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Molecular cloning of two different mannose-binding lectins from tulip bulbs

Els J. M. VAN DAMME[1], Filip BRIKÉ[1], Harry C. WINTER[2], Fred VAN LEUVEN[3], Irwin J. GOLDSTEIN[2] and Willy J. PEUMANS[1]

[1] Laboratory for Phytopathology and Plant Protection, Katholieke Universiteit Leuven, Belgium
[2] Department of Biological Chemistry, The University of Michigan, Ann Arbor, USA
[3] Center for Human Genetics, Katholieke Universiteit Leuven, Belgium

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Two lectins were isolated from the bulbs of *Tulipa* cv. Apeldoorn and their corresponding cDNA clones analyzed. The first, called TxLMII (second mannose-binding *Tulipa* hybrid lectin), is a novel mannose-binding tulip lectin. Based on its molecular structure, carbohydrate-binding specificity and amino acid sequence, TxLMII belongs to the superfamily of mannose-binding monocot lectins which are also found in representatives of the plant families Amaryllidaceae, Alliaceae, Orchidaceae and Araceae. Molecular cloning of the second lectin, called TxLCI (first *Tulipa* hybrid lectin with complex specificity), allowed determination unambiguously of the molecular structure of this previously described protein. In addition, evidence is presented that each TxLCI subunit possesses a mannose-binding site and an *N*-acetylgalactosamine-binding site, which act independently of each other. Both binding sites are located in a separate domain of the lectin polypeptide. Since the first domain of TxLCI shows sequence similarity to TxLMII, it is suggested that their genes evolved from a common ancestor.

Keywords: cDNA cloning; lectin; tulip; *Tulipa*.

The plant kingdom contains a whole variety of carbohydrate-binding proteins, which because of their specific and reversible binding and precipitation of glycoconjugates or cells, are referred to as lectins or agglutinins (Goldstein and Poretz, 1986). For historical reasons, plant lectins have, for a long time, been considered as typical seed proteins. During the last decade, however, evidence has accumulated that lectins are not confined to seeds but occur in virtually all types of vegetative tissues. In particular, storage organs of perennial species, such as, e.g. bark, bulbs, tubers, rhizomes and corms are particularly rich sources of lectins (Peumans and Van Damme, 1993). Although representatives of most taxonomic groupings contain lectins in some vegetative tissue, the research on non-seed lectins has been focused on leaf, stem and root lectins of a few legume species (Etzler, 1994), bark lectins of a few trees (Nsimba-Lubaki and Peumans, 1986; Hankins et al., 1988; Yoshida et al., 1994; Van Damme et al., 1995a) and on the bulb and tuber/corm lectins of a limited number of representatives of the monocot families Amaryllidaceae (Van Damme et al., 1987), Alliaceae (Van Damme et al., 1992b, 1993a,b), Orchidaceae (Van Damme et al., 1994), Araceae (Van Damme et al., 1995b) and, to a lesser extent, Liliaceae (Oda and Minami, 1986; Cammue et al., 1986; Oda et al., 1987; Koike et al., 1995). Surprisingly, all the lectins found thus far in storage organs of Amaryllidaceae, Alliaceae, Orchidaceae and Araceae species belong to a single superfamily of mannose-binding proteins (Van Damme et al., 1995c). However, the latter proteins are certainly not the only type of lectin in vegetative tissues of monocots, since the Liliaceae agglutinins, which have been isolated from tubers and bulbs of meadow saffron (*Colchicum autumnale*) (Peumans et al., 1986) and tulip (*Tulipa* sp.) (Oda and Minami, 1986; Cammue et al., 1986; Oda et al., 1987), respectively, have different molecular structures and specificities. Moreover, according to previously published results, tulip bulbs apparently contain at least two different lectins. In 1986, Oda and Minami reported the isolation of a first tulip lectin which bound immobilized yeast mannans and agglutinated yeast cells of the genus *Saccharomyces* but no animal erythrocytes. Further characterization of the purified lectin indicated that it was a tetramer composed of four identical subunits of 17 kDa which exhibited specificity towards mannose, mannose-6-*P*, fucose and fucosamine. Almost simultaneously, Cammue et al. (1986) reported the isolation of a second tulip lectin, which bound immobilized fetuin and readily agglutinated animal erythrocytes. Since this second lectin could not be inhibited by any simple sugar and, in addition, was a tetramer of four identical subunits of 28 kDa, there was no doubt that it differed from the first tulip lectin. To distinguish both tulip lectins the first is indicated as TxLMI (first mannose-binding *Tulipa* hybrid lectin) and the second as TxLCI (first *Tulipa* hybrid lectin with complex specificity). In a third report on a tulip lectin, Oda et al. (1987) described the isolation of an hemagglutinin, which was retained on immobilized thyroglobulin and exhibited a so-called complex specificity. This lectin strikingly resembled TxLCI described by Cammue et al. (1986) concerning its agglutination properties, specificity and amino acid composition, but was reported as a heterodimer of 26 kDa and 14 kDa subunits. Since later experiments indicated that the mitogenic activity of TxLCI on human lymphocytes (Kilpatrick et al., 1990) was very similar to that of the lectin preparation described by Oda et al. (1987), the same protein may have been described twice.

Correspondence to E. van Damme, Catholic University of Leuven, Laboratory for Phytopathology and Plant Protection, Willem de Croylaan 42, B-3001 Leuven, Belgium
Fax: +32 16 322976.

Abbreviations. TxLCI, first *Tulipa* hybrid lectin with complex specificity; TxLMI, first mannose-binding *Tulipa* hybrid lectin; TxLMII, second mannose-binding *Tulipa* hybrid lectin.

Enzyme. Pyroglutamate aminopeptidase (EC 3.4.19.3).

Note. The novel nucleotide sequence data reported in this paper have been submitted to the Genbank™/EMBL Data library and are available under the accession numbers U23041, U23042, U23043 and U23044.

To unravel the identity of the previously described tulip lectins, we isolated and analyzed cDNA clones encoding TxLCI. A comparison of the amino acid sequences of the lectin polypeptides and the deduced sequences of the cDNA clones revealed that the 28-kDa lectin monomer is partly cleaved into two smaller polypeptides of 14 kDa and 13 kDa, which strongly suggests that the same protein has been described, indeed, by Cammue et al. (1986) and Oda et al. (1987). Furthermore, cloning of the lectin cDNA demonstrated that the N-terminal domain of the lectin monomer has a remarkable similarity to the sequences of the aforementioned monocot mannose-binding lectins. Subsequent carbohydrate-binding and specificity studies confirmed the presence of a mannose-binding site which acts independently of a GalNAc-binding site. These findings not only explain the existing discrepancies regarding the specificity of the tulip lectin but also demonstrate that a plant lectin possesses two separate binding sites for two unrelated sugars. Finally, this report describes the isolation, partial characterization and molecular cloning of a novel mannose-binding tulip lectin, indicated as TxLMII (i.e. the second mannose-binding *Tulipa* hybrid lectin), which clearly belongs to the superfamily of monocot mannose-binding lectins. Interestingly, the subunits of TxLMII show a striking similarity to the first domain of the 28-kDa monomer of TxLCI. It is suggested, therefore, that both tulip lectin genes have a common ancestor.

EXPERIMENTAL PROCEDURES

Reagents. Fetuin and mannose were coupled to Sepharose 4B using activation of the matrix by divinylsulphone. After activation with divinylsulphone (1 ml/10 ml gel) in 0.5 M sodium carbonate, pH 11, for 3 h at 25 °C, the gel was washed extensively with water. Coupling was for 15 h at 37 °C in 0.5 M sodium carbonate, pH 10, containing 100 mg/ml mannose or 10 mg/ml fetuin. After coupling, the gel was washed extensively with water and the remaining activated groups blocked by incubation in 0.2 M Tris/HCl, pH 8.5, for 3 h at 25 °C.

Plant material. All experiments were carried out with bulbs of the tulip variety 'Apeldoorn'. For the isolation of the lectins, resting (dry) bulbs were used. Bulbs destined for the isolation of RNA were collected from plants immediately after wilting of the flowers. The two innermost scales of the new developing bulbs were removed, frozen in liquid nitrogen and stored at −80 °C until use.

Isolation of the lectins. Due to their different specificities, TxLCI and TxLMII could be isolated from the same extract by successive affinity chromatography on fetuin-Sepharose 4B and mannose-Sepharose 4B, respectively.

Bulbs (100 g) were stripped of their outer sclerotized layer, cut into small pieces and homogenized in a Waring blender in 1 l 0.2 M NaCl containing 0.2% ascorbic acid (adjusted to pH 6.5 with NaOH). The homogenate was filtered through cheese cloth and centrifuged (10 min, 3000 *g*). Afterwards, $CaCl_2$ (1 g/l) was added to the decanted supernatant and the pH raised to pH 9.0 (with 1 M NaOH). After standing overnight in the cold, the precipitate was removed by centrifugation (10000 *g*; 15 min) and the cleared extract filtered through filter paper. The pH was lowered to 7.0 (with 1 M acetic acid) and the extract loaded on a column (2.6 cm×10 cm; 50 ml bed volume) of fetuin-Sepharose 4B. After passing through the extract, the column was washed with 0.2 M NaCl until the A_{280} fell below 0.01, and the bound lectin was desorbed with 20 mM acetic acid. Fractions containing the lectin (TxLCI) were pooled, dialyzed against water and lyophilized.

To purify the mannose-binding lectin (TxLMII), the extract which passed through the fetuin-Sepharose 4B column (and the first 200 ml of the washing) was loaded onto a column (2.6 cm×5 cm; 25 ml bed volume) of mannose-Sepharose 4B. After washing the column with 0.2 M NaCl until the A_{280} fell below 0.01, the bound lectin was desorbed with 0.1 M mannose in 0.2 M NaCl, dialyzed against water and lyophilized.

The overall recovery of TxLCI and TxLMII was about 2 mg/g fresh mass and 0.8 mg/g fresh mass, respectively.

Gel filtration. Gel filtration of the purified tulip lectins was on a Pharmacia Superose 12 column equilibrated with 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 140 mM NaCl, pH 7.4 (NaCl/P_i) containing 0.2 M mannose and 0.2 M galactose. Samples (200 µl containing about 200 µg pure lectin) were loaded onto the column and chromatographed (using a Pharmacia Fast Protein Chromatography system) at a flow rate of 20 ml/h. Since it is known that the elution behaviour of some lectins differs from that of normal proteins, we used the well characterized monocot mannose-binding lectins from snowdrop (50 kDa) (Van Damme et al., 1987) and garlic (25 kDa) (Van Damme et al., 1992b), and the lectin (120 kDa) and toxin (60 kDa) from castor bean as molecular-mass reference markers.

Electrophoresis. Lectin preparations were analyzed by SDS/PAGE using 12.5−25% (mass/vol.) acrylamide gradient gels as described by Laemmli (1970).

Agglutination assays. Agglutination assays were carried out in microtiter plates in a final volume of 0.1 ml containing 80 µl 1% suspension of red blood cells and 20 µl crude extracts or lectin solutions (each serially diluted with twofold increments). Agglutination was controlled visually after 1 h at room temperature.

The carbohydrate-binding specificity of the lectins was determined using some glycoproteins (thyroglobulin, fetuin, asialofetuin, porcine mucin and ovomucoid) and a series of simple sugars. The sugars tested were: glucose, galactose, galactosamine, GlcNAc, GalNAc, mannose, lactose, melibiose, fucose, arabinose, amylose, ribose, fructose, trehalose, sorbose, xylose, sucrose, maltose and sorbitol (all sugars of the d configuration with the exception of L-fucose and L-sorbose).

Analytical methods. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin and lectins as standards. Total neutral sugar was determined by the phenol/H_2SO_4 method (Dubois et al., 1956), with D-glucose as standard.

Isolation and cyanogen bromide cleavage of the TxLCI 28-kDa polypeptides. The 28-kDa polypeptides composing TxLCI could readily be separated from the smaller lectin polypeptides (approximately 14 kDa) by gel filtration under denaturing conditions. 50 mg affinity-purified TxLCI were dissolved in 2 ml 8 M urea (in NaCl/P_i) and chromatographed on a column (40 cm×2.6 cm; 200 ml bed volume) of Sephacryl 100 using 8 M urea (in NaCl/P_i) as running buffer. Fractions were collected and analyzed by SDS/PAGE. The first fractions, which contained exclusively 28-kDa polypeptides, were pooled, dialyzed and lyophilized. 2 mg dry powder were dissolved in 0.1 ml 70% formic acid; 10 mg solid cyanogen bromide was added and the mixture incubated overnight at 37 °C in the dark. Peptides were recovered by evaporation under vacuo.

Amino acid sequence analysis. Protein sequencing was conducted on an Applied Biosystems model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyzer.

Equilibrium dialysis. Equilibrium dialysis was carried out in eight-chambered cells (Technilabs, Inc.). Ligands (0.15 ml) serially diluted from 10 mM (tritiated monosaccharides) or 1 mM (*p*-nitrophenylglycosides) in NaCl/P_i were placed in wells on one side of the dialysis membrane, and an equal volume of TxLCI solution in the same buffer (5.0 mg/ml; 179 mM in sub-

units) was placed in the other side. Dialysis was carried out at 4°C for 5 days. A preliminary experiment in the absence of lectin showed that 72 h was sufficient for equilibration of the ligand solution. After dialysis, aliquots from each side of each chamber were withdrawn and radioactivity determined by liquid scintillation spectrometry or, in the case of *p*-nitrophenyl-glycosides, by absorbance at 305 nm. Analysis of the ligand side also showed that no protein (A_{280}) had leaked across the membranes. Measurement of ligand binding was also attempted by determining radioactive ligand passed through an ultrafiltration membrane by centrifugal filtration, from 0.5 ml of TxLCI (7.0 mg; 0.25 µmol subunits) containing 5.0 mM tritiated mannose or *N*-acetylgalactosamine (Goldstein et al., 1981). Radioactive ligand solutions were checked for non-dialyzable radioactive impurities by passage through a column of Bio-Gel P-2. No radioactivity was detected in the excluded volume of the column.

RNA isolation, construction and screening of cDNA library. Poly(A)-rich RNA was prepared from plant material stored at −80°C as described by Van Damme and Peumans (1993). A cDNA library was constructed from poly(A)-rich RNA isolated from young developing tulip bulbs using the cDNA synthesis kit from Pharmacia. cDNA fragments were inserted into the *Eco*RI site of the multifunctional phagemid pT_7T_3 18 U (Pharmacia). The library was propagated in *Escherichia coli* XL1 Blue (Stratagene).

Recombinant lectin clones were screened using a ^{32}P-end-labeled degenerate oligonucleotide probe (20-residues, AGA/G TTG CAG TCN TNT/C TGC AT) derived from the amino acid sequence MQEDCNL which is conserved among the mannose-binding lectins from Amaryllidaceae, Alliaceae and Orchidaceae species (Van Damme et al., 1995c). In a later stage, cDNA clones encoding the tulip lectins were used as probes. Hybridization using the oligonucleotide probe was carried out overnight at 40°C as reported previously (Van Damme et al., 1991). Colonies that produced positive signals were selected and rescreened at low density using the same conditions. Plasmids were isolated from purified single colonies on a miniprep scale using the alkaline lysis method as described by Mierendorf and Pfeffer (1987) and sequenced by the dideoxy method (Sanger et al., 1977). DNA sequences were analyzed using programs from PC Gene (Intelligenetics) and Genepro (Riverside Scientific).

Northern blot. RNA electrophoresis was performed according to the method of Maniatis et al. (1982). Approximately 3 µg mRNA was denatured in glyoxal and dimethylsulfoxide and separated in a 1.2% (mass/vol.) agarose gel. Following electrophoresis, the RNA was transferred to Immobilon N membranes (Millipore) and the blot hybridized using a random-primer-labeled cDNA insert. Hybridization was performed as reported by Van Damme et al. (1992b). An RNA ladder (0.16−1.77 kb) was used as a marker.

Genomic DNA analysis. Total DNA from tulip ovaries was isolated according to the procedures described by Dellaporta et al. (1983) and de Kochko and Hamon (1990). The DNA preparation was treated with RNAse in order to remove any contaminating RNA. Approximately 50 µg DNA was digested with restriction endonucleases and subjected to electrophoresis in a 0.8% (mass/vol.) agarose gel. DNA was transferred to Immobilon N membranes (Millipore) and hybridized at 68°C using the ^{32}P-labeled cDNA insert as a probe (Van Damme et al., 1992a).

RESULTS

Isolation and characterization of a novel mannose-binding lectin TxLMII from tulip bulbs. A screening of storage tissues of different Liliaceae species for the possible presence of proteins related to the previously described monocot mannose-binding lectins revealed the presence of a mannose-binding lectin, called TxLMII in tulip bulbs. This lectin was isolated by affinity chromatography on mannose-Sepharose 4B and characterized in some detail with respect to its molecular structure and carbohydrate-binding specificity.

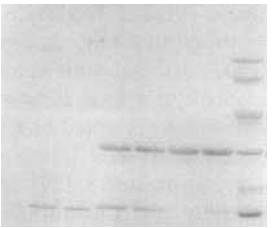

Fig. 1. SDS/PAGE of purified tulip lectins. Samples were loaded as follows, lane 1, reduced (with 2-mercaptoethanol) TxLMII; lane 2, unreduced TxLMII, lane 3, reduced (with 2-mercaptoethanol) TxLCI, lane 4, unreduced TxLCI, lane 5, purified (by gel filtration in 8 M urea) 28-kDa subunits of TxLCI, lane 6, sample of the TxLCI preparation described by Cammue et al. (1986). About 25 µg of each lectin was loaded onto the gel. Molecular-mass reference proteins shown in lane R are: lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa).

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TxLMII: KPXTP NNVLY TGESL YGGQS LT
GNA:          NNILY SGETL SAGEF LN
ACA:          RNVLV NNEGL YAGQS LV

TxLCI:  RETPN LPRMQ NLLFS GQTLY  (14 kDa polypeptide)
        QNDXN LVLYG GGFQS N      (A)
        LSSND DSTTF ELSKX QLSL   (B)
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Fig. 2. Comparison of the N-terminal amino acid sequences of the different tulip lectin polypeptides and the lectins from *Galanthus nivalis* (GNA) and *Allium cepa* (ACA). A and B denote the 11-kDa and 12-kDa fragments, respectively, obtained after cleavage of the 28-kDa polypeptide of TxLCI with cyanogen bromide. No amino acid could be determined at position 3 of TxLMII and positions 4 and 15 of the A and B fragments, respectively, obtained after cleavage of TxLCI with cyanogen bromide.

Analysis of purified TxLMII by SDS/PAGE both in the presence and absence of 2-mercaptoethanol revealed a single polypeptide of 12 kDa (Fig. 1). Since the native lectin eluted from the Superose 12 column with an apparent molecular mass of 25 kDa (results not shown), we assume that TxLMII is a dimer of two identical subunits which are not held together by disulphide bridges. Determination of the total carbohydrate content of TxLMII and staining of the protein with the glycan detection kit from Boehringer yielded negative results (results not shown), indicating that this lectin is, like all other monocot mannose-binding lectins, unglycosylated.

Amino acid sequencing of the TxLMII polypeptide yielded a unique N-terminal sequence which showed similarity (53−60%) to the N-terminal sequences of previously described monocot mannose-binding lectins (Fig. 2). Since this sequence similarity strongly suggested that TxLMII belongs to the same

superfamily of mannose-specific lectins, its agglutination and carbohydrate-binding properties were studied in some detail.

Purified TxLMII was assayed with human and rabbit erythrocytes. Even when added at a final concentration of 2 mg/ml, TxLMII failed to agglutinate untreated or trypsin-treated human red blood cells, irrespective of their blood group. Although the lectin reacted with trypsin-treated rabbit erythrocytes, its specific agglutination activity (i.e. the minimal concentration required for agglutination) was low (about 10 μg/ml) in comparison to that of most other monocot mannose-binding lectins (e.g. 0.8 μg/ml in the case of the snowdrop lectin; Van Damme et al., 1987). Untreated rabbit erythrocytes did not agglutinate in the presence of 2 mg/ml lectin.

Hapten inhibition assays with a series of simple sugars revealed that of all monosaccharides and oligosaccharides tested, only mannose had an inhibitory effect, the concentration required for 50% inhibition of trypsin-treated rabbit erythrocytes being 15 mM (which is comparable to the values obtained with e.g. Amaryllidaceae and Orchidaceae lectins). Similar assays were also carried out with some animal glycoproteins. Fetuin and ovomucoid were only inhibitory at a high concentration (500 μg/ml). Mucin and asialofetuin caused a 50% inhibition of the agglutination at 250 μg/ml and 125 μg/ml, respectively. Thyroglobulin was the most potent inhibitor being active at a concentration of 15 μg/ml.

TxLMII definitely differs from the mannan-binding tulip protein (TxLMI) described by Oda and Minami (1986). First, TxLMII agglutinates animal erythrocytes, whereas TxLMI does not. Second, TxLMII is composed of 12-kDa subunits, whereas TxLMI is a tetramer of four 17 kDa subunits. Third, the amino acid composition of both lectins differs. For instance, TxLMII contains three cysteine residues/polypeptide chain, whereas TxLMI has no cysteine.

Isolation and characterization of TxLCI. Since there are some discrepancies in the literature about the molecular structure of TxLCI, this issue has been readdressed. In addition, novel experiments have been designed to pinpoint the reasons for the apparently contradictory results.

To determine the molecular structure of TxLCI, an affinity-purified lectin preparation was analyzed by SDS/PAGE and gel filtration. SDS/PAGE of TxLCI yielded a complex polypeptide pattern showing a major band of 28 kDa and a weaker band of 14 kDa (Fig. 1). Since reduced (with 2-mercaptoethanol) and unreduced samples showed indentical patterns (Fig. 1), the 28-kDa polypeptide cannot correspond to S-S linked 14-kDa polypeptides. Native TxLCI eluted with an apparent molecular mass of 100 kDa upon gel filtration on the Superose 12 column (results not shown). These results suggest that TxLCI is a tetramer composed of four identical subunits of 28 kDa, which are at least partially cleaved into smaller polypeptides of approximately 14 kDa. To prove that the presumed cleavage of the lectin polypeptide occurs, we tried to determine the N-terminal sequence of the different (electroblotted) polypeptides (Fig. 2). Unfortunately, only the 14-kDa polypeptide yielded a sequence. Since the 28-kDa polypeptide was blocked and could not be deblocked with pyroglutamate aminopeptidase, the 28-kDa polypeptides were purified by gel filtration (of the affinity-purified lectin) in 8 M urea (Fig. 1), cleaved with cyanogen bromide and two fragments of 11 kDa and 12 kDa, respectively, sequenced (Fig. 2). These results, together with results obtained after cloning and sequencing of TxLCI, show that the 14-kDa polypeptide is derived, indeed, from the 28-kDa lectin subunit.

The above described results partly agree with the previously reported data on the molecular structure of TxLCI. According to Cammue et al. (1986), native TxLCI is a tetramer composed of four identical subunits of 28 kDa. Since a reinvestigation of the preparation used by these authors revealed the presence of small amounts of the 14-kDa polypeptides (Fig. 1), cleavage of the 28-kDa polypeptide was apparently overlooked. The preparation described by Oda et al. (1987) also contained both uncleaved and cleaved TxLCI polypeptides but eluted with an apparent molecular mass of about 40 kDa from a Sephadex G-100 column, which led to the conclusion that TxLCI was a heterodimer of one 26-kDa and one 14-kDa subunit. Most likely, the differences in the estimations of the molecular mass of the native lectin are inherent to the use of polysaccharide-based matrices (which may interact with the lectin).

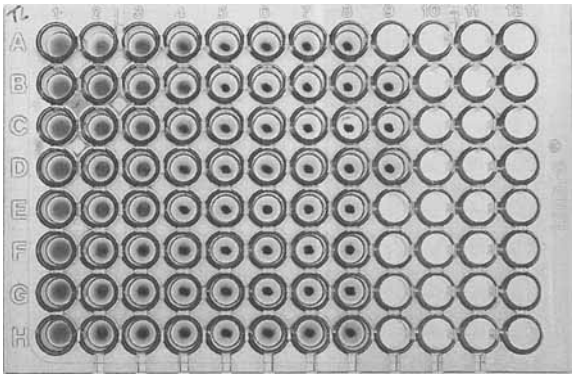

Fig. 3. Inhibition of the TxLCI-mediated agglutination of trypsin-treated rabbit erythrocytes with mannose and GalNAc. To study the combined effect of mannose and GalNAc, both sugars were added at different concentrations. The final concentrations of GalNAc in the first eight wells of rows A–H were 32, 16, 8, 4, 2, 1, 0.5 and 0.25 mM, respectively. Similarly, the final concentrations of mannose in the wells of columns 1–8 were 128, 64, 32, 16, 8, 4, 2 and 1 mM. Well B9 contained no sugar, whereas wells C9 and D9 contained 32 mM GalNAc and 128 mM mannose, respectively. TxLCI was added to a final concentration of 1 μg/ml.

Determinations of the total carbohydrate content of TxLCI and staining of the polypeptides with the glycan-detection kit from Boehringer yielded negative results (results not shown), indicating that this lectin is unglycosylated.

TxLCI possesses independent binding sites for mannose and GalNAc. TxLCI agglutinated rabbit as well as human erythrocytes (Cammue et al., 1986). Curiously, however, the agglutination of human erythrocytes was fully inhibitable by low concentrations (0.2 mM) of GalNAc, whereas that of rabbit erythrocytes could not be inhibited by any simple sugar. Since cDNA cloning of TxLCI suggested the presence of a domain with a striking sequence similarity to the monocot mannose-binding lectins, the GalNAc-inhibitable agglutination of human erythrocytes argued for the presence of a second GalNAc-specific carbohydrate-binding site. If so, one would expect that TxLCI binds to both immobilized mannose and immobilized GalNAc and that the agglutination of rabbit red blood cells is inhibited by a combination of mannose and GalNAc. Hapten inhibition assays, whereby the presumed GalNAc-binding site was blocked with GalNAc, demonstrated that mannose inhibited the agglutination of rabbit erythrocytes in a concentration-dependent manner (Fig. 3). Affinity chromatography of purified TxLCI on mannose-Sepharose 4B and GalNAc-Sepharose 4B (Fig. 4) confirmed that the lectin binds to both immobilized sugars. It should be noted here that binding occurs only in the presence of a minimal concentration of ammonium sulfate. Such a binding could be expected since most members of the monocot mannose-bind-

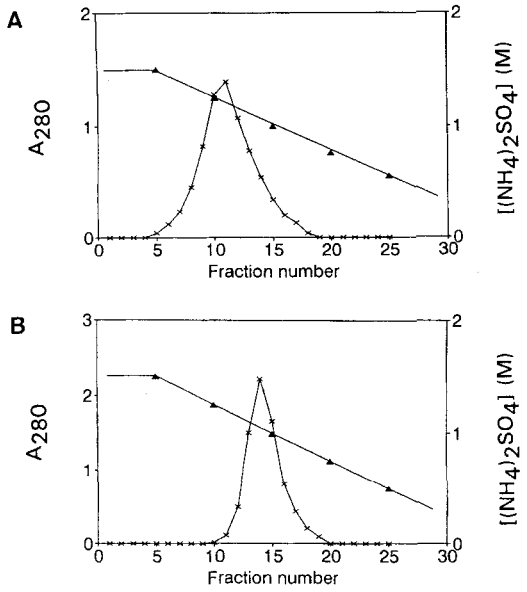

Fig. 4. Affinity chromatography of TxLCI on mannose-Sepharose 4B and GalNAc-Sepharose 4B. 100 mg purified TxLCI dissolved in 1.5 M ammonium sulfate was applied to a column (2.6 cm×10 cm; 50 ml bed volume) of mannose-Sepharose 4B (A) or GalNAc-Sepharose 4B (B) and eluted with a linear gradient of 1.5 M ammonium sulfate/water. Fractions of 10 ml were collected and the A_{280} (×) and ammonium sulfate concentration (▲) determined.

ing lectins require comparable or even higher concentrations of ammonium sulfate for binding to the same mannose-Sepharose 4B column. Only a few exceptions, such as TxLMII and some orchid lectins, are quantitatively retained on a mannose column in NaCl/P_i. Moreover, since the addition of 0.1 M mannose and 0.02 M GalNAc to the lectin TxLCI in 1 M ammonium sulfate prevented the binding of TxLCI to mannose-Sepharose 4B and GalNAc-Sepharose 4B, respectively, the observed absorption to the affinity columns is sugar specific.

The concerted action of a mannose-binding and a GalNAc binding site on TxLCI fully explains the previously published data. Since, on the analogy of the monocot mannose-binding lectins, the mannose-binding domain of TxLCI has no receptor molecules on human erythrocytes, the agglutination of human red blood cells is exclusively accomplished by the GalNAc-binding domain and, hence, is fully inhibitable by GalNAc (Cammue et al., 1986). In contrast, rabbit erythrocytes, which contain receptor molecules both for monocot mannose-binding lectins and for GalNAc-specific lectins, do interact with the mannose-binding as well as the GalNAc-binding site of TxLCI. As a result, their agglutination can only be inhibited by the combined action of mannose and GalNAc.

Equilibrium dialysis of TxLCI. By equilibrium dialysis, using either radiolabeled mannose, radiolabeled GalNAc, or the *p*-nitrophenyl-α-glycosides of each, no measurable binding to TxLCI was detected. Minimum binding detected by these methods at the highest concentration of ligand would be approximately 0.5 mol ligand/mol protein subunits. By standard Scatchard analysis (Scatchard, 1949), and assuming a single binding site/subunit, this would correspond to a maximum dissociation constant of approximately 12 mM in the case of radiolabeled substrates, or 2 mM in the case of the *p*-nitrophenylglycosides. Measurements of weaker binding is not possible by these methods, since the amount of ligand bound would be less than about 2% of the total ligand present. Likewise, no binding was detected by centrifugal ultrafiltration of a solution of TxLCI with radiolabeled monosaccharides. Finally, we observed no alteration of the ultraviolet absorption spectrum of 1 mM *p*-nitrophenyl-α-D-mannoside or *N*-acetylgalactosaminide and lectin upon mixing the two components in a split-chambered mixing cuvette, further indicating that binding of the ligands is less than about 1% at these concentrations. We, therefore, conclude that the binding of free mannose or GalNAc to the tulip lectin TxLCI is weak, having an dissociation constant of greater than about 12 mM.

Isolation and characterization of cDNA clones encoding TxLMII and TxLCI. Screening of a cDNA library constructed from mRNA isolated from young (developing) tulip bulbs using an oligonluceotide probe derived from the amino acid sequence MQEDCNL (which is a highly conserved region in the family of monocot mannose-binding lectins), resulted in the isolation of two groups of positive clones. One group of cDNA clones (indicated as LECTxLMII) could readily be identified since their deduced amino acid sequence matches the N-terminal sequence of TxLMII. As shown (Fig. 5), LECTxLMII1 contains an open reading frame of 531 bp encoding a 177-amino-acid precursor with one putative initiation codon at position 3 of the deduced amino acid sequence. Translation starting with this methionine residue results in a protein of 175 amino acids with a calculated molecular mass of 18.8 kDa which, after co-translational cleavage of the signal peptide of 36 amino acids, yields a lectin precursor polypeptide of 15.0 kDa. Cleavage of the signal peptide between amino acids 36 and 37 conforms to the rules for protein processing of von Heijne (1986) and yields a lectin polypeptide with an N-terminal amino acid sequence identical to that obtained by sequencing TxLMII. Since the molecular mass of the lectin polypeptide (after cleavage of the signal peptide) is still approximately 3 kDa larger than that of the mature TxLMII subunit, we presume that, on the analogy of the C-terminal processing of other monocot mannose-binding lectins (Van Damme et al., 1995c), a second post-translational processing takes place at the C-terminus of the TxLMII precursor.

Further screening of the cDNA library using the random-primer-labeled clone LECTxLMII1 as a probe yielded several other cDNA clones encoding TxLMII, which slightly differed from each other in their nucleotide and deduced amino acid sequences. Among these clones, there was also one cDNA clone called LECTxLMII2 (Fig. 5) which revealed an insertion of seven amino acids within the signal peptide. However, the mature lectins encoded by LECTxLMII1 and LECTxLMII2 show approximately 90% sequence similarity at the nucleotide as well as at the amino acid levels. Both LECTxLMII1 and LECTxLMII2 contain one putative glycosylation site within the C-terminal sequence.

In an attempt to isolate cDNA clones encoding TxLCI, a differential screening of the tulip cDNA library was performed using a degenerate oligonucleotide (derived from the consensus sequence MQEDCNL of the monocot mannose-binding lectins) and the cDNA insert LECTxLMII1 as probes. Following this strategy, we were able to isolate a second group of lectin cDNA clones. Sequence analysis of the clone LECTxLCI1 revealed that it encodes a 275-amino-acid protein with two possible initiation codons at positions one and three of the deduced amino acid sequence (Fig. 6). Translation starting with the first methionine yields a polypeptide with a calculated molecular mass of 29.8 kDa. Sequence analysis of this polypeptide demonstrated that it contains the N-terminal sequence of the 14-kDa polypeptide of TxLCI as well as the sequences of the peptides obtained by cyanogen bromide cleavage of the 28-kDa polypeptide of TxLCI. It is evident, therefore, that TxLCI is synthesized as a

```
LECTxLMII1:  spMALPQSSIAILLIIPTILGLLAAPS.......YSRKILSGEK   35
LECTxLMII2:  sp----H-.------------------CQAESVI----------

LECTxLMII1: A▼EPCTPNNVLYTGESLYGGQSLTWESYTFIMQTDCNLVLYEGNG   79
LECTxLMII2: - K-------------------------------------------
LECGNA1:           --I--S--T-SA-EF-NQGN-V----E-------DVDK

LECTxLMII1: PIWASGSNDLGSGCYVTMQKDGNLVIYSKSGNSVWASQTHQAEGN  124
LECTxLMII2: S-------G--------------------I---------------
LECGNA1:    -L-ETNTGG-SRR--LN--T-----V-NP-NKPI---N-GGEN--

                                              *
LECTxLMII1: YVLVLQKDRNVVIYGPSLWATNTDQFSLTSNSTTESGSGMANEGK  169
LECTxLMII2: ---------------S-------P-----------------D---
LECGNA1:    --CI----G-IA----AI---G

LECTxLMII1: IAMVTK                                        175
LECTxLMII2: ------
```

Fig. 5. Sequence alignment of deduced amino acid sequences of lectin cDNA clones encoding TxLMII. The arrowhead indicates the cleavage site for the signal peptide. (–) Sequence identity with LECTxLMII1. (·) Gaps introduced for maximal alignment. Putative glycosylation sites are indicated by an asterisk. Since the first ATG codon is most probably used as the translation-initiation site, the deduced amino acids preceding this methionine residue are shown in lower-case letters.

```
LECTxLCI1: MGMALVVALAVALASTVAG▼QQNVLLSGNTLANEESLSYGSVKFIM   45
LECTxLCI2:     F-------------- ---------------------------

LECTxLCI1: QNDCNLVLYGGGFQSNTASNGKKCTLTLNNNGHLIINDGLTGRVVW   91
LECTxLCI2: ------------------------------D-------------A--

LECTxLCI1: RNSNAQNPVGRYAAVLRPAPGTADVAIYGPAIWSTNDEFSYQESGV  137
LECTxLCI2: K-----------------------------------AG----G--A

LECTxLCI1: VPHKLGRETPNLPRMQNLLFSGQTLYGTDGMLSSNDGSTTFELSKC  183
LECTxLCI2: ---N-------------------------K--P--DN--------
                                                 *

LECTxLCI1: QLSLSLGNEVKWKVAPTVENGKENYCHVRLDHRGHLSLLNDDNVVL  229
LECTxLCI2: --------------------N-D----------------------

LECTxLCI1: FRSIPTNGVEPPIGDYVLIVEKGAGYIYGPPIWNTYDNRRYKVASA  275
LECTxLCI2: -----A-----------------T----S----------------
```

Fig. 6. Sequence alignment of deduced amino acid sequences of lectin cDNA clones encoding TxLCI. The arrowhead indicates the cleavage site for the signal peptide. (–) Sequence identity with LECTxLCI1. (·) Gaps introduced for maximal alignment. Putative glycosylation sites are indicated by an asterisk. The N-terminal sequences of the 14-kDa polypeptide as well as the A and B fragments obtained after cleavage of TxLCI with cyanogen bromide are underlined.

28-kDa precursor, which is post-translationally cleaved into smaller polypeptides. However, since TxLCI preparations contain mostly uncleaved 28-kDa subunits, it is obvious that only a part of the lectin polypeptides is processed. According to the rules for protein processing of von Heijne (1986), cleavage of the signal peptide can occur at different positions, though is most likely to occur between amino acids 19 and 20 of the lectin precursor. Cleavage of the lectin precursor at this site is in good agreement with the fact that the N-terminal sequence of the mature TxLCI is blocked. Assuming that no further processing occurs at the C-terminus of the lectin polypeptides, cleavage of the precursor between amino acids 143 and 144 will yield two lectin polypeptides (124 and 132 amino acids) with calculated molecular masses of 13.3 kDa and 14.7 kDa, respectively. These molecular masses of the lectin polypeptides predicted from the deduced amino acid sequence of the lectin precursor are in good agreement with the molecular masses of the smaller lectin polypeptides which are present after SDS/PAGE of TxLCI.

Sequence analysis of different cDNA clones encoding TxLCI revealed minor differences in their nucleotide as well as in their amino acid sequences (Fig. 6). However, all clones encode a lectin pecursor of approximately 29 kDa which contains the N-terminal sequence of the 14-kDa polypeptide of TxLCI. LECTxLCI2 differs from LECTxLCI1 in that it contains one putative glycosylation site at position 175 of the lectin precursor. However, since TxLCI is not glycosylated, this glycosylation site is probably not used.

Northern-blot analysis. To determine the total length of the RNAs encoding the tulip lectins, a blot containing mRNA isolated from the bulbs was hybridized with the different cDNA clones. Hybridization of the blot revealed that TxLMII is translated from an mRNA of approximately 800 nucleotides, whereas TxLCI is encoded by an mRNA with an estimated length of approximately 1300 nucleotides (Fig. 7).

Sequence similarities between LECTxLMII and other monocot mannose-binding lectins. Sequence alignment of LECTxLMII and previously cloned monocot mannose-binding lectins from Amaryllidaceae, Alliaceae and Orchidaceae revealed 39–

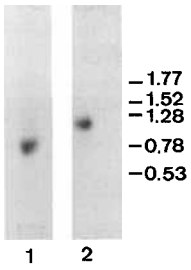

Fig. 7. Northern blot of mRNA isolated from tulip bulbs. The blot was hybridized using the ^{32}P-labeled cDNA insert LECTxLMII1 (lane 1) or LECTxLCI1 (lane 2). Numbers on the right show RNA size (kb).

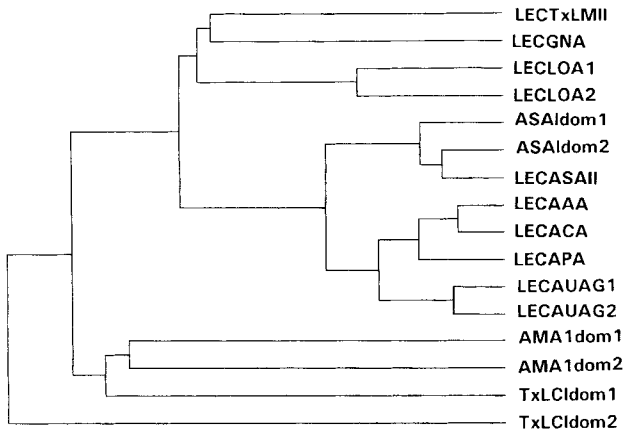

Fig. 8. Phylogeny of amino acid sequences encoding the tulip lectins and some previously cloned mannose-binding monocot lectins. The dendrogram was constructed using the simultaneous alignment and phylogeny program (CLUSTAL) from the PC Gene software package (Intelligenetics). The different sequences encode the lectins from the Liliaceae species *Tulipa* sp. (TxLMII, TxLCIdom1, TxLCIdom2), the Amaryllidaceae species *Galanthus nivalis* (LECGNA) (Van Damme et al., 1991), the Orchidaceae species *Listera ovata* (LECLOA1 and LECLOA2) (Van Damme et al., 1994), the Alliaceae species *Allium sativum* (ASAIdom1, ASAIdom2 and LECASAII) (Van Damme et al., 1992b), *Allium ascalonicum* (LECAAA) (Van Damme et al., 1993a), *Allium cepa* (LECACA) (Van Damme et al., 1993a), *Allium porrum* (LECAPA) (Van Damme et al., 1993a) and *Allium ursinum* (LECAUAG1 and LECAUAG2) (Van Damme et al., 1993b), and the Araceae species *Arum maculatum* (AMA1dom1 and AMA1dom2) (Van Damme et al., 1995b).

60% sequence identity (54% and 67% sequence similarity) within the mature lectins. It can be concluded, therefore, that TxLMII also belongs to the superfamily of monocot mannose-binding lectins. Furthermore, the dendrogram of alignment of the sequences of the mature polypeptides indicates that the Amaryllidaceae lectins are the closest relatives of TxLMII followed by the Orchidaceae and Alliaceae lectins (Fig. 8).

Sequence similarities between LECTxLMII and LECTxLCI. A detailed comparison of the deduced amino acid sequences of the cDNA clones encoding TxLMII and TxLCI showed an overall sequence identity of only 25% (34% sequence similarity). However, when the two domains of TxLCI (coinciding with the N-terminal and C-terminal part of the mature lectin) are considered as separate entities, the similarity between the first domain of TxLCI and mature TxLMII is much more prominent, especially at the position of the sequences which are known as highly conserved between the monocot mannose-binding lectins (Fig. 9). It is also evident that the sequence identity between the first domains of TxLCI and TxLMII (41% and 57% sequence similarity) is higher than that between the first and the second domain of TxLCI (which is only 27%). Similarly, the sequence similarity between the second domain of TxLCI and TxLMII is also low (28%).

Sequence similarities between LECTxLCI and other monocot mannose-binding lectins. From the previous results, it is obvious that the sequence similarity between LECTxLCI and previously characterized monocot mannose-binding lectins is mainly restricted to the N-terminal part of the mature TxLCI polypeptide. The highest degree of sequence similarity was found between the N-terminal domain of TxLCI and the first domain of the *Arum maculatum* lectin, showing 44% sequence identity (53% sequence similarity). Screening of the EMBL database revealed no similarity between the second domain of TxLCI and any other known protein, except for the second domain of the *A. maculatum* lectin (Van Damme et al., 1995b) which shows 31% sequence identity to TxLCI.

Evidently, TxLCI consists of two different domains. In this respect, TxLCI clearly differs from the previously described two-domain monocot lectins from garlic (*Allium sativum*) bulbs (Van Damme et al., 1992b) and *A. maculatum* tubers (Van Damme et al., 1995b) which are encoded by cDNA clones containing two distinct domains which show 85% and 50% sequence similarity, respectively.

TxLMII and TxLCI are encoded by gene families. Since sequence analyses of different clones encoding TxLMII and TxLCI, respectively, revealed minor differences both in their nucleotide and deduced amino acid sequences, the possible presence of multiple genes was checked by Southern-blot analysis of genomic tulip DNA. To visualize the genomic restriction fragments containing LECTxLMII and LECTxLCI sequences, the blots were hybridized at 68 °C using the labeled cDNA inserts as probes. The results shown (Fig. 10) clearly demonstrate that each cDNA insert hybridizes with a set of restriction fragments. Since the restriction enzymes used do not cut the cDNA insert (except for *Eco*RI which cuts LECTxLCI), it can be concluded that LECTxLMII as well as LECTxLCI is encoded, indeed, by (small) families of genes.

DISCUSSION

This paper describes the isolation and characterization of two tulip lectins TxLMII and TxLCI, and their corresponding cDNA clones. Based on its molecular structure, carbohydrate-binding specificity and amino acid sequence, TxLMII clearly belongs to the superfamily of monocot mannose-binding lectins. Thus, the occurrence of these proteins, which was documented previously in the families Amaryllidaceae, Alliaceae, Orchidaceae and Araceae (Van Damme et al., 1995c), has to be extended to the family Liliaceae. This finding is interesting from a chemotaxonomic point of view, since it demonstrates that monocot mannose-binding proteins occur in all major families of the Liliales. It should be mentioned here that a similar mannose-binding lectin has also been isolated recently from leaves of *Aloe arborescens* (which also belongs to the Liliaceae; Koike et al., 1995).

TxLCI is an unusual plant lectin since its subunits contain two different carbohydrate-binding domains, which recognize structurally unrelated sugars. In the past, several plant lectins have been described which possess two different carbohydrate-binding sites for related monosaccharides or oligosaccharides.

```
LECTxLCI1dom1:          QQNVLLSGNTLANEESLSY---GSVKFIMQNDCN
                         .::: .: .:     ::..    :  :::: :::
LECTxLMII1:          EPCTPNNVLYTGESLYGGQSLTW---ESYTFIMQTDCN
                        .:.:..: .:::    .       : :: . . :.
LECTxLCI1dom2: RETPNLPRMQNLLFSGQTLYGTDGMLSSNDGSTTFEL-SKCQ

LECTxLCI1dom1: LVLYGGG---FQSNTASNGKK---CTLTLNNNGHLIINDGLT
               :::: :    . : .   :     : .:..  : :.:    .
LECTxLMII1:    LVLYEGNGPIWASGSNDLGSG---CYVTMQKDGNLVIYSK-S
               : :  ::   :                : : .   : : .
LECTxLCI1dom2: LSLSLGNEVKWKVAPTVENGKENYCHVRLDHRGHLSLLND-D

LECTxLCI1dom1: GRVVWRNSNAQN---PVGRYAAVLRPAPGTADVAIYGPAIWS
               :  ::  :.. .     : :  ::         : ::::..:.
LECTxLMII1:    GNSVW.ASQTHQ---AEGNYVLVLQKDR---NVVIYGPSLWA
                  .. .  :       : :::.. :        :::: .:
LECTxLCI1dom2: NVVLFRSIPTNGVEPPIGDYVLIVEKGAG----YIYGPPIWN

LECTxLCI1dom1: T-N-DEFSYQES
               : : : ::
LECTxLMII1:    T-NTDQFSLTSNSTTESGSGMANEGKIAMVTK
               :     . ..:
LECTxLCI1dom2: TYDNRRYKVASA
```

Fig. 9. Sequence comparison of cDNA-derived amino acid sequences of the two TxLCI domains encoded by LECTxLCI1 and TxLMII encoded by LECTxLMII1. The amino acid sequences have been aligned by introducing gaps (−) to maximize identity. Colons between lines indicate identical amino acid residues, whereas single dots indicate similar amino acids.

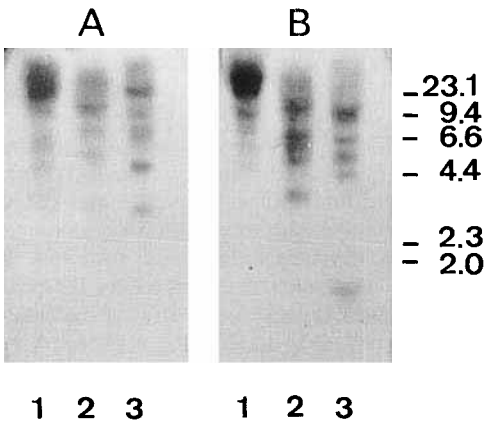

Fig. 10. Southern blot of genomic DNA isolated from tulip ovaries. DNA was digested with *Bam*HI (lane 1), *Hin*dIII (lane 2) and *Eco*RI (lane 3), and hybridized with the ^{32}P-labeled cDNA insert LECTxLMII1 (A) or LECTxLCI1 (B). Numbers on the right show DNA size (kb).

However, in most cases studied thus far, the two different carbohydrate-binding sites are located on different subunits, which are encoded by separate genes. For instance, the E and L subunits of *Phaseolus vulgaris* hemagglutinin, which exhibit a different specificity towards complex oligosaccharides, are encoded by separate, though related, genes (Hoffman and Donaldson, 1985). Similarly, the GalNAc/galactose-binding and galactose-binding sites of the *Bandeiraea* lectin I are associated with the A and B subunits, respectively (Murphy and Goldstein, 1979). There are also some exceptions. For instance, the lectin from stinging nettle (*Urtica dioica*) possesses two binding sites with intrinsically different affinities for *N*,*N*′,*N*″-triacetylchitotriose on a single polypeptide chain (Shibuya et al., 1986). However, since both binding sites recognize the same trisaccharide and, in addition, are located on two tandemly arranged highly similar hevein domains, the nettle lectin contains two very similar rather than different domains (Beintema and Peumans, 1992). Similarly, the two carbohydrate-binding sites of the ricin B chain (which are located on a single polypeptide) display a different specificity since site 1 binds only galactose whereas site 2 binds galactose and GalNAc (Yamasaki et al., 1985; Hatakeyama et al., 1986). Again, however, both sites recognize structurally related sugars.

A closer examination of the sequence of TxLCI indicates that this lectin is built up of a mannose-binding domain, which is clearly related to the basic subunit of the monocot mannose-binding proteins, and a second GalNAc-binding domain, which has no apparent similarity to any known protein. Basically, two possible mechanisms can explain the origin of the TxLCI gene. First, it may have evolved as a fusion product of two genes encoding a monocot mannose-binding protein and a GalNAc-binding protein of yet unknown origin. Second, the gene may have arosen by gene duplication. However, since the mannose-binding domain of TxLCI is much closer related to that of the lectins from the Araceae family than to TxLMII (Fig. 8), it seems unlikely that both tulip lectins evolved from each other. Most probably, the TxLCI gene or its ancestor originated before the Liliales and Arales diverged from each other.

Although TxLCI is a unique lectin in that it possesses two different carbohydrate-binding domains, gene duplication (or fusions) have taken place at several instances during evolution of the monocot lectins. For instance, the genes of the Araceae lectins are built up of two separate (but homologous) domains, each of which gives raise to a mannose-binding lectin subunit (Van Damme et al., 1995b). Similarly, the high degree of sequence similarity (84%) between the two subunits of the heterodimeric garlic lectin, which are both derived from a single precursor polypeptide encoded by a two-domain mRNA, argue for an (evolutionary) recent gene duplication (Van Damme et al., 1992b). At present, we can only speculate on the role of gene fusion in the evolution of the monocot lectins, since only a few examples are known.

It is also noteworthy in this context that there are other examples of fusions in which genes of carbohydrate-binding proteins are involved. For instance, the genes of the type-2 ribo-

some-inactivating proteins are considered as fusion products of a toxin gene and a gene encoding a carbohydrate-binding protein (Barbieri et al., 1993). Similarly, the genes of the class-I plant chitinases are believed to be the fusion products of two genes which encode a (chitin-binding) hevein domain and a catalytic domain, respectively (Collinge et al., 1993).

A final point to discuss concerns the unique capability of TxLCI to bind two different, unrelated carbohydrates. In contrast to all other plant lectins, which recognize and bind only one type (or two closely related types) of sugars, TxLCI can associate with both mannose-containing and GalNAc-containing glycan chains. As a result, the tulip lectin TxLCI is, at least in principle, capable of cross-linking totally different carbohydrate chains of glycoconjugates or cell surfaces. At present, the biological significance (in terms of the physiological role) of this dual binding is not understood. However, since TxLCI is a potent mitogen (Kilpatrick et al., 1990), there is no doubt that the lectin is able to provoque very specific processes in animal cells.

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