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Carbohydrate Research 277 (1995) 67–85

CARBOHYDRATE RESEARCH

Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of *Nicotiana alata*

Alison M. Gane [a,1], David Craik [b,2], Sharon L.A. Munro [b], Geoffrey J. Howlett [c], Adrienne E. Clarke [d], Antony Bacic [d,*]

[a] *Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia*

[b] *Victorian College of Pharmacy, Monash University, Parkville, Victoria 3052, Australia*

[c] *Department of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia*

[d] *Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia*

Received 28 December 1994; accepted 11 April 1995

Abstract

Arabinogalactan-proteins (AGPs) from the female reproductive tissues (stigmas and styles) of *Nicotiana alata* were isolated from the saturated ammonium sulfate supernatant of buffer-soluble extracts by precipitation with the β-glucosyl Yariv reagent, followed by gel-filtration chromatography under dissociating conditions. The AGPs had characteristics typical of other AGPs: a high proportion of carbohydrate (95%) with a high ratio of Gal*p* to Ara*f* (2:1), and a low protein content (5%) with high levels of alanine, serine, and hydroxyproline. The AGPs consisted of a major species which was almost neutral, and a minor species which was more negatively charged. Sedimentation equilibrium experiments showed that the purified AGPs had a weight-average molecular weight of 143 kD. Linkage analysis showed that the AGPs contained a highly branched backbone of 3-, 6-, and 3,6-linked Gal*p* residues, bearing terminal Gal*p* and terminal Ara*f* residues. Analysis by one-dimensional and two-dimensional ^{1}H and ^{13}C NMR spectroscopy confirmed the presence of these glycosyl linkage types, and showed a high mobility of the terminal Ara*f* residues consistent with their location on the periphery of the molecules. This

* Corresponding author.

[1] Present address: Department of Consumer Sciences, University of Otago, PO Box 56, Dunedin, New Zealand.

[2] Present address: Centre for Drug Design and Development, University of Queensland, St Lucia, Queensland 4072, Australia.

SSDI 0008-6215(95)00197-2

analysis represents the most complete ^1H assignment for AGP molecules in solution. No difference in the carbohydrate analyses was found between AGPs isolated separately from stigmatic or stylar tissue, or between AGPs isolated from stigmas and styles of plants of different self-incompatibility genotypes.

Keywords: Arabinogalactan-proteins; Structural analysis; *Nicotiana alata*

1. Introduction

Arabinogalactan-proteins (AGPs) are proteoglycans rich in carbohydrate (usually > 90%) which contain a high proportion of D-galactopyranosyl (D-Gal*p*) and L-arabinofuranosyl (L-Ara*f*) residues, but they may also contain smaller and variable levels of a range of other neutral and acidic monosaccharides (for a review see ref. [1]). AGPs have been isolated from many tissues of both lower and higher plants covering a wide range of taxonomic groups [2,3]. Within plant tissues, AGPs are mostly found in the extracellular space [2–5], while less is found in cytoplasmic vesicles [6–8] and at the protoplast surface [3,4,9–11]. They are also found in the medium of suspension-cultured cells [6–8]. AGPs have been ascribed a range of functions such as developmental regulation, maintenance of humidity, adhesion, nutrition, and response to wounding [1,12]. More recently reports have implicated AGPs in plant growth and development [11] as determinants of cellular identity [13,14], and in the control of somatic embryogenesis [15,16].

The structure of AGPs from a number of species has been studied using a range of chemical and physical techniques (see [1]). In a few cases linkage analysis by methylation and selective chemical and enzymic degradation techniques have been used in conjunction with NMR spectroscopy to determine fine structural details of their carbohydrate moiety [17–22]. The carbohydrate portion of AGPs comprises a framework of 3-, 6-, and 3,6-linked β-D-Gal*p* residues which are substituted primarily with α-L-Ara*f* residues, and other less-abundant monosaccharides, including α-L-Ara*p*, α-L-Rha*p*, β-D-Glc*p*, and β-D-Glc*p*A, often in terminal positions [1].

The protein core of AGPs (< 10%) is typically rich in hydroxyproline, serine, alanine, threonine, and glycine. Until recently, there was little information available on the amino acid sequence of the protein core of AGPs, apart from *N*-terminal amino acid sequences from AGPs of carrot, ryegrass, and rose [23–25] and some peptide sequences of ryegrass AGP [24]. cDNA clones encoding protein backbones of AGPs from styles of *Nicotiana alata* [26] and suspension-cultured cells of pear [27] have now been isolated and sequenced. These studies show that AGPs are a family of molecules that differ not only in their carbohydrate moieties, but also in the nature of their protein cores. The linkage between the carbohydrate and protein domains has been unequivocally identified only for the AG-peptide from wheat endosperm, where the carbohydrate chains are linked to hydroxyproline via an alkali-stable β-D-Gal*p* linkage [28,29]. Other linkage types have also been detected in AGPs, such as α-L-Ara*f* linked to hydroxyproline in Timothy grass [30], β-D-Gal*p* to serine in radish and oil seed rape leaves [31,32] and β-D-Gal*p* to threonine in radish seeds [33].

In the female sexual tissues of *N. alata*, AGPs are a family of molecules which comprise the major high-molecular-weight carbohydrate component of the extracellular mucilage secreted by cells of the stigma and style [26,34,35]. In this paper we report the detailed structures of the carbohydrate moiety of AGPs isolated from stigmas and styles of *N. alata*, using linkage analysis and NMR spectroscopy. Full carbon and an extensive proton assignment was achieved by a combination of one-dimensional (1D) and two-dimensional (2D) homonuclear and heteronuclear NMR techniques.

2. Experimental

Plant material.—Seeds of *N. alata* Link et Otto (self-incompatibility genotype S_2S_2 and S_6S_6) were the kind gift of the late Dr K.K. Pandey, Genetics Unit, Grasslands Division, D.S.I.R., Palmerston North, New Zealand. The plants were grown in individual pots in a glasshouse under natural light conditions. Flowers of *N. alata* were emasculated with fine forceps at the beginning of petal coloration, collected when the pistils were fully mature (usually 48 h after flower opening), and stigmas and styles excised and stored immediately at −70°C.

Preparation of stigma and style extracts.—Stigma and styles were ground to a fine powder in liquid nitrogen, and Polyclar AT (insoluble, BDH) was added (0.1 g per g FW tissue), and the grinding continued. Soluble components were extracted at 4°C in 100 mM Tris-HCl, pH 7.8, containing 14 mM 2-mercaptoethanol (5 mL buffer per g FW tissue). The extract was filtered through two layers of Miracloth (Calbiochem), and centrifuged (10 000*g*, 20 min). The supernatant was treated sequentially with increasing amounts of solid ammonium sulfate to saturation, and the insoluble material removed by centrifugation (10 000*g*, 10 min). The saturated ammonium sulfate supernatant, which contained the AGPs, was then dialysed extensively against distilled water (M_r cut-off 12 000), freeze dried, and then purified as described later.

Purification of AGPs.—AGPs were purified by precipitation with the β-glucosyl Yariv reagent followed by gel-filtration chromatography. The AGP-containing material was dissolved in 1% w/v NaCl to give an AGP concentration of 2 mg mL^{-1} and AGPs precipitated by addition of an equal volume of a solution of 2 mg mL^{-1} β-glucosyl Yariv reagent (Biosupplies Australia Pty Ltd, Melbourne, Australia) in 1% w/v NaCl. This is a modification of the procedure of Jermyn and Yeow [2], as the optimum weight ratio of β-glucosyl Yariv reagent to AGP for precipitation was determined as 1:1. The mixture was left to precipitate overnight at 4°C, and the insoluble β-glucosyl Yariv–AGP complex was collected by centrifugation (10 000*g*, 20 min, 4°C), washed three times with 1% w/v NaCl to remove excess β-glucosyl Yariv reagent, and three times with MeOH. The pellet was dried, dissolved in a minimum volume of deionised water, and solid sodium dithionite was added to 30% w/v; the flask was then purged with nitrogen gas, sealed, and stirred for 1–2 h at 20°C. The resultant clear yellow solution was concentrated by ultrafiltration (M_r cut-off 10 000), and chromatographed on a Biogel P6 column (10 cm × 1.0 cm i.d., Bio-Rad) equilibrated with 10 mM NH_4HCO_3, pH 8.5. Fractions which contained AGP, as determined by the β-glucosyl Yariv diffusion assay, were pooled and freeze dried.

The material precipitated by β-glucosyl Yariv was dissolved in 6 M guanidine hydrochloride, 20 mM DTT to give 1 mg AGP mL^{-1}, incubated for 2 h at 20°C, and then dialysed into deionised 6 M urea, 10 mM Tris-HCl, pH 9.6. The sample was chromatographed on a gel-filtration column of Toyopearl HW-65(S) (Toyo Soda Manufacturing Co. Ltd; 90 × 2.2 cm i.d.), equilibrated and eluted in 6 M urea, 10 mM Tris-HCl, pH 9.6. The column was calibrated with the dextran standards T2000 (V_0), T500, T250, T110 (Pharmacia), and galactose (Vt; Sigma). Fractions (4 mL) were collected and tested for AGP by the β-glucosyl Yariv diffusion assay and for protein by A_{280}. The AGP-containing fractions were pooled, dialysed against distilled water and freeze dried.

Analytical methods.—Total carbohydrate was determined using the colorimetric assay of Dubois et al. [36], using galactose (10–80 μg) as the standard.

Protein was determined colorimetrically by the method of Bradford [37] using the Bio-Rad protein microassay, using bovine serum albumin (0.5–20 μg) as the standard.

AGP was determined with the radial diffusion assay of van Holst and Clarke [38], using gum arabic (Sigma) as the standard (0.25–3.0 μg). Since the β-glucosyl Yariv reagent interacts with different AGPs to differing degrees, a correction factor was determined to calculate the amount of *N. alata* AGP. The amount of AGP measured using gum arabic as a standard (y) was plotted versus the amount of pure *N. alata* AGP measured gravimetrically (x), giving a straight line with the equation $y = 0.89x - 0.37$. This equation was used to calculate the corrected amount of AGP in extracts from *N. alata* assayed by diffusion against β-glucosyl Yariv reagent.

Neutral monosaccharides were analysed as their alditol acetates, following acid hydrolysis (TFA; 2.5 M, 2 h, 100°C), by GLC–MS as described in Gane et al. [39]. Acidic monosaccharides were identified as their trimethylsilylated methyl glycosides (1 M methanolic-HCl, 16 h, 80°C) by GLC–MS as described by McConville et al. [40].

Linkage analysis was by methylation using the NaOH method of Ciucanu and Kerek [41] as modified by McConville et al. [40]. The partially methylated alditol acetates were separated, identified and quantified by GLC–MS as described by Gane et al. [39].

SDS-PAGE was performed as described by Laemmli [42] using a Mini-Protean II system (Bio-Rad). 15% Polyacrylamide separating gels with 5% polyacrylamide stacking gels were used. The gels were stained with silver (Bio-Rad silver stain kit), or Coomassie Blue, or 0.2 mg mL^{-1} β-glucosyl Yariv reagent in 1.0% w/v NaCl overnight followed by washing in 1% w/v NaCl (4 × 10 min).

Crossed electrophoresis of AGP extracts was performed as described by van Holst and Clarke [43].

The amino acid composition of the purified AGPs was analysed, as their phenylthiocarbamyl (PTC) derivatives, following acid hydrolysis (6 M HCl, 110°C, 18 h) using reversed-phase HPLC based on the method of Bidlingmeyer et al. [44], as described by Oxley and Bacic [45].

Molecular-weight analysis of AGPs by ultracentrifugation.—The molecular weight of the purified AGP samples was determined by ultracentrifugation using a Beckman Optima XLA ultracentrifuge. The samples (1 mg mL^{-1}) were dissolved in 0.1 M sodium phosphate buffer, pH 7.2. Sedimentation equilibrium experiments were performed at 20°C using a rotor speed of 5000 rpm for 18 h. The data were analysed on the

basis of two components, using the assumption that each component sedimented as an ideal solute according to $d\ln c/dr^2 = M(1 - v\rho)\omega^2/2RT$, where M is the molecular weight of the component, ω the angular velocity, ρ the solution density, T the absolute temperature, and v the partial specific volume of AGPs taken as 0.62 mL g^{-1} [46].

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra of the AGPs were recorded on Bruker AMX-300 WB, AMX-500 and AMX-600 spectrometers at 30°C. Samples were dissolved in solutions of D_2O (99.6 atom %; 60 mg AGP in 0.6 mL), and transferred to 5 mm tubes (Wilmad 535 PP). Me_2SO (50 μL) was added to each sample as an internal standard. ^{1}H and ^{13}C chemical shifts of AGPs were measured relative to Me_2SO (^{1}H, 2.71 ppm; ^{13}C, 40.4 ppm).

Typical ^{1}H NMR spectra at 300.13 MHz were acquired with a spectral width of 2.8 kHz, 16 K data points, 90° pulse (5.9 μs), an acquisition time of 2.9 s, a relaxation delay of 1.5 s and sufficient scans to give good signal-to-noise ratio (typically 64 scans). Assignments were made by comparing the chemical shifts and coupling constants to reference AGPs [21] and using 2D DQF-COSY [47], TOCSY [48,49], and NOESY [50] spectra. Typical conditions for the 2D spectra included a spectral width of 5.1 kHz, 4 K data points, an acquisition time of 0.41 s, a relaxation delay of 1.5 s and 32 scans per increment. For the TOCSY experiment an 80 ms spin-lock pulse (using the DIPSI-2 spin-lock sequence [51]) was used and 688 increments in the F_1 dimension were recorded. For the DQF-COSY and NOESY experiments 512 increments were recorded. A mixing time of 100 ms was used in the NOESY experiment. Solvent suppression was achieved by a low-powered irradiation during the relaxation delays (and in the NOESY during the mixing time as well), with the water resonance being placed on the transmitter offset.

Proton-decoupled ^{13}C NMR spectra were recorded at 75.5 MHz. A typical acquisition used a spectral width of 22.7 kHz, 60° pulse (6.4 μs), an acquisition time of 0.36 s, a relaxation delay of 2 s, and 2000–3000 scans. Free induction decays (FIDs) of 16 K data points were acquired, and prior to Fourier transformation the FIDs were multiplied by an exponential function (line broadening of 12 Hz) to improve the signal-to-noise ratio. Confirmation of the chemical shifts for the methylene carbons (C-6 of Gal*p* and C-5 of Ara*f* residues) was by the *J*-modulated spin-echo experiment [52]; referred to as the *J*-mod or attached proton test (APT) pulse sequence. These spectra were acquired with a spectral width of 16.1 kHz, 16 K data points, 90° pulse, a relaxation delay of 1.5 s, an evolution time (τ) of 7.1 ms, and approximately 8000 scans.

A HSQC (heteronuclear single quantum coherence [53]) spectrum was used, in conjunction with the proton assignments, to assign the carbon spectra. Conditions included a relaxation delay of 1.7 s, evolution delay of 1.79 ms, 4096 complex data points in F_2, 32 scans per increment, an acquisition time of 0.5 s, 207 increments in F_1, spectral width of 4098 Hz in F_2 and 13582.4 Hz in F_1 on the AMX-600 spectrometer.

All data were processed using the standard Bruker software (UXNMR) or using the FELIX program (Hare Research Inc.) on a Silicon Graphics 4D/30 Personal Iris. In the homonuclear 2D spectra the F_1 dimension was zero-filled to 2048 real data points, and either a 90° or 60° phase-shifted sine bell or sine bell squared window function applied. In heteronuclear 2D spectra the F_1 dimension was zero-filled to 1024 real data points, with a line-broadening of 3–6 Hz applied prior to Fourier transformation.

3. Results and discussion

Purification of N. alata *stigma and style AGPs.*—Buffer-soluble extracts of stigmas and styles of *N. alata* contain a range of components, including the S-glycoproteins (S-RNases [54]), a (1 → 3)-β-D-glucanase [55], a 120-kD style-specific glycoprotein [56], and proteinase inhibitors [57] as well as AGPs (Fig. 1). On SDS-PAGE analysis the AGPs electrophoresed as a smear in the stacking gel extending approximately to the 94 kD marker in the running gel (Fig. 1). AGPs constitute approximately 4–5% of the total carbohydrate in the crude extracts and represent 36.5 μg per stigma and style (Table 1). The major neutral monosaccharide (Table 1) in these crude extracts was Glc; Gal, Rha and Ara were also present at significant levels. Methanolysis showed that the only uronic acid present in the crude extracts was GalA, at levels of 1.2% of the total carbohydrate. Fractionation of the buffer-soluble crude extracts with saturated ammonium sulfate precipitated most of the protein, while effectively all (99%) of the AGP remained soluble. Dialysis of the saturated ammonium sulfate supernatant removed all the low-molecular-weight material, including oligosaccharides (primarily sucrose), and proteins such as the proteinase inhibitor peptides [57] (Fig. 1). During dialysis 25–35%

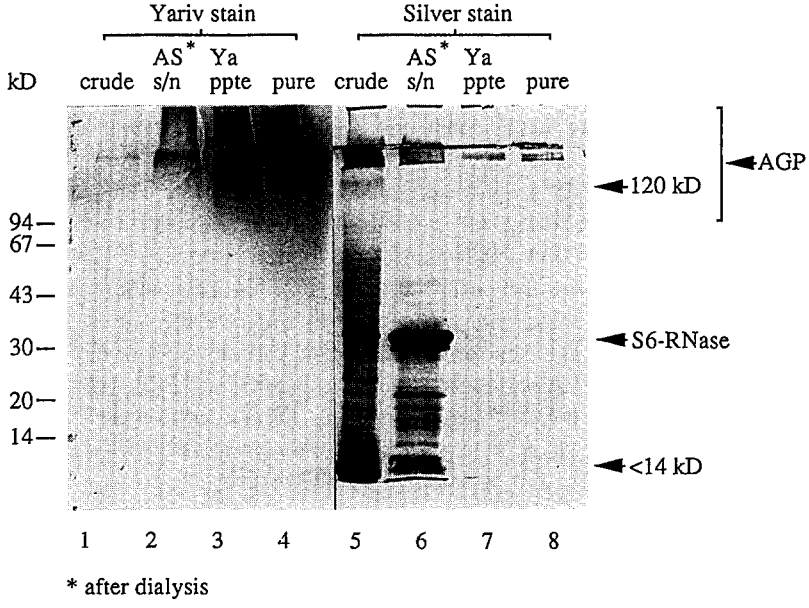

Fig. 1. SDS-PAGE of different stages of purification of AGP from stigmas and styles of *Nicotiana alata*. Samples were separated on a 5–15% polyacrylamide gel. Lanes 1–4 were stained with β-glucosyl Yariv reagent, and lanes 5–8 were stained with silver. Markers are in kilodaltons (kD); 120–120 kD glycoprotein; S_6-RNase-self-incompatibility ribonuclease; 14 kD—proteinase inhibitor. The same amount of carbohydrate (20 μg) was loaded onto each lane. Lanes 1 and 5, buffer-soluble crude style extract from genotype S_6S_6 (1 μg AGP, 6 μg protein); lanes 2 and 6, saturated ammonium sulfate supernatant after dialysis (7 μg AGP, 5 μg protein); lanes 3 and 7, material after precipitation with β-glucosyl Yariv reagent and reduction with sodium dithionite (20 μg total carbohydrate, 20 μg AGP; trace protein); lanes 4 and 8, after gel-filtration chromatography (20 μg AGP; trace protein).

of the AGP was routinely lost, although no selective losses of particular charged families of AGP occurred as judged by crossed-electrophoresis (Fig. 2). AGPs were not detected in the dialysate, and thus losses may have been due to adherence of AGPs to the dialysis membrane. Treatment of the buffer-soluble extracts with saturated ammonium sulfate and subsequent dialysis resulted in an 8.5-fold enrichment of AGP when calculated as μg AGP per mg dry weight.

SDS-PAGE of the extracts following dialysis (Fig. 1) confirmed that most of the proteins, including the 120 kD glycoprotein and the majority of the S-RNase, were removed by precipitation with ammonium sulfate (see also Table 1). The S-RNase, however, remained the most abundant silver-stained component of the ammonium sulfate supernatant.

AGPs were then purified from the dialysed supernatant by precipitation with the β-glucosyl Yariv reagent. SDS-PAGE analysis of the Yariv-precipitated material indicated that only AGPs and a small amount ($< 2\%$ of the AGP by weight) of S-RNase was present (Fig. 1). After treatment with guanidine hydrochloride and DTT the AGPs could be separated from the S-RNase by gel-filtration chromatography under dissociating conditions (Fig. 3). The co-precipitation of a trace of S-RNase with the AGPs therefore resulted from a non-covalent association between these molecules. This interaction is likely to be due to their widely differing pIs: the S-RNases are basic molecules with pI values over 9 [54], whereas AGPs are acidic. The AGPs eluted as a single symmetrical peak from the gel filtration column, and on SDS-PAGE staining with silver and β-glucosyl Yariv reagent coincided (Fig. 1). The SDS-PAGE analysis indicated that no contaminating protein was present and the AGPs were thus considered pure.

The isolated AGP preparation represented 55% of the AGP present in the initial extract from stigmas and styles (Table 1). The ratio of Gal:Ara in the purified AGP was the same as that in the ammonium sulfate supernatants, indicating that there were no selective losses of particular classes of AGP during purification. Crossed electrophoresis showed that the charge patterns of the purified AGPs was the same as in the crude extract (Fig. 2), again indicating that no selective losses had occurred.

Composition of stigma and style AGPs.—The AGPs isolated from the stigmas and styles of *N. alata* have a high proportion of carbohydrate (95% w/w) compared with protein (5% w/w). The amount of protein was calculated from the amino acid analyses. Gal and Ara comprised over 90% of the monosaccharide constituents of the purified AGP with minor amounts of Man, Glc, Xyl, and Rha (Table 1). No uronic acid residues were detected (data not shown). The monosaccharide composition of AGPs isolated from stigmas and styles by precipitation with β-glucosyl Yariv reagent is very similar to that of AGPs purified by J539-affinity chromatography from stigmas alone [35]. In contrast, AGPs isolated separately from stigmas and styles of *Gladiolus gandavensis* had different Gal:Ara ratios, although they contained the same minor sugars [58]. AGPs from *N. alata* had a lower Gal:Ara ratio than did AGPs from both stigma and style tissue of *G. gandavensis*, but had a similar Gal:Ara ratio to AGPs isolated from the stigma of *Lilium longiflorum* [59]. However, the stigmatic AGPs from *L. longiflorum* also contained a significant amount of GlcA (11 mol%), which was absent in the AGPs from *N. alata*.

Table 1
Composition of stigma and style fractions during purification of AGPs from *Nicotiana alata*. (a) Recovery of AGP, carbohydrate, and protein during purification of AGP. Data are the amounts of each component extracted per style. (b) Neutral monosaccharide composition at different stages of purification of AGP. (tr = trace, – = not detected)

(a)	Component					
	AGP		Carbohydrate		Protein	
Fraction	μg	% recovery	μg	% recovery	μg	% recovery
Crude extract	36.5	100	780.0	100	265.0	100
Saturated $(NH_4)_2SO_4$ supernatant						
Before dialysis	36.0	99	695.0	89	35.0	13
After dialysis	26.5	73	80.0	10	20.0	8
Yariv precipitate	22.0	60	22.0	3	tr	tr
Gel filtration	20.0	55	20.0	3	tr	tr

(b)	mol %				
		Saturated $(NH_4)_2SO_4$ supernatant			
Monosaccharide	Crude extract	Before dialysis	After dialysis	Yariv precipitate	Gel filtration
Rha	11.0	12.4	12.1	1.6	1.8
Fuc	2.8	4.5	–	–	–
Rib	0.9	–	–	–	–
Ara	8.2	6.0	21.8	30.5	29.7
Xyl	1.9	–	3.3	tr	1.1
Man	3.3	4.4	3.0	tr	2.3
Gal	12.9	12.5	45.4	66.4	63.5
Glc	59.0	60.2	14.3	0.8	1.6
Gal/Ara	1.57	2.08	2.08	2.18	2.14

The amino-acid composition of the protein component of the AGPs (data not shown) was essentially the same as that previously reported for stigmatic AGPs [35] and typically rich in alanine, serine and hydroxyproline. In crossed electrophoresis experiments (Fig. 3) the AGPs were diffuse with respect to charge and consisted of a major (~ 70%), almost neutral species and a minor (~ 30%) more negatively charged species. AGPs isolated from stigmas and styles of plants of different self-incompatible genotypes (S_2S_2 and S_6S_6) were essentially indistinguishable with respect to monosaccharide and amino acid composition, charge, and electrophoresis on SDS-PAGE.

Molecular-weight determination of stigma and style AGPs.—Analysis of the sedimentation velocity and sedimentation equilibrium behaviour of AGPs isolated from stigma and styles of *N. alata* indicated the presence of more than one component (Fig. 4). The sedimentation equilibrium data were analysed assuming two major components. The best fit to the data was obtained for species of molecular weight 143 and 1910 kD representing 94 and 6% of the sample, respectively. The high-molecular-weight component probably represents aggregated AGP resulting from the freeze-drying of the

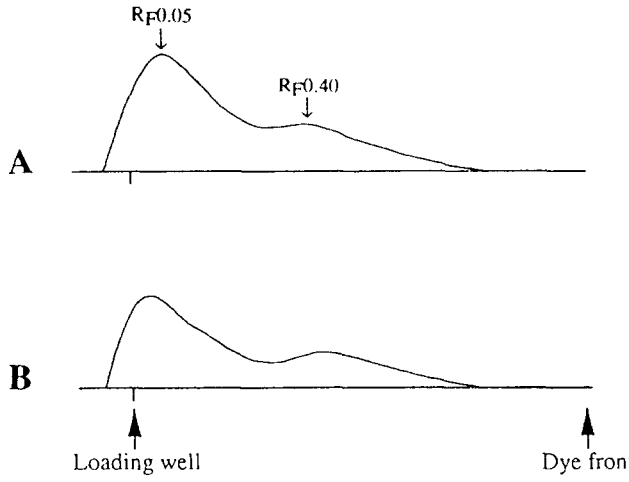

Fig. 2. Crossed-electrophoresis of AGPs from stigmas and styles of *Nicotiana alata*. Crossed electrophoresis of AGPs from crude extracts of stigmas and styles of *N. alata*, and after purification. R_f values were measured against a dye front of bromophenol blue. (A) Dialysed buffer-soluble crude extracts made from stigmas and styles, containing 20 μg AGP as determined by the Yariv diffusion assay. (B) Purified AGP (20 μg).

samples prior to ultracentrifugation, since the AGPs had been previously purified on a gel-filtration column that excluded material with a molecular weight of over 1000 kD (Fig. 2).

The molecular weight of the AGPs calculated from ultracentrifugation (143 kD) contrasts markedly with that estimated from gel-filtration chromatography under dissociating conditions (500 kD). A similar difference in molecular weight has been reported for AGPs purified from *Brassica napus*, which had a molecular weight of 500 kD estimated from gel filtration, and a molecular weight of 126 kD calculated from ultracentrifugation [2]. The relationship between the shape of a macromolecule in solution and its molecular weight must be known to interpret gel-filtration data correctly [60]. Unlike gel-filtration chromatography, sedimentation equilibrium is independent of the shape of the molecule.

Linkage analysis of stigma and style AGPs.—AGPs were subjected to linkage analysis by methylation (Table 2). Ara was present in the furanose form and most was terminally linked, although small amounts of 2- and 5-linked Ara*f* were also detected. Gal was present in the pyranose form and was predominantly 3,6-linked. Smaller amounts of terminal, 3- and 6-linked Gal*p* were also detected. The molar recovery of non-reducing terminal residues (Ara*f* and Gal*p*) was approximately equal to the molar recovery of branched residues (3,6-linked Gal*p*), indicating complete methylation. Rha, Xyl, Man, and Glc present in small amounts in monosaccharide analyses (see Table 1) were not detected in the linkage analyses. The glycosyl linkage composition of the AGPs from stigmas and styles of plants of different self-incompatibility genotypes were essentially identical (data not shown).

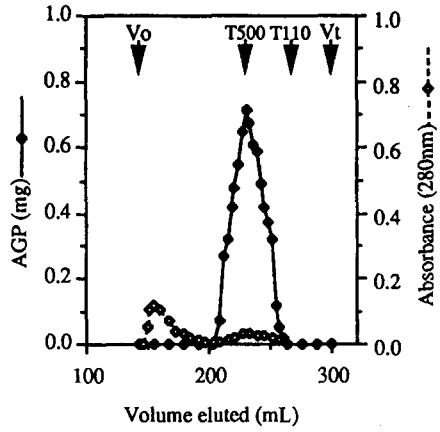

Fig. 3. Gel-filtration chromatography of material precipitated by β-glucosyl Yariv reagent from stigmas and styles of *Nicotiana alata*. Material that had been precipitated by β-glucosyl Yariv reagent and subsequently reduced with dithionite was dissolved in 6 M guanidine-HCl, 20 mM DTT and dialysed into 6 M urea, 10 mM Tris-HCl, pH 9.6. Approximately 10 mg AGP was applied to a Toyopearl HW-65(S) gel filtration column (90 cm × 2.2 cm i.d.) and eluted with the same buffer. Fractions (4 mL) were collected and assayed for A_{280}, and for the ability to bind β-glucosyl Yariv reagent. The column was calibrated with dextran standards T2000 (V_0), T500, T110, and gal (V_t), as indicated by the arrows. Fractions which contained AGP were pooled and used for further analysis.

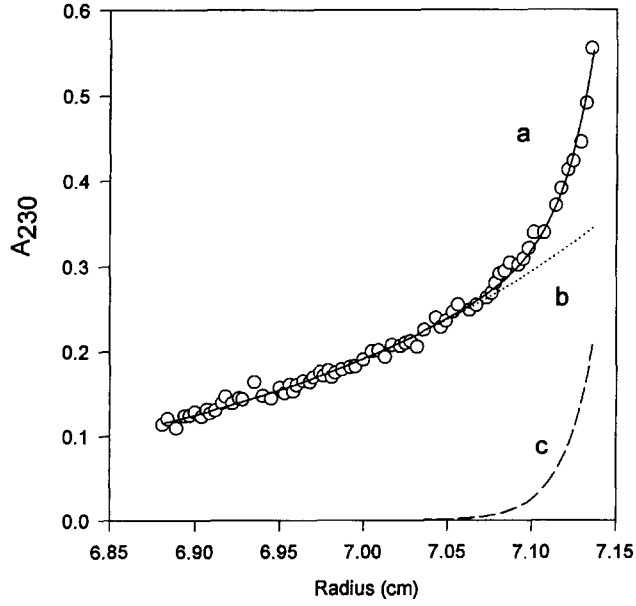

Fig. 4. Sedimentation equilibrium distribution of AGPs purified from stigmas and styles of *Nicotiana alata*. The buffer used was 0.1 M sodium phophate buffer, pH 7.2 and sedimentation was at 5000 rpm for 18 h and 20°C. The data (A_{230} versus radial distance) was analysed assuming two major components. The upper curve (a) represents the line of best fit and corresponds to the sum of the lower two curves (b and c) calculated for species of 143 (94%) and 1910 kD (6%), respectively.

Table 2
Linkage analysis of AGPs purified from stigmas and styles of *Nicotiana alata*

Deduced glycosidic linkage [a]		mol % [b]
Ara*f*	Terminal	29
	2-	tr [c]
	5-	1
Gal*p*	Terminal	11
	3-	9
	6-	9
	3,6-	41

[a] Terminal Ara*f* is deduced from 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylpentitol, etc.
[b] Average of duplicate determinations.
[c] tr, trace (< 1 mol%).

Comparison of AGPs from stigmas and styles of *N. alata* with AGPs from stigmas and styles of the monocotyledons *G. gandavensis* [58] and *L. longiflorum* [59] shows that there are differences in their glycosyl linkage compositions. The core structure of the AGPs in the stigma of these three species appears to be similar as they all contain similar amounts of 3-, 6- and 3,6-linked Gal*p*, and the variation is confined to the terminal glycosyl residues. The AGPs from the stigma of *G. gandavensis* contain significant amounts of terminal Glc*p*, and the AGPs from the stigma of *L. longiflorum* contain significant amounts of terminal Rha*p* and terminal and 4-linked GlcA*p*. None of these residues were detected in the AGPs purified from stigmas and styles of *N. alata*.

NMR spectroscopy of stigma and style AGPs.—The ^{1}H and ^{13}C NMR spectra of the AGPs purified from *N. alata* stigmas and styles are shown in Figs 5–8, and the assignments reported in Tables 3 and 4, respectively. The 1D and 2D proton spectra for AGPs isolated from self-incompatibility genotypes S_2S_2 and S_6S_6 at 500 MHz were compared and no significant differences found, therefore, most of the NMR characterization was carried out on AGPs isolated from genotype S_6S_6. ^{1}H assignments were obtained from homonuclear COSY, TOCSY (Fig. 6) and NOESY spectra. The ^{13}C assignments were established from a combination of *J*-modulated spin-echo experiments (Fig. 7, which distinguished between methine and methylene resonances) and the HSQC spectrum (Fig. 8).

An extensive proton assignment of all the major linkage types detected by methylation analysis was made. In the 1D ^{1}H NMR spectrum (Fig. 5) H-1 signals corresponding to the D-Gal*p* residues (4.44–4.52 ppm) and L-Ara*f* residues (5.07–5.25 ppm) were detected. There were numerous signals from 3.53 to 4.22 ppm which corresponded to H-2 to H-6 of the β-D-Gal*p* residues, and H-2 to H-5 of the α-L-Ara*f* residues. To resolve and assign these signals to particular sugar residues 2D homonuclear experiments were performed. A 2D TOCSY spectrum is shown in Fig. 6 and the chemical shifts are summarized in Table 3. Signals for all the Ara*f* and Gal*p* linkage types could be assigned except for signals corresponding to H-2 to H-5 of 2-linked Ara*f*, and H-3 to H-5 of the 5-linked Ara*f*, which were not detected in the 2D spectrum due to the low abundance of these residues (see Table 2). As an example, the spin system of the

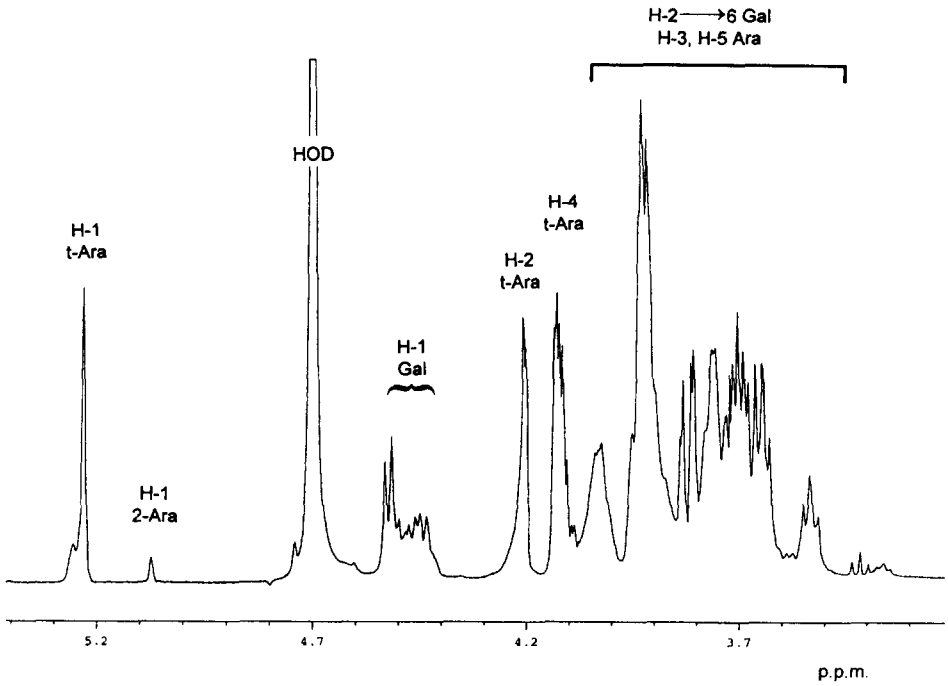

Fig. 5. ^{1}H NMR spectrum of AGPs purified from stigmas and styles of *Nicotiana alata*. This spectrum was recorded on a Bruker AMX-500 MHz spectrometer with a spectral width of 4.0 kHz, 32 K data points, 90° pulse (5.6 μs), an acquisition time of 4.1 s, a relaxation delay of 2.0 s and 32 scans.

terminal Ara*f* residue is marked on the TOCSY spectrum in Fig. 6. Assignment of the different linkage types to each of the spin systems identified in the 2D spectra was done both by consideration of the methylation data in Table 2 and comparison with the NMR data reported on grape berry AGPs by Saulnier et al. [21].

Akiyama and Kato [17] used ^{1}H NMR to investigate the structure of AGPs from the extracellular medium of suspension cultures of *N. tabacum*, but could not assign all the signals observed. More recently, Saulnier et al. [21] studied the structures of AGPs from grape berries, and were able to identify NMR peaks arising from α-L-Ara*f* as well as H-1 and H-2 of β-D-Gal*p*, but did not report a complete assignment. The current study therefore represents the first full carbon and most complete proton assignment of an AGP.

In the anomeric region in the ^{13}C NMR spectrum (Fig. 7), a sharp signal at 110.9 ppm was assigned to C-1 of terminal α-L-Ara*f*. The signals at 104.8–105.3 ppm were not completely resolved, and assignments were made using the 2D HSQC spectrum (Fig. 8) and the relative abundances of Gal*p* residues in methylation analyses (Table 2). The signal at 104.8 ppm was assigned to C-1 of 3,6-linked Gal*p*, and the remaining peaks at 105.1 and 105.3 ppm could not be unequivocally assigned but are due to the C-1 of terminal, 3- and 6-linked Gal*p*. Resonances corresponding to C-1 of 2- and 5-linked Ara*f* were not detected due to their low abundance.

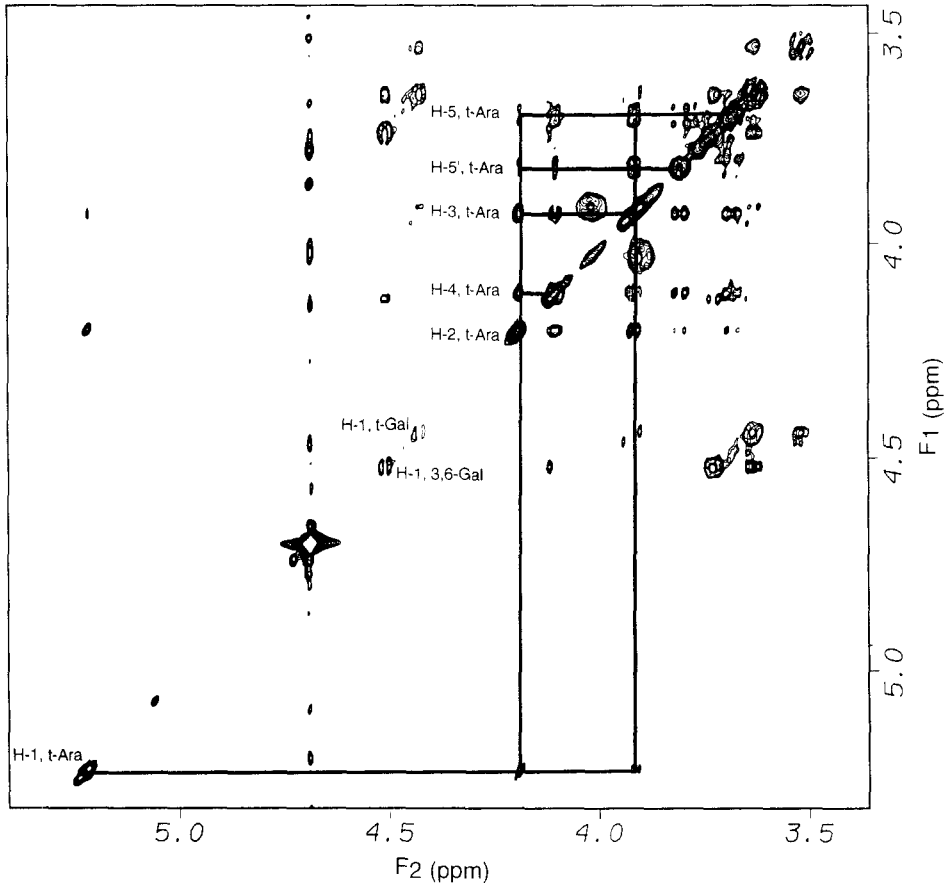

Fig. 6. ^{1}H Homonuclear 2D TOCSY spectrum of AGPs purified from stigmas and styles of *Nicotiana alata*. The TOCSY spectrum was recorded on a Bruker AMX-500 MHz spectrometer with a spectral width of 5.1 kHz, 4 K data points, an acquisition time of 0.41 s, a relaxation delay of 1.5 s, and 32 scans. An 80 ms spin-lock pulse (using the DIPSI-2 mixing scheme) was used and 688 increments were recorded in F_1.

The methylene signals of C-5 of the Ara*f* residues and C-6 of the Gal*p* residues were identified in the *J*-mod spectrum, in which they appeared as negative peaks (Fig. 7). Therefore, the resonances at 62.9 and 62.7 ppm were assigned to C-5 of terminal Ara*f* and to C-6 of terminal and 3-linked Gal*p*, respectively. A component of the multiplet at ~ 71.0 ppm was identified as arising from a methylene group and assigned as C-6 of 6- and 3,6-linked Gal*p*. This assignment is consistent with the large downfield shift (+8.3 ppm) associated with substitution at the C-6 position. The region corresponding to C-2 to C-5 of the β-D-Gal*p* residues and to C-2 to C-4 of α-L-Ara*f* (70.1–85.5 ppm) was crowded and required 2D NMR techniques for a complete assignment. The HSQC spectrum (Fig. 8) resolved most of the resonances in this region, and when used in conjunction with the proton assignments resulted in the unambiguous

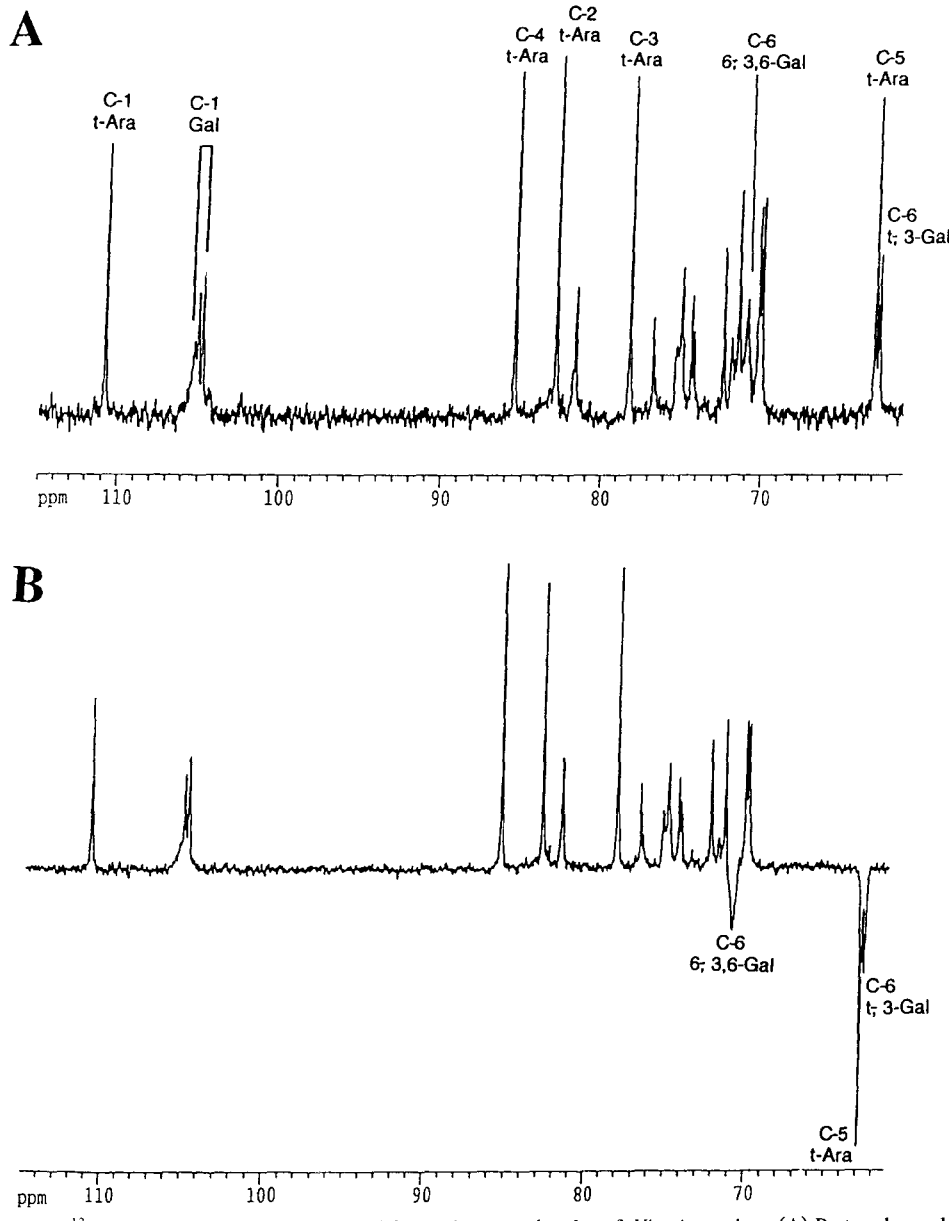

Fig. 7. ^{13}C NMR spectra of AGPs purified from stigmas and styles of *Nicotiana alata*. (A) Proton-decoupled ^{13}C NMR spectrum of AGPs. The spectrum was recorded on a Bruker AMX-600 MHz spectrometer at a ^{13}C frequency of 150 MHz, with a spectral width of 15.0 kHz, 16 K data points, 60° pulse (6.4 μs), an acquisition time of 0.36 s, a relaxation delay of 2 s, and approximately 2500 scans. (B) *J*-modulated spectrum of AGPs. The spectrum was recorded on a Bruker AMX-300 MHz spectrometer with a spectral width of 16.1 kHz, 16 K data points, 90° pulse, an acquisition time of 0.36 s, a relaxation delay of 1.5 s, a τ of 7.1 ms, and approximately 8000 scans.

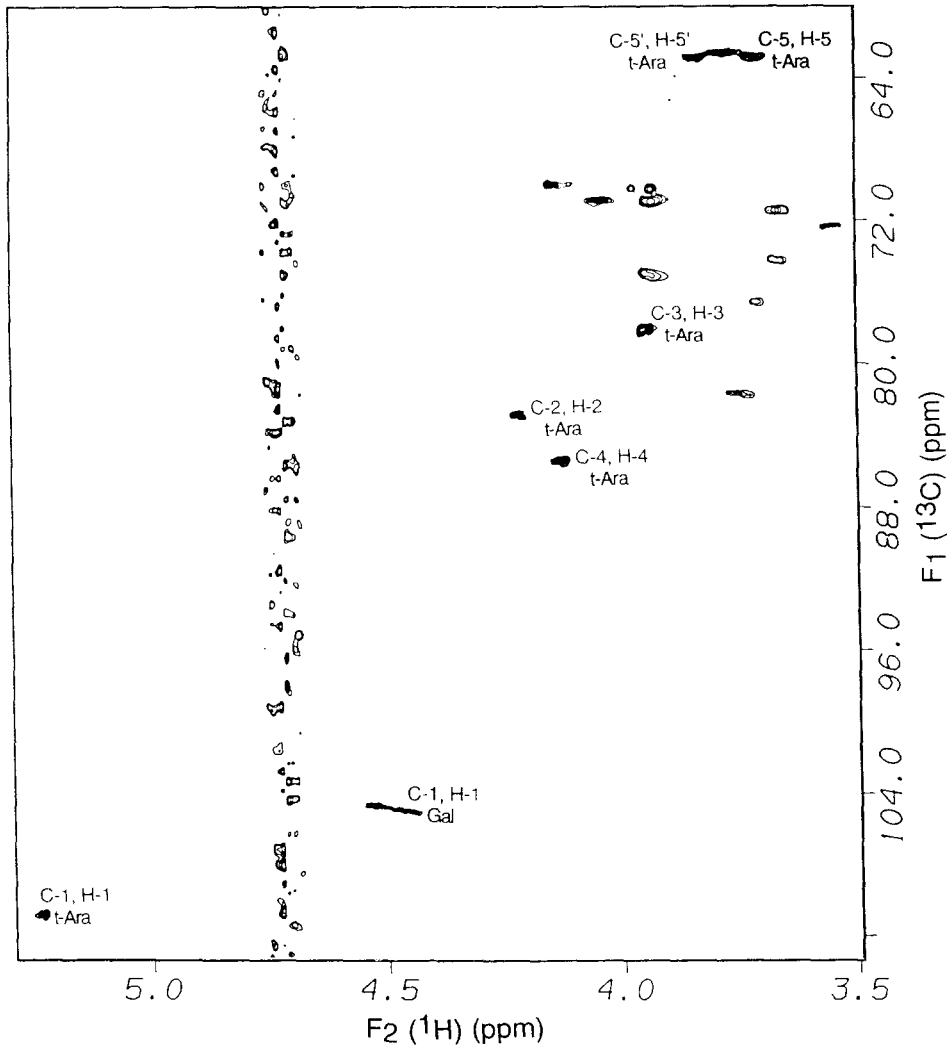

Fig. 8. HSQC spectrum of AGPs purified from stigmas and styles of *Nicotiana alata*. This HSQC spectrum was recorded on a Bruker AMX-600 MHz spectrometer with a ^{13}C frequency of 150 MHz, a spectral width of 4.1 kHz in F_1 and 13.6 kHz in F_2, a relaxation delay of 1.7 s, an evolution delay of 17.9 ms, 4096 complex data points in F_2, 32 scans per increment, an acquisition time of 0.5 s and 207 increments in F_1. The spectrum was processed with line-broadening of 6 Hz in F_1 and a sine-squared window function in F_2.

assignments given in Table 4. Assignments are listed for all the linkage types detected by methylation analysis, except the minor 2- and 5-linked Ara*f* components.

^{13}C NMR studies of several other AGs and AGPs have been reported previously [17,18,20–22]. The ^{13}C NMR data for AGPs from *N. alata* obtained in this study were in close agreement with the data reported for grape berries [21], with only the assignments for the C-2 of 3-, 6- and 3,6-linked Gal*p* differing between the two studies.

Table 3
Summary of ^{1}H chemical shifts for AGPs purified from stigmas and styles of *Nicotiana alata*

Glycosidic linkage	Chemical shift (ppm) [a]					
	H-1	H-2	H-3	H-4	H-5	H-6
α-L-Ara*f* Terminal-	5.23	4.20	3.93	4.12	3.82/3.70	
2-	5.07	– [b]	– [b]	– [b]	– [b]	
5-	5.25	4.22	– [b]	– [b]	– [b]	
β-D-Gal*p* Terminal-	4.44	3.53	3.65	3.92	3.70	3.77
3-	4.48	3.54	3.67	4.09	3.70	3.77
6-	4.46	3.54	3.66	3.96	3.93	3.90/4.03
3,6-	4.52	3.65	3.74	4.13	3.93	3.90/4.03

[a] Chemical shifts measured relative to sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) (30°C).
[b] –, not detected.

For *N. alata* AGPs the signals for C-2 of Gal*p* were assigned as 71.6 (3- and 3,6-linked) and 72.4 (terminal and 6-linked) ppm, from a combination of the DQF-COSY, TOCSY, and HSQC spectra (relative to internal Me_2SO which had been calibrated as 40.4 ppm relative to DSS). For the AGPs from grape berries, resonances between 76.43 and 76.65 ppm were assigned as C-2 of 3-, 6-, and 3,6-linked Gal*p* (relative to internal Me_2SO at 39.5 ppm) [21]. For *N. alata* a signal at 76.8 ppm was assigned as the C-5 of terminal and 3-linked Gal*p*, a position which was not assigned by Saulnier et al. [21] for grape berry native AGP. Following dearabinosylation, however, C-5 of terminal and 3-linked Gal*p* was assigned at 76.6 and 75.2 ppm [21].

No ^{1}H or ^{13}C resonances from amino acid moieties of the AGP were observed in any of the NMR spectra for *N. alata* AGPs. Weak ^{1}H signals have been reported which were thought to arise from amino acid residues of the AGP isolated from grape berries [21], however, cross-peaks from these peaks were not observed in the 2D spectra.

Proposed structure of the AGPs from stigmas and styles of N. alata.—The structure of the carbohydrate moiety of the AGPs from stigmas and styles from *N. alata* is consistent with the model proposed by Fincher et al. [1]. In this model, the molecule is composed of a backbone of 3-linked β-D-Gal*p* residues which is highly branched at O-6

Table 4
Summary of ^{13}C chemical shifts for AGPs purified from stigmas and styles of *Nicotiana alata*

Glycosidic linkage	Chemical shift (ppm) [a]					
	C-1	C-2	C-3	C-4	C-5	C-6
α-L-Ara*f* Terminal-	110.9	83.0	78.2	85.5	62.9	–
β-D-Gal*p* Terminal-	105.1 [b]	72.4	74.4 [c]	70.3	76.8	62.7
3-	105.3 [b]	71.6	81.8	70.1	76.8	62.7
6-	105.3 [b]	72.4	74.3 [c]	70.3	75.0	71.0
3,6-	104.8	71.6	81.8	70.1	75.0	71.0

[a] Chemical shifts measured relative to DSS (30°C).
[b] Assignments may be interchanged.
[c] Assignments may be interchanged.

to short sidechains of 6-linked β-D-Gal*p* residues. These 6-linked Gal*p* residues are then substituted mainly with terminal α-L-Ara*f* and some terminal Gal*p* residues. This model is supported by the ^{13}C NMR spectra in which the signals corresponding to α-L-Ara*f* residues were much sharper than those for β-D-Gal*p* residues, indicating that they had an enhanced mobility consistent with their location at the periphery of the AG structure. Saulnier et al. [21] have also concluded that 3-linked Gal*p* forms the central core of the AGPs from grape berries by NMR spectroscopic data. The model is also consistent with the proposed structures of AGPs from *Acacia senegal* [1], *Raphanus sativus* [31], *Brassica campestris* [32] and *Lolium multiflorum* [19]. The *N. alata* AGPs appear to have a simpler structure than AGPs from *A. senegal*, *R. sativus* and *B. campestris* in that they lack extended sidechains of 2-, 3- or 5-linked Ara*f*, or the complexity of terminal Rha*p*, Fuc*p*, Xyl*p* or GlcA*p* residues.

In the proposed structures of AGPs from *A. senegal* and *L. multiflorum*, the 3-linked Gal*p* backbone is interspersed with periodate-sensitive residues, such as 6-linked Gal*p* or 5-linked Ara*f* [19,61]. These residues result in flexible "kinks" in the galactan chains. In *L. multiflorum* Bacic et al. [19] found that Smith degradation resulted in (1 → 3)-β-linked Gal*p* oligomers which were mono-disperse (dp 7) with respect to molecular size, indicating that periodate-oxidisable residues were at regular intervals along the galactan backbone. The well-resolved ^{13}C NMR spectra of the *N. alata* AGPs suggests that there is some kind of regularity in their galactan core, and it is possible that the low proportion of 5-linked Ara*f* detected in the analyses of the *N. alata* AGPs may contribute repetitive periodate-sensitive linkages in the galactan backbone.

In the "wattle-blossom" type structure of AGPs proposed by Fincher et al. [1] the AG substituents are linked to a polypeptide backbone. In wheat endosperm AGP this linkage is through an alkali-stable β-D-Gal*p*-Hyp linkage [28,29]. Saulnier et al. [21] also concluded that this is the most likely carbohydrate–protein linkage in grape AGPs. However, linkages through serine and threonine have also been proposed in AGPs from *R. sativus* and *B. campestris* [31–33]. An alternative model for AGPs from *A. senegal*, the "twisted hairy rope", has been proposed by Qi et al. [62]. They predicted that AGPs are rod-like molecules with numerous small polysaccharide substituents, regularly arranged along a highly repetitive polypeptide backbone based on a 10–12 residue motif. The hypothetical 7 kD subunits would organize the polysaccharide side-chains along the long axis of the peptide to give a "twisted hairy rope". Qi et al. [62] proposed in addition that *A. senegal* AGPs also contain hydroxyproline arabinosides similar to extensins.

From aminoacid analysis the AGPs from the stigma and style of *N. alata* contain ~ 5% protein, and from ultracentrifugation their molecular weight is estimated to be approximately 143 kD. Thus, the molecular weight of the protein portion of the *N. alata* AGPs is estimated to be ~ 7 kD, which represents ~ 60 amino acids, and the M_r of the carbohydrate portion is approximately 136 kD, which represents ~ 850 glycosyl residues. Recently, a cDNA clone encoding a protein backbone of a style AGP from *N. alata* was isolated and sequenced [26]. The mature protein had a predicted molecular weight of 10 kD, which is in close agreement with that deduced from the analytical data described in this paper; it contained 23 proline residues, of which 87% were hydroxylated. The AGPs contained 15% hydroxyproline from amino acid analyses (data not

shown [26]) which is equivalent to nine hydroxyproline residues per molecule, and if these are all glycosylated then each AGP molecule contains nine carbohydrate side-chains, each of approximately 95 sugar residues. Similarly, Fincher et al. [1] proposed that the galactan chains of AGPs may be 120 residues or more long, composed internally of 10 or more blocks of 12 residues separated by periodate-sensitive residues. Carbohydrate–protein linkages through serine and threonine would, however, result in the AGPs from *N. alata* possessing more but shorter carbohydrate side-chains.

It is now possible to separate AGPs from *N. alata* stigmas and styles into individual components by reversed-phase HPLC [26]. This will allow the glycosylation patterns of these individual components to be examined.

Acknowledgements

This work was supported by funds from a Special Research Centre grant from the Australian Research Council. AG acknowledges the support of an Australian Postgraduate Research Award. We are grateful to Ms S. Mau for collection of plant material and Drs M.-L. Liao, S.M. Read and I. Sims for fruitful discussions. We thank Ms J. Noble for typing the manuscript.

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