Proteins isolated from lucerne roots by affinity chromatography with sugars analogous to Nod factor moieties

Zoran MINIC[1], Lydie LEPROUST-LECOESTER, Jean LAPORTE, Yaroslav DE KOUCHKOVSKY and Spencer C. BROWN[2]
Institut des Sciences Végétales (CNRS-UPR40), Bâtiment 24, F-91198 Gif-sur-Yvette, France

Nod factors are important elicitors in legume–bacterium symbiosis. Any candidate plant receptor(s) for these lipo-oligosaccharides can be expected to show some lectin-like properties. A novel protein (P60), a native tetramer with 60 kDa monomers, has been isolated from a membrane fraction of *Medicago sativa* (lucerne, alfalfa) roots by using affinity chromatography with either GlcNAc or *N*,*N*′,*N*″-triacetyl-(1 → 4)-β-D-chitotriose [$(GlcNAc)_3$] grafted to agarose beads as the matrix and, in a second step, Sephadex G-200 gel filtration. With $(GlcNAc)_3$–agarose an additional protein of 78 kDa was isolated. P60 showed haemagglutination activity with specificity for GalNAc, GalN, GlcNAc and GlcN. Binding experiments with radioactive GlcNAc gave a K_d of 95 nM and one binding site per monomer of P60; Nod factor competed strongly for this binding. In native PAGE, protein incubated with O-sulphated Nod factors had a higher electrophoretic mobility as a consequence of binding. However, the largest modification was observed with a natural mixture of Nod factors, containing the O-acetylated and O-sulphated tetrasaccharidic NodRm-IV(Ac,S) (in which Ac stands for an O-acetylated group at the non-reducing end and S for O-sulphation at the reducing end) in addition to the non-O-acetylated NodRm-IV(S) (which alone had little effect) and NodRm-V(S). The native PAGE study was also performed with known lectins from other sources, but only the 34 kDa lectin of *Phytolacca americana* (pokeweed) showed any such interaction, although without discrimination between Nod factors. Finally, one peptide of each isolated protein was sequenced; the peptide from P60 showed some similarity with dihydrolipoamide dehydrogenase and ferric leghaemoglobin reductase, whereas the peptide from P78 was identical with an analogous region of 70 kDa heat shock protein.

Key words: lectin-type protein, heat shock protein, native PAGE, *Medicago sativa* L.

INTRODUCTION

Certain plant elicitors such as oligosaccharides can act as infection signals and can initiate various defence responses, including phytoalexin biosynthesis, oxidative burst and the production of protease inhibitors, of pathogenesis-related proteins, of cell wall glycoproteins and of lignin [1,2]. Similarly, the lipo-oligosaccharidic Nod factors produced by rhizobial soil bacteria can elicit not only the formation of root nodules (reviewed in [3]) but also, at high concentrations, defence responses [4]. These Nod factors, first isolated from *Sinorhizobium meliloti* [synonym *Rhizobium meliloti* (Rm)] on the basis of their ability to cause root hair deformation in *Medicago sativa* (lucerne, alfalfa) [5], were found to be derivatives of tetra- and penta-*N*-acylated glucosamine. They carry a fatty *N*-acyl group instead of an *N*-acetyl group on the glucosamine at the non-reducing end, and can be O-acetylated on this end and O-sulphated on the reducing end [6,7] (see Figure 1A, in which the distinctive *N*- and *O*-acetyl groups are circled). Depending on modifications and on the length of the chito-oligosaccharide backbone, Nod factors are perceived differently in different legumes [3,4] and have variable biological stability [7,8]. It is evident that if proteins were to serve as receptors for these lipo-oligosaccharidic signals, by definition they would be classified as lectins if they were recognized by their carbohydrate backbone rather than by the lipid chain.

Chitin oligomers structurally similar to Nod factors are active as elicitors in rice [9], pea [10] and tomato [11] cells. Chitin-binding proteins retaining binding affinity and specificity have been solubilized from membranes of suspension-cultured tomato [12] and rice [9] cells by using the cross-linking of a radiolabelled *N*-acetylchitopentaose or photo-affinity and chemical-affinity cross-linking respectively. Lectins and chitinases can bind GlcNAc, its oligomers and chitin, which is a polymer of GlcNAc residues [13]. Lectins are an important determinant of host specificity in the rhizosphere [14]. However, their role might not be in the actual species-specific recognition, which is apparently mediated by Nod factors, but rather in serving to agglutinate large numbers of bacteria at the root hair surface. In contrast, a sequence similar to the legume lectin domain has been identified as part of putative receptor kinase genes in *Arabidopsis thaliana* that encode receptor-like serine/threonine kinases [15], although the functional role of the lectin-like domain has not been established.

The low concentrations (1 pM to 1 nM) of Nod factor that have been found to induce physiological responses in legumes [7] suggest that Nod factor receptors have a high affinity for their ligands. Research into putative receptors or binding proteins of Nod factors has met with limited success [16,17]. Niebel et al. [16] reported potential binding sites for Nod factors in the microsomal fraction from a cell suspension culture of *Medicago varia* by using synthetic NodRm factor labelled to a high specific radio-

Abbreviations used: DTT, D,L-dithiothreitol; FLbR, ferric leghaemoglobin reductase (EC 1.6.2.6); $(GlcNAc)_3$, *N*,*N*′,*N*″-triacetyl-(1 → 4)-β-D-chitotriose; HSP70, 70 kDa heat shock protein; NodRm, nodulation factors (Nod) from the symbiotic soil bacterium *Sinorhizobium meliloti* (synonym *Rhizobium meliloti*, Rm) [lipochito-oligosaccharides (number of GlcNAc moieties indicated by a roman numeral) that bear an *N*-acetylated unsaturated fatty acid ($C_{16:2}$) and possibly an O-acetylated group (Ac) at the non-reducing end, and that can be O-sulphated (S) at the reducing end]; P60 and P78, 60 and 78 kDa proteins (this study).

[1] Present address: Biochimie des Signaux Régulateurs Cellulaires et Moléculaires, CNRS-Université Paris 6, 96 boulevard Raspail, 75006 Paris, France.
[2] To whom correspondence should be addressed (e-mail spencer@isv.cnrs-gif.fr).

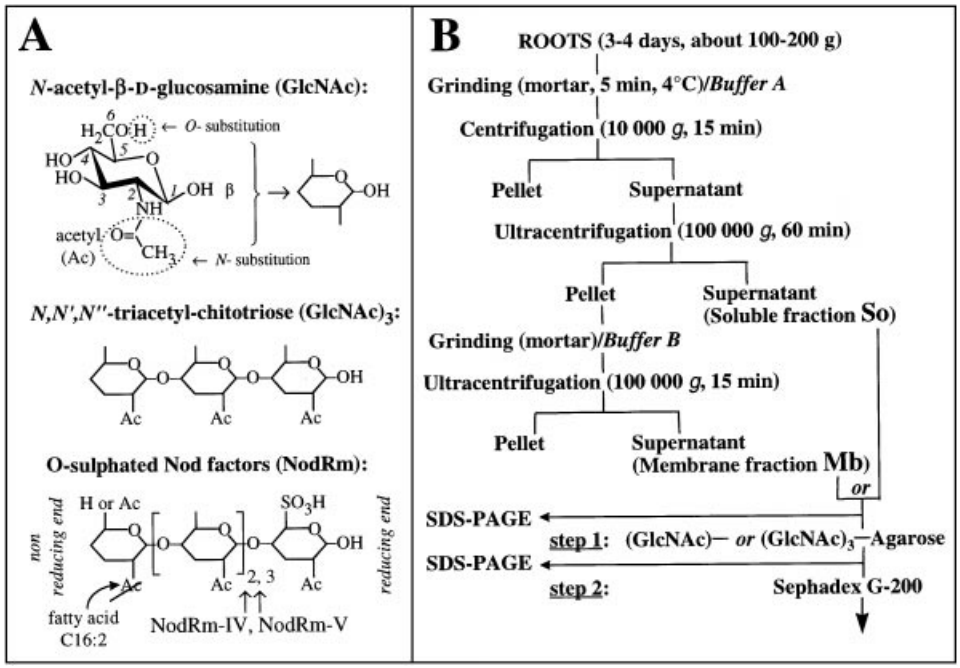

Figure 1 Chemical structure of GlcNAc, $(GlcNAc)_3$ and Nod factors used in this work (A) and flow chart of protein extraction (B)

See the Experimental section for a description of the buffers used.

activity (29.6 TBq, i.e. 800 Ci/mmol). These authors, using [^{35}S]NodRm-IV(Ac,S,$C_{16:2}$) (in which Ac stands for an O-acetylated group at the non-reducing end and S for O-sulphation at the reducing end), have characterized two types of site, one with high affinity (K_d 1.9 nM) and the other with lower affinity (K_d 70 nM).

We have chosen to select a protein fraction from a membrane extract of lucerne roots by affinity chromatography on agarose gels grafted with GlcNAc or *N*,*N*′,*N*′′-triacetyl-(1 → 4)-*β*-D-chitotriose [$(GlcNAc)_3$] because these sugars correspond to structural elements of Nod factors. The proteins selected by this procedure were then tested for their ability to bind Nod factors produced by *S. meliloti* by looking for changes in the electrophoretic mobility of native protein. This experimental strategy is applicable if the ligands are not metabolized during the assay, which is not always true [18].

EXPERIMENTAL

Materials

Sugars, lectins, GlcNAc–agarose, $(GlcNAc)_3$–agarose and reduced Triton X-100 (rather than Triton X-100, which absorbs at 280 nm) were products of Sigma (St. Louis, MO, U.S.A.). Sephadex G-200 was from Pharmacia (Uppsala, Sweden). Nod factors used (all with $C_{16:2}$ side chain) were tetrameric O-sulphated NodRm-IV(S) and a natural extract of mixed NodRm factors, NodRm(mix), produced by *S. meliloti* and containing 72 % NodRm-IV(S), 20 % NodRm-V(S) and 8 % NodRm-IV(Ac,S). These were prepared in our laboratory by Dr. M. Schultze; being amphiphilic, they could be used with water as solvent.

Seeds (150 g) of *M. sativa* L. cv. Sitel (Tourneur, Montauban, France) were surface-sterilized [8] and germinated for 24 h in the dark at 24 °C. The seedlings were dispersed over a plastic mesh that covered 15 cm × 15 cm plastic Petri dishes filled with 25 ml of sterile Jensen medium [19]; they were then grown under sterile conditions in the dark at 24 °C.

Membrane protein extract from roots

The 3–4-day-old roots (approx. 150 g) on the mesh were shaved over 300 ml of ice-cold extraction buffer and blended for 5 min. Buffer A contained 20 mM Bis-Tris, pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 1 mM D,L-dithiothreitol (DTT), 5 % (v/v) glycerol, 1.5 % (w/v) 40 kDa poly(vinylpyrrolidone), 4 μM sodium cacodylate and the following protease inhibitors: 1 mM PMSF, 1 mM pepstatin, 10 mM leupeptin and 10 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64'; Boehringer, Mannheim, Germany). The grinding juice was centrifuged at 4 °C for 15 min at 10000 ***g***; the supernatant was centrifuged for 60 min at 100000 ***g***. The resulting supernatant, containing 'soluble proteins' (So), was kept and the pellet was solubilized by treatment with 10–20 ml of ice-cold extraction buffer B [buffer A plus 0.1 % (w/v) reduced Triton X-100] and blended for 5 min. The suspension was centrifuged at 4 °C for 15 min at 100000 ***g*** and the supernatant, containing solubilized 'membrane proteins' (Mb), was used for further isolations. The protein content of all eluates was estimated by A_{280}. The procedure is summarized in Figure 1(B).

Step 1: affinity chromatography

A 1 cm × 6 cm column was filled with 5 ml of GlcNAc or $(GlcNAc)_3$ immobilized on agarose beads and washed with 20 ml of 0.1 M NaCl. Protein extract (3 ml) was run on to the column and allowed to stand for 30 min at 4 °C. The matrix was then rinsed with 15 ml of buffer containing 25 mM Bis-Tris, pH 7.0, 50 mM NaCl and 0.1 % (w/v) reduced Triton X-100; the retained proteins were eluted by the same buffer plus 50 mg/ml GlcNAc.

The eluates were collected, then pooled and concentrated by use of a YM3 Diaflo membrane (Amicon-Grace, Bedford, MA, U.S.A.).

Step 2: gel-filtration chromatography

The concentrated fraction from affinity chromatography (4 ml) was injected into a 2.6 cm × 75 cm column of Sephadex G-200 (Pharmacia) precalibrated with the following molecular mass markers (Pharmacia): vitamin B_{12} (1.32 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), γ-globulin (158 kDa), catalase (232 kDa) and thyroglobulin (670 kDa). Equilibration and elution were performed with 25 mM Bis-Tris (pH 7.0)/0.2 M NaCl.

Protein-denaturing electrophoresis

SDS/PAGE was performed by using slab gels with a 4–18 % (w/v) polyacrylamide gradient [20]. Standard markers (Bio-Rad) were phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and egg-white lysozyme (14.4 kDa). The gels were stained with either Coomassie Brilliant Blue R-250 or silver nitrate [20].

Haemagglutination assay

Haemagglutination assays were conducted as described [21], the reference lectin being concanavalin A (Sigma). One haemagglutination unit is arbitrarily defined as the amount of material that is required to cause a decrease of 50 % in the pseudo-absorbance of the erythrocyte suspension under given conditions. Assays were performed here with 5 μg/ml protein (2 haemagglutination units) mixed with rabbit erythrocyte suspension (14 μl of packed cells/ml) in 25 mM Bis-Tris, pH 7.0, containing 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Sugar inhibition was measured by a serial dilution of the sugar at a fixed final lectin concentration. The minimal sugar concentration for complete inhibition of agglutination of rabbit erythrocytes was measured by light scattering at 640 nm [21], with a starting pseudo-absorbance of 1.0 given by the present erythrocyte concentration.

Ligand binding assays

The binding of the labelled ligand [*N-acetyl*-^{3}H]GlcNAc, with a specific radioactivity of 166 GBq/mmol, with 0.5 μg of isolated protein was assayed after preincubation for 2 h at 4 °C in 1 ml of binding buffer [25 mM Bis-Tris (pH 7.0)/0.1 M NaCl/1 mM $CaCl_2$] containing increasing concentrations of labelled ligand in the range 1–140 nM. L-[6-^{3}H]Fucose, with a specific radioactivity of 1.8 TBq/mmol, was used as control. Incubations were performed on Centricon 30 membrane filters (Millipore, Bedford, MA, U.S.A.). At the end of the incubation, the radioactivity of 200 μl samples before ultrafiltration and 200 μl ultrafiltrates after centrifugation were measured by scintillation spectrometry. Radioligand binding experiments were processed by the computer program Prism (GraphPAD, San Diego, CA, U.S.A.).

Nod factor binding assay by native electrophoresis

Aliquots (20 μl) containing 0.2–2 μg of purified protein or of commercial lectins were incubated for 2 h at 4 °C in the absence or the presence of different concentrations of Nod factors in 25 mM Bis-Tris, pH 7.0, containing 0.1 M NaCl, 1 mM $CaCl_2$ and 0.05 % (w/v) reduced Triton X-100. The migration of protein bands was analysed by native PAGE in 7 % (w/v) slab polyacrylamide gels with 0.35 M Tris/HCl (pH 8.9) or 30 mM L-histidine plus 30 mM Mes (pH 6.1) buffers containing 0.05 % (w/v) reduced Triton X-100. Protein samples (10 μl) were applied to the gel.

Protein determination and microsequencing

The protein content was determined with bicinchoninic acid, by using BSA as standard (BCA method; Pierce, Rockford, IL, U.S.A.).

Amino acid sequences of selected peptides from proteins obtained by affinity chromatography of the soluble and membrane-associated fractions were obtained with a model 610A protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.) with the use of the automated Edman-degradation chemistry. The two proteins (P60 and P78, of molecular masses 60 and 78 kDa respectively) were separated by SDS/PAGE [10 % (w/v) gel], with gel dimensions 15 mm × 12 mm × 1.5 mm, and digested at 35 °C for 18 h with 1:500 (w/v) endolysin C in 200 μl of 0.05 M Tris/HCl buffer, pH 8.6, containing 0.03 % SDS. The resulting peptides were separated by HPLC (DEAE-C_{18} column, 0.1 cm × 3.5 cm) with a linear gradient from 2 % to 70 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid. After separation, selected peptides were sequenced.

RESULTS

Extraction of proteins

Figure 1(B) summarizes the extraction and purification methods used. We chose to focus here on possible membrane-associated Nod-factor-binding proteins, adapting a procedure [22] for the isolation of membrane proteins of roots. Approx. 120 mg of crude protein extract was recovered in this way from 150 g of fresh roots of 3-day-old lucerne.

Step 1: proteins with affinity for GlcNAc and $(GlcNAc)_3$

The affinity chromatography of soluble and membrane-associated protein fractions was performed with GlcNAc-agarose and $(GlcNAc)_3$–agarose. Figure 2 illustrates the different bands

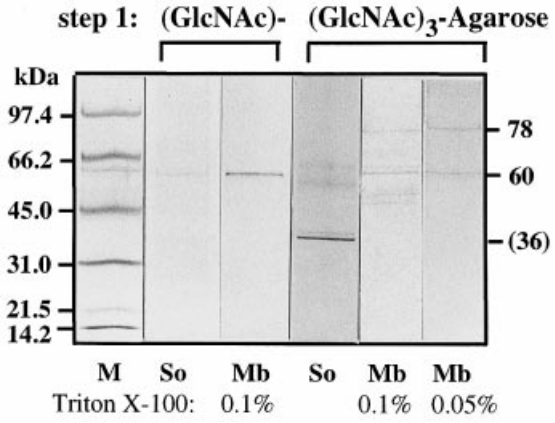

Figure 2 SDS/PAGE analyses of soluble (So) and membrane-associated (Mb) protein fractions after affinity chromatography with GlcNAc or $(GlcNAc)_3$

Each fraction (30 mg of protein) was injected on the column and the eluates after chromatography were concentrated 10-fold with a YM3 Diaflo membrane. Protein sample (10 μl) was applied to the gel in each case (3 mM DTT was present in all). Lane M, molecular mass standards (molecular masses indicated at the left).

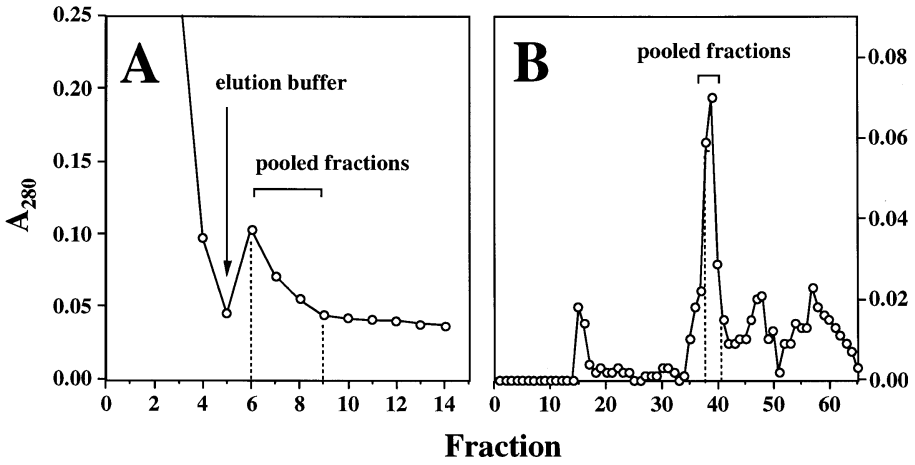

Figure 3 Two-step chromatography for root protein purification

(**A**) Enrichment (step 1): elution profile from affinity chromatography on GlcNAc–agarose. (**B**) Purification (step 2): gel-filtration chromatography on Sephadex G-200 of pooled fractions 6–9 from step 1 (see Figure 1B).

obtained by SDS/PAGE of equal quantities of proteins after affinity chromatography. With the GlcNAc–agarose column, a major band of 60 kDa (P60) was clearly seen in the membrane-associated fraction (Mb) but it was essentially absent from the soluble fraction (So). After affinity chromatography with a $(GlcNAc)_3$–agarose column, the same, although much thinner, P60 band was obtained with the membrane-associated fraction, whereas two faint bands above and below P60 were seen in the soluble fraction. In addition to these and other minor bands, the soluble fraction also revealed a band of 36 kDa, corresponding to the chitinase that we had previously isolated with the same technique and characterized [18]. The membrane-associated fraction treated with two different concentrations of detergent revealed a major band (P78) in addition to that of P60.

Step 2: protein purification

The affinity chromatography profile of the protein extract on GlcNAc–agarose gave one broad peak (Figure 3A). It was recovered and subjected to Sephadex G-200 gel filtration, which yielded another major peak (Figure 3B); this was retained for further analysis and several minor peaks were discarded. After this step, 50 μg of purified protein was obtained in the major peak from 120 mg of total protein extract. In contrast with the large amounts in legume seeds, lectins normally constitute only a small percentage of the soluble protein extracted from vegetative tissues [23].

SDS/PAGE of the fractions obtained by gel filtration

After affinity chromatography (step 1), several bands were revealed, the denser having a molecular mass of 60 kDa (Figure 4A, lane labelled 'step 1'). This major fraction, after treatment with 0.20% SDS/3 mM DTT and being heated for 3 min at 90 °C, showed a single 60 kDa band in silver staining (Figure 4A, lane labelled 'yes'). An unheated aliquot yielded two bands: one minor, at 60 kDa, and one major, of much higher molecular mass (Figure 4A, lane labelled 'no') that was found to be approx. 240 kDa by Sephadex G-200 gel filtration (Figure 4B). The 240 kDa band was identical in the presence of 0.1% (w/v) reduced Triton X-100, indicating that the binding of Triton to this protein was negligible and that the latter was not dissociated into monomers by the detergent (results not shown). Therefore the apparent molecular masses of the native protein obtained by gel-filtration chromatography and SDS analysis are consistent with a tetramer that is dissociated to its monomers by being heated in the presence of DTT. The recognition of specific saccharides by this protein, indicated by its affinity for GlcNAc and $(GlcNAc)_3$ during affinity chromatography, and its tetrameric nature are typical of a lectin [13,23].

Haemagglutination assays

Haemagglutination was tested with the native 240 kDa protein, this tetramer being termed here $(P60)_4$ on the basis of the molecular mass of its monomer. Agglutination of rabbit erythrocytes by $(P60)_4$ was inhibited by GalNAc and GalN slightly more effectively than by GlcNAc and GlcN; D-mannose, L-fucose and D-fructose had essentially no effect (Table 1). From the relative efficiencies of D-glucosamine and D-galactosamine compared with those of D-glucose and D-galactose for inhibiting haemagglutination, the amino substituent seemed to be important for the binding of $(P60)_4$, whereas the *N*-acetyl function was inconsequential. However, the limited competition of ethanolamine with GlcNAc (Figure 5B) indicated that, by itself, the charge carried by the amine group was not sufficient. In addition, the similar efficiencies of GalN and GlcN showed that the hydroxy position on C-4 (see Figure 1A) is not important here for monomers, in contrast with oligomers such as Nod factors, in which this determines the backbone structure.

Ligand binding assays

Although it probably has a higher affinity (Table 1), radioactive GalNAc was not used because the available isotope was of low specific radioactivity. GlcNAc was used instead; its binding to $(P60)_4$ is demonstrated in Figure 5(A), which also shows the limited interaction with a non-specific sugar such as L-fucose. After subtraction of the background binding in the absence of $(P60)_4$, the K_d (mean $\pm$ S.D.) was 95 ± 43 nM and the number of binding sites (B_{max}) was extrapolated to 15.4 ± 3.4 pmol/μg of protein. From a knowledge of the molecular mass of P60, it can be calculated that there are 0.92 binding sites per monomer. As shown in Figure 5(B), ethanolamine competed poorly with

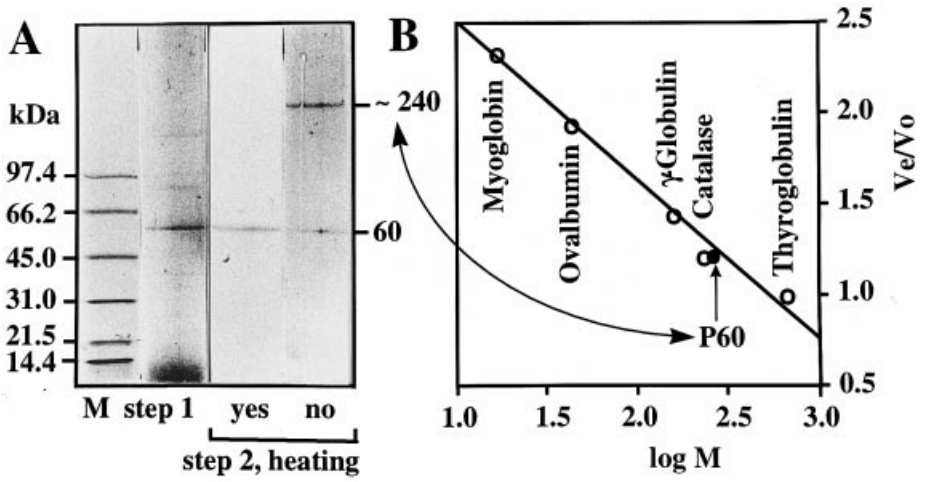

Figure 4 Analytical characterization of P60

(**A**) SDS/PAGE after affinity chromatography and further gel filtration (silver staining): lane M, molecular mass standards (2 μg) (molecular masses indicated at the left); lane labelled 'step 1', pooled fractions 6–9 from GlcNAc–agarose chromatography (2 μg); lanes labelled 'step 2', pooled fractions 38–41 from Sephadex G-200 gel filtration (1 μg), after treatment with SDS (0.20%, w/v) and DTT (3 mM), with (lane labelled 'yes') or without (lane labelled 'no') being heated for 3 min at 90 °C. The right-hand lane (unheated) shows an additional upper band. (**B**) Gel-filtration chromatography of this unheated fraction yielded a molecular mass of 240 kDa for the P60 native form, in agreement with the estimated molecular mass for the upper band of the right-hand lane in (**A**).

Table 1 Sugar concentrations required for full inhibition of haem-agglutination

Agglutination of rabbit erythrocytes (14 μl of packed cells/ml) by $(P60)_4$ at 5 μg/ml (i.e. 2 haemagglutination units) was assayed by light scattering at 640 nm.

Sugar tested	Sugar concentration (mM)
GalNAc	0.1
GalN	0.1
GlcNAc	0.25
GlcN	0.25
D-Galactose	1
D-Glucose	1
D-Mannose	5
L-Fucose	5
D-Fructose	5

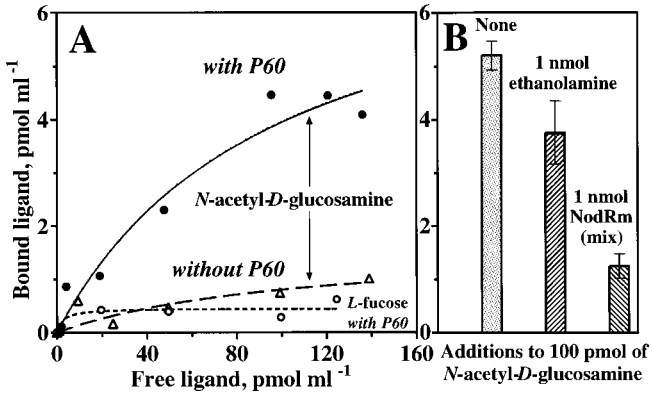

Figure 5 Binding experiments with P60

(**A**) Saturation curves for [*N-acetyl*-^{3}H]GlcNAc and [6-^{3}H]fucose binding to the lucerne root protein P60 isolated in the present study. P60 (0.5 μg; approx. 8.3 pmol) was incubated in 1 ml of binding buffer with increasing concentrations of [^{3}H]GlcNAc or [^{3}H]fucose for 2 h at 5 °C. The best fit drawn by using non-linear regression with the Marquardt algorithm gave a K_d of 95 ± 43 nM and a B_{max} of 7.7 pmol/ml (i.e. 15.4 ± 3.4 pmol/μg of P60 protein), with $r^2 = 0.97$, after subtraction of background (line labelled 'without P60'). (**B**) Competitive inhibition of binding of [^{3}H]GlcNAc (100 pmol/ml) was tested in the presence of biologically produced NodRm(mix) (1 μg/ml, i.e. approx. 1 nmol/ml, assuming an average molecular mass of Nod factors mixture of 1 kDa) or ethanolamine (1 nmol/ml).

GlcNAc, whereas NodRm(mix) decreased binding virtually to background levels (Figure 5A): this shows the strong affinity of $(P60)_4$ for this natural bacterial product.

Native PAGE

The mobility of a macromolecule through a gel under an electric field depends upon its charge and also on its molecular mass, size and shape [24]. The presence or absence of a ligand can change these parameters, which in turn would affect the protein's electrophoretic mobility [24]. Comparative native PAGE of $(P60)_4$ in the presence or absence of Nod factors as possible ligands is shown in Figure 6. The addition of mixed Nod factors produced by rhizobia [see the Experimental section for the composition of NodRm(mix)] induced a large increase in the mobility of the protein band (Figure 6A). This cannot be attributed to any detergent effect of the different Nod factors, because all were added at approximately equimolar ratios to the protein itself. In fact, at a similar concentration, pure non-O-acetylated NodRm-IV(S) had only a marginal effect except for a dispersion of the migrating front. The degree of mobility was dependent on the concentration of Nod factors [Figure 6(B), in which the mobility change is less than in Figure 6(A) because of the lower protein amount, which favours the dynamic dissociation and reassociation of the ligand–protein complex]. Some control experiments with other lectins are shown in Figures 6(C)

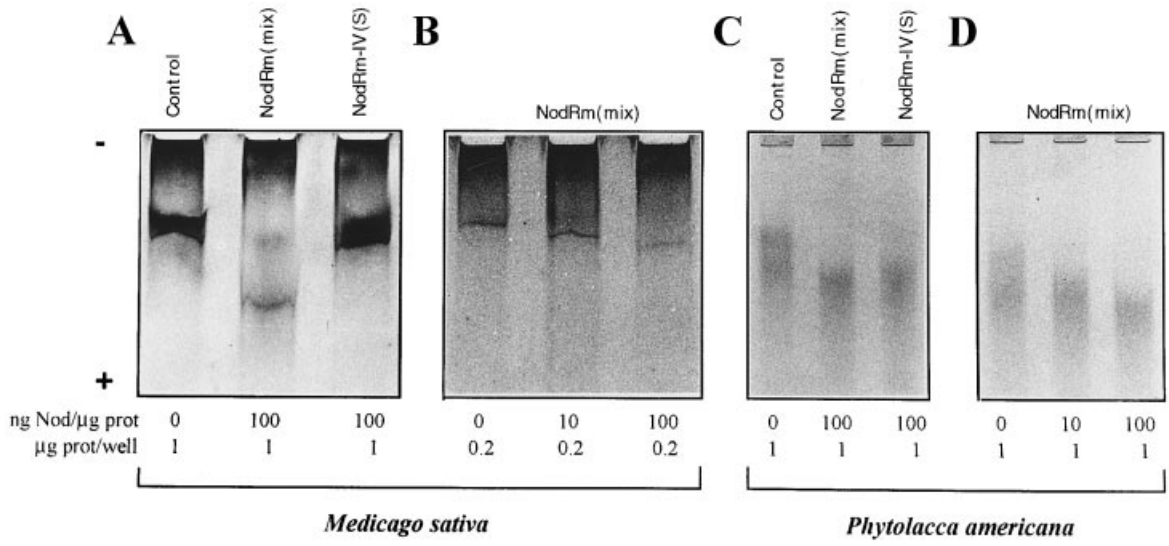

Figure 6 Native PAGE

Migration was performed for 1 h at pH 8.9 of lectins in 10 μl of buffer, preincubated for 2 h at 4 °C, in the presence or absence of various amounts of Nod factors (silver staining). (**A**) P60 (1 μg): alone, with 100 ng of NodRm(mix) or with 100 ng of NodRm-IV(S). (**B**) P60 (0.2 μg): alone, with 10 ng of NodRm(mix) or with 100 ng of NodRm(mix). (**C**) As in (**A**) but with *Phytolacca americana* (pokeweed) lectin (1 μg). (**D**) As in (**B**) but with pokeweed lectin (1 μg).

Table 2 Interaction of Nod factors with commercial lectins and with the novel lectin-like lucerne protein $(P60)_4$

Interaction was probed by mobility change in non-denaturing PAGE. Results in (**b**) are means $\pm$ S.D. for three experiments.

(a) Qualitative effect of NodRm (1 μg of lectin)

Lectin	Known specificity [38]	Nod factor added	PAGE mobility
Concanavalin A	αMan > αGlc > GlcNAc	NodRm(mix)	Unmodified
Arachis hypogaea	Galβ1-3GalNAc > α and βGal	NodRm(mix)	Unmodified
Glycine max	α and βGalNAc > α and βGal	NodRm(mix)	Unmodified
Ricinus communis (RCA120)	α and βGal > GalNAc	NodRm(mix)	Unmodified
Triticum vulgaris	$GlcNAc(\beta1\text{-}4GlcNAc)_{1,2}$ > βGlcNAc > NeuNAc	NodRm(mix)	Unmodified
Datura stramonium	$GlcNAc(\beta1\text{-}4GlcNAc)_{1-3}$ = Galβ1-4GlcNAc	NodRm(mix)	Unmodified
Solanum tuberosum	$GlcNAc(\beta1\text{-}4GlcNAc)_{1-4}$	NodRm(mix)	Unmodified
Phytolacca americana	$GlcNAc(\beta1\text{-}4GlcNAc)_{1-5}$ = $(Gal\beta1\text{-}4GlcNAc)_{2-5}$	NodRm(mix)	Faster
P60 (this study)	GalNAc, GalN > GlcNAc, GlcN (this study)	NodRm(mix)	Faster

(b) Quantitative effect of NodRm (1 μg of lectin)

Lectin	Nod factor added	PAGE mobility (%)
P60	None	100
	NodRm(mix)	164 $\pm$ 31
	NodRm-IV(S)	121 $\pm$ 5
Phytolacca americana	None	100
	NodRm(mix)	140 $\pm$ 4
	NodRm-IV(S)	137 $\pm$ 7

and 6(D) and Table 2. Despite some known affinities for oligoglucosamines, these eight lectins did not generally undergo changes in their electrophoretic mobility in the presence of mixed Nod factors. Only the lectin of *Phytolacca americana* (pokeweed) had a shifted electrophoretic migration in the presence of mixed Nod factors (Figures 6C and 6D); however, an identical effect was also observed with pure NodRm-IV(S), at variance with the selectivity observed with $(P60)_4$. The electrophoretic mobility was higher for the higher concentration of Nod factors incubated with the pokeweed 34 kDa lectin (Figure 6D). The mean relative electrophoretic mobilities of $(P60)_4$ and pokeweed lectin in the presence of NodRm(mix) are detailed in Table 2.

Amino acid microsequencing

SDS/PAGE of the membrane-associated fraction of root proteins purified by affinity chromatography with $(GlcNAc)_3$-

A

Peptide	Origin	N°																					N°	kDa
P78	Lucerne		**(K)**	**X**	**X**	**G**	**V**	**I**	**A**	**G**	**L**	**N**	**V**	**A**	**R**	**I**	**I**	**N**	**E**	**P**	**T**	**A**	**A**	**A**	**I**		**78**
HSP70	Soybean	188	**K**	**D**	**A**	**G**	**V**	**I**	**A**	**G**	**L**	**N**	**V**	**A**	**R**	**I**	**I**	**N**	**E**	**P**	**T**	**A**	**A**	**A**	**I**	210	**80**
"	Tobacco	193											(identical sequences)													215	**75-80**
	Spinach	192																								214	**71**

B

Peptide	Origin	N°															N°	kDa	Subunits
P60	Lucerne		**(K)**	**X**	**I**	**P**	**S**	**I**	**A**	**Y**	**T**	**E**	**P**	**E**	**V**	**A**		**60**	4
FLbR	Soybean	379	**D**	**K**	**V**	**P**	**G**	**V**	**V**	**Y**	**T**	**N**	**P**	**E**	**V**	**A**	392	**54-56**	2
DLDH	Pea	380	**D**	**K**	**V**	**P**	**G**	**V**	**V**	**Y**	**T**	**N**	**P**	**E**	**V**	**A**	393	**60-61**	2

Figure 7 Amino acid sequences of P78 and P60

After SDS/PAGE, digestion with endolysin C and separation by reverse-phase HPLC, two peptides were sequenced, one each of P78 and P60. (**A**) Comparison of the partial amino acid sequence of P78 with the corresponding amino acid sequences deduced from cDNA clones of HSP70 from different plants. (**B**) Comparison of the partial amino acid sequence of P60 with the corresponding amino acid sequences of FLbR from *Glycine max* and of dihydrolipoamide dehydrogenase from *Pisum sativum*. In both (**A**) and (**B**) the initial and final amino acid numbers (N °) of sequences are indicated, together with the molecular mass of each monomer determined by SDS/PAGE (P78, P60, soybean and tobacco HSP70, 54 kDa soybean FLbR, pea dihydrolipoamide dehydrogenase) or deduced from cDNA (spinach HSP70, 56 kDa pea FLbR). Dark grey boxes, identical amino acids; light grey boxes, conservative amino acid replacements.

agarose gave two major bands, P60 and P78. They were digested with endolysin C and separated by reverse-phase HPLC. The sequences of one peptide each for P60 and P78 are shown in Figure 7. Scanning of data banks (Fasta Search Results) showed identity between the peptide of P78 and a motif of putative proteins deduced from the cDNA of the 70 kDa heat shock protein (HSP70) [25–27]. In contrast, the sequence of 13 residues of P60 had some similarity to ferric leghaemoglobin reductase (FLbR; EC 1.6.2.6) of soybean [28] and dihydrolipoamide dehydrogenase (DLDH; EC 1.8.1.4) of pea [29].

DISCUSSION

We have undertaken affinity chromatography to isolate oligochitin-binding and putative Nod-factor-binding proteins. This is a more direct and convenient method than conventional purification on Sephadex [18]. Because the 36 kDa band of the soluble fraction and the 78 kDa band of the membrane-associated fraction apparently correspond to a known chitinase [18] and to a heat shock protein (Figure 7) respectively, we focused our attention on P60, which seemed to be novel.

Affinity chromatography, radioactive binding and agglutination assays show that P60 binds with GlcNAc, the building block of Nod factors. Moreover, various Nod factors modified the electrophoretic mobility of a native protein fraction enriched in P60. This lectin-type protein was isolated as a homotetramer, $(P60)_4$, of approx. 240 kDa as determined by gel filtration, with apparently identical subunits of approx. 60 kDa on SDS gels. The tetramer was dissociated by reduction with DTT and by being heated to 90 °C in presence of SDS. Our measurements with radioactive GlcNAc gave approx. 0.9 binding site per P60 monomer; Nod factor competed for this binding. This is very close to the value of 1 expected for a lectin monomer. Even though protein quantification with the BCA method cannot be considered as absolute, this calculation also suggests that P60 was not greatly contaminated with other proteins.

In *Medicago* species, no lectins with specificity for GalNAc or GlcNAc have been isolated until now. Three members of the legume lectin gene family have been identified in *M. truncatula* [30,31] but their sugar specificity is unknown. These are the only reports of lectins in *Medicago* species. In the present study, proteins were obtained from the membrane fraction in the presence of the detergent Triton X-100. After native PAGE we noticed partial protein precipitation in the absence of detergent. This is consistent with the proposal of certain authors that some of the lectins solubilized by Triton may still be membrane-bound or perhaps trapped in membrane vesicles [23]. Indeed, immunofluorescence studies of lectins have shown staining throughout the cytoplasm and also in the membrane [23,32].

The carbohydrate structure of the Nod signal has led to the suggestion that a lectin could be a Nod-signal receptor [17]. Interest in this idea was stimulated by the studies of Diaz et al. [14,33] demonstrating that a pea lectin can affect nodulation, but they do not prove that a lectin is the Nod-signal receptor.

Native PAGE is a technique sensitive to ligand binding because electrophoretic mobility is determined by multiple factors including charge and structure [24]. The electrophoretic migration of lectins was modified by Nod factors, demonstrating a binding of this ligand to lectin. Nod factors carry an O-sulphate group, which should change the net charge of a protein on binding, but it seems that not all Nod factors are consequential here (in much the same way as they also differ in biological activity). Our experiments indicate that, whereas N-acetylation does not modulate the haemagglutinating property of P60 (Table 2), O-acetylation is a predominant factor in its interaction with Nod factor (Figure 6). Thus NodRm(mix) containing NodRm-IV(Ac,S) had a much higher binding efficiency than pure NodRm-IV(S). These sources had been purified to the same level except for the final fractionation of Nod species. Therefore we discount any artifact due to trace contaminants, whose concentration would anyway be very low in comparison with that of P60. The effectiveness of Nod factors in early symbiotic interactions [8] between *M. sativa* and *S. meliloti* is in the order NodRm-IV(Ac,S) > NodRm-IV(S) > NodRm-V(S). NodRm(mix) has these constituents in the proportion 8:72:20, giving a molar ratio of NodRm-IV(Ac,S) to $(P60)_4$ of approx. 2:1 (in Figure 6A). Because NodRm-IV(S) was inactive in binding to P60 (Figure 6A) and NodRm-V(S) has little biological activity [7], our working hypothesis is that NodRm-IV(Ac,S) is responsible for the mobility shift of P60 induced by NodRm(mix). It can also be seen that in native PAGE, the band of P60 was less intense in the presence of Nod factors. This can be attributed to a partial release of the Nod factors from the putative complex during

electrophoresis, so that the P60 was distributed between displaced and undisplaced bands.

We have extended this PAGE strategy to the interaction between a series of lectins from different sources and Nod factors (Table 2). In fact, only one lectin from pokeweed roots changed electrophoretic mobility in the presence of Nod factors. This suggests the binding of Nod factor to that lectin. However, in contrast with their distinctive effects on P60, NodRm(mix) and NodRm-IV(S) had indiscriminate effects on the electrophoretic mobility of pokeweed lectin (Figure 6C). This lectin is composed of seven repetitive chitin-binding domains that have 48–79% sequence similarity with each other; these domains are absolutely conserved in other chitin-binding domains of plant lectins and class I chitinases [34].

We have previously reported the purification of a chitinase from a soluble protein lucerne root extract by using a $(GlcNAc)_3$–agarose column [18]. This 36 kDa chitinase (CHIT36), which hydrolyses the pentameric Nod factor NodRm-V(S) to the lipotrisaccharide, has the functional property of class I chitinases. It seems that the specificity of the Nod-signal perception system relies both on the ability of the Nod signal to withstand root chitinases [8] and on also having a Nod factor receptor.

The peptide sequence of P60 indicated some similarity to members of a superfamily of pyridine nucleotide:disulphide oxidoreductases: FLbR of soybean roots and nodules [28] and dihydrolipoamide dehydrogenase of pea mitochondria [29], both membrane-associated proteins [28,35], as is P60. However, FLbR and dihydrolipoamide dehydrogenase are homodimers, whereas native P60 seems to be a tetramer.

We have also isolated P78 in the membrane fraction. Sequence analysis suggests that it might be an HSP70 such as those found in the lumen of the endoplasmic reticulum, in which molecular chaperones facilitate the assembly and folding of polypeptides *in vivo* without themselves becoming part of the final folded protein [36]. The HSP70 genes of plants are activated by a variety of stresses such as heat shock, water stress or abscisic acid [37]. Their induction by diverse stresses suggests that HSP70 has a general role in metabolic adaptations [36,37]. Their retention with $(GlcNAc)_3$–agarose, but not with GlcNAc–agarose, implies an interaction of this protein with the oligochitin.

Our study of lectins by native electrophoresis in the presence and absence of Nod factors supports the hypothesis that a lectin-type protein might constitute part of the plant perception or transport mechanism for Nod factors or for rhizobia with Nod factor on their surface. It suggests an alternative strategy for cloning Nod-factor-related proteins (receptors, lectins): to target chitin-binding domains such as those from pokeweed lectin.

In conclusion, this study has demonstrated an interaction between a root protein of lectin nature, P60, and a bacterial Nod factor acting as a ligand. A striking illustration of this was given by altered protein mobility seen in native PAGE. It would be important to characterize the specificity of that interaction, with the use of purified factors as well as their mixture or NodRm-IV(S) alone, notably NodRm-IV(Ac,S), NodRm-V(S) and modified chitins (O-sulphated and O-acetylated), not to mention other effectors or conditions that might enhance these interactions.

We thank Philippe Muller and Michael Schultze (Institut des Sciences Végétales, Gif-sur-Yvette, France), Jean d'Alayer (Institut Pasteur, Paris, France), and Michel Hours and Jean-Pierre Tenu (Université Paris-Sud, Orsay, France) for advice on radioactivity experiments, the provision of Nod factors, protein microsequencing, and discussions on binding experiments respectively. Z.M. also personally thanks Lucienne Bloch for her constant help. This work was supported by CNRS and the European INCO-COPERNICUS contract PL966067 'Somatic embryogenesis in Alfalfa'.

REFERENCES

1 Côte, F. and Hahn, M. G. (1994) Plant Mol. Biol. **26**, 1379–1411
2 Farmer, E. E., Moloshok, T. D., Saxton, M. J. and Ryan, C. A. (1991) J. Biol. Chem. **266**, 3140–3145
3 Dénarié, J. and Cullimore, J. (1993) Cell **74**, 951–954
4 Savouré, A., Sallaud, C., El-Turk, J., Zuanazzi, J., Ratet, P., Schultze, M., Kondorosi, A., Esnault, R. and Kondorosi, E. (1997) Plant J. **11**, 277–287
5 Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C. and Dénarié, J. (1990) Nature (London) **344**, 781–784
6 Dénarié, J., Debelle, F. and Rosenberg, C. (1992) Annu. Rev. Microbiol. **46**, 497–531
7 Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J. N., Endre, G., Gero, S. D. and Kondorosi, A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 192–196
8 Staehelin, C., Schultze, M., Kondorosi, E., Mellor, R. B., Boller, T. and Kondorosi, A. (1994) Plant J. **5**, 319–330
9 Ito, Y., Kuku, H. and Shibuya, N. (1997) Plant J. **12**, 347–356
10 Collmer, A. and Keen, N. T. (1986) Annu. Rev. Phytopathol. **24**, 383–409
11 Felix, G, Baureithel, K. and Boller, T. (1998) Plant Physiol. **117**, 643–650
12 Baureithel, K. and Boller, T. (1995) J. Cell Biochem. Suppl. **19B**, 151
13 Chrispeels, M. J. and Raikhel, N. V. (1991) Plant Cell **3**, 1–9
14 Diaz, C. L., Melchers, L. S., Hooykass, P. J. J., Lugtenberg, B. J. J. and Kijne, J. W. (1989) Nature (London) **338**, 579–581
15 Hervé, C., Serres, J., Dabos, P., Canut, H., Barre, A., Rougé, P. and Lescure, B. (1999) Plant Mol. Biol. **39**, 671–682
16 Niebel, A., Bono, J. J., Ranjeva, R. and Cullimore, J. V. (1997) Mol. Plant–Microbe Interact. **10**, 132–134
17 Bono, J. J., Riond, J., Nicolaou, K. C., Bockovich, N. J., Estevez, V. A., Cullimore, J. V. and Ranjeva, R. (1995) Plant J. **7**, 253–260
18 Minic, Z., Brown, S., de Kouchkovsky, Y., Schultze, M. and Staehelin, C. (1998) Biochem. J. **332**, 329–335
19 Van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E. and Wijffelman, C. A. (1982) Plant Sci. Lett. **27**, 317–325
20 Westermeier, R. (1993) Electrophoresis in Practice. A Guide to Theory and Practice, VCH, Weinheim, Germany
21 Sharon, N. and Lis, H. (1972) Methods Enzymol. **28**, 360–368
22 Hall, J. L. (1983) in Isolation of Membranes and Organelles from Plant Cells (Hall, J. L. and Moore, A. L., eds.), pp. 55–81, Academic Press, London
23 Etzler, M. E. (1986) in The Lectins. Properties, Functions and Applications in Biology and Medicine (Liener, I. E., Sharon, N. and Goldstein, I. J., eds.), pp. 371–435, Academic Press, New York
24 Goldenberg, D. P.(1989) in Protein Structure, a Practical Approach (Creighton, T. E., ed.), pp. 225–250, IRL/Oxford University Press, Oxford
25 Kalinski, A., Rowley, D. L., Loer, D. S., Foley, C., Buta, G. and Herman, E. M. (1995) Planta **195**, 611–621
26 Denecke, J., Goldman, M. H. C., Demolder, J., Seurinck, J. and Botterman, J. (1991) Plant Cell **3**, 1025–1035
27 Anderson, J. V., Neven, L. G., Li, Q. B., Haskell, D. W. and Guy, C. L. (1994) Plant Physiol. **104**, 303–304
28 Ji, L., Becana, M., Sarath, G. and Klucas, R. V. (1994) Plant Physiol. **104**, 453–459
29 Bourguignon, J., Macherel, D., Neuburger, M. and Douce, R. (1992) Eur. J. Biochem. **204**, 865–873
30 Bauchrowitz, M. A., Barker, D. G. and Truchet, G. (1996) Plant J. **9**, 31–43
31 Hirsch, A. M., Brill, L. M., Lim, P. O., Scambray, J. and Van Rhijn, P. (1995) Symbiosis **19**, 155–173
32 Brewin, N. J. and Kardailsky, I. V. (1997) Trends Plant Sci. **2**, 92–98
33 Diaz, C. L., Logman, T. J. J., Stam, H. C. and Kijne, J. W. (1995) Plant Physiol. **109**, 1167–1177
34 Yamaguchi, K., Yurino, N., Kino, M., Ishiguro, M. and Funatsu, G. (1997) Biosci. Biotechnol. Biochem. **61**, 690–698
35 Danson, M. J., Conroy, K., McQuattie, A. and Stevenson, K. J. (1987) Biochem. J. **243**, 661–665
36 Ellis, R. J. and van der Vies, S. M. (1991) Annu. Rev. Biochem. **60**, 321–347
37 Heikkila, J. J., Pap, J. E. T., Schultz, G. A. and Bewley, J. D. (1984) Plant Physiol. **76**, 270–274
38 Goldstein, I. J. and Poretz, R. D. (1986) in The Lectins. Properties, Functions and Applications in Biology and Medicine (Liener, I. E., Sharon, N. and Goldstein, I. J., eds.), pp. 45–47, Academic Press, New York

Received 2 August 1999/17 September 1999; accepted 1 November 1999