

Review

Lectins as possible candidates towards anti-microbial defense in silkworm, *Bombyx mori* L

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Unlike vertebrates, insects do not have acquired immunity and therefore rely totally on innate immunity towards defense against invading microorganisms. Insect innate immunity consists of cellular and humoral reactions and both reactions work in concert in preventing insects acquiring infections. The most likely candidates for recognizing foreign material in insects are the lectins, which have already been shown to be important in mammalian innate immunity. Several reports of endogenous serum lectins having opsonic activity for invading pathogens have been circumstantiated in several insect specimens and therefore have been continuously explored for binding to wide range of microorganisms, obviating the necessity of antibodies in these animals. Silkworm, *Bombyx mori* L. is an important economic insect with unparalleled significance to the prosperity of weaker sections of the society and also has been promoted as a powerful laboratory model involving basic research in biology. It therefore merits immediate attention towards proper understanding of host-pathogen interactions, defensive mechanisms evolved in the host body in response to infection, anti-defensive molecules released by pathogen to suppress host immunity before reflecting on aspects of disease control. In this regard, lectins have been implicated as pattern recognition molecules serving as biosensors for detecting carbohydrate components on the microbial cells, thus triggering signaling cascade for immune activation. Understanding of such silkworm agglutinins, most specifically their binding specificities and pattern of recognition with identifiable gene families have been discussed towards establishment of its candidate role as immune defense molecules.

Key words: *Bombyx mori*, lectins, innate immunity, carbohydrate-binding domains.

INTRODUCTION

Insects are the earliest and most diverse taxon of animals accounting for more species than all other animals put together because of their high reproductive potential and varied niche. Also, insects are the most successful group of animals that exist in a myriad of environment where the potential for infection by microorganisms and parasites are great. Insects do not possess the ability to produce antibodies (immunoglobulins) and do not use

immunoglobulin as recognition molecules against foreign antigens and hence antigenic memory is lacking. Also, they do not produce alpha/beta interferons (α/β IFN) in response to viral infections. Insects possess innate immunity which is characterized by non-specific immune reactions against foreign materials. The defense mechanism in insects as a part of survival strategy against invading pathogens includes physical blockades such as cuticle and peritrophic matrix, epithelial barriers, protease cascades leading to coagulation and melanization, cellular responses such as phagocytosis and encapsulation and also the production of certain antimicrobial

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peptides (Lavine and Strand, 2002; Ligoxygakis et al., 2002; Meister and Lagueux, 2003; Lehane et al., 2004).

One characteristic of insect immunity is rapid and transient activation of immune genes upon microbial infection to produce effectors. When microorganisms penetrate the hemocoel, foreign invaders are first recognized by pattern recognition factors for cellular (Kocks et al., 2005) and humoral immune reactions (Kurata et al., 2006). Innate immune system in both mammals and arthropods utilize pattern recognition proteins or receptors, which performs surveillance function by binding to molecules (classes of polysaccharides, such as lipopolysaccharide, lipoteichoic acid, peptidoglycan and β -1,3-glucans) common to the groups of microorganisms (Yu et al., 2002; Hoffmann, 2003). This type of recognition of non-self leads to rapid and broad responses to infection, evoking stimulation of modulating factors in turn, provokes signal transduction in specific tissues as fat body or hemocytes (Ferrandon et al., 2007). Such signaling cascades activate genes coding effector molecules and a battery of these molecules are produced in specific tissues and secreted into haemolymph (Hoffmann, 2003).

GENOME-WIDE ANALYSIS OF INNATE IMMUNITY GENES AND GENE FAMILIES IN INSECTS

Genome-wide analytical screening of immunity related genes and gene families has been conducted in 14 dipteran insects, *Drosophila melanogaster* (Irving et al., 2001), 11 other *Drosophila* species including *Drosophila simulans*, *Drosophila yakuba*, *Drosophila erecta*, *Drosophila annanasse*, *Drosophila persimilis*, *Drosophila pseudoobscura*, *Drosophila willistoni*, *Drosophila virilis*, *Drosophila mojavensis*, *Drosophila sechellia* and *Drosophila grimshawi* (Sackton et al., 2007), *Anopheles gambiae* (Christophides et al., 2002) and *Aedes aegypti* (Waterhouse et al., 2007), a hymenopteran insect *Apis mellifera* (Evans et al., 2006) and a coleopteran insect *Tribolium castaneum* (Zou et al., 2007). These studies have shed focus into immune related gene expansion and diversification reflecting different pressures from different pathogenic microorganisms in different life environments from different life styles of insects.

Lepidopterous insects have life and feeding styles different from dipteran, hymenopteran and coleopteran insects. The silkworm, *Bombyx mori* has been exploited both as a powerful biological model system and also as a tool to convert leaf protein into silk. Recent success of transgenesis of the silkworm has opened new prospects for this insect species (Tamura et al., 2000). Also very lately, the combination of whole genome sequencing and gene silencing has been explored as a tool to analyze the functions of insects and most specifically *B. mori* immune genes (Table 1, Figure 1) (Dietzl et al., 2007; Tanaka et al., 2008; Sagisaka et al., 2010).

AGGLUTININS (LECTINS)

Lectins are ubiquitous proteins found in plants, animals and microorganisms. In vertebrates, the role of lectins as mediators of non-self recognition in the innate immune response has been well documented, with reports of several mammalian lectins having an opsonic function. The best studied of these proteins are the mannose-binding lectins (MBLs), which are essential component of vertebrate innate immune system, since MBL deficient individuals are prone to recurrent infections during infancy (Turner, 1996). In invertebrates, due to lack of antibody-based immunity, lectins are vital for non-self recognition and clearance of invading microorganisms. As earlier categorically stated, in invertebrates, phagocytosis with intimate interaction of humoral substances, particularly as recognition factors has been seriously implicated in innate defense against foreign invaders. Such humoral factors, naturally occurring or formed after antigenic stimulation includes invertebrate agglutinins and have been extensively studied due to their functional similarities to vertebrate antibodies, serving a defensive function (Jayasree, 2001; Jayaraj et al., 2008a).

Agglutinins (lectins) are proteins having the ability to agglutinate cells with complementary carbohydrates on their surfaces specifically and reversibly. They may specifically recognize whole sugar, a specific site in a sugar, a sequence of sugars or their glycosidic linkages. The carbohydrate specificity of lectins is exploited conventionally by the hapten inhibition technique, in which different monosaccharides, oligosaccharides, or glycopeptides are tested for their ability to inhibit either hemagglutination or polysaccharide precipitation by the lectin. Agglutination by lectins is also inhibited differentially by some oxidizing agents and chelating agents. The serum agglutinin was found to be heat-labile and susceptible to pH extremes. Such hemagglutinating proteins have been detected in mucus as well as in certain tissues of invertebrates (Suzuki and Mori, 1991), its immunogenic potential is best understood in haemolymph and recent studies have shown that purified, haemolymph-derived agglutinins served as opsonins in few insects (Kawasaki et al., 1993). Insect haemolymph contains hemagglutinating activities which agglutinates vertebrate erythrocytes and certain microorganisms and are important for the recognition of non-self and cellular defense and are involved in immune surveillance. Due to the involvement of lectins in the recognition of non-self, a number of studies have concentrated on the role of lectins in parasite-host relationships (Grubhoffer and Matha, 1991; Mohamed et al., 1992; Volf et al., 1993).

Apart from natural hemagglutinins, inducible forms of haemolymph agglutinin by wounding or microorganisms have been reported in flesh fly, *Sarcophaga peregrina* (Takahashi et al., 1986), the velvetbean caterpillar, *Anticarsia gemmatalis* (Pendland and Boucias, 1985) and the tobacco hornworm, *Manduca sexta* (Minnick et al.,

Table 1. Immunity-related gene and gene families of *B. mori*.

Recognition		Modulation		Signaling		Effector		Others	
Gene family	No.	Gene family	No	Gene family	No.	Gene family	No.	Gene family	No
PGRP-S	6	CLIP		<u>Toll genes</u>		PPO	2	Caspase	4
PGRP-L	6	Serine Protease	15	Toll	14	POI	1		
B-GRP	4			Spatzle	3	Lysozyme	1	Argonaute-2	1
Hemolin	1			Tollip	2	Lysozyme-like protein	3		
FRP	3	Serpin	26	Others*	8	Cecropin	13		
SC-A	4					Attacin	2		
SC-B	13			IMD genes**	10	Defensin	1		
SC-C	1					Gloverin	4		
C – lectin	21			JNK genes***	4	Moricin	1		
Hemocytin	1					Moricin-like protein	8		
Galectin	4			JAK/STAT genes****	4	Lebocin	1		
TEP	3					NOS	2		
Nimrod-A	1								
Nimrod-B	1								
Nimrod-C	2								
Draper	1								
Eater	0								
Dscam	1							Total	203

PGRP-S, Peptidoglycan recognition proteins-short; PGRP-L, peptidoglycan recognition proteins-long; β -GRP, β -glucan recognition protein; FRP, fibrinogen-related proteins; SC, scavenger receptor; TEP, thioester containing proteins; PPO, polyphenol-oxidase; POI, phenoloxidase inhibitor; NOS, nitric oxide synthase. **MyD88, Tube, Pellino, Pelle, TRAF-2, ECSIT, Cactus, Dif/Dorsal* genes. ***IMD, Dredd, TAK1, FADD, Tab2, IAP2, IKK β , IKK γ , Ubc13, Relish* genes. ****Hem, JNK, Fos, Jun* genes. *****Upd3, PIAS, SOCS, Domeless, Hopscotch, STAT* genes.

1986). Inducible hemagglutinating activity (a lectin like protein) in the haemolymph of fifth instar larvae of the silkworm, *B. mori* by infection with cytoplasmic polyhedrosis virus (CPV) was confirmed by western blotting (Mori et al., 1989). It was noticed that 280 and 82 K subunits of haemolymph accumulated appreciably, suggesting increase of hemagglutinating activity by CPV infection. Hemagglutinating activity of such lectin-like proteins may be required for the exclusion of virion in the haemolymph, derived from their multiplication in midgut cells or

scavenging midgut tissue fragments due to infection. It is therefore critical to elucidate the quantitative interactions between lectin-like proteins and microbes during the process of infection to examine their significant role as candidates in insect immunity against microbes. Multiple lectins have been purified from insects, namely, the silkworm, *B. mori*; the American cockroach, *Periplaneta americana*; West Indian leaf cockroach, *Blaberus discoidalis* and desert locust, *Schistocerca gregaria*. Two of the lectins from *P. americana*, that is, the lipopolysaccharide

(LPS)-binding protein (Jomori and Natori, 1992) and the *Periplaneta* lectin (Kawasaki et al., 1993) have been reported to bind to and increase the *in vivo* clearance of *Escherichia coli* from the haemolymph.

In *B. discoidalis*, four lectins have been reported, namely, BDL1, BDL2, BDL3 and GSL, of which three, BDL1, BDL2 (Chen et al., 1993), and GSL (Chen et al., 1995), have been purified. BDL1 has shown to have properties similar to MBLs, in terms of specificity, structure and activation of complement and GSL has been

shown to be similar to invertebrate C-reactive proteins. These endogenous lectins are also capable of enhancing the activation of the prophenoloxidase system. BDL1 induced high level phagocytosis of yeast, as *Saccharomyces cerevisiae*, is known to have abundant supply of polymannans on its cell surface, which provides numerous binding sites for mannose-binding lectins. GSL binds exclusively to β -1,3-glucans, another group of polysaccharides, abundant in yeast cell walls and *N*-acetyl-D-galactosamine-specific BDL3 enhances the phagocytosis of *Bacillus cereus*. Chitin, a polysaccharide composed of repeating units of N-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid, is also present, in small amounts on the yeast cell surface providing binding opportunities for BDL2. Significant increase in phagocytosis of *B. cereus*, a Gram-positive bacterium, by BDL2, revealed specificity towards GlcNAc (Wilson et al., 1999). Three lectins, designated as Sg₁, Sg₂ and Sg₃ have been identified in the serum of the desert locust, *Schistocerca gregaria* (Dorrah et al., 2009), which were found to be highly specific for rabbit red blood cells (RBCs) than those of other vertebrates. The hemagglutinating activity of these lectins was calcium (Ca²⁺) dependent, heat-labile and is strongly inhibited by D-galactosides.

SILKWORM LECTINS

Innate immunity is a primary defense mechanism based on innate pattern recognition, in which molecular structures common to invading pathogens, known as pathogen-associated molecular patterns (PAMPs), are recognized by the host. The host molecules that recognize PAMPs are called pattern recognition proteins (PRPs), which function in both mammalian and insect innate immunity (Royet, 2004). Such pattern recognition receptors bind to certain molecular patterns present in the array of carbohydrate components on the surface of microorganisms. These microbial patterns include lipopolysaccharides and peptidoglycan in bacterial cell walls and β -1,3-glucans from fungal cell walls (Begum et al., 2000; Zhu et al., 2006).

Hemagglutinating activity purified from haemolymph of silkworm, *B. mori* by ammonium sulfate fractionation, gel-filtration on sephacryl S-300 and affinity chromatography revealed that *Bombyx* hemagglutinin is a tetramer of two different subunits with molecular weights of 88,000 and 90,000 (Amanai et al., 1990). Hemagglutinating activity of the haemolymph changed developmentally in the 4th and 5th instars; with observed increase in the activity during larval molting associated with renewal of larval tissues as well as at the times of pupal ecdysis. Also, the naturally occurring hemagglutinins were shown to be dependent on Ca²⁺ for their activity and were proved to be heat-labile and susceptible to pH extremes (Patnaik et al., 2012).

Immunohistological observations have established the occurrence of lectin (protein of 49 K) in the cuticular intima of *Bombyx* anterior silk glands suggesting important role in histolysis during molting and metamorphosis (Amanai et al., 1991).

Partial purification of the hemagglutinating activity from haemolymph of *B. mori* revealed a protein with a molecular weight of 260 K that binds preferentially to fixed sheep RBC's (Suzuki and Natori, 1983). In *Bombyx*, the hemagglutinating activity increased significantly in the haemolymph after infection with CPV, and that a lectin-like protein with a subunit molecular weight of 280 K was induced concomitantly after infection (Mori et al., 1989). A series of isolectins from giant silkworm, *Hyalophora cecropia* was purified by affinity chromatography with a molecular weight of around 160 K and the sizes of the subunits were 41 and 38 kD for A and B chains, respectively. Animal C-type lectins (calcium-dependent lectins) have been reported as important candidates in pathogen recognition and cellular interactions. Collectins, a sub-group of C-type lectin superfamily, play a first line of defense against infections. Collectins contain a carbohydrate-recognition domain (CRD) connected to a collagen-like domain. Mannose-binding protein (MBP), the most extensively studied collectin, can activate the complement system through the lectin pathway (Matsuhita, 1996).

Seven C-type lectins from lepidopteron insects have been described, suggesting their importance in insect defense against invading microorganisms, especially Gram-negative bacteria. It includes immuelectin-1, -2, -3, and -4 from the tobacco hornworm, *Manduca sexta* (Yu et al., 1999; Yu et al., 2000), a lectin from the fall webworm, *Hyphantria cunea*, designated *H. cunea* lectin (Shin et al., 2000); LPS-binding lectin [*B. mori* LPS-binding protein (Bm-LBP)] from the silkworm, *B. mori* (Koizumi et al., 1999; 1997) and *B. mori* immuelectin (Kim et al., 2003). Immuelectin-1 and -2, *H. cunea* lectin and BmLBP bind to gram-negative bacteria via LPS, a gram-negative PAMP. Immuelectin-3 specifically binds to LPS, lipoteichoic acid and β -1,3-glucan. Immuelectin-4 binds to N-galactosamine (GalNAc) and glucose. BmLBP have been reported to trigger hemocyte-mediated nodule formation and plays a role in elimination of invading Gram-negative bacteria from the hemocoel.

It is essentially clear that two C-type lectins from *B. mori* have been reported. BmLBP recognizes Gram-negative bacteria, as stated above; however, the recognition target of *B. mori* immuelectin (BmIML) is unknown. These C-type lectins have two different carbohydrate recognition domains (CRDs) arranged in tandem and are different from most animal C-type lectin that have a single CRD. The insect lectins with tandem CRDs have increased binding affinity to carbohydrates on the surface of pathogens. Also the CRDs have been divided into two types, a 'short form' approximately 115 residues long and a 'long form' approximately 130 residues long which

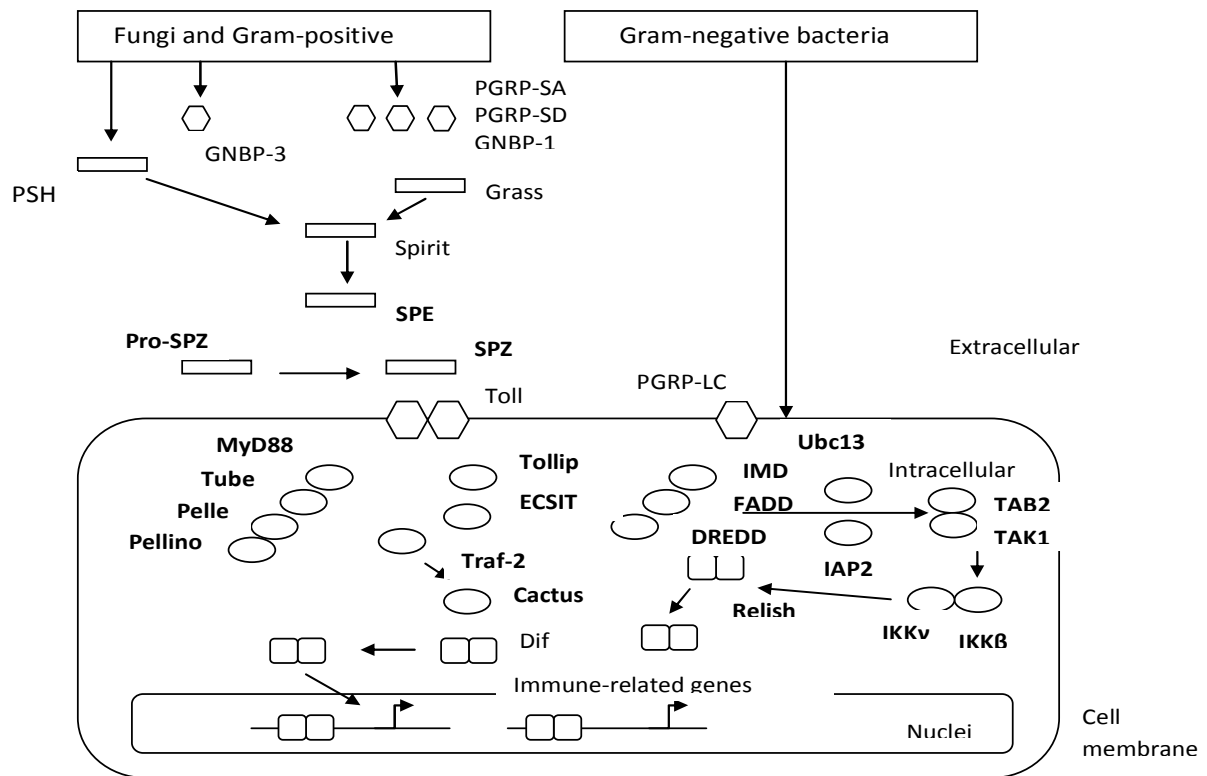


Figure 1. Toll and IMD pathways for immune regulation in *Drosophila*. The factors represented in bold letters indicate *B. mori* ortholog of genes encoding these factors. The factors represented in normal letters indicate no *B. mori* ortholog of genes for them. - Represent proteins involved in recognition Represent proteins involved in extracellular pathway - Represent proteins involved in intracellular pathways - Represent transcription factors. PGRP & GNBPs- Peptidoglycan Recognition Protein & Gram-negative bacteria binding protein (Pattern Recognition Proteins), NEC- Necrotic (Serine Proteinase inhibitor), PSH- Persephone (Serine Proteinase), SPE- Spatzle Processing Enzyme, SPZ- Spatzle (Cytokine like function), ECSIT- Evolutionarily conserved intermediates in Toll pathway, TRAF2- Tumor Necrosis factor receptor-associated factor 2, IAP-2- Inhibitor of Apoptosis 2, TAK-1- Transferring growth factor-activated kinase 1 (serine/threonine protein kinase), TAB-2- TAK-1 binding protein, IKKβ- IκB kinase β, IKKγ- IκB kinase γ.

includes two additional disulfide-bond cysteine residues at the amino terminus. In the insect two-domain lectins, including immulectin-2, the amino-terminal CRD1 is the short form whereas the carboxyl-terminal CRD2 is the long form. It has been shown that the CRD in the C-terminal half activates prophenoloxidase.

Lately, C-type lectin (*B. mori* multibinding protein (BmMBP) have also been reported that can bind to Gram-positive bacteria, Gram-negative bacteria and yeasts (Watanabe et al., 2006). This unique C-type lectin has two dissimilar CRDs, a short form (CRD1) and a long form (CRD2) with broad, dissimilar spectra for binding target sugars. These properties enable BmMBP to bind to two sites on a microorganism, facilitating high-affinity. CRD1 of BmMBP bound to teichoic acid and mannan and CRD2 bound to teichoic acid, peptidoglycan and mannan, PAMPs of Gram-positive bacteria and yeasts. High-affinity binding of BmMBP was attributable to two-point binding to teichoic acid and mannan. It is therefore remarked that BmMBP acquired its capacity to bind

multiple microorganisms by virtue of having two CRDs, each of which demonstrated wide range of sugar specificities. BmLBP, a C-type lectin recognizing LPS, is reported to exist as a polymer in the haemolymph of *B. mori* (Koizumi et al., 1997). BmLBP was found to be bound to Gram-negative bacteria, enhancing nodule formation by hemocytes resulting in elimination of bacteria.

Phylogenetic studies of the sequences encoding the short and long form CRDs have been conducted (Figure 2). The short-form CRDs from BmLBP, immulectin-1, immulectin-2 and *H. cunea* lectin, which binds LPS, clustered in the same group, whereas, CRDs from immulectin-3 and immulectin-4, which bind GalNAc and glucose clustered together, as did the long-form CRDs from these lectins (Yu et al., 2000). CRDs of BmMBP, though, were not grouped with any of these lectins, were therefore placed separate from all groups. BmMBP was suggested to function as a trigger for the nodule reaction, atypical of cellular reaction in insect immune responses.

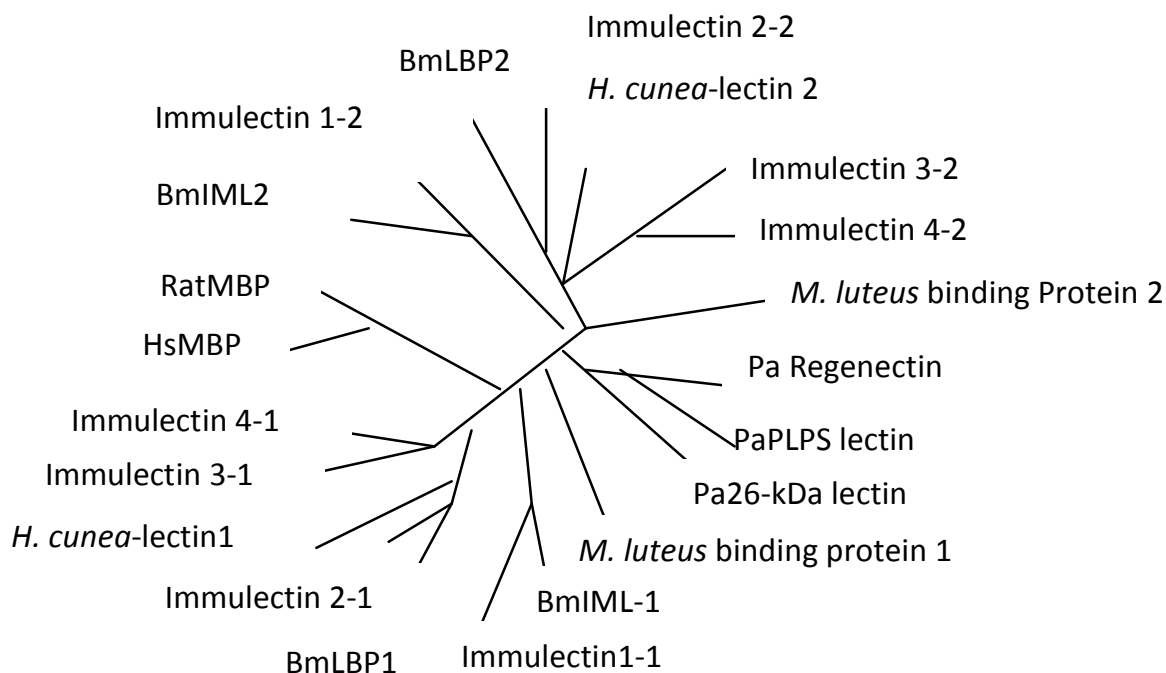


Figure 2. Phylogenetic relationship tree of *B. mori*, *M. luteus*-binding lectin and various C-type lectins.

Three *B. mori* low-expression lectins, designated BmLEL-1, BmLEL-2 and BmLEL-3 were identified by silkworm genome database search and it was observed that these are expressed in testis and ovary, while BmLEL-2 expression was upregulated after bacterial infection and were shown to bind rough and smooth strains of Gram-negative bacteria (Takase et al., 2009). This again indicated that novel C-type lectins might play a role in the innate immunity in these tissues as PRPs.

Hemocytin, a *B. mori* humoral lectin gene with significant homology with mammalian von Willebrand factor and coagulation factor V and VII was cloned having a sequence of 10477 bp encoding 3133 amino acids (Kotani et al., 1995). cDNA encoding the C-terminal sequence region of the gene was shown to induce hemagglutinating properties in recombinant virus infected cells and was inhibited by D-mannose, N-acetyl-D-galactosamine and D-maltose. Transcription of the gene was evident in hemocytes of silkworm at larval-pupal metamorphosis and after injection of *E.coli* and LPS. Sialic acid (N-acetylneuraminic/N-glycolylneuraminic acid) binding specificity was noticeable in the natural agglutinin derived from the haemolymph of *B. mori* (Patnaik et al., 2012). 30-kDa lipoproteins, major components of *B. mori* haemolymph (Ujita et al., 2002), specifically bind to glucose, maltose and glucans. Such lipoprotein formed *in vivo* in haemolymph is an endogenous signal of immune activation. These lipoproteins form a complex with lipopolysaccharide of bacteria and this complex strikingly reduces the cecropin

inducibility of lipopolysaccharide. Hemocytin is synthesized as 343 kDa precursor and posttranslationally processed into a 280 kDa mature protein and comprises of a lectin domain homologous to mannose binding protein. It is known to stimulate aggregation of *B. mori* hemocytes and is induced after whole-bacterial, LPS or peptidoglycan challenge.

CONCLUSION

It is therefore apparent that the isoforms associating with separate microorganisms are different, suggesting unique roles lectins play in recognition and differentiation of microbes. The functional diversity of lectins in invertebrates with different carbohydrate specificities therefore seems to evolutionarily compensate for lack of acquired immunity. Most specifically, the role of insect C-type lectins in defense and their prominent role in recognition have to be further validated, though the identification of several new lectin-like molecules in silkworm may provide justifiable basis to the recognition network against all the invading microbes.

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