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Isolation of Two cDNAs Encoding a Putative Peptidoglycan Recognition Protein Gene from the Domestic Silkworm, *Bombyx mori*

Sang Hyun Kim*, Heui Sam Lee, Jin Won Kim, Young Sin Lee, Iksoo Kim, Kang Sun Ryu, Jun Yang[1] and Minoru Yamakawa[1]

Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-100, Korea.

[1]National Institute of Animal Science, Ohwashi 1-2, Tsukuba 305-8634, Japan.

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Peptidoglycan recognition protein (PGRP) is one of the pattern recognition proteins in innate immunity of insect. We isolated differentially expressed two cDNAs, BTL-LP1 and BTL-LP2, in the fat body of *Bombyx mori* larvae injected with bacteria by subtractive hybridization method. These two clones showed amino acid sequence divergence of 30.4%. In the comparison with other insect PGRP genes, BTL-LP2 showed 48.8% and 45.2% of sequence homology to the known PGRP genes of *Bombyx mori* and *Tricoplusia ni*, respectively, and BTL-LP2 was 31.8% and 30.9%, respectively. Phylogenetic analysis showed relatively close relationship of the BTL-LP2 to the known insect PGRP, unlike BTL-LP1, which was equidistant both to insect and mammals, suggesting a divergent relationships of the two newly cloned *B. mori* PGRP genes. Northern blot analyses confirmed an induction of the expression of BTL-LP2 by the bacterial infection in the fat body of *B. mori*, suggesting the involvement of the gene in the insect immunity.

Key words : Peptidoglycan recognition protein gene, PGRP, Silkworm, *Bombyx mori*, Insect immunity

Introduction

Insects possess highly effective defense mechanisms against invading micro-organisms such as bacteria, fungi and viruses. The respective defense mechanism consists of cellular and humoral responses. The former involves phagocytosis, encapsulation and nodule formation by plasmatocytes and granulocytes (Dunn, 1986), whereas the latter includes the phenol oxidase activating cascade and immune proteins such as lysozymes, lectins, antibacterial proteins and antifungal proteins (Boman and Hultmark, 1987).

Immune response in insects begins with the recognition of the pathogens as foreigners. This type of recognition is termed pattern recognition, as opposed to clonal recognition, in which clonally selected immunoglobulins are employed. Three types of cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PG) and β-1,3 glucan (βG) presented in the bacteria and fungus were reported and these are recognized by pattern recognition proteins that are present in the insect plasma as free-floating molecules or on the cell surface as receptors (Ashida *et al.*, 1983; Yoshida *et al.*, 1986; Ratcliffe *et al.*, 1991). LPS binding molecules and the cellular receptor CD14 are well known to elicit immune reactions (Schumann *et al.*, 1990; Gegner *et al.*, 1995), but the pattern recognition molecules for PG and βG have not been characterized as thoroughly as those for LPS (Jomori *et al.*, 1990). In the innate immune system of insects, bacterial cell envelope substances activate both the phenol oxidase cascade and the genes for humoral antibacterial effector proteins (Ashida *et al.*, 1983; Kawabata *et al.*, 1995; Yoshida *et al.*, 1996; Chosa *et al.*, 1997; Jiang *et al.*, 1998; Ochiai *et al.*, 1988; Satoh *et al.*, 1999).

In the present study we used a subtractive hybridization method to identify new components of the immune system in the domestic silkworm, *Bombyx mori*, and report presence of two PGRP homologue cDNAs, BTL-LP1 and BTL-LP2 , that is up-regulated after bacterial infection.

*To whom correspondence should be addressed.
Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea. Tel: +81-31-290-8463; Fax: +82-31-295-2176; E-mail: SH.Kim@rda.go.kr

Materials and Methods

Animals, immunization and preparation of poly(A)+ RNA

The domestic silkworm, *Bombyx mori* (Tokai x Asahi strain) was reared on an artificial diet (Nihonnosanko) at 25 as usual. The larvae on the 3rd day of the 5th instar were injected with 20 μl (1×10^5 cells) of the heat-killed (120, 20 min) *Escherichia coli* (JM 109 strain) suspended in the physiological saline (150 mM NaCl, 5 mM KCl). Fat bodies were excised from the larvae 2 h after injection. Untreated larvae were used as a control experiment. Poly (A)+ RNAs were isolated from the fat bodies using Quick Prep mRNA Purification kit (Pharmacia, Piscataway, NJ).

Subtractive hybridization

cDNA subtraction was essentially performed according to the manufacturer's protocol using PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA). Briefly, first-strand cDNAs were synthesized from 2 μg of poly (A)+ RNA from the fat body of the immunized larvae for tester and from control larvae for driver. Following second-strand cDNA synthesis, both tester and driver cDNAs were digested with *Rsa*I, and adaptors 1 and 2 were ligated only to the tester. The tester cDNA was hybrdized twice with an excess driver cDNA. After hybridizations, differentially expressed cDNAs were selectively amplified utilizing nucleotide sequences of both adaptor 1 and 2 as primers for suppression PCR amplification. Nested PCR amplicons were subcloned into pCR 2.1 Vector using an Original T/A Cloning kit (Invetrogen). A subtracted plasmid library was constructed and the clone inserts were checked using colony PCR with nested primers. All clones containing inserts were sequenced using an automatic sequence analyzer (ABI model 377 DNA sequencer; Applied Biosystems).

3'-and 5'-RACE

The 3'-and 5'-rapid amplifications of cDNA ends (3'-and 5'-RACE) were performed on a GeneAmp PCR System 2400 (Perkin Elmer) to obtain longer sequences of the cDNA fragments of interest. At first, mRNA was isolated from the fat body of larvae 3 hrs after immunization as described above. The first strand cDNA was synthesized using a First Strand cDNA Synthesis kit (Pharmacia), and the 3'-RACE PCR was carried out using a specific oligonucleotide primer corresponding to the nucleotide sequence of each cDNA clone and *Not*I d(T)18 primer (Pharmacia). PCR was performed for 35 cycles under the following conditions: 94 for 30 s, 55 for 30 s and 72 for 30 s. The 5'-RACE was carried out according to manufacture's instruction using a 5'-RACE kit (GIBCO). The specific antisense oligonuclotides from the clones were used as primers in the 5'-RACE.

Northern blot analysis

B. mori larvae (3 days of the 5th instar) were injected with lipopolysaccharide (50 μl of 1 mg/ml). Fat bodies were excised for 2~12 hrs after injection. Fat bodies from untreated larvae were used as controls. Total RNA was isolated from the samples using an Isogen kit (Nippon Gene Co.). Northern blotting was carried out according to manufacture's protocol (Boehringer Mannheim, Mannheim, Germany). Total RNA samples were electrophoresed on 1.2% agarose containing 6.6% formaldehyde and transferred onto a Gene Screen Plus membrane (Dupont). The strip was prehybridized at 50 for 1h and hybridized overnight at the same temperature with cDNA probes from the clones of interest. The probes were labelled with digoxigenin using a PCR DIG Labeling Mix (Boehringer Mannheim).

Phylogenetic analysis

PAUP (Phylogenetic Analysis using Parsimony) ver. 3. 0 (Swofford, 1990) was used to infer possible phylogenetic relationships among peptidoglycan recognition protein (PGRP) gene sequences obtained newly from this study and GenBank search. GenBank accession numbers of the sequences utilized for phylogenetic analysis are as follows: the fruit fly, *Drosophilla melanogaster* PGRP (AAF48056; Adames *et al.*, 2000), cabbage looper, *Trichoplusia ni* PGRP (AAC31820; Kang *et al.*, 1998), *B. mori* PGRP (BAA77209, Ochiai and Ashida, 1999), Arabian camel, *Camelus dromedarius* PGRP (CAC19553; unpublished), and Norway rat, *Rattus norvegicus* PGRP (NP_445825; unpublished). The phylogenetic tree was obtained by bootstrap analyses (1,000 replications) with the tree presentation option of "retain groups compatible with 50% majority-rule consensus" in the heuristic search.

Results and Discussion

To identify immune-related clones, we employed the subtractive hybridization technique based on PCR amplification. After two consecutive rounds of subtractive hybridization and reamplification, the tracer-enriched cDNA fragments were subcloned and further complemented to full-length sequences by 3'-and 5'-RACE. In this manner, we have identified two different clones, designated BTL-LP1 and BTL-LP2. BTL-LP1 is composed of 208 amino acids residues (627 base pairs) with a calculated molecular mass of 23,120 Da (Fig. 1). On the other hand, BTL-LP2 had 195 amino acid residues containing a putative

```
1     GAATTCGGCTTGCGGATCCATGTTTCTGTC ATTTTGTATTTTCATAGTTTTTTGTGCTTA
                        M  F  L  S   F  C  I  F  I  V  F  C  A  Y     14

61    CACTTCGAGCCATCCTCGGCTTATCGAAAA AGATCATCTATCCGTGGATTTCCCGGTTTG
        T  S  S  H  P  R  L  I  E  K   D  H  L  S  V  D  F  P  V  C     34

121   CTCACGCGACTGCTGGGGCGCTGTTCCTTC AAAAGACACAAGACCTCTGAACAAGCCAGT
        S  R  D  C  W  G  A  V  P  S   K  D  T  R  P  L  N  K  P  V     54

181   GCCCTACGTCATTATACATCACACCGCTAT TCCGACTGTATGCAACACCACAACCCGATG
        P  Y  V  I  I  H  H  T  A  I   P  T  V  C  N  T  T  T  R  C     74

241   TATGAGAGATATGCGAAGCATGCAGAAATA CCACAATTCCTTAGGCTGGGGTGACATTGG
        M  R  D  M  R  S  M  Q  K  Y   H  N  S  L  G  W  G  D  I  G     94

301   ATACCATTTCTGCGTGGGCGGCGATGGGGT GGCGTACGAAGGCCGCGGGTGGAACGTCAT
        Y  H  F  C  V  G  G  D  G  V   A  Y  E  G  R  G  W  N  V  I    114

361   AGGTATTCATGCTGGACCAGCCAATAAATT GAGCATCGGCATTTGCCTGACTGGAGATTG
        G  I  H  A  G  P  A  N  K  L   S  I  G  I  C  L  I  G  D  W    134

421   GAGAGTCGAGACACCTTCAGCTGAGCAGTT GGCTACGACAAAAAAGCTTCTGTCCACGGG
        R  V  E  T  P  S  A  E  Q  L   A  T  T  K  K  L  L  S  T  G    154

481   AGTGGAAATGGGAGCTATTAGCTCCGATTA CAAGCTAATAGGACACAATCAGGCTATGAC
        V  E  M  G  A  I  S  S  D  Y   K  L  I  G  H  N  Q  A  M  T    174

541   GACAGAATGTCCGGGAGGAGCGCTGCTGGA AGAGGTTTCAACCTGGGATAATTATCATCC
        T  E  C  P  G  G  A  L  L  E   E  V  S  T  W  D  N  Y  H  P    194

601   TGGACACGTTAATTTTAGAGAACTAAATAA ACAGACAAAATTTTGACTCGAGGCAAGCCG
        G  H  V  N  F  R  E  L  N  K   Q  T  K  F  *                  208

661   AATTC
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Fig. 1. Nucleotide and deduced amino acid sequence of *B. mori* BTL-LP1 gene. The amino acid sequence is numbered at right, beginning at the N terminus of the protein, and the nucleotide sequence is numbered at left. The start codon of ATG and termination codon of TGA are marked as M and asterisk, respectively, with an individual underline.

signal peptide (21 amino acids) and mature peptide (the rest of 174 amino acids) with a calculated molecular mass of 23,120 Da (Fig. 2). The cDNA sequence of BTL-LP2 was deposited in the GenBank under the accession number AF441723.

The nucleotide and amino acids sequences were subjected to Blast Search. It turned out to have a high homology to peptidoglycan recognition protein (PGRP) of both *Bombyx mori* (Ochiai and Ashida, 1999) and, cabbage lopper, *Tricoplusia ni* (Kang *et al.*, 1998) at the amino acid sequence levels (Table 1). The deduced amino acid sequence of BTL-LP2 had ~45% of identity to the sequences of *B. mori* PGRP and ~49% of identify to *T. ni* PGRP, respectively. On the other hand, BTL-LP1 had ~31% of identity to the sequences of *B. mori* PGRP and ~32% of identify to *T. ni* PGRP, respectively. The amino acid sequence divergence between the two clones was ~30%, suggesting somewhat a larger divergence than those between species.

Phylogenetic analysis of the two clones with a few GenBank-registered sequences confirmed the divergent relationships between the two newly cloned *B. mori* BTL-LP1 and BTL-LP2, and relationships with the PGRP gene of mammals and other insects (Fig. 3). In the PAUP analysis using Norway rat (*Rattus norvegicus*) as an outgroup, all the insect PGRP sequences (*T. ni*, *B. mori*, and *Drosophilla malanogester*) formed relatively strong group with the newly cloned *B. mori* BTL-LP1 and BTL-LP2 (99% bootstrap value; Fig. 3a). However, when BTL-LP1, which showed a least sequence homology to others (Table 1), was utilized as an outgroup, BTL-LP1 was obviously excluded from the other insect PGRP gene, even from BTL-LP2 (Fig. 3b). This result suggests that the insect PGRP gene is highly divergent even within insects and between vertebrate and invertebrate during its evolution. Further investigation on the isoforms of the PGRP genes among insect species will illustrate further detailed information on the evolution of the gene.

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1    GAATTCGGCTTCAGGTTGCAAAGATGTTGG TTGCACCGTCTCTATTATTGTTGGTGTTCT
                                                                  12

61   TGGTGAGTTTCGGAACCCTGAATGCAGCGT CCGAATGCGGCGAAATTCCCATCACCGAAT
                                A  S  E  C  G  E  I  P  I  T  E   32

121  GGAGTGGCACGGAGTCACGTCGTAAACAGC CCCTGAAGAGTCCTATTGACTTGGTGGTGA
     W  S  G  T  E  S  R  R  K  Q  P  L  K  S  P  I  D  L  V  V   52

181  TACAACACACGGTATCCAACGATTGCTTTA CAGATGAAGAGTGTTTGCTAAGCGTAAATT
     I  Q  H  T  V  S  N  D  C  F  T  D  E  E  C  L  L  S  V  N   72

241  CTCTACGACAACATCATATGCGTCTGGCTG GGTTCAAGGACTTGGGCTATTCATTCGTGG
     S  L  R  Q  H  H  M  R  L  A  G  F  K  D  L  G  Y  S  F  V   92

301  CTGGAGGCAACGGAAAAATTTATGAAGGAG CGGGTTGGAACCATATCGGTGCTCACACAT
     A  G  G  N  G  K  I  Y  E  G  A  G  W  N  H  I  G  A  H  T  112

361  TGCACTACAATAATATATCCATAGGGATCG GTTTCATTGGCGACTTTAGGGAGAAGCTGC
     L  H  Y  N  N  I  S  I  G  I  G  F  I  G  D  F  R  E  K  L  132

421  CGACCCAGCAGGCACTGCAGGCGGTCCAAG ACTTTTTAGCCTGTGGGGTTGAAAATAACT
     P  T  Q  Q  A  L  Q  A  V  Q  D  F  L  A  C  G  V  E  N  N  152

481  TATTGACTGAAGACTACCACGTCGTTGGTC ACCAGCAGTTGATAAACACGCTAAGTCCTG
     L  L  T  E  D  Y  H  V  V  G  H  Q  Q  L  I  N  T  L  S  P  172

541  GAGCTGTACTGCAATCAGAAATCGAAAGTT GGCCCCATTGGCTTGATAATGCTCGAAAAG
     G  A  V  L  Q  S  E  I  E  S  W  P  H  W  L  D  N  A  R  K  192

601  TACTGGGTTAATATTTTATTTACACAACGA ATAGATCGAGTATTACTATAATCGGACTTT
     V  L  G  *                                                   195

661  ATATTACATTGAATAAAATTAATAGAGTAA AAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequence of *B. mori* BLT-LP2 gene. The amino acid sequence is numbered at right, beginning at the N terminus of the protein, and the nucleotide sequence is numbered at left. The start codon of ATG, termination codon of TAA, and polyadenylation signal are marked as M, asterisk, and two asterisks, respectively, with an individual underline. The amino acid sequences boxed with gray are a putative signal peptide consisted of 21 amino acids. GeneBank accession number of the sequence is AF441723.

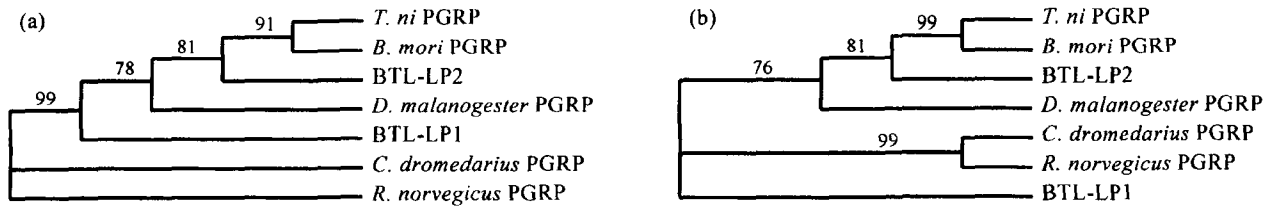

Fig. 3. PAUP analyses among the amino acid sequences of Peptidoglycan recognition protein (PGRP) from *B. mori* derived from this study and the homologous GenBank-registered sequences. GenBank accession number of each sequence is recorded next to the species name in the parentheses. The shown sequences, except for *B. mori* BTL-LP1 and BTL-LP-2, were obtained from GenBank: the fruit fly, *Drosophilla melanogaster* PGRP (AAF48056), cabbage looper, *Trichoplusia ni* PGRP (AAC31820), *B. mori* PGRP (BAA77209), Arabian camel, *Camelus dromedarius* PGRP (CAC19553), and Norway rat, *Rattus norvegicus* PGRP (NP_445825). The tree was obtained by bootstrap analyses with the option of heuristic search using (a) *R. norvegicus* PGRP and (b) *B. mori* BTL-LP2 as an outgroup, respectively. The option for tree presentation was "retain groups compatible with 50% majority-rule consensus and the numbers on the branches represent values for 1,000 replicates.

Table 1. Pairwise comparison among amino acid sequences of the PGRP genes

	1	2	3	4	5	6	7
1. *T. ni* PGRP	-	0.369	0.512	0.571	0.539	0.507	0.682
2. *B. mori* PGRP	80	-	0.548	0.608	0.618	0.613	0.691
3. BTL-LP2	111	119	-	0.622	0.604	0.613	0.696
4. *D. malanogester*	124	132	135	-	0.599	0.627	0.719
5. *C. dromedarius*	117	134	131	130	-	0.323	0.687
6. *R. norvegicus*	110	133	133	136	70	-	0.664
7. BTL-LP1	148	150	151	156	149	144	-

Numbers above the diagonal are mean values; numbers below the diagonal are absolute distance values.

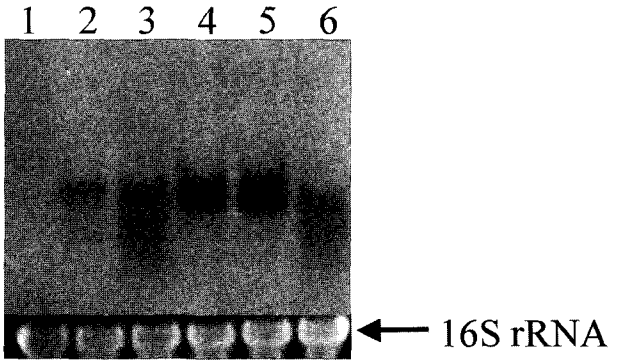

Fig. 4. A time-dependent gene expression pattern of *B. mori* PGRP analyzed by Northern blot. Total RNA was extracted from naive, *B. mori* (3 days of 5th instar) (lane 1), and at 2 hrs (lane 2), 4 hrs (lane 3), 6 hrs (lane 4), 8 hrs (lane 5), and 12 hrs (lane 6) after injection with dead *E. coli*. The RNA samples were separated in a 1.2% agarose gel containing 6.6% formaldehyde, transferred on to a nylon membrane, and hybridized with the radio-labeled probe.

To confirm an expression of the BTL-LP1 and BTL-LP2 genes in the *B. mori* fat body Northern blot analysis was carried out. Although the gene transcript for BTL-LP1 was not detectable in the untreated controls, it was detectable 3h after injection of the dead *E. coli* (data not shown), demonstrating that gene expression of this protein is truly induced by bacterial infection. In addition, we investigated a time-course of accumulation of BTL-LP2 gene transcripts after immunization in *B. mori*. The transcripts were detectable by 2 h, reached a maximum abundance at 6~8 hrs, and then gradually decreased, but were not detectable in the naive larvae (Fig. 4). In terms of induction time, Ochiai and Ashida (1999) reported an induction at 6 h and maximum at 24 h after bacterial injection into the larvae on day 5 of the 5th instar. The difference in induction time between studies, with the sequence homology of the newly cloned sequence to the known *B. mori* PGRP suggest that the newly cloned *B. mori* BTL-LP2 maybe another type of *B. mori* PGRP. In summary, the result obtained in this study suggest that the protein plays a role in non-self recognition in insect immune response, and the recognition proteins in insects are diverse and ancient.

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