Immune responses against bacterial infection in *Bombyx mori* and regulation of host gene expression

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In silkworm *Bombyx mori*, innate immune mechanisms such as phagocytosis, cellular encapsulation, prophenol oxidase cascade, and synthesis of antimicrobial proteins with antimicrobial activity are effectively engaged in defence reaction against invading pathogens. Antimicrobial proteins appear to be ubiquitous and multi-components of the innate immune mechanisms existing in *B. mori*. Several such immune proteins have been isolated from *B. mori* and their amino acid sequences determined. Apart from the earlier reported antibacterial proteins like cecropin, attacin and lysozyme, three novel proteins such as lebocin, moricin and hemocytin were isolated from *B. mori*. The antibacterial mechanisms of lebocin and moricin have been analysed and their ability to form ion channels in bacterial membranes shows their important role in defence against bacterial infection. Interestingly, hemocytin plays a dual role in immune mechanism against bacterial infection as well as in metamorphosis. cDNAs and genes encoding different antibacterial proteins have been cloned to examine their induction mechanisms upon bacterial infection. Regulatory motifs such as kB-like sequence and GATA sequence have also been identified in *B. mori* antibacterial protein genes. These findings suggest that the insect immune system is able to recognise the bacterial cell wall components and responds by higher level activation of appropriate protein genes to repel the infection. Lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, induces specific nuclear proteins to bind to the 5′ upstream regulatory region for the activation of lebocin gene expression. Similarly, a novel transcription factor F2BPI binds to the LPS responsive *cis*-element in cecropin promoter for the full activation of cecropin gene. This review describes the recent findings on antibacterial protein gene expression in *B. mori* with special focus on role of specific regulatory motifs for antibacterial gene expression and utilization of cecropin gene for the development of bacterial disease-resistant transgenic rice.

INSECTS are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection. Insects possess a complex and efficient system of biological defense against pathogens and parasites. This involves the following: (a) the integument and gut as physical barrier to infection, (b) activation of prophenol oxidase cascade and co-ordinated responses of several subpopulations of hemocytes when these barriers are breached, and (c) the induced synthesis of antimicrobial proteins, primarily by the fat body and their synthesis induced within hours following a septic injury[1]. Vertebrates have the acquired immunity with 'immunological memory', whereas invertebrates lack this immune system. Instead, they possess innate immunity, which is characterized by non-specific immune reactions against foreign materials. In general, the insect immunity consists of cellular and humoral reactions. Cellular reactions involve phagocytosis, nodule formation and encapsulation by plasmatocytes and granulocytes. On the other hand, humoral reactions involve activation of the prophenol oxidase cascade and induction of immune proteins such as lysozymes, lectins, antibacterial proteins and antifungal proteins[2]. Both immune reactions work in concert to prevent insects from acquiring infections with microorganisms. During late 1970s, Boman and his associates in Stockholm began a series of investigations on the immune reaction in *Drosophila melanogaster* and, later in the giant silkmoth, *Hyalophora cecropia*[3]. They observed that injection of bacteria into pupae of this moth resulted in the synthesis of immune proteins. They purified several of these proteins and characterized novel classes of antibacterial proteins, which they called cecropins and attacins. The time was then ripe for large-scale isolation of immune-induced molecules in various insect species and, in the years that followed, an interestingly wide variety of inducible antimicrobial proteins were isolated in several laboratories from various insect sources and their number now exceeds 150 (ref. 4).

Though several reviews on *D. melanogaster* have been published in this field in recent years[5], information available on *Bombyx mori* antibacterial proteins, various mechanisms interacting in antibacterial defense and their gene regulation remain scanty. Hence this article focuses on humoral reactions in the silkworm, *B. mori* and des-

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cribes the structural and functional characteristics of the immune proteins and the regulatory mechanisms of their gene expression.

Inducible antibacterial proteins of *B. mori*

Antibacterial proteins are known to be rapidly induced, mainly in the fat body and hemocytes upon bacterial infection or a wounding. Insect antibacterial proteins are heat stable and have a broad antibacterial spectrum. Many antibacterial proteins have been isolated from various insects and at least four types (cecropin, attacin, lebocin and moricin) have been identified in *B. mori*.

Cecropin consists of about 40 amino acid residues (ca. 4 kDa) and is heat stable. Three subtypes of cecropin (A, B and D) have been reported from *B. mori*[6–8]. Cecropins contain two α-helices and are rich in basic amino acid residues at the N-terminus and in hydrophobic amino acid residues at the C-terminus (Figure 1). Cecropins are active mainly against Gram-negative bacteria. It was demonstrated that cecropin forms ion channels in bacterial membranes and, as a result, bacteria are killed[9].

Attacin is glycine rich and has a molecular mass of ca. 20 kDa (ref. 10). Attacin acts against growing Gram-negative bacteria and was shown to inhibit synthesis of the bacterial outer membrane.

Lebocin consists of 32 amino acid residues and is proline rich[11]. An amino acid residue of lebocin (15-Thr) is glycosylated and this sugar is important for expression of antibacterial activity. The primary structure and antibacterial character of lebocin resembles those of abaecin (41% identity in amino acid sequence), an antibacterial protein of the honeybee although abaecin is not O-glycosylated. Three lebocin analogues (lebocin 1, 2 and 3) have been isolated and it was found that lebocin 1 and 2 have common amino acid sequences (Figure 1). Moreover, the glycosylated threonines of these antibacterial proteins contain different sugars, namely *N*-acetylgalactosamine and galactose (lebocin 1) and *N*-acetylgalactosamine (lebocin 2) (Figure 1). The amino acid sequence of lebocin 3 shows one amino acid sequence replacement (16-Leu) and 15-Thr is modified with *N*-acetylgalactosamine (Figure 1). Recently, a new member of the lebocin gene family (lebocin 4) has been cloned[12] and its deduced amino acid sequence reveals a high similarity to other lebocin members (Figure 1). The nature of the glycosylation at 15-Thr of lebocin 4 remains unknown. Incubation of lebocins with a liposome preparation causes leakage of entrapped glucose under low-ionic strength conditions, suggesting that the bacterial membrane is a target for lebocin. Since lebocins show very weak antibacterial activity under physiological conditions and require low ionic strength for full expression for their activity[11], their biological significance in the immune mechanism remains obscure. These complicated features of lebocin suggest that although they have very weak antibacterial activity, they might primarily serve to reduce the mini-

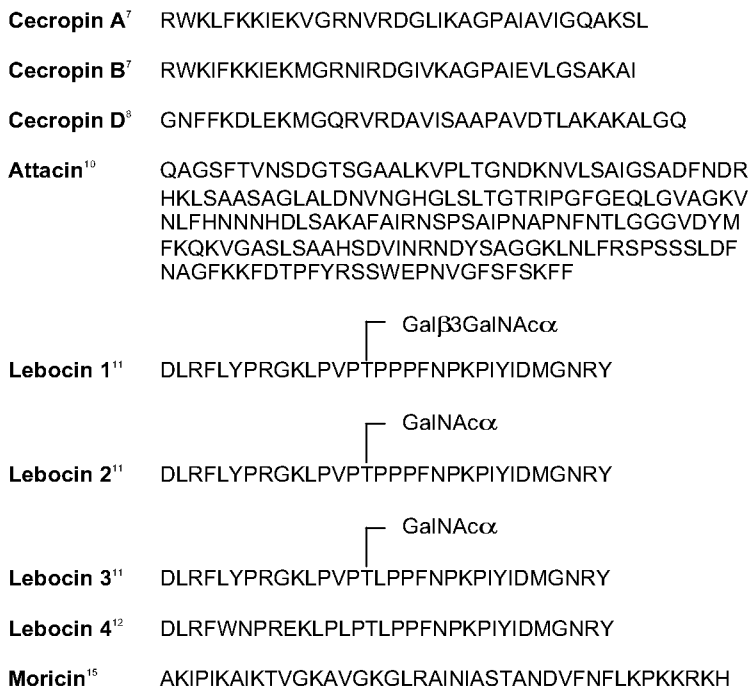

Figure 1. Amino acid sequences of antibacterial proteins from *B. mori*. Amino acid sequences of attacin and lebocin 4 were deduced from nucleotide sequences of cDNA and gene, respectively. Gal, Galactose; GalNAc, *N*-acetylgalactosamine.

mum inhibitory concentration of other antibacterial proteins under physiological conditions. Indeed, lebocin 3 was shown to have a synergistic effect with cecropin D, suggesting that antibacterial proteins work co-operatively in the immunity of *B. mori*[13]. Such a synergistic effect was also observed between an antibacterial protein (Sarcotoxin IA) and an antifungal protein in a flesh fly, *Sarcophaga peregrina*[14].

Another novel antibacterial protein designated moricin was isolated from the hemolymph of *B. mori* and it showed antibacterial activity against *Staphylococcus aureus*[11]. This protein consists of 42 amino acid residues and is highly basic. It had no significant similarity with other antibacterial proteins. Moricin has antibacterial activity against several Gram-negative and positive bacteria, has a higher activity against Gram-positive bacteria than cecropin B and is inducible upon bacterial injection. These results suggest that the protein is responsible for antibacterial activity against Gram-positive bacteria in *B. mori*. The effects of the protein on bacterial liposomal membranes indicate the target of the protein is the bacterial cytoplasmic membrane. The results also suggest that the N-terminal portion of this protein, containing a predicted α-helix, is responsible for an increase in the membrane permeability[15].

A cDNA of *B. mori* lysozyme was cloned and the deduced amino acid sequence revealed that it is highly homologous to that of chicken C-type lysozyme[16]. The lysozyme gene is constitutively expressed at a low level in the fat body, hemocytes and epidermal cells and is strongly expressed upon bacterial infection. In addition, lysozyme is considered to contribute significantly to the production of soluble peptidoglycan (PG), a strong inducer of antibacterial proteins[17].

Two lectins have been reported from *B. mori*; one has a molecular mass of ca. 260 kDa and another designated hemocytin ca. 280 kDa (ref. 18). Deduced amino acid sequence of hemocytin cDNA shows that it is related to a mammalian coagulation factor, von Willlebrand factor[19]. The characteristic features of the carbohydrate recognition domain of C-type animal lectins were detected at the C-terminal sequence of hemocytin. When cDNA encoding this region was introduced to a baculovirus vector, hemagglutinating activities were detected in the culture fluid of recombinant virus-specific cells. These activities were inhibited by D-mannose, *N*-acetyl-D-galactosamine, and D-maltose, which are haptenic saccharides of authentic hemocytin. Analysis of dot and Northern blot hybridization revealed that the hemocytin gene is transcribed in hemocytes of the silkworm at the larval–pupal metamorphosis and/or after injection of *Escherichia coli* and lipopolysaccharide (LPS). After injection of the C-terminal protein of hemocytin into *B. mori* hemolymph, aggregation of hemocytes was observed in the hemolymph. The results suggest that hemocytin plays an important role in both immunity and metamorphosis. Hemocytin also has a homologous region with coagulation factor, V and VIII (ref. 19).

Molecules triggering antibacterial protein synthesis

In most of the cases the insect antibacterial proteins are not synthesized under instinctive conditions, but are rapidly synthesized upon bacterial infection. Bacterial cell wall components, such as LPS and PG, are known to be strong triggers for the induction of antibacterial proteins. Moreover, LPS components, such as 2-keto-3-deoxyoctonate and lipid A, are also efficient triggers[20]. These elicitors were assumed to be produced upon bacterial infection.

In our laboratory extensive work has been carried out to study the molecular mechanism of antibacterial protein induction by LPS in *B. mori.* A primary culture containing adhering hemocytes, mainly granulocytes from *B. mori*, was used to investigate *in vitro* phagocytosis of *E. coli*[21]. Phagocytosis was confirmed to occur in this system by microscopic observation. The LPS concentration in the culture medium was determined by *Limulus* test. A higher LPS concentration was detected in an *in vitro* culture medium, where phagocytosis was observed, compared to that in control samples, which excluded either *E. coli* or adhering hemocytes. Moreover, it was found that culture medium containing a high LPS concentration, but not control medium, strongly induced gene expression of cecropin B after injection of culture medium into *B. mori* larvae[21]. These results suggest that bacterial cell wall components, such as LPS released by phagocytosis, play an important role in the induction of insect antibacterial proteins.

In invertebrate immunity, LPS plays a role in the initial stage of signal transduction to activate acute phase protein genes. In this case, LPS binds to LPS-binding protein (LPB) and CD14 recognizes this LPS–LBP complex, leading to the stimulation of signal transduction pathway[22]. In *B. mori*, a ca. 11-kDa membrane protein on the surface of hemocytes was identified to specifically bind LPS[23]. Likewise, 40 and 43 kDa proteins were purified as LPB from hemolymph of *B. mori*[24]. Furthermore, a Gram-negative bacteria-binding protein (GNBP) (50 kDa) was purified and the cDNA was also cloned[25]. The *GNBP* gene is constitutively expressed in the fat body and CD 14 antibodies can recognise *GNBP*. The role of *GNBP* in the activation of antibacterial protein genes still remains unclear.

It is a general phenomenon that antibacterial gene expression reaches maximum level a few hours after bacterial infection and gradually declines with time. This decrease in antibacterial protein gene expression was shown to be related with LPS clearance mechanism[26]. In an *in vitro* culture of fat body, cecropin B mRNA

appeared 20 min after incubation with LPS and its accumulation persisted for more than 72 h. No significant desensitization was observed for at least 24 h *in vitro*. On the contrary, cecropin B mRNA completely disappeared 24 h after injection of LPS *in vivo*. These results suggest that the concentration and/or biological activity of LPS decrease in hemolymph with time. LPS concentration assays at various time intervals after injection revealed two phases of LPS clearance from the hemolymph, an initial rapid phase, which was followed by a very slow phase[26]. The threshold concentration of LPS to induce cecropin B mRNA *in vivo* was over ten-fold higher than that *in vitro*. Although no cecropin mRNA accumulation was detected after 24 h of LPS injection, it appeared again with a reinjection of LPS. This result suggests that the reduction of LPS concentration directly terminates cecropin induction *in vivo*. In addition, the formation of the lipophorin–LPS complex in *B. mori* hemolymph and its role in LPS detoxification have been explored[27]. Analytical density gradient ultracentrifugation revealed that after LPS injection into *B. mori* larvae, the LPS peak shifted to a zone of lower density with time. The shifted peak was identified as the lipophorin–LPS complex. This complex formation was also achieved in an *in vitro* mixture of cell free hemolymph and LPS at 25°C, but not at 1°C. The lipophorin–LPS complex had a significantly lower capacity to elicit the mRNA of cecropin B. The biological activity of re-extracted LPS from the complex was slightly reduced in the *Limulus* test and no structural modification was observed in sodium dodecylsulfate-polyacrylamide gel electrophoresis. These results suggest that the formation of lipophorin–LPS complexes strikingly reduces the cecropin inducibility without any structural change in LPS.

Similar serum lipophorin–LPS complex formation and reduction of biological activities of LPS were also observed in mammals. Therefore, it is suggested that the formation of the serum lipophorin–LPS complex is a common pathway to inactive LPS both in insects and in mammals.

Signal transduction pathways were examined using an *in vitro* culture of plasmatocytes and granulocytes[28]. Expression of *B. mori* cecropin B gene in this *in vitro* culture was triggered by LPS, dibuyrylcyclic AMP (dcAMP), *Vibrio cholerae* (cholera) toxin, ionomycin and 4β-phorbol 12-myristate 13-acetate (PMA), suggesting that cyclic AMP, G protein and protein kinase C (PKC) are involved in expression of the cecropin B gene. Inhibitors such as H-7 and H-89, but not genistein, greatly suppressed cecropin gene activation by LPS, strongly suggesting that PKC and protein kinase A (PKA), but not tyrosine kinase, participate in signal transduction. Conversely, other serine/threonine kinase inhibitors such as W-7 and KN-62 showed only weak suppression. This result suggests that myosin light chain kinase and Ca^{2+}/calmodulin-dependent protein kinase did not play a significant role in cecropin B gene activation. The inhibition of induction by cyclohexamide indicated that a certain protein factor with a high turnover rate might be indispensable for cecropin B gene expression triggered by LPS.

The same *in vitro* system described above was also used to examine the induction of PKC and PKA activities by various stimuli[29]. The PKC activity was clearly detected after the hemocytes were treated with LPS from *E. coli*, ionomycin, cholera toxin or PMA. However, the activity was not detected in the absence of these stimuli. These results suggest that not only LPS, but also Ca^{2+} and a G protein may be involved in the induction of PKC activity. Likewise, the PKA activity was observed in the hemocytes treated with LPS, dcAMP, ionomycin or cholera toxin, whereas non-treated control cells did not show activity. Thus, LPS, cyclic AMP, Ca^{2+} and a G protein likely participate in the signal transduction for induction of PKA activity. Western blot analysis using monoclonal antibodies against rabbit brain PKC-α showed hemocyte samples treated with LPS contained a ca. 90 kDa protein, which cross reacted with the antibodies, but the protein was not present in a LPS non-treated sample[29]. LPS may activate PKC and PKA in *B. mori* hemocytes *in vivo* upon bacterial infection, resulting in triggering of the immune reactions such as antibacterial protein gene expression.

Concerning the induction of insect immune protein gene expression including cecropin genes by an effective stimulus such as LPS, at least two different possible pathways for intracellular signalling were suggested. One is PKC-mediated pathway in a lepidopteran insect, *H. cecropia*[30], and the other is PKC non-involved pathway in two dipteran insects, *D. melanogaster*[31] and *S. peregrina*[32]. Our results suggest that signal transduction for *B. mori* cecropin gene activation by LPS belongs to the former pathway. There is probably a common pathway in lepidopteran insects, which differs from that in dipteran insects. In the case of *D. melanogaster*, however, G protein has been demonstrated to participate in the cecropin gene induction[31], implying that there should also be a common signalling factor in both dipteran and lepidopteran insects.

On the other hand, eicosanoids were shown to be involved in signal transduction for antibacterial gene expression triggered by PG in *B. mori*[33], suggesting that there are different signal transduction pathways, which are involved in the activation of cecropin gene expression.

Regulatory motifs essential for antibacterial protein gene expression

The inducible and tissue-specific regulation of immune genes expression in insects seems to be at the level of transcriptional induction. To understand the mechanisms underlying this control it is crucial to identify the *cis-*

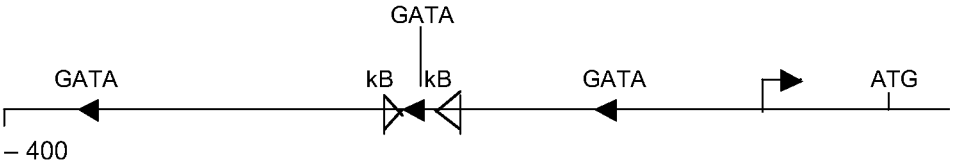

Figure 2. Location and orientation of some putative regulatory motifs in the upstream region of *B. mori* cecropin B1 gene. Numbers refer to the distances from the transcription starting site and translational start site is indicated by ATG. White arrows indicates kB motif and black arrows indicate GATA motif.

Table 1. kB-like sequence present in the 5′-upstream region of *B. mori* antibacterial protein genes. Position of the nucleotide sequence is calculated from the starting site of transcription (+ 1) and in the case of lebocin, it is calculated from the beginning site of translation (+ 1)

Gene	No.	Position	Sequence
Cecropin A1	1	–195 ~ –186	GGGATCTCCC
Cecropin A2	1	–191 ~ –182	GGGTAGCCCC
Cecropin B1	2	–147 ~ –138	GGGGATTAAC
		–119 ~ –110	GGGAAGTACC
Cecropin B2	2	–143 ~ –134	GGGGATTAAC
		–118 ~ –109	CGGTACTTCC
Attacin	1	–99 ~ –90	GGGGATAATT
Lebocin 3	3	–376 ~ –385	GGGGAAGTTC
		–128 ~ –137	GGGGAAGTCC
		–92 ~ –83	GGGAAGTACC
Lebicin 4	1	–128 ~ –137	GGGGAATACC
Consensus sequence		–92 ~ –83	GGGRANNNNY

Table 2. GATA sequence present in the 5′-upstream region of *B. mori* antibacterial protein genes. Position of the nucleotide sequence is calculated from the starting site of transcription (+ 1) and in the case of lebocin, it is calculated from the beginning site of translation (+ 1)

Gene	No.	Position	Sequence
Cecropin A1	1	–105 ~ –110	TGATAA
Cecropin A2	1	–105 ~ –110	TGATAA
Cecropin B1	3	–243 ~ –248	AGATAA
		–130 ~ –135	TGATAA
		–70 ~ –75	AGATAA
Cecropin B2	3	–234 ~ –239	AGATAA
		–121 ~ –126	TGATAA
		–70 ~ –75	AGATAA
Lebocin 3	1	–121 ~ –126	TGATAA
Lebocin 4	2	–122 ~ –127	TGATAA
		–147 ~ –142	TGATAA
Consensus sequence			WGATAA

regulatory elements involved and such studies have been initiated by several groups[34]. Knowledge of transcriptional control of immunoglobulins and acute phase genes in mammals led to the important discovery of the striking conservation among some of the insect antibacterial protein genes of a sequence motif with homology to the mammalian kB motif. This binding site was first identified in the enhancer region of the immunoglobulin light kappa-gene, and has later been found in promoter and enhancer elements in many mammalian genes that are involved in immune, inflammatory and acute phase responses[34]. The kB motif has subsequently been identified in the promoter region of most of the inducible immune genes of insects including *B. mori* (Figure 2). The kB-like sequence present in the 5′-upstream region of *B. mori* antibacterial genes is shown in Table 1. Comparison of kB sites in the inducible immune genes of *B. mori* lead to the definition of a consensus sequence for this motif[4], GGGRANNNNY.

Transcription factors belonging to the Rel family were involved in this gene activation mechanism in *D. melanogaster*[35,36] and *H. cecropia*[37]. Of these factors, dorsal-related immune factor (DIF) was identified in *D. melanogaster* and DIF was demonstrated to bind to the kB sequence in the 5′-upstream of antibacterial protein genes. DIF is known to be retained in the cytoplasm by cactus under non-infection conditions, but it is dissociated from cactus and moves into nuclei upon bacterial infection. In addition, dorsal involved in the dorsoventral determination in embryogenesis of *D. melanogaster* is also revealed to activate transcription of kB containing reporter gene[36]. Dorsal gene expression is stimulated in the fat body by bacteria, suggesting that dorsal is involved in the activation of antibacterial protein gene expression[38,39]. The role of dorsal and DIF in *B. mori* antibacterial gene activation is unclear.

Apart from kB motif, another DNA sequence, GATA sequence, present in the 5′-upstream regulatory region plays important role in the induction of antibacterial protein genes[40]. GATA sequences were also detected in the 5′-upstream region of *B. mori* genes encoding cecropin A1, A2, B1, B2, attacin and lebocin[12,41–43] (Table 2). The consensus sequence of GATA region was WGATAR; a similar type of sequence was also reported in *D. melanogaster*. It is assumed that the GATA sequence is important for insects' immunity. It is also speculated that since the GATA sequence is located in the vicinity of the kB sequence, the factors related to these sequences work co-operatively to activate antibacterial protein genes.

Induction of the binding of nuclear proteins to the 5′-upstream regulatory region of lebocin gene by LPS

A cDNA clone encoding lebocin 1/2 was isolated from a fat body cDNA library of *B. mori* larvae immunized with

E. coli[44] and two genomic DNA clones encoding lebocin 3 and 4 were screened from a *B. mori* genomic library[12]. Lebocin gene expression in *B. mori* larvae was shown to be induced by injection of *E. coli* LPS and chitin oligomers, and occurred tissue specifically in the fat body and hemocytes[20]. The cloning and sequencing of two lebocin genes showed that the 5′-upstream regulatory region of lebocin 3 and 4 genes contains kB and IL6 sites. To analyse lebocin gene expression induction mechanisms, electrophoretic mobility shift assay (EMSA) was performed with nuclear proteins[45]. They performed EMSA with 12 different probes of different regulatory sites of lebocin genes and crude nuclear extracts from the fat body of LPS-injected or non-injected silkworm larvae. Nuclear proteins formed DNA–protein complexes with two kB probes, indicated that LPS induces or enhances binding of nuclear proteins to specific sequences containing a kB site and other regions upstream of the lebocin 3 and 4 genes. Furukawa *et al.*[12] also reported that lebocin 3 and lebocin 4 genes contained one or two GATA motifs near kB sites. Interestingly, only two kB sites adjacent to a GATA motif(s) were found to interact with nuclear proteins and no such interaction was observed with kB site having no GATA motifs. Adjacent GATA motifs and kB sites are known to regulate the antibacterial peptide gene expression of *D. melanogaster*[46]. The GATA motifs play an important role in regulating lebocin gene expression cooperating with a kB site and L3kB1 in lebocin 3 and L4kB in lebocin 4 are structurally and functionally identical. Furukawa *et al.*[45] also suggested that these two kB sites have close evolutionary relationship and play an important role in induction of lebocin gene expression by LPS.

LPS response element in cecropin B promoter

The structure of cecropin B gene has been analysed to elucidate the bacteria-specific gene activation mechanism[41]. At least four copies of the genes exist in each individual. Two cloned genes, *CecB1* and *CecB2*, revealed 90% identity within the upstream region spanning 800 bp, suggesting that the genes are regulated by the same transcription factors. In the proximal region of the promoters, kB-like decamer motifs, three GATA sites and one mammalian type II interleukin-6 response element (IL-6 RE) were found. The electrophoresis mobility shift assay (EMSA) identified three different DNA-binding proteins that bind to 235 bp of the *CecB1* promoter, of which one of the proteins is probably a kB-related factor because competition with a kB-like sequence inhibited the binding and other proteins are F2BPI and F4BP. Further EMSA analyses indicated that the F2BPI-binding site was CATTA, and that F2BI translocated from the cytoplasm to the nucleus after infection[47]. In a recently established *B. mori* cell line, NISES-BoMo-DZ, 235 bp of *CecB* promoter linked to reporter luciferase was activated 6-fold by stimulation with LPS, which is a major trigger of *CecB* expression in larvae. Truncation of the F2BPI-binding site from the promoter reduced the activation two fold. Deletion of either of two kB motifs also reduced promoter activation two folds. Elimination of both the F2BPI-binding site and the kB motifs resulted in the complete loss of LPS inducibility. In *B. mori*, two CATTA are conserved in *CecB1* and *CecB2*, and one is conserved in *CecA1* and *CecA2*. Other *B. mori* genes like attacin, lebocin, moricin and hemocytin contain 1 to 4 copies of CATTA on both or either strand in the proximal promoter region, although the number and position vary (Table 3). These results indicate that the F2BPI-binding site is an LPS responsive *cis*-element that is necessary for full activation of *CecB*[47]. Further, CATTA is a common LPS responsive element in mammals and insects, in addition to the kB motifs and GATA site.

Strategies to improve plant resistance to bacterial diseases in transgenic plants expressing cecropin genes

Bacterial diseases are of great economic significance for many crop plants, with the highest losses occurring in cereals, vegetables and fruits. In most cases, applications of protective agrochemicals, when feasible, are not sufficient to control bacterial diseases. Furthermore, the use of chemicals is subject to increasing restrictions because of their potentially harmful impact on the environment, and so the control of bacterial diseases is often limited to prophylaxy. However, the existence of natural source of resistance to bacterial diseases has enabled selection of a number of resistance varieties through conventional breeding. Classical hybridization is obviously restricted within species (or between closely related species). In addition to that it may be difficult and time consuming to introduce resistance from a wild species into commercial cultivars. Recent improvements in plant-transformation

Table 3. Distribution of CATT (A/T) in LPS-inducible promoters in *B. mori* antibacterial protein genes

Gene	Motif	No.	Distance from TATA box (bp)
CecB1	CATTA	2	–125, –140
CecB2	CATTA	2	–120, –135
	CATTT	1	–173
CecA1	CATTA	1	–23
CecA2	CATTA	1	–23
Attacin	CATTT	1	–35
	CATTA	1	–300
Lebocin 3	CATTA	3	–130, –177, –434
	CATTT	2	–108, –329
Lebocin 4	CATTA	1	–131
Moricin	CATTA	4	–154, –244, –295, –329
	CATTT	2	–182, –205

Figure 3. Enhanced resistance to bacterial disease caused by *Pseudomonas syringae* pv. *oryzae* in cecropin B transgenic rice plants. Disease symptom on detached leaflet of rice transformed with pRSPcec19-2 (Cecropin B) gene construct and untransformed control lines (Machii, H., Sharma, A. and Yamakawa, M., unpublished data).

techniques and progress in the understanding of plant–pathogen interaction enable the use of genetic engineering for the rational creation of disease-resistant plants[48]. In our laboratory, effort has been devoted to the creation of transgenic rice expressing cecropin B, that is resistant to bacterial leaf blight disease in rice caused by bacteria *Xanthomonas oryzae* pv. *oryzae*[49].

As we discussed earlier, cecropin, a small family of lytic peptide was found in *H. cecropia*. These proteins are small and strongly basic, and comprise three major forms A, B and D. Cecropins exhibit a broad spectrum of antibacterial activity against both Gram-negative and positive bacteria by adopting α-helical structure on interaction with bacterial membranes resulting in the formation of ion channels[9]. This observation prompted scientists to propose the idea of using the genes cloned from insects to enhance bacterial disease resistance in plants[50]. Also, genes encoding cecropins and their analogs have been expressed in transgenic tobacco with contradictory results regarding pathogen resistance[51]. The attempts made towards developing transgenic plants using cecropin B genes did not meet with success because of cellular degradation of this protein by plant endogenous peptidases, thereby limiting a critical level sufficient to kill the pathogen[50]. Interestingly, the rate of degradation of cecropin B and its structural analog reduced in intercellular fluids, indicating enhanced stability of cecropin B in plant intercellular fluid[49]. These findings strongly suggested the possibility of tailoring cecropin B genes for plants showing a reduced rate of protein degradation. In order to make this protein effective for the control of bacterial leaf blight in rice, it was imperative that this protein must be translocated into the intercellular space at the earliest to prevent cellular degradation. This was achieved by fusing the coding sequence of mature protein of *B. mori* cecropin B to the sequence of signal peptide of chitinase gene of rice which is known to direct the secretion of chitinase gene product into the intercellular spaces in rice[49]. This transgenic rice plant developed in our study, which was carrying *B. mori* cecropin B gene fused with signal peptide sequence of chitinase, showed strong resistance to *X. oryzae* pv. *oryzae*[49] and also to *Pseudomonas syringae* pv. *oryzae* (Machii, H., Sharma, A. and Yamakawa, M., unpublished data) (Figure 3). These findings provide an exciting approach for effectively utilizing them in developing bacterial-resistant transgenic crop plants.

Perspectives

As stated in this review, silkworms *B. mori* have developed an efficient host defense against invading microorganisms, which involves three major components such as phagocytosis, cellular encapsulation, prophenol oxidase cascade, and synthesis of antimicrobial proteins. Unlike the well-established interrelationships among immune mechanisms and other mechanisms maintaining homeostasis in higher vertebrates, there is a paucity of information concerning such interrelationships in insects. Satoh *et al.*[52] established the existence of prophenol oxidase cascade and molecular mechanisms involved in the recognition of bacterial cell wall components in *B. mori*. In our laboratory a special emphasis was given to *B. mori* antibacterial proteins and their gene regulation. Several research papers have been published in the past year on signal transduction and role of Rel proteins Dorsal, Dif and Relish in the regulation of antimicrobial peptides of *D. melanogaster* whereas this interesting mechanism is yet to be investigated in *B. mori*. Moreover, recent advances in the molecular characterization of innate immunity in insects have revealed striking similarities with

mammalian innate immunity, suggesting that signal transduction mechanisms share a common evolutionary ancestry. Due to the enormous use of antibiotics, most of the clinical pathogens are becoming resistant to those antibiotics. Promising alternative antibiotics are the antibacterial peptide antibiotics from insect source, which can kill their specific targets by damaging the bacterial cell membrane.

In India, the indigenous tropical polyvoltine races showed more resistance to diseases than temperate bivoltine races. In order to understand the differential response at molecular level, it may be an ideal approach to compare the expression level of antibacterial genes in hardy polyvoltine races like Pure Mysore and Nistari with temperate races.

1. Hoffmann, J. A., Reichchart, J. M. and Ezekowitz, R. A., *Science*, 1999, **284**, 1313–1318.
2. Gillepe, J. P., Kanost, M. R. and Trenczeck, T., *Annu. Rev. Entomol.*, 1997, **42**, 611–643.
3. Hoffmann, J. A. and Reichchart, J. M., *TICB*, 1997, **7**, 309–316.
4. Yamakawa, M. and Tanaka, H., *Dev. Comp. Immunol.*, 1999, **23**, 281–289.
5. Boman, H. G., *Annu. Rev. Immunol.*, 1995, **13**, 61–92.
6. Teshima, T. *et al.*, *Tetrahedron*, 1986, **42**, 829–834.
7. Morishima, I., Suginaka, S., Ueno, T. and Hirano, H., *Comp. Biochem. Physiol.*, 1990, **B95**, 551–554.
8. Hara, S., Taniai, K., Kato, Y. and Yamakawa, M., *Comp. Biochem. Physiol.*, 1994, **B108**, 303–308.
9. Christensen, B., Fink, J., Merifield., R. B. and Mauzerall, D., *Proc. Natl. Acad. Sci. USA*, 1998, **85**, 5072–5076.
10. Sugiyama, M. *et al.*, *Insect. Biochem. Mol. Biol.*, 1995, **25**, 385–392.
11. Hara, S. and Yamakawa, M., *Biochem. J.*, 1995, **310**, 651–656.
12. Furukawa, S., Taniai, K., Ishibashi, J., Hara, S., Shono, T. and Yamakawa, M., *Biochem. Biophys. Res. Commun.*, 1997, **238**, 769–774.
13. Hara, S. and Yamakawa, M., *Appl. Entomol. Zool.*, 1995, **30**, 606–608.
14. Iijima, R., Kurata, S. and Natori, S., *J. Biol. Chem.*, 1993, **268**, 12055–12064.
15. Hara, S. and Yamakawa, M., *J. Biol. Chem.*, 1995, **270**, 29923–29927.
16. Lee, W. J. and Brey, P. T., *Gene*, 1995, **161**, 199–203.
17. Dunn, P. E., Wei, D., Kanost, M. R. and Geng, C., *Dev. Comp. Immunol.*, 1985, **9**, 559–568.
18. Suzuki, T. and Natori, S., *J. Biochem.*, 1983, **93**, 583–590.
19. Kotani, E. *et al.*, *Biochem. Biophys. Acta*, 1995, **1260**, 245–258.
20. Taniai, K., Furukawa, S., Shono, T. and Yamakawa, M., *Biochem. Biophys. Res. Commun.*, 1996, **226**, 783–790.
21. Taniai, K., Wago, H. and Yamakawa, M., *Biochem. Biophys. Res. Commun.*, 1997, **231**, 623–627.
22. Ulevitch, R. J. and Tobias, P. S., *Annu Rev. Immunol.*, 1995, **13**, 437–457.
23. Xu, J. *et al.*, *Insect. Biochem. Mol. Biol.*, 1995, **25**, 921–928.
24. Koizumi, N., Morozumi, A., Imamura, A., Tanaka, E., Iwahana, H. and Sato, R., *Eur. J. Biochem.*, 1997, **248**, 217–224.
25. Lee, W. J., Lee, J. D., Kravchenko, V. V., Ulevitch, R. J. and Brey, P. T., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 7888–7893.
26. Kato, Y., Motoi, Y., Taniai, K., Kadono-Okuda, K., Hiramitsu, M. and Yamakawa, M., *Insect. Biochem. Mol. Biol.*, 1994, **24**, 539–545.
27. Kato, Y. *et al.*, *Insect Biochem. Mol. Biol.*, 1994, **24**, 547–555.
28. Shimabukuro, M. *et al.*, *Appl. Entomol. Zool.*, 1996, **31**, 135–143.
29. Choi, H. K. *et al.*, *J. Seric. Sci. Jpn.*, 1995, **64**, 450–456.
30. Sun, S. C., Lindstrom, I., Lee, J-Y. and Faye, I., *Eur. J. Biochem.*, 1991, **196**, 247–254.
31. Samakovlis, C., Asling, B., Boman, H. G., Gateff, E. and Hultmark, D., *Biochem. Biophys. Res. Commun.*, 1992, **188**, 1169–1175.
32. Kobayashi, A., Matsui, M., Kubo, T. and Natori, S., *Mol. Cell Biol.*, 1993, **13**, 4046–4056.
33. Morishima, I., Yamano, Y., Inoue, K. and Matsuo, N., *FEBS Lett.*, 1997, **419**, 83–86.
34. Engstrom, Y., in *Molecular Mechanism of Immune Responses in Insects* (eds Brey, P. T. and Hultmark, D.), Chapman and Hall, London, 1997, pp. 213–244.
35. Ip, Y. T. *et al.*, *Cell*, 1993, **75**, 753–763.
36. Petersen, U. M., Bjorklund, G., Ip, Y. T. and Engstrom, Y., *EMBO J.*, 1995, **14**, 3146–3158.
37. Sun, S. C. and Faye, I., *Eur. J. Biochem.*, 1992, **204**, 885–892.
38. Imler, J. l. and Hoffmann, J. A., *Curr. Opin. Microbiol.*, 2000, **3**, 16–22.
39. Reichhart, J. M., Georgel, P., Meister, M., Lematre, B., Kappler, C. and Hoffmann, J. A., *C.R. Acad. Sci.*, 1993, **316**, 1218–1224.
40. Sun, S. C. and Faye, I., *Comp. Biochem. Physiol.*, 1992, **B103**, 225–233.
41. Taniai, K. *et al.*, *Gene*, 1995, **163**, 215–219.
42. Yamano, Y., Matsumoto, M., Sasahara, K., Sakamoto, E. and Moroshima, I., *Biosci. Biotechnol. Biochem.*, 1998, **62**, 237–241.
43. Taniai, K., Ishii, T., Sugiyama, M., Miyanoshita, A. and Yamakawa, M., *Biochem. Biophys. Res. Commun.*, 1996, **220**, 594–599.
44. Chowdhury, S. *et al.*, *Biochem. Biophys. Res. Commun.*, 1995, **214**, 271–278.
45. Furukawa, S., Tanaka, H., Nakazawa, H., Ishibashi, J., Shono, T. and Yamakawa, M., *J. Seric. Sci. Jpn.*, 1999, **68**, 289–294.
46. Kadalayil, L., Peterson, U. M. and Engstrom, Y., *Nucleic Acids Res.*, 1997, **25**, 1233–1239.
47. Taniai, K. and Tomita, S., *J. Biol. Chem.*, 2000, **275**, 13179–13182.
48. Mourgues, F., Brisset, M. N. and Chevreau, E., *TIBTECH*, 1998, **16**, 203–209.
49. Sharma, A., Sharma, R., Imamura, M., Yamakawa, M. and Machii, H., *FEBS Lett.*, 2000, **484**, 7–11.
50. Salmeron, M. J. and Vernooij, B., *Curr. Opin. Plant Biol.*, 1998, **1**, 347–352.
51. Osusky, M., Zhou, G., Osuska, L., Hancock, E. R., Kay, W. W. and Misra, S., *Nature Biotechnol.*, 2000, **18**, 1162.
52. Satoh, D., Horii, A., Ochiai, M. and Ashida, M., *J. Biol. Chem.*, 1999, **274**, 7441–7453.

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