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Expression of cDNA for a bark lectin of *Robinia* in transgenic tobacco plants

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Abstract A cDNA encoding a bark lectin of *Robinia pseudoacacia* was introduced into tobacco plants. The expression of the lectin cDNA under control of the 35S promoter was confirmed by Western blot analysis and a hemagglutination assay of extracts of transgenic plants. Western blot analysis revealed that the subunit of the lectin from tobacco had a molecular mass of 29 kDa. The sequence of nine amino acids from the N-terminus of the lectin from transgenic tobacco plants was identical to that of the bark lectin from *Robinia*, indicating that the lectin had been processed correctly at its N-terminus in tobacco. The molecular mass of the purified native lectin produced by tobacco plants was estimated to be 112 kDa by gel filtration on a column of Superdex 200. It is suggested that the lectin subunits assembled to form tetramers in transgenic tobacco plants.

Key words: Robinia pseudoacacia; Bark lectin; Transgenic tobacco; Hemagglutinating activity

1. Introduction

Lectins are proteins that recognize and bind to carbohydrate moieties of glycoproteins and glycolipids and to polysaccharides. They are widely distributed in animals, plants and microorganisms. The best-characterized lectins are those isolated from the seeds of leguminous plants [1]. Lectins have also been purified from leaves [2–4], roots [5,6] and bulbs [7–9], and cDNAs that encode such lectins have been cloned [4,10,11]. The bark of woody plants is also a source of lectins [12–15]. In a previous study, we isolated a lectin from the bark of *Robinia pseudoacacia*, a leguminous woody plant [15]. We cloned the cDNA for this lectin and determined its nucleotide sequence [16].

Chrispeels and Raikhel proposed that plant lectins most probably function as part of defense systems [17], and that lectins eaten by predators bind to glycoproteins that line the intestinal tract and inhibit the absorption of nutrient. Pusztai and co-workers showed that *Robinia* lectin binds to the epithelial cells of the small intestine of the rat [18]. This finding led us to attempt to use the cDNA for the bark lectin of *Robinia* for the genetic engineering of plants. It is essential for such an application that the cDNA be expressed at adequate levels and that the encoded lectin subunits be folded into a biologically active form in the heterologous system. Some genes and cDNAs for plant lectins have been introduced into heterologous plants and the synthesis and sub-cellular localization of such lectins have been studied extensively [19–25]. However, little is known about the properties of lectins produced in transgenic plants.

In this study, we introduced the cDNA for the bark lectin of *Robinia* into tobacco plants and characterized some of the properties of the lectin that was produced.

2. Materials and methods

2.1. Construction of chimeric gene

DNA was manipulated by the methods described by Sambrook et al. [26]. The cDNA for bark lectin of *R. pseudoacacia* was cloned at the *Xba*I and *Sac*I sites of pBI-121 or pBI-101 [27] after removal of the gene for β-glucuronidase (GUS). The resultant plasmids were designated 35S::LEC and promoterless LEC, respectively.

2.2. Production and analysis of transgenic tobacco plants

The plasmids, namely 35S::LEC and promoterless LEC, were introduced into disarmed *Agrobacterium tumefaciens* LBA4404. The leaf-disk cocultivation procedure was used for generation of transgenic tobacco plants [28]. Regenerated transformants were grown on moist vermiculite in a growth chamber (12 h light/12 h darkness, 25°C). 35S::LEC transgenic plants were identified by the presence of lectin, as revealed by a hemagglutination assay and immunoblotting experiments. Promoterless LEC transgenic plants were identified by polymerase chain reaction (PCR) with total DNA from leaves and appropriate primers. Transgenic tobacco plants that produced lectin were raised to flowering and flowers were self-polinated to produce T2 seeds.

2.3. Extraction of proteins and Western blotting

Proteins were extracted from transgenic tobacco plants with 0.1 M phosphate-buffered saline (PBS, pH 7.2) that contained 0.15 M NaCl, and the lectin was detected by Western blotting with polyclonal antibodies raised against the bark lectin, as described previously [15]. For the purification of the lectin, proteins were extracted from the leaves of transgenic tobacco plants (T2 plants) with PBS and the lectin was purified by affinity chromatography on a column of lacto-agarose (E-Y Laboratories, San Mateo, CA, USA) as described previously [15].

2.4. Indirect immunofluorescence staining

Indirect immunofluorescence staining was performed with polyclonal antibodies against the bark lectin of *Robinia* and with FITC-conjugated goat antibodies against rabbit IgG, as previously reported [29]. A barrier filter (CDF; Vacuum Optics Corporation of Japan, Tokyo, Japan) was used to eliminate interference by the red fluorescence from chlorophyll.

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Abbreviations: CaMV, cauliflower mosaic virus; CBB, Coomassie brilliant blue; FITC, fluorescein isothiocyanate; GUS, β-glucuronidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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2.5. Hemagglutination assay

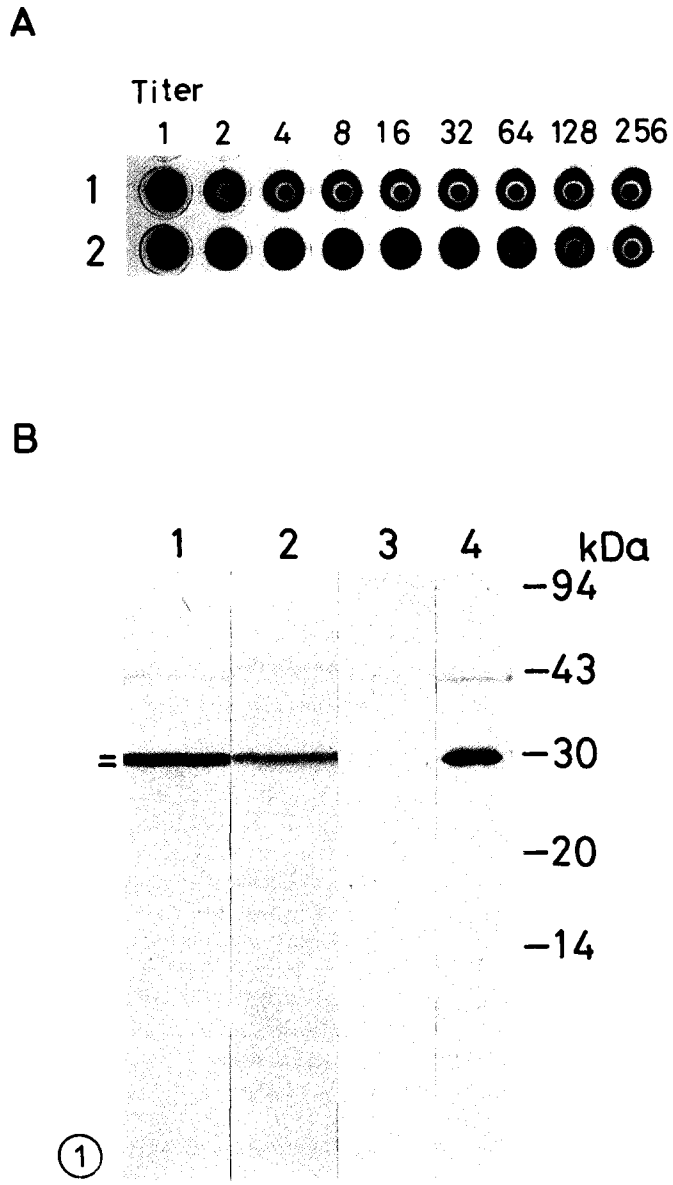
The hemagglutinating activity of the lectin was assayed by the serial- dilution method in microtiter plates with rabbit erythrocytes [14]. The extent of agglutination was expressed as the titer, namely, the reciprocal of the highest dilution that gave detectable agglutination.

2.6. N-terminal amino acid sequencing

Lectin, isolated from transgenic tobacco plants, was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, Beford, MA, USA). The membrane was stained with Coomassie brilliant blue (CBB) and the bands that corresponded to lectin subunit were cut out from the membrane. N-terminal amino acid sequences were determined with a protein sequencer (model 477A; Applied Biosystem, CA, USA), equipped with an on-line PTH-analyzer.

2.7. Estimation of molecular mass

The molecular masses of the native lectin and that produced in transgenic plants were estimated by gel filtration chromatography on a column of Superdex 200 (Pharmacia P-L Biochemicals, Uppsala, Sweden). The lectin was chromatographed in 0.02 M phosphate buffer (pH 7.2) that contained 0.15 M NaCl in the presence or absence of 0.3 M lactose. For the calibration of the column, the following proteins were used: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).



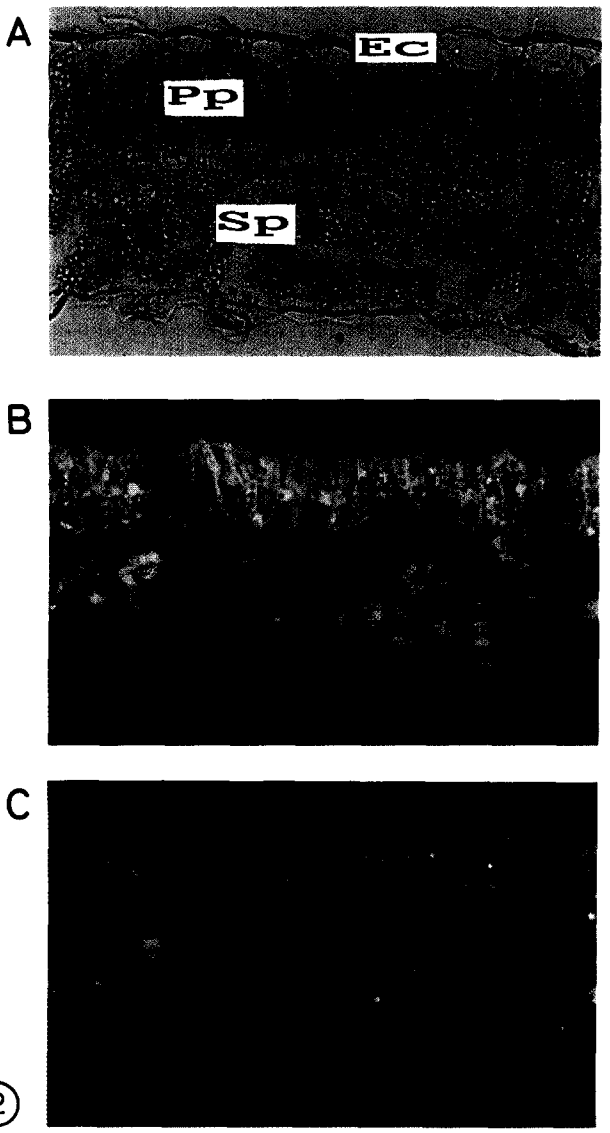


Fig. 1. Detection of lectin in transgenic tobacco plants. (A) Hemagglutinating activity in extracts of leaves of transgenic tobacco plants. Hemagglutinating activity was assayed with rabbit erythrocytes. Soluble proteins from the leaves of transgenic (row 2) and non-transgenic tobacco (row 1) plants were subjected to the assay. (B) Western blot analysis of lectin from the leaves of transgenic tobacco. Soluble proteins from the leaves of transgenic (lane 1) and non-transgenic (lane 3) tobacco and the bark lectin of *Robinia* (lane 4) were subjected to Western blot analysis on a 15% polyacrylamide gel with antibodies against the bark lectin of *Robinia*. Leaf proteins of a transgenic tobacco plant were also extracted with denaturing buffer and analyzed by Western blotting (lane 2). =, double of proteins of about 29 kDa.

Fig. 2. Specific expression of lectin in mesophyll cells of the leaves of transgenic tobacco plants. The localization of lectin was examined by indirect immunofluorescence staining. (A) Light microscopic view of a section of a leaf from a transgenic tobacco plant. (B) Immunofluorescence microscopy of the same section as in A. (C) Immunofluorescence microscopy of the same section as in A with a barrier filter in place to eliminate the red fluorescence of chlorophyll. Ec, epidermal cell; Pp, palisade parenchymal cell; Sp, spongy parenchymal cell.

3. Results

3.1. Expression of the cDNA for bark lectin in transgenic tobacco plants

Seven independent 35S::LEC transgenic tobacco plants were obtained. The expression of the cDNA for bark lectin was demonstrated in T1 transgenic plants. The hemagglutinating activity of tissue extracts with rabbit erythrocytes was examined. Extracts of leaves, stems and roots of transgenic tobacco plants had the hemagglutinating activity, whereas those of promoterless LEC transgenic and non-transgenic tobacco plants did not exhibit any activity. There was about a four-fold difference in hemagglutinating activities in the leaf extracts between seven transformants. The results with a typical transformant are shown in Fig. 1A. Western blot analysis of proteins from the leaves of transgenic tobacco plants revealed a doublet of proteins that cross-reacted with antibodies against bark lectin. This doublet migrated in the region that corresponded to a molecular mass of 29 kDa (Fig. 1B). The doublet was also found in the analyses of stems and roots (data not shown). The proteins from non-transgenic and promoterless LEC transgenic tobacco plants did not cross-react with the antibodies. The doublet raised the possibility that the extraction from tissues had caused a change in the lectin protein. When proteins were extracted from the leaves of transgenic tobacco plants with a denaturing buffer that contained 0.28 M Tris buffer (pH 6.8), 4.4% SDS and 22% glycerin, a single protein cross-reacted with the antibodies and this protein corresponded to the larger protein in the doublet (Fig. 1B, lane 2). This result suggests that the larger protein was converted to the smaller protein in the doublet during the extraction of tissues with PBS. Phytohemagglutinin, a lectin from the seeds of *Phaseolus vulgaris*, was similarly produced as a doublet in cultured tobacco cells that had been transformed by a gene for the lectin [30]. The mechanism responsible for the production of the doublet is unknown.

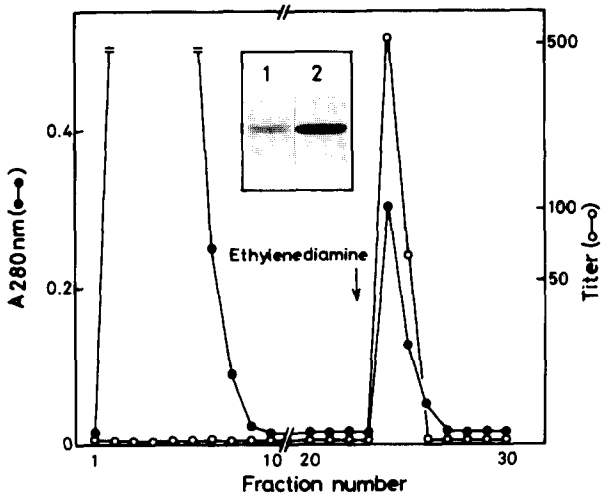


Fig. 3. Purification of the lectin from a transgenic tobacco plant on an affinity column. The crude extract of leaves of a transgenic tobacco plant was loaded onto a lacto-agarose column and the adsorbed lectin was eluted with 20 mM ethylenediamine. Hemagglutinating activity was assayed with rabbit erythrocytes. Insert: Fractions containing hemagglutinating activity were combined and proteins were subjected to SDS-PAGE and stained with CBB (lane 1) or analysed by Western blotting (lane 2).

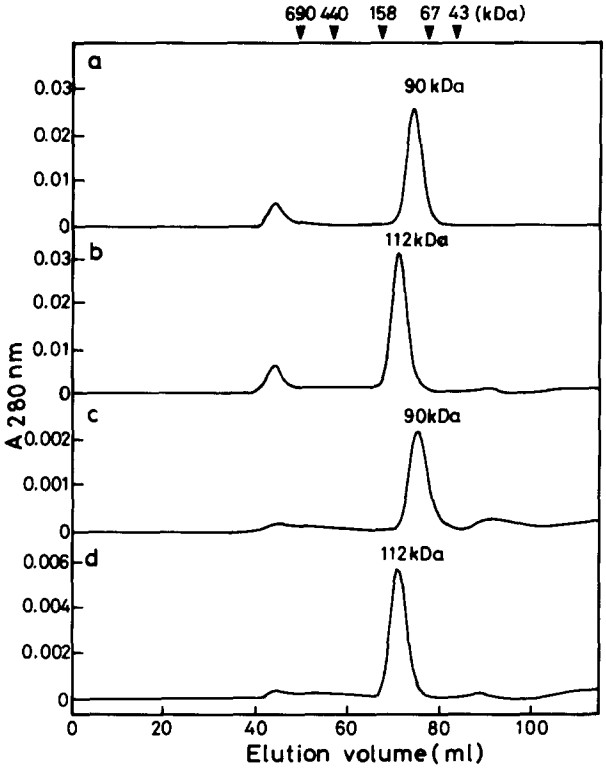


Fig. 4. Determination of the molecular mass of lectins on a column of Superdex 200. Lectin from *Robinia* bark (a,b) and lectin purified from transgenic tobacco plants (c,d) were chromatographed on a column of Superdex 200 in the presence (b,d) and in the absence (a,c) of 0.3 M lactose. Arrowheads indicate elution positions of molecular mass markers.

3.2. Localization of the lectin in the leaves of transgenic tobacco plants

The expression of the cDNA for the bark lectin in the leaves of transgenic tobacco plants was examined by indirect immunofluorescence staining (Fig. 2). Red autofluorescence of chlorophyll and yellow fluorescence of FITC were observed in the chloroplasts and in the cytoplasmic regions of the mesophyll cells, respectively, but not in the epidermis. When non-immune rabbit serum was used, the yellow fluorescence was not observed in the cells (data not shown). These results indicate that the lectin protein is located in the mesophyll cells but not in the epidermis. Pea and barley lectins have also been produced in the leaves of heterologous systems under the contol of the CaMV 35S promoter [21,22,25].

3.3. Purification of the lectin from transgenic tobacco plants

When a crude extract of the leaves of a T2 transgenic plant was applied to a column of lacto-agarose gel, all of the hemagglutinating activity in the crude extract was adsorbed to the affinity column (Fig. 3). The lectin was eluted from the column by 20 mM ethylenediamine as is the actual bark lecin of *Robinia* [15]. After SDS-PAGE, the fractions with hemagglutinating activity gave only a doublet of proteins upon staining of the gel with CBB and both proteins cross-reacted with the antibodies raised against the bark lectin. Thus the lectin was purified to homogeneity (Fig. 3, insert). Typically, about 1 mg of purified

active lectin was obtained from 50 g fresh weight of leaves of a transgenic tobacco plant.

3.4. Determination of the N-terminal sequence

The N-terminal amino acid sequence the lectin from transgenic tobacco plants was determined. The larger lectin protein in the doublet had the N-terminal sequence Thr-Gly-Ser-Leu-Ser-Phe-Ser-Phe-Pro, which is identical to that of the bark lectin of *Robinia* [15]. Although the reason was unknown, it was difficult to determine the N-terminal sequence of the smaller protein in the doublet because of the lower level of this protein. However, the partial sequence Thr-Gly-X-Leu-X-Phe-X-Phe-Pro (X, not determined) was obtained. This result strongly suggests that the sequence was also identical to that of the bark lectin of *Robinia*.

3.5. Estimation of molecular mass

In a preliminary experiment, we found that there were weak interactions between the bark lectin of *Robinia* and the gel matrix of Sephacryl S-200 and that these interactions could be abolished when 0.3 M lactose was included in the filtration buffer. The bark lectin of *Robinia* was eluted at a position corresponding to a protein of approximately 90 kDa from a column of Superdex 200 in the absence of lactose (Fig. 4a). However, the molecular mass was estimated to be 112 kDa when 0.3 M lactose was present in the filtration buffer (Fig. 4b), indicating that there were also some interactions between the bark lectin of *Robinia* and the matrix of Superdex 200. The behavior of the lectin from transgenic tobacco plants during chromatography on a column of Superdex 200 was the same as that of the native bark lectin of *Robinia* (Fig. 4c,d). The molecular mass of this lectin was determined to be 112 kDa in the presence of lactose.

4. Discussion

In our previous report [16], we proposed a pathway for the production of lectin in the cells of bark tissue, as follows: (1) lectin mRNA is translated into a precursor protein of 286 amino acid residues (31,210 Da); (2) the precursor is processed to the mature protein, which consists of 255 amino acid residues (27,600 Da); (3) the mature protein is glycosylated at a putative site for N-glycosylation to yield the mature subunit (29 kDa); and finally, (4) four identical subunits assemble to form the native lectin that is active in hemagglutination.

In the present study, the lectin produced in transgenic tobacco plants migrated with the mobility of a 29-kDa protein during SDS-PAGE (Fig. 1B). Moreover, the sequence of nine amino acids from the N-terminus of the lectin was identical to that of the bark lectin of *Robinia*. These results suggest that the precursor to bark lectin is correctly processed at the N-terminal end to produce a mature protein of 27 kDa and that this protein is converted into the mature subunit of 29 kDa by glycosylation in transgenic tobacco plants.

The agglutination of red blood cells requires the multivalent interaction of a lectin with cell-surface carbohydrates [31]. Furthermore, since the lectin subunit contains only one carbohydrate-binding site, multimeric forms of lectin are necessary for the agglutination of the cells. Therefore, the hemagglutinating activity demonstrated in the present study suggests that the lectin subunits assembled to form homotetramers in the transgenic tobacco plants. The tetrameric form of the lectin in transgenic tobacco plants was confirmed by gel filtration chromatography, which revealed that the molecular mass of lectin is about 112 kDa (Fig. 4d).

Affinity chromatography on lacto-agarose gel showed that the lectin from transgenic tobacco plants bound to lactose (Fig. 3). Furthermore, as in the case for the bark lectin of *Robinia*, the lectin purified from tobacco leaves interacted weakly with the gel matrix of Superdex 200 and this interaction was abolished in the presence of 0.3 M lactose (Fig. 4). These results indicate that the binding specificity of the lectin produced in transgenic tobacco plants was the same as that of the bark lectin of *Robinia*.

In the present study, we used the CaMV 35S promoter for expression of the cDNA for the bark lectin in tobacco. The level of expression of the lectin cDNA was sufficient to permit detection of the lectin as hemagglutinating activity in leaf extracts of transgenic tobacco plants (Fig. 1A). However, the amount of the lectin in tobacco was not as high as that in bark tissues of *Robinia* trees [16]. For example, in a typical experiment, at least 50 mg of the lectin were purified from 50 g fresh weight of *Robinia* bark by affinity chromatography. However, in the present study, only 1 mg of purified lectin was obtained from the same quantity of fresh leaves of transgenic tobacco plants. Although several possibilities might explain the difference in lectin content between *Robinia* and tobacco, one possibility is that the lectin produced in the heterologous system is not as stable as that produced in the bark tissues.

Van Damme at al. reported that the bark of *R. pseudoacacia* contains a complex mixture of lectins [32]. Furthermore, they cloned three cDNAs that encoded lectin subunits. One of these cDNAs was identical to the one that we had already cloned and that we used in this study. The presence of a small family of genes for the lectin was suggested from the results of Southern blot analysis of *Robinia* DNA [16,32]. Therefore, it will be of interest to investigate the molecular mechanisms that are responsible for the bark-specific expression of a gene for lectin in *Robinia* trees. We are now cloning genes for lectins from *R. pseudoacacia*.

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