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Cyborg Lectins: Novel Leguminous Lectins with Unique Specificities[1]

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***Bauhinia purpurea* lectin (BPA) is one of the β-galactose–binding leguminous lectins. Leguminous lectins contain a long metal-binding loop, part of which determines their carbohydrate-binding specificities. Random mutations were introduced into a portion of the cDNA coding BPA that corresponds to the carbohydrate-binding loop of the lectin. An library of the mutant lectin expressed on the surface of lambda foo phages was screened by the panning method. Several phage clones with an affinity for mannose or *N*-acetylglucosamine were isolated. These results indicate the possibility of making artificial lectins (so-called "cyborg lectins") with distinct and desired carbohydrate-binding specificities.**

Key words: *Bauhinia purpurea* lectin, lectin, phage display system, specificity.

Lectins, a class of carbohydrate-binding proteins, that are widespread among animals, plants, and prokaryotes (*1*), are classified into several groups based on their structures (*2, 3*). Among them, both C-type lectins in animal cells and leguminous lectins in plant seeds show a variety of carbohydrate-binding specificities despite their similar primary and tertiary structures (*2, 3*). Previously, we purified a nonapeptide with an affinity for lactose from galactose-binding *Bauhinia purpurea* lectin (BPA) by means of lactose-Sepharose column chromatography. The synthetic nonapeptide, DTWPNTEWS, from BPA was found to posess lactose-specific binding activity in the presence of calcium (*4*). Similar studies were carried out on several anti-H(O) lectins using Fuc-Sepharose for *Lotus tetragonolobus* lectin (LTA) and *Ulex europeus* lectin I (UEA-I), and oligo-GlcNAc-Sepharose for *U. europeus* lectin II (UEA-II) and *Labrnum alpinum* lectin I (LAA-I). Peptides that showed affinity for haptenic sugars were shown to be homologous to one another and to form part of the metal-binding region of the lectins (*5–9*). In order to confirm the assumption that the metal binding region, especially the calcium-binding portion, defines the carbohydrate-binding specificities of leguminous lectins, we constructed a chimeric lectin gene using a cDNA clone coding β-galactose–binding BPA in which the nonapeptide sequence, DTWPNTEWS, was replaced by the corresponding region from the α-mannose–binding *Lens culinaris* lectin (LCA). The chimeric lectin expressed in *Escherichia coli* cells was found to bind to α-mannosyl-bovine serum albumin (BSA) instead of to β-galactosyl-BSA (*10*). Interestingly, the chimeric lectin was shown to have a distinctly different carbohydrate-binding specificity than those of either BPA or LCA.

Filamentous bacteriophages can display foreign peptides or proteins on the surface of virus particles by fusing to vial coat proteins, and have proven to be useful for selecting particular clones from a large ensemble. This technique has been used to select particular clones by constructing libraries of various peptides that can be screened *in vitro* by affinity selection (*11–13*). Other surface expression schemes also have been devised for bacterial cells and animal viruses. All of the these systems rely on the ability of the fusion proteins to translocate across the plasma membrane. We previously reported a bacteriophage vector, λfoo, for the display of foreign proteins fused to the V protein, a major protein in the phage tail. This vector has been used to display the homo-tetrameric leguminous lectin BPA on the surface of lambda phage (*14*).

Here we introduced random mutations in the cDNA corresponding to the carbohydrate-binding nonapeptide of BPA lectin, and expressed these mutants on the tail of λfoo phages as fusion proteins. Among recombinant phages, several phage clones expressing specific sugar-binding properties were screened and cloned successfully. Using these techniques, we demonstrate the possibility of making artificial lectins with desired carbohydrate-binding specificities.

MATERIALS AND METHODS

Materials—Restriction enzymes were purchased from Boehringer (Mannheim, Germany) or New England Biolabs (Bevely, MA, USA). Taq DNA polymerase was from Perkin-Elmer (Branchburg, NJ, USA). α-Mannosyl-BSA (Man-BSA), β-galactosyl-BSA (Gal-BSA), α-fucosyl-BSA (Fuc-BSA), *N*-acetylglucosaminyl-BSA (GlcNAc-BSA), and *N*-acetylgalactosaminyl-BSA (GalNAc-BSA) were obtained from Sigma (St. Louis, MO, USA).

Construction of the Fusion Vectors—λfoo vector was con-

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Abbreviations: BPA, *Bauhinia purpurea* lectin; LCA, *Lens culinaris* lectin; Gal, galactose; Fuc, fucose; Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; BSA, bovine serum albumin.

structed as described elsewhere (*14*). For the construction of λBPA, BPA cDNA was amplified by polymerase chain reaction (PCR) with a pair of oligonucleotide primers, 5′-CCCGGATCCCGACAAGCTCAACCTTA (B-primer) and 5′-CCCGGAATTCTGATTACATACTGGAATA (E-primer), as described previously. These primers were designed to obtain a cDNA encoding mature BPA lectins flanked by artificial sites for *Bam*HI and *Eco*RI at the 5′ and 3′ ends, respectively. After digestion with *Bam*HI and *Eco*RI, the amplified fragments were ligated into the *Bam*HI and *Eco*RI sites of λfoo vector. *In vitro* packaging of the ligated λfoo-BPA DNAs (λBPA) was carried out using Max Plax (Epicentre Technologies, Madison, WI, USA) as described by the manufacturer. A schematic diagram of the construction of a BPA lectin gene library is shown in Fig. 1a. To construct cDNAs of the BPA lectin library (BPA whose nonapeptide sequence, DTWPNTEWS, is replaced with rando-mized nonapeptide sequences), first the cDNA coding the NH_2-terminal fragment of BPA, encompassing nucleotides 108 to 408 of the BPA coding region with *Bam*HI and *Afl*II sites at the 5′ and 3′ ends, was amplified by PCR using primers 5′-CCCGGATCCCGACAAGCTCAACCTTA (A-primer), and 5′-CCCATCTAAGTCMNNCCAMNNMN-NATTMNNCCAAGTGTCAAA (A-random primer, Fig. 1a) (N; A, T, G, or C, M; A or C). In this fragment, randomized nucleotides corresponding to the nonapeptide from Asp-135 to Ser-143 were introduced. In all cases of Asp-135, Asn-139, and Trp-142 were conserved because these amino acid residues are necessary for the binding of calcium ions in all leguminous lectins tested. The cDNA encoding the COOH-terminal half of the BPA lectin from nucleotide 429 to 789 flanked by artificial sites for *Afl*II and *Bam*HI was also generated by PCR using primers 5′-GGGGACTTAAGATATC-CACATATT (A-primer) and 5′- CCCGGAATTCTGATTAC-ATACTGGAATA (E-primer). The cDNAs encoding the NH_2-terminal and COOH-terminal halves thus prepared were ligated to each other. The ligated DNA was further digested with *Eco*RI and *Bam*HI, and then inserted between the *Bam*HI and *Eco*RI sites of λfoo vector to yield a λfoo/BPA lectin library (Fig. 1b). *In vitro* packaging of these lectin library DNAs was performed as described above. 7.0×10^6 independent clones were constructed as the λfoo/BPA lectin library. The vector permits color selection for recombinants so that the quality of the library can be assessed by plating on an agar plate supplemented with X-gal; 53% of the recombinants formed white plaques.

Amplification of the λfoo/BPA Lectin Library—To display recombinant lectins on phage particles fused with coat protein pV, the λfoo/BPA lectin librariy (3.7×10^6 independent clones) was propagated on *E. coli* strain with an amber suppressor mutation (*supE*) TG1 cells in 1.0 ml of CY medium at 0.1 multiplicity of infection. After adsorption at 37°C for 10 min, the culture was diluted 100-fold with CY medium containing 10 mM $MgSO_4$ and incubated with vigorous agitation at 37°C until the cells were completely lysed. After complete lysis of the cells, the supernatants were collected by centrifugation at 3,000 rpm for 10 min, and the recombinant phages were precipitated with polyethylene glycol. The precipitated recombinant phages were suspended in 1 ml of 50 mM Tris/HCl, pH 7.6, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and used as follows.

Affinity Selection of the λfoo/BPA Lectin Library—To select phages with weak affinity, we used the mild panning method. Fifty microliters of phage suspension [10^{10} plaque forming units (pfu) in 50 μl] was applied to microtiter plates that had been coated with 0.1 mg/ml Man-BSA or GlcNAc-BSA at 4°C for 18 h. After washing the wells three times with 100 μl of 50 mM Tris/HCl, pH 7.6, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ at room temperature for 30 min, the bound phages were eluted with 50 μl of 0.1 M mannose or 0.1 M *N*-acetylglucosamine in 50 mM Tris/HCl, pH 7.6, at room temperature for 1 h. Eluates were assayed by infection into TG1 and plated in top agar on a plate. The rest of the eluates were amplified by infection into *E. coli* TG1 cells. Five rounds of affinity selection, titration, amplification, and concentration of the recombinant phages were carried out as described above. After the fifth round of affinity panning, the eluted phage clones were isolated independently.

Specificities of Recombinant Phage Clones—Clones of recombinant phages showing an affinity for Man-BSA or GlcNAc-BSA were applied to the wells coated with Man-BSA, Gal-BSA, Fuc-BSA, GalNAc-BSA, or GlcNAc-BSA at 4°C for 18 h. Unbound and nonspecific binding phage particles were removed by washing three times with 50 mM Tris/HCl, pH 7.6, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and the bound phages were specifically eluted with 0.1 M haptenic sugar. The specificities of the phage clones were compared to the number of phages (pfu) bound to the neoglycoproteins.

DNA Sequencing of Recombinant Phage Clones—The DNA sequences of phage clones with carbohydrate-binding activities were determined directly from the plaques with a Cyclist Ex-Pfu DNA sequencing kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction using 5′-ATGTAGCAAGCTTTTACACTTCC oligonucleotide as a sequencing primer.

RESULTS

Expression of BPA Lectin on the Surface of λfoo Phage Particles—The cDNA coding BPA lectin was ligated to λfoo vector at the 3′ end of the pV gene. As reported previously, BPA lectin is expressed on phage particles that have carbohydrate-binding activity (*4*). To confirm the carbohydrate-binding specificity of the BPA fusion proteins on phage tails, recom-binant λBPA prepared from TG1 was tested for its ability to bind to Man-BSA, Gal-BSA, Fuc-BSA, GalNAc-BSA, or GlcNAc-BSA. Phage suspension was applied to the wells of microtiter plates coated with neoglycoproteins, and the adsorbed phages were eluted with haptenic sugars and recovered. The phage titer of each fraction is shown in Fig. 2. The λfoo phage did not bind to wells coated with Man-BSA, Gal-BSA, Fuc-BSA, GalNAc-BSA, or GlcNAc-BSA. In contrast, the recombinant λBPA phage bound specifically to wells coated with Gal-BSA and was eluted with 0.1 M lactose, indicating that BPA expressed on the surface as λfoo phage particles has the same carbohydrate-binding specificity as BPA purified from seeds.

Construction of a BPA Lectin Library Using λfoo Phage—To verify the carbohydrate-binding specificity of BPA lectin, randomly synthesized oligonuculeotides were introduced into the metal-binding portion corresponding the amino acid sequence DTWPNTEWS (from Asp-135 to Ser-143). We have shown that this loop binds directly to lactose in the presence of calcium ions and have also defined the car-

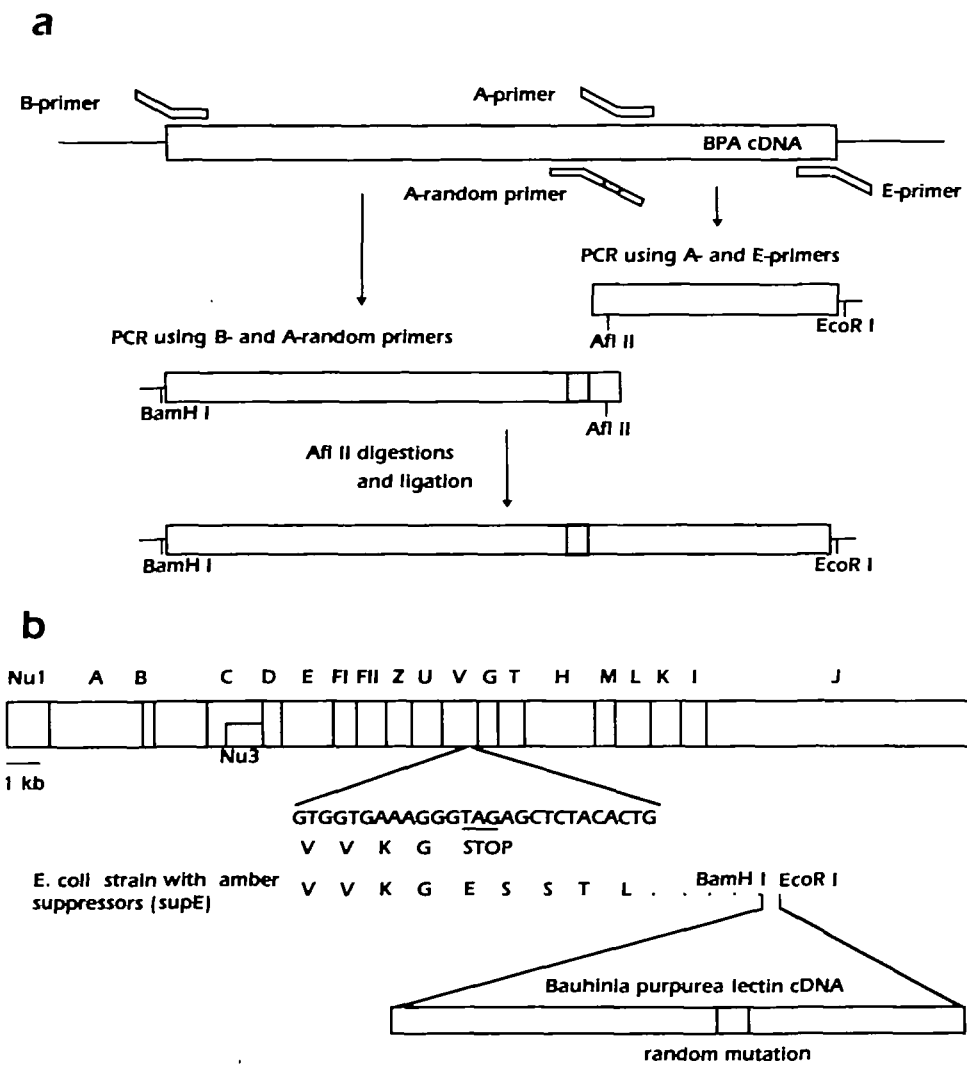

Fig. 1. **a: Schematic diagram of the construction of a BPA lectin library.** Randomized oligonucleotides are indicated by the dotted areas. **b: Genetic map of λfoo and the cloning site of the lectin gene.** Mutated BPA lectin cDNA generated as above was inserted between the *Bam*HI and *Eco*RI sites of λfoo phage vector. The position of the amber stop codon is underlined.

bohydrate-binding specificity of the BPA lectin. In synthesizing random oligonucleotides, the nucleotide sequences corresponding amino acid residues Asp-135, Asn-139, and Trp-142 were conserved since X-ray analyses of several leguminous lectins have suggested that Asp-135 and Asn-139 may bind directly to calcium and maintain the correct conformation of the carbohydrate-binding loop, and that Trp-142 interacts with sugar moieties *via* van der Waals forces. We made a library consisting of 7.0×10^6 plaque-forming phage units, of which 53% formed white plaques on indicator plates as fusion recombinants.

Affinity Selection of a Lectin Library Using Man-BSA—The propagated phage library of about 10^{10} pfu was applied to the wells of a microtiter plate coated with Man-BSA for affinity selection. After extensive washing, the bound phages were eluted with 0.1 M mannose. Five rounds of affinity panning were performed and the efficiency of enrichment after several panning steps was measured. The ratios of white to blue plaques were 11, 67, and 84% after the 1st, 3rd, and 5th pannings, respectively, and the ratio increased after each panning steps (data not shown). This indicates that the number of phages with affinity for mannose was increasing. To confirm that these recombinant phages have an affinity for mannose, we picked up 10 plaques at random after the fifth round of affinity panning using Man-BSA. Each phage clone was amplified and subjected to binding assays for Man-BSA, Gal-BSA, Fuc-BSA, GalNAc-BSA, and GlcNAc-BSA as shown in Fig. 3. Of these five neoglycoproteins, 9 out of 10 clones specifically bound to Man-BSA. This indicates that phage clones expressing recombinant BPA lectins specific for mannose were selected by microtiter wells coated with Man-BSA. The affinities for the other sugars varied among the clones. Clones 1, 3, 4, 6, and 8 showed a weak affinity for galactose, whereas clones 5, 7, 9, and 10 did not. Clone 6 also showed a weak affinity for fucose. We further determined the encoded sequences by DNA sequencing from which the amino acid sequences of the carbohydrate-binding loops of the recombinant BPA lectins were deduced. Clones 1, 3, and 8 were shown to be the same with the deduced amino acid sequences of DSPENTSWE in the carbohydrate-binding loop instead of DTWPNTEWS. Although clones 5 and 9 were identified as the same clone with the sequence DSATNAEWG in the carbohydrate-binding loop, their recoveries in recombinant phages differed, perhaps due to a difference in the efficiency of expression on the surface of the recombi-

nant phages under the culture conditions used. The deduced amino acid sequences of the carbohydrate-binding loops of the other recombinant phage clones are shown in Fig. 4. These sequences were neither similar to one another nor to sequences in mannose-binding leguminous lectins including concanavalin A, *Lens culinaris* lectin, *Pisum sativum* lectin, *Vicia fava* lectin, and *Latirus ochrus* lectin (*15–19*).

Affinity Selection of a Lectin Library Using GlcNAc-BSA—The same strategy for selection was employed to pick up recombinant phage clones with an affinity for GlcNAc, except that the microtiter wells were coated with GlcNAc-BSA. When GlcNAc-BSA used as a ligand, 3 out of 30 clones showed an affinity for GlcNAc after 7 rounds of panning under the same conditions as in the case of Man-BSA (data not shown). However, the enrichment and recovery of recombinant phages with an affinity for GlcNAc were lower than the case of Man-BSA.

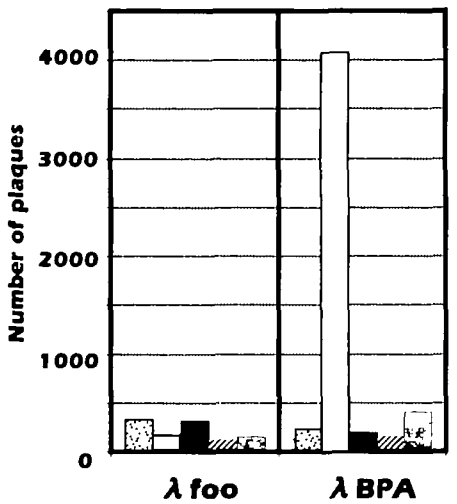

Fig. 2. **Binding of λfoo or λBPA phages to Fuc-, Gal-, Man-, GalNAc-, or GlcNAc-BSA.** λfoo phage particles (10^{10} pfu) with or without BPA on their surface were applied to microtiter wells coated with Fuc-, Gal-, Man-, GalNAc-, or GlcNAc-BSA. Phages bound specifically to the sugars were measured as described in "MATERIALS AND METHODS". Phages bound to Fuc-BSA (dotted bars), phages bound to Gal-BSA (white bars), phages bound to Man-BSA (black bars), phages bound to GalNAc-BSA (hatched bars), and phages bound to GlcNAc-BSA (gray bars) are indicated.

DISCUSSION

Previously, we purified peptides with lactose-binding activity from the Asp-N endoproteinase fragments of lactose-binding leguminous BPA lectin (*4*). The peptide thus purified had the amino acid sequence DTWPNTEWS, and formed part of the metal-binding region of the lectin. Chimeric BPA lectin, whose nonapeptide sequence DTWPNTEWS was replaced with the corresponding sequence from mannose-binding *Lens culinaris* lectin, showed an affinity for mannose instead of galactose indicating that the nonapeptide of BPA lectin has carbohydrate-binding activity and also determines the specificity of the lectin (*10*). This carbohydrate-binding loop appears to be similar to the complementary determining region of immunoglobulin, although rearrangement of the genes encoding lectins does not occur in leguminous plant cells. To produce novel lectins with unique carbohydrate-binding specificities, we introduced randomly synthesized oligonucleotides into the BPA cDNA corresponding to the carbohydrate-binding nonapeptide. Recombinant BPA cDNA was successfully expressed on the surface of λfoo phage particles as described before (*14*), and BPA expressed on the surface of phages was demonstrated to bind to Gal-BSA. BPA lectin purified from seeds shows an affinity for both β-galactosyl and β-*N*-acetylgalactosaminyl residues as reported previously (*20*). In those experiments, we did not confirm the failure of the recombinant BPA on the phages to bind GalNAc-BSA, probably because the GalNAc-BSA was a mixture of α- and β-GalNAc, or the length of the spacer was different from

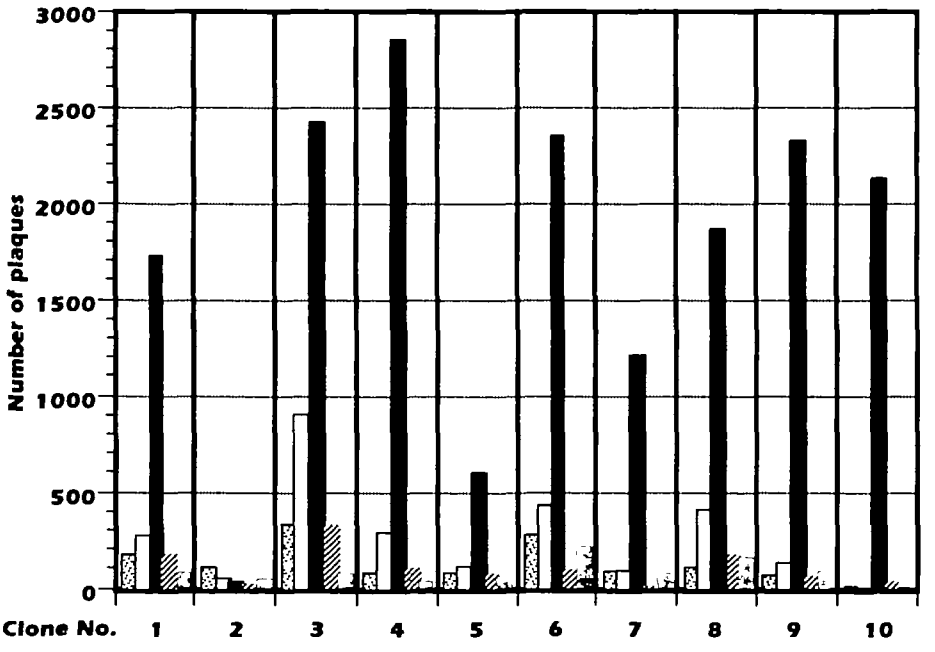

Fig. 3. **Carbohydrate-binding specificities of recombinant λfoo/BPA phage clones selected by binding to Man-BSA.** Ten recombinant λfoo/BPA phage clones selected by binding to Man-BSA were applied to microtiter wells coated with Fuc-, Gal-, Man-, GalNAc-, or GlcNAc-BSA. Phages bound specifically to each sugar were measured as described in "MATERIALS AND METHODS." Phages bound to Fuc-BSA (dotted bars), phages bound to Gal-BSA (white bars), phages bound to Man-BSA (black bars), phages bound to GalNAc-BSA (hatched bars), and phages bound to GlcNAc-BSA (gray bars) are indicated.

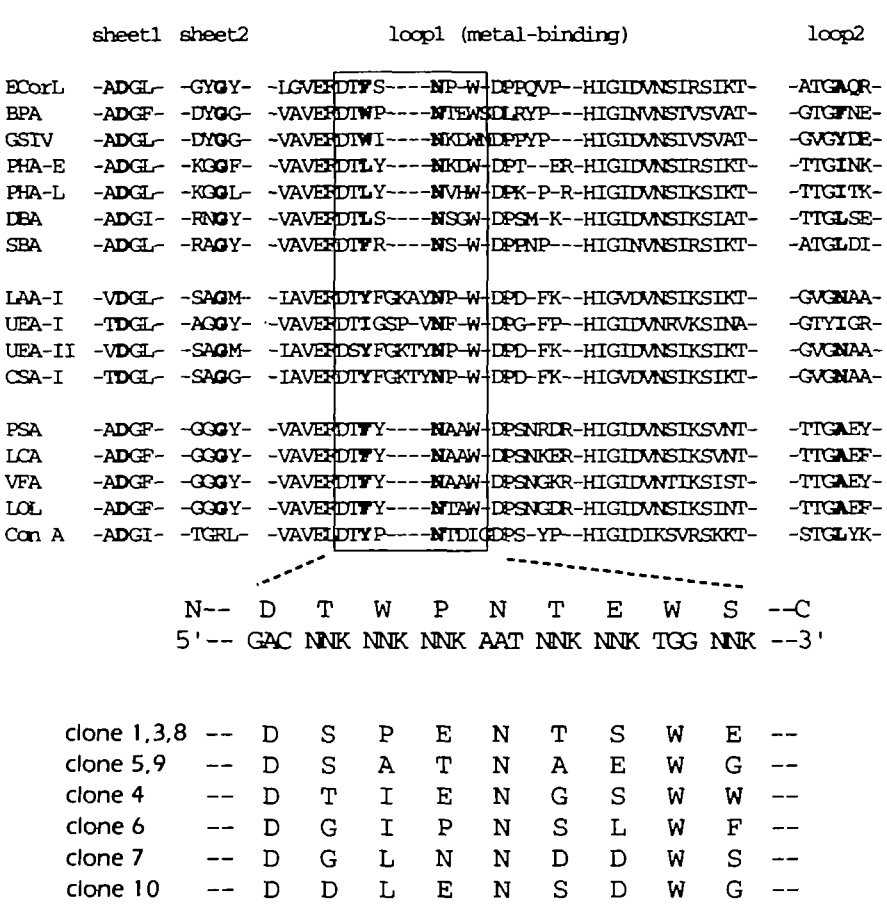

Fig. 4. **Deduced amino acid sequences of the carbohydrate-binding loops of recombinant λfoo/BPA phage clones selected by the binding of Man-BSA.** The deduced amino acid sequences of the carbohydrate-binding loop1 of ten λfoo/BPA phage clones (listed in Fig. 3) are aligned with those in other leguminous lectins. The upper group comprises galactose-binding lectins, the middle group *N*-acetylglucosamine–binding lectins, and the lower group mannose-binding lectins. X-ray studies have shown that the carbohydrate-binding sites of leguminous lectins consist of four peptide strands, sheet1, sheet2, loop1, and loop2.

that of Gal-BSA.

In this study, we used λfoo vector to construct the lectin library. The λfoo vector encodes the amber translation stop codon, TAG, between phage coat protein pV and its foreign fusion partner to produce fusion proteins that are expressed only in *E. coli* with the amber suppresser mutation. We tried several affinity selection methods to screen the library, and affinity panning was found to be able to enrich and recover recombinant phages with affinities for the sugars used as ligands. By using microtiter plates coated with Man-BSA, mutated BPA lectins whose carbohydrate-binding specificities were changed from galactose to mannose were expressed on the surface of phage particles. The cloning and sequencing of these mutated lectins showed the amino acid sequences of the carbohydrate-binding portions to be different from that of mannose-binding leguminous lectins. Previously, we demonstrated that a BPA chimeric lectin, in which the carbohydrate-binding nonapeptide sequence is replaced by the corresponding region from *Lens culinaris* lectin, binds Man-BSA; however, this chimeric lectin was not included in the lectin library used in these experiments. In these experiments, we used a lectin library in which Asp-135, Asn-139, and Trp-142 were fixed, because these charged and aromatic amino acid residues were found to be essential for sugar binding. Conservation of these amino acid residues may increase the chance of obtaining clones with carbohydrate-binding activity. In fact, several mutated BPA lectins with mannose-binding activity have been cloned. The amino acid sequences of the carbohydrate-binding loops of the recombinant lectins are listed in Fig. 4. Among these clones, no conserved amino acid residue(s) were found in distinct positions of carbohydrate-binding loop. There are 20^6 (6.4×10^7) different possibilities when random mutations are introduced at each of 6 amino acid positions. In this study, we used 3.7×10^6 pfu independent phage clones as a lectin library, representing only about 6% of the total. To determine the essential motif for mannose binding, it is important to use a much larger lectin library, and further structural analyses involving X-ray, NMR, and computer-assisted homology modeling are necessary.

When GlcNAc-BSA was used as a ligand, the enrichment and recovery of the mutated BPA lectins with affinity for this sugar were much lower. In the case of GlcNAc-binding leguminous lectins, the carbohydrate-binding loop is longer than in mannose- and galactose-binding lectins. It has been suggested that a long carbohydrate-binding loop is necessary to bind the *N*-acetyl group of this sugar (*6, 7, 21, 22*). In constructing the lectin library, longer randomized peptides inserted in the carbohydrate-binding loop may be necessary for the efficient production of mutant GlcNAc-, GalNAc-, or NeuAc-binding lectins.

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