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ARABINOGALACTAN PROTEINS FROM STIGMAS OF *NICOTIANA ALATA*

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Key Word Index—*Nicotiana alata*; Solanaceae; stigma exudate; arabinogalactan protein; structural studies.

Abstract—Stigmas of *Nicotiana alata*, an ornamental tobacco, contain arabinogalactan proteins (AGPs) as major components (*ca* 65%) of the buffer-soluble high M_r carbohydrate. Glycosyl linkage analyses are compatible with a model of an arabinogalactan with a 3-linked galactan backbone substituted at C(0)6 with chains of 6-linked galactopyranosyl residues terminating in arabinofuranosyl and galactopyranosyl residues. The stigma AGPs have a low content of uronic acids (0.5–1.0% glucuronic acid). As isolated, they contain only a small amount of protein that is rich in hydroxyproline, serine, alanine, threonine, glycine, glutamate/glutamine and aspartate/asparigine. They migrate as single components with low charge (R_f 0.06) on crossed-electrophoresis. Only minor differences are apparent between AGPs isolated from different genotypes based on monosaccharide, amino acid and glycosyl linkage analyses.

INTRODUCTION

A class of proteoglycans, arabinogalactan proteins (AGPs), are present in pistils of *Nicotiana alata*, an ornamental tobacco [1]. The synthesis of these AGPs is developmentally regulated in that both the concentration and the class of AGPs (separated according to their charge on crossed-electrophoresis) in the stigma change during flower-development [1]. AGPs are common components of plant tissues and secretions [2] and are present in the buffer-soluble extracts of the pistils of many flowering plants [3]. Detailed structural analyses have been performed only on AGPs from stigma exudates of the monocotyledons *Lilium longiflorum* [4] and *Gladiolus gandavensis* [5]. The core structure of these AGPs is similar but there are differences in the fine structure of their side-chains, a characteristic of this class of proteoglycans [2].

During a compatible pollination, pollen germinates on the stigma surface to produce a pollen tube which grows extracellularly through the transmitting tissue of the style to the ovary where fertilization occurs. The function of AGPs in stigma exudates is not known, although several possibilities have been suggested (see [2] for a review). In this paper we describe the isolation and structural characterisation of stigma AGPs from *N. alata* as part of a programme directed towards understanding their function in the fertilization process.

RESULTS

The material present in the 80% (v/v) ethanol-insoluble stigma extracts gave a strong precipitin band in gel-diffusion experiments against J539-myeloma protein. This myeloma protein binds specifically to β-(1→6)-linked galacto-oligosaccharides [6]. This indicated the possibility of purification of AGPs from crude stigma extracts by affinity chromatography using J539-Sepharose 4B. The elution profile for a crude stigma extract of genotype S_2S_3 on this material is shown in Fig. 1. Unbound material was eluted with 0.15 M NaCl. The bound material, eluted with 0.1 M citric acid containing 0.3 M NaCl, pH 2.5, represented *ca* 67% of the total material recovered and accounted for 71 and 17%, respectively, of the total carbohydrate and protein applied to the column. Some carbohydrate (12%) was not recovered after affinity chromatography.

The bound and unbound fractions from affinity chromatography were pooled, dialysed against distilled water, and tested for the presence of AGPs by gel diffusion against the β-glucosyl Yariv reagent; a reagent which specifically binds to and precipitates many AGPs [7]. AGPs are exclusively present in the bound fraction. For other genotypes, bound and unbound materials were batch collected. Bound material represented 69–72% of the total material recovered and reacted with the β-glucosyl Yariv reagent. Between 85 and 90% of the carbohydrate was recovered from the affinity column.

The monosaccharide analysis of crude stigma extracts and unbound and bound fractions from J539-Sepharose 4B are shown in Table 1. Only data for genotype S_2S_3 are presented as the analyses for genotypes S_1S_3, S_2S_2 and S_3S_3 are similar to that of genotype S_2S_3. Both the crude stigma extract and the J539-Sepharose 4B bound fraction contained arabinose and galactose as the major monosaccharides. Rhamnose, xylose, mannose and glucose were present in minor amounts in both fractions. These minor sugars were present in higher amounts in the crude stigma fraction than in the J539-Sepharose 4B bound material. In contrast, the J539-Sepharose 4B unbound fraction was enriched in xylose and glucose, was significantly depleted in arabinose and galactose and contained less carbohydrate (47%) than the J539-Sepharose 4B bound (95%) and crude (82%) fractions.

Gel permeation chromatography of the J539-Sepharose 4B bound fractions from each genotype on Toyo Pearl HW-55 gave essentially the same profile as that illustrated in Fig. 2 for genotype S_2S_3. Two peaks of carbohydrate were eluted from the column, a major peak containing *ca* 72% of the carbohydrate applied and a minor peak containing *ca* 4%. Some carbohydrate (*ca* 24%) was not recovered from the column. Of the two

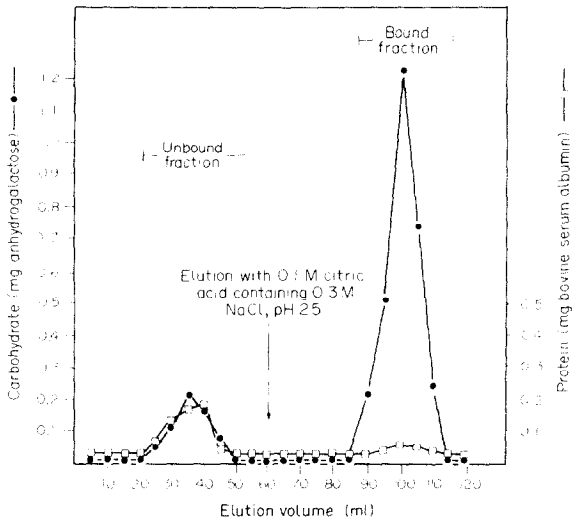

Fig. 1. Affinity chromatography of *Nicotiana* stigma extracts on J539-Sepharose 4B.
Crude stigma extract (300 stigmas; 5 ml, 1 mg carbohydrate/ml) in 0.15 M NaCl was applied directly onto a J539-Sepharose 4B column, (22.5 × 2 cm) which had been pre-equilibrated in 0.15 M NaCl and operated at a flow rate of 0.5 ml/min. Unbound fractions (12 × 5 ml) were eluted with 0.1 M citric acid containing 0.3 M NaCl, pH 2.5 (arrow). Fractions were tested for protein (□), carbohydrate (●) and their capacity to bind the β-glucosyl Yariv reagent. The bound and unbound fractions were pooled as indicated.

Table 1. Monosaccharide composition of *Nicotiana* stigma extract and fractions obtained by affinity chromatography (genotype S_2S_3)

Monosaccharide	Crude stigma	J539-Sepharose 4B unbound	J539-Sepharose 4B bound
	(% w/w)*		
Rha	3.5	11.5	1.0
Ara	28.5	15.5	34.0
Xyl	9.5	29.5	tr
Man	3.0	8.5	1.0
Gal	47.0	10.0	62.0
Glc	8.5	25.0	2.0
Gal/Ara	1.65	0.65	1.82
Carbohydrate	82	47	95

*Average of duplicate determinations.
tr = Trace.

carbohydrate-containing peaks, only the major peak, corresponding to a M_r of *ca* 110000, contained material which interacted with the β-glucosyl Yariv reagent. For extracts from genotypes S_2S_2 and S_3S_3, the fractions in this peak were pooled, dialysed, redissolved in 6 M urea and rechromatographed on the same column pre-equilibrated in 6 M urea. A single peak of carbohydrate containing material which interacted with the β-glucosyl Yariv reagent was obtained from the column (profile not shown). In contrast, two protein peaks were obtained, one which co-chromatographed with the carbohydrate peak and another of lower M_r (*ca* 20% of total protein loaded) which was devoid of carbohydrate. The AGP containing fractions were pooled, dialysed, concentrated and stored frozen at −20° prior to analysis.

The electrophoretic homogeneity of the isolated stigma AGPs from the four self-incompatibility genotypes (S_1S_3, S_2S_2, S_2S_3, S_3S_3) was examined by crossed-electrophoresis [8]. The AGPs of each genotype behaved similarly on crossed-electrophoresis and comprised a single component with an average R_f of 0.06.

The monosaccharide analyses of the stigma AGPs from the four self-incompatibility genotypes were similar in that galactose and arabinose were the major sugars with only minor amounts of rhamnose, xylose, mannose and glucose (Table 2). The ratio of galactose to arabinose was higher for genotype S_1S_3 (2.45) than for the other genotypes (1.80–1.85). Both the colorimetric method of Blumenkrantz and Asboe-Hansen [9] and the GC/MS determination show that uronic acids were minor components, although the colorimetric method consistently gave higher results (Table 2). The uronic acid was identified as glucuronic acid.

The amino acid analyses of the AGPs, for genotypes S_2S_2 and S_3S_3, were performed on material obtained from J539-Sepharose 4B chromatography and Toyo Pearl HW-55 chromatography in 6 M urea (Table 3). The analyses for both genotypes were similar: serine, threonine, glutamate/glutamine, apartate/asparagine, glycine, alanine and hydroxyproline were the major amino acids. The percentage (by wt) of protein and carbohydrate in the isolated stigma AGPs was calculated from the recovery of amino acids from known amounts of carbohydrate analysed. The relative proportions of carbohydrate and protein for genotypes S_2S_2 and S_3S_3 were 88:12 and 92:8, respectively.

Glycosyl linkage analyses of the stigma AGPs of

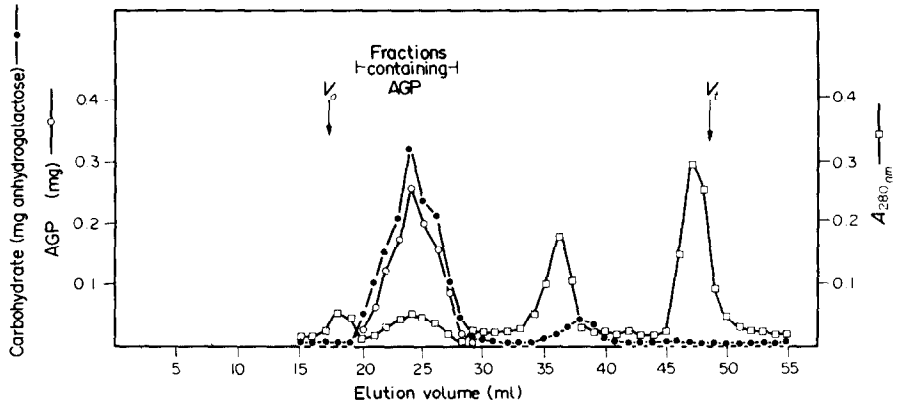

Fig. 2. Gel-permeation chromatography of *Nicotiana* J539-bound fraction on Toyo Pearl HW-55. Material from stigma extracts which bound to J539-Sepharose 4B (0.5 ml, 5 mg/ml carbohydrate) was rechromatographed on Toyo Pearl HW-55, (22 × 2 cm) equilibrated in 0.15 M NaCl and operated at a flow rate of 12 ml/hr. Fractions (100 × 0.5 ml) were analysed for total carbohydrate (●), A_{280} nm (□) and the amount of AGP(○) by the method of Van Holst and Clarke [22]. V_0 (void vol.) and V_t (bed vol.) were determined using Dextran T2000 and galactose, respectively, as indicated (arrows). The fractions that contained AGP were pooled as indicated.

Table 2. Monosaccharide analyses of *Nicotiana* stigma AGPs prepared by affinity and gel chromatography

Monosaccharide	Genotype			
	S_1S_3	S_2S_2	S_2S_3	S_3S_3
	(%w/w)*			
Neutral				
Rha	1	1	1	1
Ara	28	33.5	34	33
Xyl	—	tr	tr	tr
Man	tr	1	1	0.5
Gal	68.5	61	62	61
Glc	1.5	3.5	2	4.5
Gal/Ara	2.45	1.82	1.82	1.85
Acidic				
GlcA	1	0.5	0.5	0.5
	(1.3)†	(1.8)	(2.2)	(1.0)

*Average of duplicate determinations.
tr = Trace.
†Figures in parentheses were obtained by the colorimetric method of ref. [9].

Table 3. Amino acid analyses of *Nicotiana* stigma AGPs prepared by affinity and gel chromatography in 6 M urea

Amino acid	Genotype	
	S_2S_2	S_3S_3
	(mol %)	
Hyp	6.7	5.7
Asx	10.1	9.1
Thr	5.1	5.2
Ser	10.9	14.3
Glx	14.5	15.0
Pro	3.4	4.0
Gly	12.5	16.0
Ala	9.3	9.6
Val	3.8	3.8
1/2 Cys	1.5	0.5
Met	1.8	1.4
Ile	3.2	2.7
Leu	5.0	2.6
Tyr	2.3	2.3
Phe	3.0	—
Trp	nd	nd
Lys	3.3	3.8
His	1.1	2.0
Arg	2.6	2.1
GlcNAc	0.5	0.3
GalNAc	0.4	0.5

nd: Not detected.

genotypes S_2S_2 and S_3S_3, prepared by J539-Sepharose 4B and Toyo Pearl HW-55 chromatography, are shown in Table 4. The glycosyl linkage composition for the two genotypes was similar. Arabinose was present in the furanose form and was predominantly terminally linked although small amounts of 2- and 5-linked Ara*f* were also present. Galactose was present in the pyranose form and was predominantly 6- and 3,6-linked with smaller, but significant, amounts of terminal- and 3-linked Gal*p*. Small but variable amounts (< 1%) of 3,4,6- and 2,3,6-linked Gal*p* residues were occasionally observed in methylation analysis of both genotypes. The molar recovery of non-reducing terminal residues approximated to the molar recovery of branched residues (3,6-linked Gal*p*) for genotype S_3S_3, but a slightly higher proportion of terminal residues was observed in genotype S_2S_2.

DISCUSSION

A range of chemical components have been found in stigma exudates. They include lipids, phenolic substances, amino acids, proteins, mono-, oligo- and polysaccharides (see [10] and references cited therein). AGPs have been found as major components in the stigma exudates of *Gladiolus gandavensis* [5] and *Lilium longiflorum* [4, 5]. Gell *et al.* [1] showed that mature stigmas of *N. alata*

Table 4. Methylation analyses of *Nicotiana* stigma AGPs prepared by affinity and gel chromatography

Deduced glycosidic linkage	Genotype S_2S_2 (mol %)*	Genotype S_3S_3 (mol %)*
t-Ara*f*	33.0	23.0
2-Ara*f*	tr	0.7
5-Ara*f*	2.5	2.5
t-Gal*p*	8.0	10.0
3-Gal*p*	5.0	4.0
6-Gal*p*	21.0	21.0
3,6-Gal*p*	28.5	35.0
3,4,6-Gal*p*	†	†
2,3,6-Gal*p*	†	†

*Average of duplicate determinations.
tr = trace.
†Present in minor but variable proportions (< 1%).

contain *ca* 9–10 μg AGP. This represents *ca* 65% of the total high M_r carbohydrate of the buffer-soluble stigma extract that could be precipitated with 80% ethanol. We report here on the isolation and structural characterisation of AGPs from mature stigmas of *Nicotiana alata*

The stigma AGP preparations isolated from each genotype of *N. alata* by the combination of affinity chromatography and gel-permeation chromatography were apparently chemically homogeneous. They eluted as a single symmetrical peak in gel-permeation chromatography on Toyo Pearl HW-55 and migrated as a single component of low charge in crossed-electrophoresis experiments. Although a covalent association between protein and carbohydrate can only be unequivocally demonstrated by isolation of a glycopeptide, co-elution of protein with carbohydrate during gel-permeation chromatography under strong dissociating conditions (6 M urea) indicates such an association.

Glycosyl linkage analyses of the isolated stigma AGP preparations of *N. alata*, genotypes S_2S_2 and S_3S_3, are consistent with a model in which a 3-linked galactan backbone is branched through C(0)6 to side branches of 6-linked galactopyranosyl residues which terminate in arabinofuranosyl and galactopyranosyl residues. Thus, the stigma AGPs of *N. alata* are similar in structure to a widely distributed class of secreted proteoglycans whose biological function remains undefined [2]. The high levels of hydroxyproline, serine and alanine in the stigma AGP preparations is also a feature common to other AGPs [2].

Analysis of the isolated AGPs from stigmas of *N. alata* has not revealed any significant differences between incompatibility genotypes (e.g. S_2S_2 vs S_3S_3). Thus, the monosaccharide, amino acid and glycosyl linkage analyses, in addition to their migration on crossed-electrophoresis of the S_2S_2 and S_3S_3 isolated AGPs are similar. However, we do not know that the arrangement of the different glycosyl linkages, are the same in AGPs from different genotypes without sequencing the polysaccharide chains. In addition, differences may exist in the sequence of the protein backbone. Differences are however, evident between the AGPs of the stigma of the dicotyledon *N. alata* and the monocotyledons *Gladiolus* [5] and *Lilium* [4] (Table 5). Although arabinose and galactose are the major monosaccharides for the AGPs of all three plants, *Lilium* contains significant amounts of rhamnose (6%) and glucuronic acid (12%). It is likely that *Nicotiana* AGP differs markedly from that of *Gladiolus* and *Lilium* in the core structure of the polysaccharide portion as it contains a substantially higher proportion of 6-linked galactopyranosyl residues and less 3-linked galactopyranosyl residues. This indicates that there are longer stretches of unsubstituted side-chains, at more regular intervals, on the 3-linked galactan backbone in the *Nicotiana* AGP. Arabinofuranosyl and galactopyranosyl residues are the major non-reducing terminal residues of *Nicotiana*, *Lilium* and *Gladiolus* AGPs. The *Lilium* AGP contains, in addition, terminal rhamnopyranosyl and glucuronosyl residues whereas *Gladiolus* AGP contains terminal glucopyranosyl residues. The distribution of the terminal galactopyranosyl residues on the *Gladiolus* AGP must be different to that of the *Nicotiana* AGP since the *Nicotiana* AGP does not interact with the tridacnin lectin

Table 5. Methylation analyses of stigma AGPs

Deduced glycosidic linkage	*Nicotiana alata* (S_3S_3)	*Gladiolus gandavensis**	*Lilium longiflorum*†
t-Rha*p*	–	–	6.0
t-Ara*f*	23.0	17.0	26.0
2-Ara*f*	0.7	–	–
5-Ara*f*	2.5	–	–
t-Glc*p*	–	7.0	–
t-Gal*p*	10.0	16.0	8.0
3-Gal*p*	4.0	13.0	8.0
6-Gal*p*	21.0	6.0	6.0
3,4-Gal*p*	–	–	2.0
3,6-Gal*p*	35.0	41.0	28.0
3,4,6-Gal*p*	< 1.0	–	5.0
2,4,6-Gal*p*	< 1.0	–	–
t-GlcA	–	–	5.0
4-GlcA	–	–	6.0

*From ref. [5].
†From ref. [4].

whereas the *Gladiolus* AGP does. The inability of the *Nicotiana* AGP to interact with the tridacnin lectin, which is specific for terminal-β-D-galactopyranosyl residues [11] suggests that the terminal galactopyranosyl residues are inaccessible to the lectin and may occupy positions close to the 3-linked galactan backbone. Variability in the fine structure of the arabinogalactan side chains is a feature common to this class of macromolecules [2] and has been used by Anderson and Dea [12] as a chemotaxonomic character to distinguish different groupings of *Acacia* species.

In contrast, a monosaccharide analysis of the polysaccharide fraction of *Aptenia cordifolia* stigma exudate shows that the uronic acids, galacturonic (35.8%) and glucuronic (23.1%) are the major sugars together with glucose, fructose, galactose and arabinose [10]. Xylose, mannose and rhamnose are present in only minor amounts. Therefore, it appears that pectic polysaccharides are present in high amounts and in this respect the stigma exudate of *Aptenia* differs from that of *Nicotiana*, *Lilium* and *Gladiolus* stigma exudates.

The biological significance of the stigma AGPs remains undefined. They may be responsible for generating conditions conducive to the adhesion and germination of pollen [1] or alternatively, they may provide nutrients for the growing pollen tube [13, 14].

EXPERIMENTAL

Plant material. Seeds of *N. alata* Link and Otto (self-incompatibility genotypes S_1S_2 and S_2S_3) were a generous gift from Dr K. K. Pandey, Genetics Unit, Grasslands Division, DSIR, Palmerston North, New Zealand. Homozygous (genotypes S_2S_2 and S_3S_3) plants were obtained as described in ref. [15]. Flowers were emasculated before anthesis and pistils harvested at maturity. Pistils were stored frozen at −70° prior to use.

Preparation of stigma extracts. Stigmas were excised and ground in a mortar and pestle in 0.2 M Tris–HCl buffer, containing 10 mM NaCl, 10 mM EDTA, 1 mM DTT, pH 8 (ext. buffer) and insol. PVP (0.5 g/10 ml buffer) at 4° (400 stigmas extd per 10 ml ext. buffer). The extract was centrifuged at 10 000 *g*, 4° for 20 min and the supernatant made to 80% (v/v) EtOH and left to ppt. overnight at 4°. The ppt. was collected by centrifugation (10 000 *g*, 4°, 20 min) and then washed with 80% EtOH (20 ml). The ppt. was then redissolved in 0.15 M NaCl (7 ml per 400 stigmas) and stored at −20° prior to use.

Affinity chromatography. A Balb/c mouse bearing a myeloma protein J539 ascites tumour was kindly provided by Dr Michael Potter (National Institutes of Health, Bethesda, U.S.A.). The myeloma protein J539 was purified from ascities fluid as described in ref. [16] and covalently coupled to CNBr-activated Sepharose 4B as specified by the manufacturers (Pharmacia); the swollen gel contained *ca* 2 mg of bound J539/ml. The J539-Sepharose 4B was then equilibrated in 0.15 M NaCl.

Stigma AGP was isolated by affinity chromatography on a J539-Sepharose 4B column (22.5 × 2 cm) operated at a flow rate of 0.5 ml/min. Stigma extracts (5 ml; 1 mg/ml carbohydrate in 0.15 M NaCl) were applied to the affinity column. Unbound material (12 × 5 ml) was eluted with 0.15 M NaCl. Bound material (12 × 5 ml) was eluted with 0.1 M citric acid, pH 2.5, containing 0.3 M NaCl. Fractions were immediately dialysed and assayed for protein (A_{280}) carbohydrate and for the presence of AGP by gel diffusion (see General methods). Fractions containing AGP were combined and stored at −20° prior to use. Collection of individual fractions of bound and unbound material from J539-Sepharose 4B was restricted to stigma exts of the genotype S_2S_3. For other genotypes (S_1S_3, S_2S_2 and S_3S_3), the bound and unbound fractions were batch collected in the same total vol. (60 ml each), then dialysed against dist. H_2O at 4° and stored at −20°.

Gel chromatography. Material from stigma exts which bound to J539-Sepharose 4B (0.5 ml, 5 mg/ml total carbohydrate) was rechromatographed on a Toyo Pearl HW-55 (Toyo Soda Co., Japan) column (22 × 2 cm) equilibrated in 0.15 M NaCl. The flow rate of the column was 12 ml/hr and 0.5 ml fractions were collected. Fractions were tested for AGP, protein (A_{280}) and carbohydrate (see General methods). V_0 (void vol.) and V_t (bed vol.) were determined using dextran T2000 and galactose, respectively. Chromatography was also performed on the same column in 6 M urea.

Analytical methods. Neutral monosaccharides were identified by GC/MS and quantified by GC as their alditol acetates following hydrolysis with 2.5 M TFA [17]. Uronosyl residues in AGPs were identified and quantified by GC/MS following conversion to their corresponding neutral glycosyl residues by a modification of the method of ref. [18]. AGPs (0.1 mg) were treated with anhydrous MeOH–HCl (1 ml, 0.08 M, 24 hr, room temp.). Methyl esterified uronosyl residues were subsequently reduced with $NaBD_4$ (1 ml, 0.25 M, 24 hr, 4°) to their corresponding 6,6′-dideuterolabelled neutral sugar residue. The uronosyl residues were identified and quantified by GC/MS from the 6,6′-dideuterated portion of the corresponding alditol acetate. The ratio of peak heights at *m/z* of 217/(217 + 219) was used to determine the proportion of each uronic acid as described in ref. [19].

The glycosyl linkage composition was established by GC/MS of the per-*O*-methylated alditol acetates generated by methylation analysis using the method of ref. [20]. The methylsulphinyl-anion was prepared by the method of ref. [21]. Conditions for GC/MS were as described in ref. [17].

Amino acid analyses on purified AGPs (0.3–0.5 mg; 6 M HCl, 110°, 24 hr) were performed on an amino acid analyser.

AGPs were quantified by the single radial diffusion assay of ref. [22] with gum arabic as std. Electrophoretic separation of AGPs was by the crossed-electrophoresis method of ref. [8].

General methods. Total carbohydrate was determined colorimetrically by the phenol–H_2SO_4 method [23], using glucose as the std. Total protein was determined as BSA using the Bio-Rad Bradford micro-assay method [24]. Uronic acids were estimated colorimetrically by the method of ref. [9] using galacturonic acid as a std. Gel diffusion was performed in 1% (w/v) agarose containing 1% (w/v) NaCl and 0.02% (w/v) NaN_3 by the microslide method [25].

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