of insect species such as coleopteran and orthopteran species (Pontoh and Low 2002, Yapi et al. 2009) and function as digestive enzymes in Lepidoptera *Spodoptera frugiperda* (Marana et al. 2001), *B. mori* (Byeon et al. 2005), Blattodea *Leucophaea maderae* (Cornette et al. 2003), and Coleoptera *Tenebrio molitor* (Ferreira et al. 2001).

RT-PCR analysis showed that *BmpreBG* could be detected from egg to the fifth-instar larvae at about the same level, except in the pupa and moth. The reason may be that the pupa and moth barely take mulberry leaves and do not require digestion for energy, while the larval stage is significant period for growth (Byeon et al. 2005). In this report, the extremely low mRNA expression level of *BmpreBG* in the *l*-4*i* mutant verified that *BmpreBG* is associated with digestion or even energy metabolism. There have been several relevant studies on the relationship between downregulated β-glucosidase levels and the no-diet stage or starvation on Lepidoptera, such as *B. mori* (Byeon et al. 2005) and *S. frugiperda*, *Diatraea saccharalis* (Ferreira et al. 1997). Analysis of *BmpreBG* mRNA in different tissues showed that it could be detected in almost all tissues, moreover, it is the midgut that has the highest expression level, which is an important organ for digestion and absorption in the silkworm (Jiang et al. 2013). In most insects, however, the midgut is the major site of β-glucosidase expression (Terra 1996).

qPCR was conducted to examine the transcription pattern of the *BmpreBG* in midgut of the wild and *l*-4*i* mutant larvae. Remarkably, we found that the mRNA expression level of *BmpreBG* in the midgut of wild type was significantly higher than in the *l*-4*i* mutant, while the transcription of *BmpreBG* gene in the mutant can hardly be detected, suggesting that the digestive function of the mutant would be severely damaged, which was consistent with the mutant phenotype of not taking mulberry leaves, exhaustion and death. Indeed, the β-glucosidase usually have derived from the midgut or salivary glands and its main function is involved in processing of dieting and feeding for digestion in other insects, such as *Nephotettix cincticeps* (Uhler) and *Nasutitermes takasagoensis* (Shiraki) (Tokuda et al. 1997, 2002), which might be implied that the *l*-4*i* mutant dead from wear-out of energy.

To analyze the reason for the decreased transcription level of *BmpreBG* in *l*-4*i* mutant, we obtained the ORF and putative promoter sequences by cloning and the 5′-UTR by RACE. However, there was no sequence difference between the wild-type and *l*-4*i* mutant, which suggested that the down-regulation of *BmpreBG* transcription in *l*-4*i* mutant may be affected or regulated by other genes or factors in the mutant. After all, a phenotype is result of a series of pathways.

These results hinted that *BmpreBG* might be associated with the death of the mutant by switching off the energy supply in the mutant, although no evidence is available to clarify its mechanism at present. Taken together, it is suggested that attenuated *BmpreBG* expression may be related to the *l*-4*i* mutant phenotype in this study; however, its precise biochemical function and possible role in this process remain to be determined.

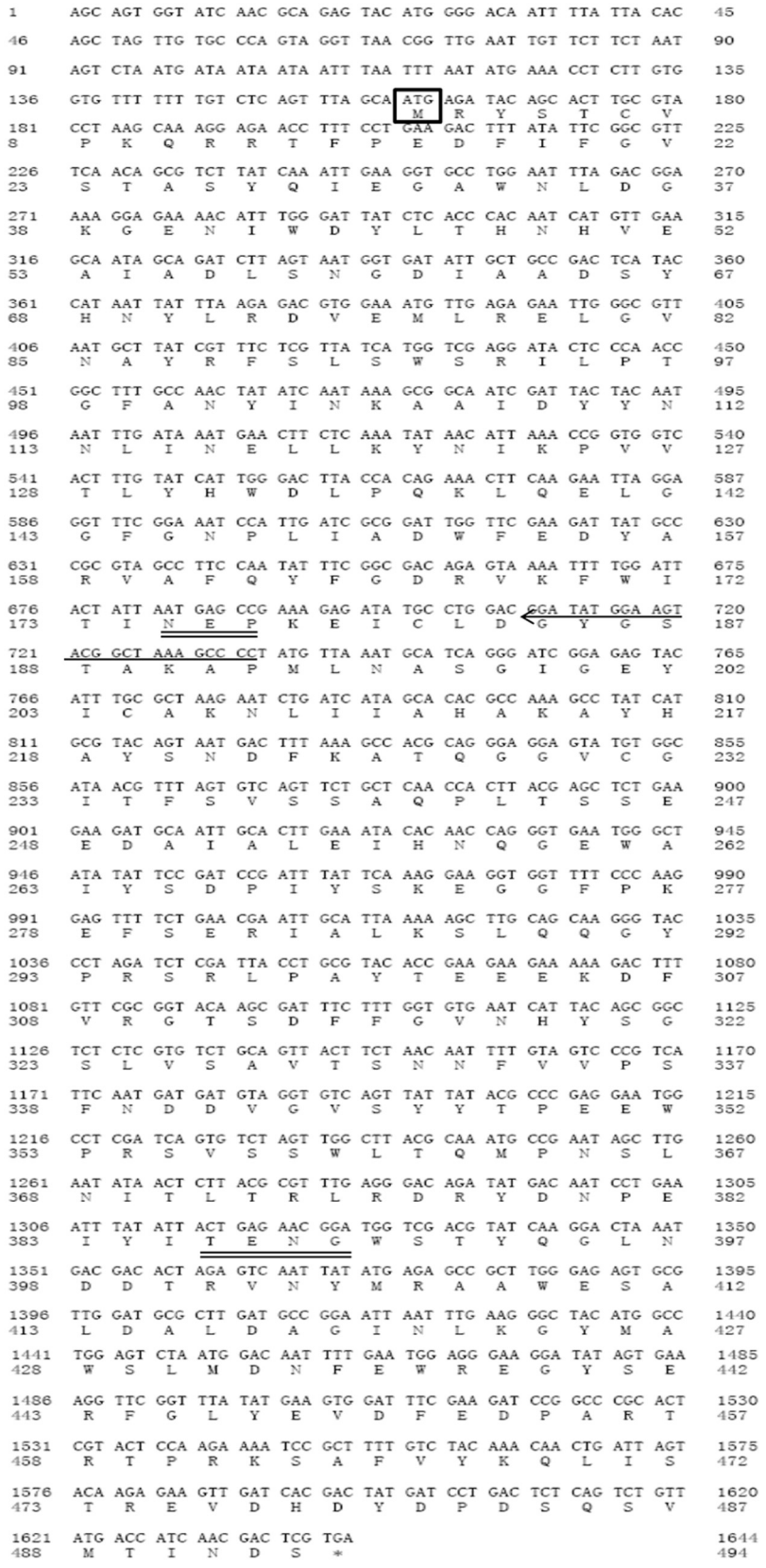

Fig. 5. ORF, 5′-UTR sequences, and deduced amino acid sequence of *BmpreBG*. The start codon is boxed. An asterisk (*) represents the stop codon. An arrow indicates the position and direction of the 5′-RACE primer. The amino acid residues marked with double underlines indicate glutamic acid residues responsible for catalysis.

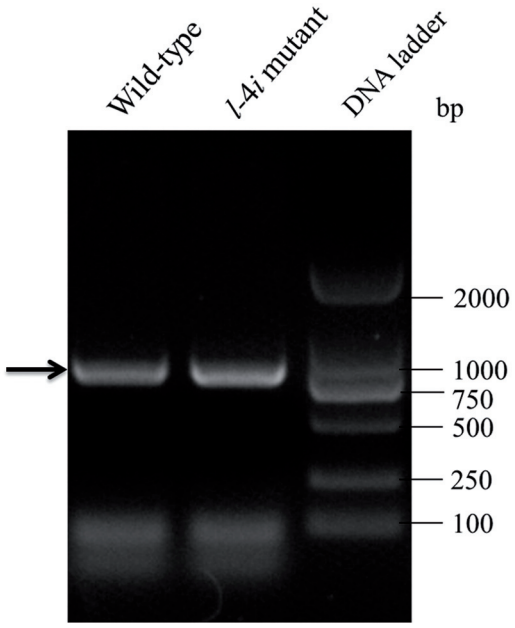

Fig. 6. PCR product from the *BmpreBG* promoter. M represents the DL2000 marker and an arrow indicates the PCR product. Lines 1 and 2 represent the wild-type and *l*-4*i* mutant.

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