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Trends in Carbohydrate Research

β-Trefoil Lectins of the Family Mytilidae from a Comparative Perspective

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This paper has been dedicated to Professor Bishnu P. Chatterjee on his 80th birthday.

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Abstract

Structural and functional differences are compared among lectins with β-trefoil folds, an essential and representative framework of proteins. The fundamental framework of this folding is the triple tandem repeat of subdomains consisting of 40 amino acids making four β-sheets to construct barrel and clover-shaped lid parts. Lectin databases UniLectin and TrefLec classified 12 categories of lectins with the β-trefoil fold in all biological domains and viruses. Each group appeared with a different proportion of distributions in each biological domain. SeviL and MytiLec represented β-trefoil lectins in the family Mytilidae. Nevertheless, each lectin had different primary structures, converging the same β-trefoil. The structural properties of MytiLec have also been shared with that of bacterial lectins. Furthermore, each mussel lectin bound to glycans of glycosphingolipid though the structures differed. Their glycan-binding properties seem to be helpful for a few applications of lectins because they recognized characteristic glycans relating to cell regulation of NK cells (GA1), auto-immune diseases (GM1b), cancers (Gb3), and regenerations (SSEA-1).

Keywords: Lectin; β-trefoil fold; Mytilidae; SeviL; MytiLec

1. Comparison of Protein Folds Among Lectins

Vast numbers of structures of lectins have been compared in a database, UniLectin3D (https://www.unilectin.eu/unilectin3D/).[1] The database classified around 30 structural folds that consisted of lectins. β-Sandwich folds, including Concanavalin A (ConA)-like lectin in *Canavalia ensiformis* (jack bean) seeds is the largest category of lectins. The group includes L(Leguminosae)-type lectin (ConA), galectin, calnexin-calreticulin-like, and Rotavirus spike protein, which all have β-sandwich fold consisting of two extended anti-parallel β-sheets. The second largest lectin group is β-trefoil lectins initially found in a plant toxin ricin (**Figure 1A**),[2] also called R(Ricin)-type lectins, as mentioned later. It consisted of triple tandem repeating subdomain containing β-sheets in a polypeptide. These β-sheets made knits a 3-leaf clover-like shape. The lectin is presented in diversified organisms. The review focused on this topic. Twenty-five percent of lectins recognize galactose (**Figure 1B**), and animal lectins represent 17% of the total (**Figure 1C**). D-Galactose (Gal) seemed to originate as an important monosaccharide later in evolutionary time than

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glucose, fructose, or mannose and therefore appears to play critical roles in cellular recognition.[3]

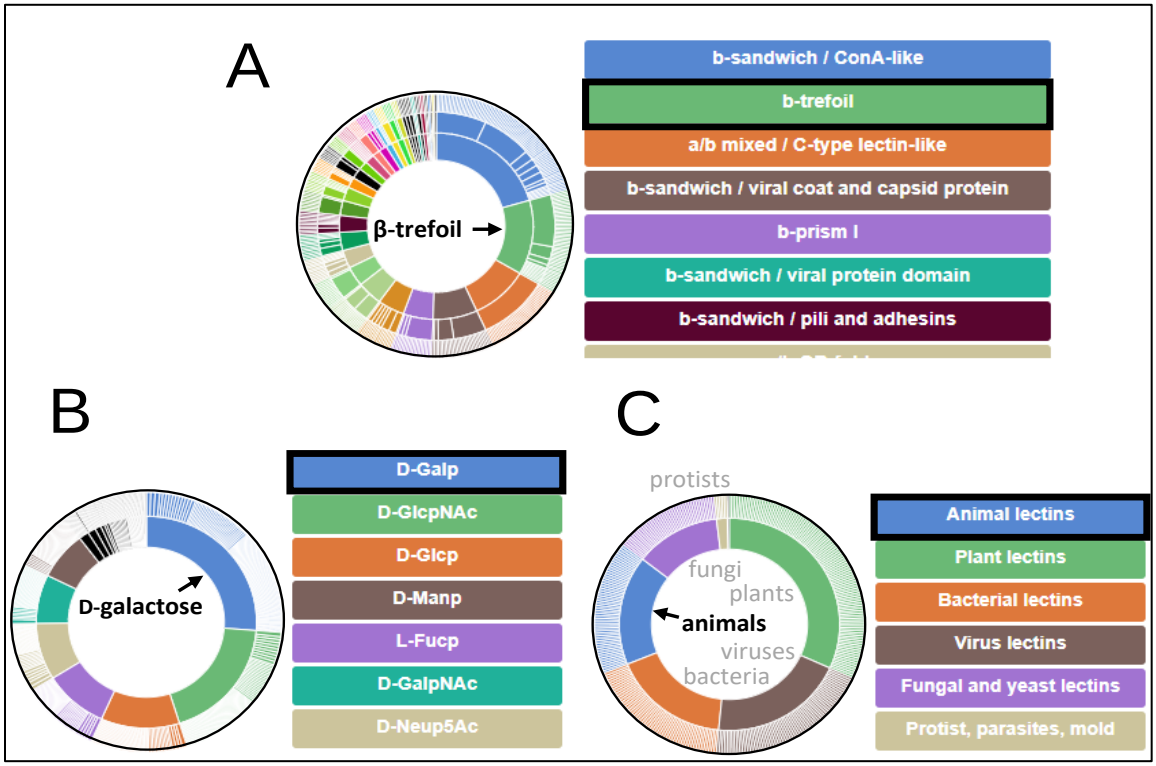

Figure 1. The comparison of structural folding of lectins browsed by a database of UniLectin3D (https://www.unilectin.eu/unilectin3D/). The percentages of protein folds (A); carbohydrate-binding properties (B); distribution among organisms and viruses (C) of lectins.

Another database, UniLectin (https://www.unilectin.eu/),[1] has recently updated data from UniLectin3D to improve the classification and prediction of lectins. LectomeXplore (https://www.unilectin.eu/predict/)[1] classified the proportions of organisms having lectins using data from over 1.2 million lectins in 26 thousand biological species. Viral and bacterial lectins occupied 15 and 10 % in total, respectively. It would be the next research trend to characterize the detail of archaeal lectins to clarify the relationship between Prokaryotes (domains of Bacteria and Archaea) and Eukaryotes via glycobiology (**Figure 2A**). This software taught us those organisms had significantly different ratios of each structural fold of lectins.

ConA-like lectins, galectins, and C-type lectins are representative lectin families of plants (ConA-like) and animals (galectin and C-type lectin). Compared to β-trefoil and ConA, C-type lectin contains two α-helices and two small β-sheets. These folds are shared mainly in Eukaryota. The motif of L-type also appeared in the domain bacteria (**Figure 2C-E**).

The pattern that shares the same lectin structure in bacteria and eukaryotes was also seen in the distributions of cyanovirin-N, which has a triple-stranded β-sheet with a β-hairpin (**Figure 2F**). Cyanobacteria and algae have this mannose-binding lectin and a wide range of antiviral activity. Almost the protein folds that make oyster mannose-binding lectin and cholera toxin lectin had only expressed within the domains of Eukaryota and Bacteria, respectively (**Figure 2G** and **H**). The distribution of influenza hemagglutinins (lectins) was almost conserved within viruses (**Figure 2I**). A few proportions of lectin had appeared beyond domains. It may suggest that they happen through horizontal gene transfer by evolution. Lectins with β-trefoil fold had been widely distributed among bacteria and eukaryotic domains (**Figure 2B**).[2]

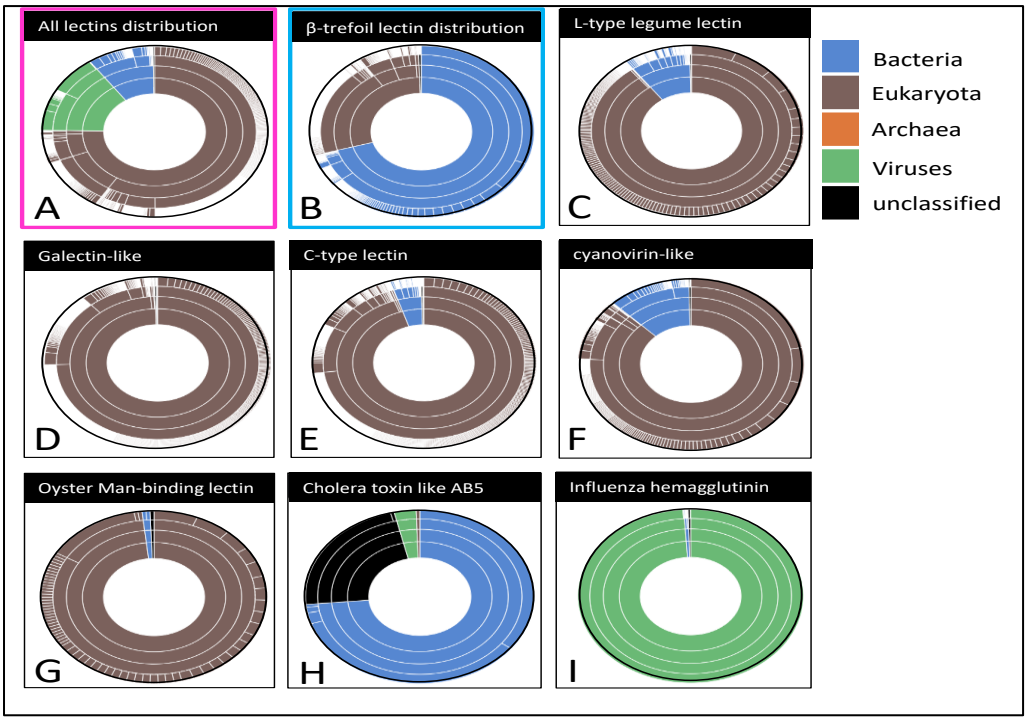

Figure 2. The comparison of primary structure families of lectins browsed by a database of LectomeXplore (https://www.unilectin.eu/predict/). Distributions of all lectins (A); β-trefoil lectin (B); L-type lectin (C); galectin-like (D); C-type lectin (E); cyanovirin-like lectin (F); oyster mannose-binding lectin (G); cholera toxin-like (H); and influenza-hemagglutinin (I) in biological domains (Bacteria, Archaea, and Eukaryota) and viruses.

2. Fundamental Properties and Diversity of Ricin and β-Trefoil Lectins

The castor seed oil plant (*Ricinus communis*) is the resource of ricin toxin, believed to originate from India and Africa.[4] H. Stillmark in 1888 found agglutination of different kinds of red cells by castor seed extract what he called ricin, the structure of it was determined after 100 years.[2] Two subunits, A and B, construct the ricin, and the B-subunit contains two β-trefoil folds of ca. 120 amino acids, including triple tandem repeating subdomains of ca. 40 amino acids. They bind to LacNAc (Galβ1-4GlcNAc). Subdomains have a Q-x-W motif. The sequences of the ricin-B chain were found in lectins from all biological domains, called "R-type lectins".[2] β-Trefoil fold architects various kinds of proteins such as toxins, enzymes, cytokines, and protease inhibitors in addition to lectins of all the biological domains and viruses.[3] This evidence suggests that the protein framework of the β-trefoil has the potential to function in various roles, such as protease inhibition and catalysis, in addition to carbohydrate-binding.[5] The fold believes to be tinkered with in the evolution and producing different proteins.

An UniLectin database, TrifLec (https://www.unilectin.eu/trefoil/),[1] is a tool for the β-trefoil fold. This software could classify β-trefoil fold lectins into 12 subgroups (**Figure 3A**): 1) Ricin-like (plants and others, 48%), 2) Cys-rich mannose receptor (mammals, 27%), 3) earthworm (Annelida, 12.3%), 4) entamoeba (5.4%), 5) mytilectin (mussels, 2.3%), 6) *Boletus* and *Laetiporus* (fungi, 2.1%), 7) *Sclerotinia* (fungi, 1.1%), 8) clostridial toxin (bacteria 0.7%), 9) *Coprinus* (fungi, 0.7%), 10) Amaranthin (plants, 0.3%), 11) SeviL, (mussels, 0.2%) and 12) *Clitocybe* (fungi, 0.04%).

This archive amazed us that bacteria were the primary ruler of genes of the ricin-B chain, even though ricin toxin was found in caster beans. Evidence that many bacteria had similar primary structures with the ricin B-chain suggests that the plant β-trefoil lectin derived from Prokaryotes (**Figure 3D**, the blue in the pie chart). This property was very different from other plant lectin genes, such as ConA-like lectin (L-type lectin) (**Figure 2C**), almost distributed in Eukaryota. The primary structure of the entamoeba lectin also has a pattern similar to that of the ricin B-chain (**Figure 3D** vs **3E**), suggesting that most of its ancestors were

prokaryotes. Similarities to the genes of Archaea were also observed for some entamoeba lectins (**Figure 3E**, the orange in the pie chart). The most potentially harmful toxin, like ricin, botulinum toxin produced by *Clostridium botulinum*, has β-trefoil subunits. This group is limitedly located explicitly in both the Bacteria domain and unknown (**Figure 3I**). These bacteria seemed to be gained the toxin by a horizontal gene transfer by bacteriophages.[6] Mammalian mannose receptors on macrophages have a β-trefoil at the N-terminus domain. The sibling of the domain differs from plant and bacteria lectins that seemed to be derived from prokaryotes. The mammalian lectin was seemed to originate from eukaryotes (**Figure 3F**). The distribution pattern of the mannose receptor was similar to that of fungus β-trefoils (**Figures 3HF** and **3H**).

3. β-Trefoil Lectins in the Family Mytilidae

Invertebrates show entirely different characteristics. The two trefoil lectins of earthworm and mussels also contain similarities to bacterial, archaeal, and viral sequences (**Figures 3B** and **3C**). They are classified as phylum Annelida and Mollusca of Protostomia, respectively. Some Protostome invertebrates are categorized into Lophotrochozoa (a trochophore larval stage with a crown of ciliated tentacles) and Ecdysozoa (animals that grow by molting their exoskeleton). The Annelida and Mollusca classify as Lophotrochozoa. Through the structural glycobiology of β-trefoil lectins of mussels (family Mytilidae) in the phylum Mollusca, we have studied characteristics of lectins.

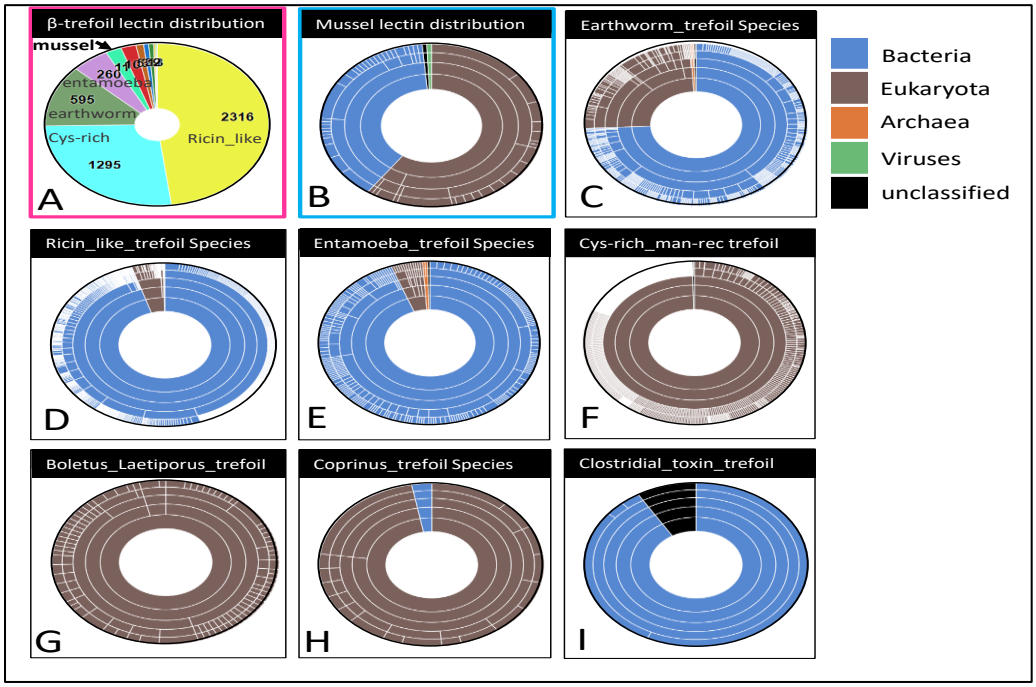

Figure. 3. The comparison of primary structures in the β-trefoil lectin family browsed by a database of TrifLec (https://www.unilectin.eu/trefoil/). Distributions of all β-trefoil lectins (A); mussel β-trefoil lectin (B); earthworm β-trefoil lectin (C); ricin like β-trefoil lectin (D); entamoeba β-trefoil lectin (E); Cys-rich mannose-receptor β-trefoil lectin (F); *Boletus* and *Laetiporus* fungal β-trefoil lectin (G); *Coprinus* β-trefoil lectin (H); and Clostridial toxin β-trefoil lectin (I).

The family Mytilidae is a valuable resource for lectin studies in addition to foods. In 1998, a GalNAc/Gal-binding lectin termed CGL (*Crenomytilus grayanus* lectin) was purified from Gray's mussel.[7] In 2010s, the characteristic β-trefoil lectins in the family Mytilidae were identified in mussels of the Mediterranean mussel (*Mytilus galloprovincialis*),[8,9] the purple bifurcate mussel (*Mytilisepta virgata*),[10,11] Gray's mussel (*Crenomytilus grayanus*),[12] *Mytilus calfornianus*,[13] and *Mytilus trossulus*[14] and their valuable properties were reported.

4. The Property of β-Trefoil Fold in Mytilidae

M. virgata and *M. galloprovincialis* are common mussels distributed at the Pacific Ocean seashore in the far East. SeviL and MytiLec both D-galactose-binding lectins were purified from each species of the mussels, respectively. SeviL with 15 kDa polypeptide

(129 a.a.) is a typical R-type lectin. The lectin consists of a triple tandem subdomain. SeviL is involved in Q-x-W motifs in the polypeptide as same as ricin (**Figure 4** upper).[15,16] On the other hand, MytiLec has a slightly larger 17 kDa polypeptide (149 a.a.) than SeviL (**Figure 4** lower).[17,18] Each lectin had a characteristic sequence with less than 10% similarity from the common R-type lectin family. Besides, SeviL contains Q-x-W motifs, a typical character of R-type lectins, whereas such motif has been found absent in MytiLec (**Figure 4** SeviL vs. MytiLec).

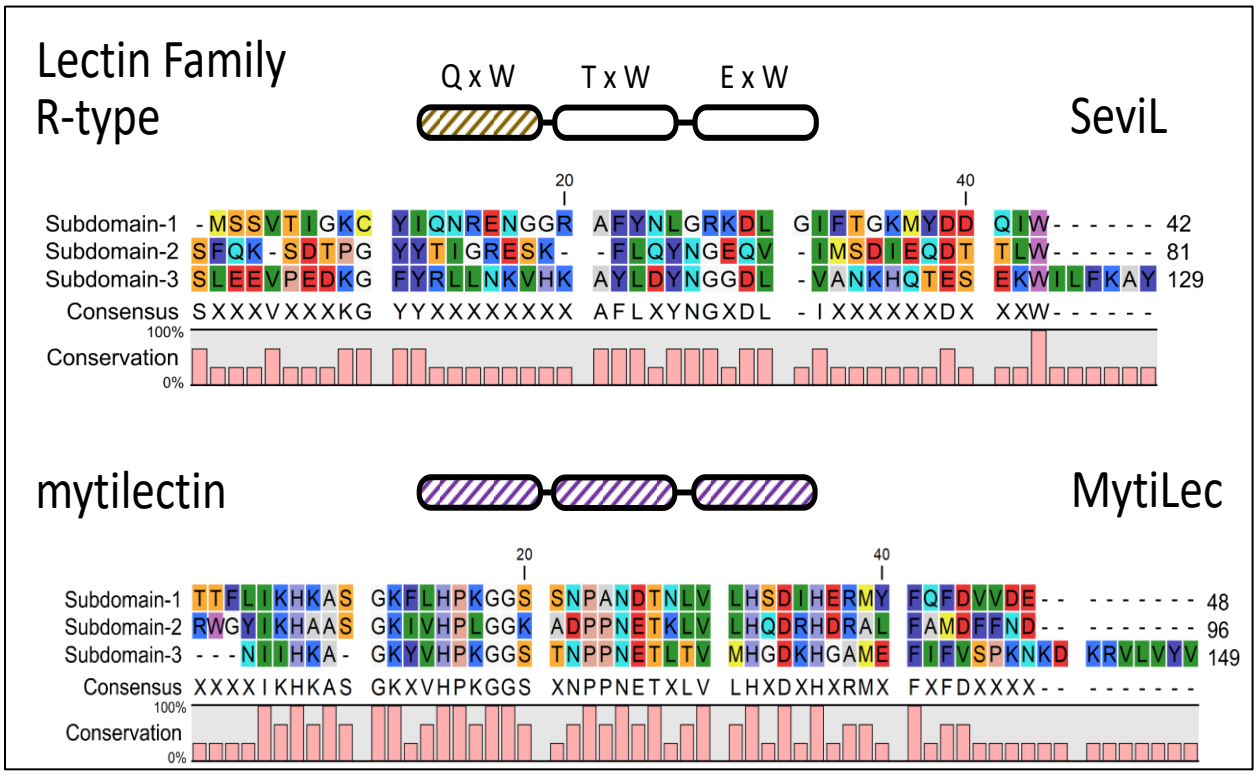

Figure 4. Comparison of primary structures of R-type lectin (SeviL) and mytilectin (MytiLec) families in the family Mytilidae. Subdomains having glycan-binding activity are indicated by a dotted scheme (subdomain-1 in SeviL and subdomain-1 to 3 in MytiLec). Subdomain-1 to 3 correspond to subdomain-α to γ in Figure 5. Subdomains colored slash indicate to have glycan-binding property

Crystallography study elucidated that these mussel lectins converged to have a β-trefoil fold even though they have no similarity in the primary structure. Twelve regions of a few amino acids make β-sheets in a polypeptide, folding lid and barrel parts of subunits. Trefoil-shaped lid part binds to glycans, and the barrel part makes a dumb-bell shaped dimer. Three conserved Q-x-W sequences (indicated in green) fold in the barrel (**Figure 5**). SeviL (PDB 6LF1)[11] has carbohydrate-binding activity only in the α-subdomain, the same is found in ricin B-chain)[2] MytiLec (PDB 3WMU)[9] as similar overviews. TrefLec database already announces nine relatives of SeviL R-type lectins. Still, their distributions are within Mytilidae, which will expand the finding among other organisms and domains. On the other hand, the software analysis showed the structure of MytiLec and its orthologs are present among some other relative marine mussels of different phyla, such as Dinoflagellate,[19] Brachiopoda,[20] Rotifera,[21] and Porifera,[22] respectively **Figure 5**).

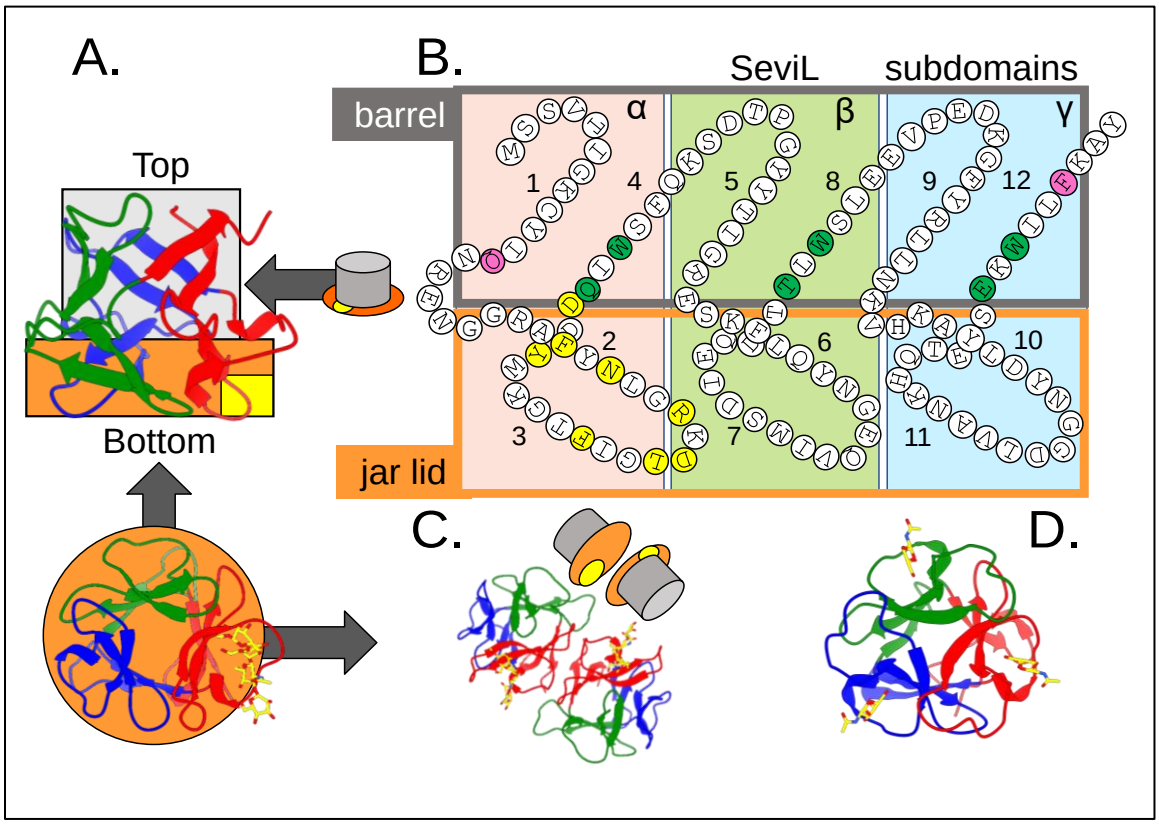

Figure 5. Structure of mussel β-trefoil lectins.

A: 3-D structure of SeviL. The barrel and jar lid parts in a polypeptide indicate gray and orange, respectively. The yellow square in the jar lid (orange) indicates the part of carbohydrate-binding. β-trefoil forms in the jar lid part and binds to glycans. **B**: Primary and secondary structure of SeviL. Numbers indicate the positions of the twelve β-sheet in the polypeptide. Each subdomain is indicated by the N-terminal side (α: red area), mid (β: green area), and C-terminal side (γ: blue area). Yellow residues indicate amino acids relating the glycan-binding. Green residues indicate the Q-x-W sequence in each subdomain. Blue residues (Q(Gln)12 and F(Phe)126) indicate amino acids related to dimerization. **C**: Dimeric form of SeviL. Two subunits non-covalently face binding the lids of each other. One glycan can bind to one subdomain in the subunit. **D**: 3-D structure of β-trefoil of MytiLec. Figures like hats consisting of gray and orange respond to the β-trefoil in A and B.

5. Different Glycan-binding Properties within SeviL and MytiLec

The array and scanning technology showed SeviL (R-type lectin), and MytiLec (mytilectin) specifically bind to glycans of glycosphingolipids. This will be a specific trait on mussel lectins, comparing that many other lectins bind to glycoproteins and glycolipids. Besides, the structures of target glycans recognized by each lectin were characteristic (**Figure 6**). SeviL bound to glycans as **01:** GM1b (Neu5Acα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc), **02:** GA1 (Galβ1-3GalNAcβ1-4Galβ1-4Glc), **03:** stage specific embryonal antigen (SSEA)-4 (Neu5Acα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc).[15] They have a core structure indicated by Galβ1-3GalNAcβ1-3/4Gal. On the other hand, MytiLec bound to **04:** heparin, **05:** Lacto-*N*-tetraose (LNT: Galβ1-3GlcNAcβ1-3Galβ1-4Glc), **06:** GA2 (GalNAcβ1-4Galβ1-4Glc), **07:** Gb3 (Galα1-4Galβ1-4Glc), **08:** Gb4 (GalNAcβ1-3Galα1-4Galβ1-4Glc), and **09:** Forssman antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc).[17]

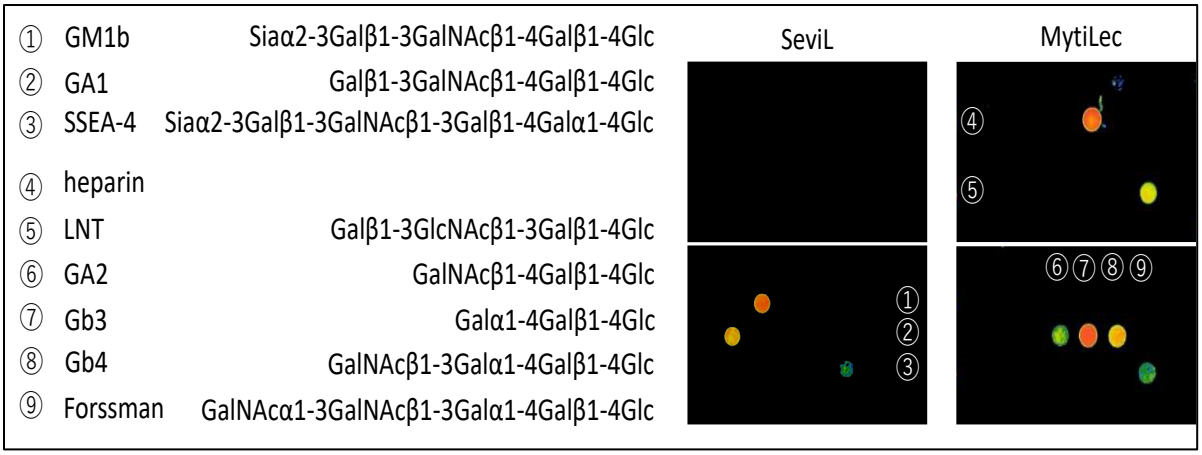

Figure 6. Comparison of glycan-binding properties of SeviL and MytiLec.

Each lectin was administrated to a glycan-immobilized microarray, and a surface plasmon resonance biosensor detected the binding. Left: Names and their structures of glycans. Right: Numbers indicate the recognized glycans by SeviL (1-3) and MytiLec (4-9), respectively.

These glycan structures recognized by mussel lectins are closely related to biomedical phenomena and diseases. SeviL recognized GA1 (**01**) that expresses on the NK (natural killer) cells explicitly. Lectin binding to the glycan would be able to regulate the growth of NK cells for clinical studies. GM1b (**02**) is a glycan that is captured by the autoantibody generated by Guillain-Barré syndrome, which happened by the infection of *Campylobacter jejuni*. Molecular engineering to generate monomer SeviL stably, the artificial lectin may block the auto-antibody's injury. SSEA-4 (**03**) on the surface of iPS (induced pluripotent stem) cells. The binding SeviL would be available to apply for a biomarker of differentiations and regenerations. On the other hand, MytiLec bound to heparin (**04**), an anti-coagulant consisting of glycosaminoglycan. The applications to LNT (**05**) in milk, GA2 (**06**) related neuro diseases, and Gb4 (**08**), which is known to bind Toll-like receptor-4, are still considerable. Gb3 (**07**), recognized by the lectin, is a target of Vero-toxin and is expressed on Burkitt lymphoma cells. MytiLec and SeviL induced apoptosis of cancer cells expressing Gb3 and GA1, respectively. The binding specificity of MytiLec to glycosphingolipids, such as against Forssman antigen (**09**), will benefit future studies.

6. Cellular regulative activities of mussel lectins and other β-trefoil lectins

Mussel lectins act as anti-cancer agents to cancer cells, governs cell growth, and cell differentiation depending on the concentrations and glycan structures. Various kinds of kinases localize at the inside of the glycosphingolipid-enriched microdomain. The administration of mussel lectins to cancer or normal cells with target glycans of the lectin-activated MAP kinases such as MEK and ERK induces biological regulations such as cell growth, differentiation, and apoptosis.[23-26] A dimer form of lectins is essential to regulate cells. Mitsuba-1, a monomerized MytiLec developed by genetic engineering, has been found to lose its apoptotic activity against Burkitt's lymphoma cells compared to the dimeric form.[27]

A scheme of the induction of signal transduction and cell growth regulations by mussel lectins binding to the ligand glycans on the cell surface is presented as **Figure 7**.

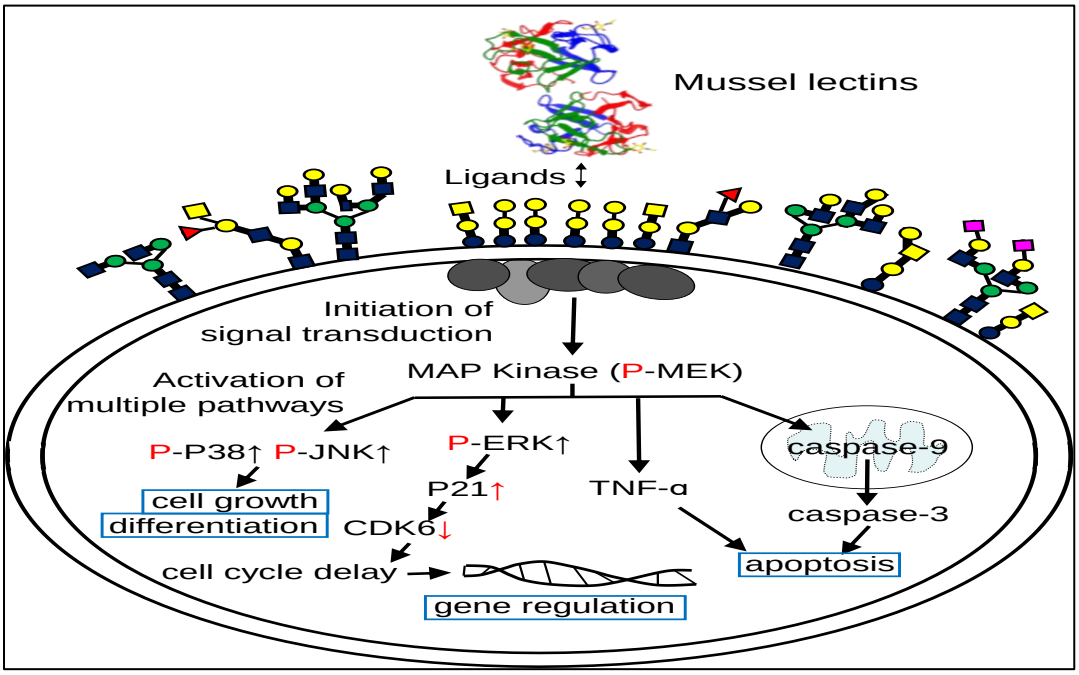

Figure 7. A scheme of the induction of signal transduction and cell growth regulations by mussel lectins. Lectins bind to the ligand glycans on the cell surface, activating kinases to regulate such as cell growth, differentiation, gene regulation, and apoptosis indicated by blue boxes.

7. Conclusion

The members of β-Trefoil lectins are growing including typical R-type lectins with the Q-x-W sequence and new categories as mytilectin has been described here. These data are available to the archive by a carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/CBM13.html).[28] The recent findings of the lectin sequence from the Archaeal domain in the deep sea and Mimivirus, in addition to phage, will develop the environmental sciences and pharmaceutical applications through this lectin family.[28] In addition to integrating molecular design by computer sciences and gene recombination to develop synthetic lectin drugs like Mitusba-1[27], the β-trefoil lectin is being tried to make "Lectibody" by combination with antibodies.[29] The β-Trefoil fold can potentially be applied to generate lectins for manufacturing future new drugs.

Acknowledgments

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