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Isolation of the Protein Backbone of an Arabinogalactan-Protein from the Styles of *Nicotiana alata* and Characterization of a Corresponding cDNA

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Arabinogalactan-proteins (AGPs) from the styles of *Nicotiana alata* were isolated by ion exchange and gel filtration chromatography. After deglycosylation by anhydrous hydrogen fluoride, the protein backbones were fractionated by reversed-phase HPLC. One of the protein backbones, containing mainly hydroxyproline, alanine, and serine residues (53% of total residues), was digested with proteases, and the peptides were isolated and sequenced. This sequence information allowed the cloning of a 712-bp cDNA, AGP*Na*1. AGP*Na*1 encodes a 132-amino acid protein with three domains: an N-terminal secretion signal sequence, which is cleaved from the mature protein; a central sequence, which contains most of the hydroxyproline/proline residues; and a C-terminal hydrophobic region. AGP*Na*1 is expressed in many tissues of *N. alata* and related species. The arrangement of domains and amino acid composition of the AGP encoded by AGP*Na*1 are similar to that of an AGP from pear cell suspension culture filtrate, although the only sequence identity is at the N termini of the mature proteins.

INTRODUCTION

Arabinogalactan-proteins (AGPs), a family of proteoglycans, are components of extracts from most plant tissues and many secretions and are also produced by plant cells in suspension culture (Clarke et al., 1979; Fincher et al., 1983; Showalter and Varner, 1989; Roberts, 1990; Showalter, 1993). They commonly occur as extracellular components but are also present on the plasma membrane of cells from several plant species (Knox, 1992; Pennell, 1992) and have been proposed to have a range of functions within plant cells and tissues (for example, see Stacey et al., 1990; Kreuger and van Holst, 1993).

We have a particular interest in the structure and function of AGPs of the female sexual tissues of flowering plants as they are extracellular components of styles from all angiosperm families examined (Hoggart and Clarke, 1984). Detailed structural studies of style AGPs have been undertaken for two species, *Gladiolus gandavensis* (Gleeson and Clarke, 1979) and *Nicotiana alata* (Gell et al., 1986; Bacic et al., 1988; Gane, 1994). The AGPs of *N. alata* pistils are present on the stigma surface, in the extracellular matrix of the transmitting tract tissue cells (Sedgley et al., 1985; Sedgley and Clarke, 1986), and are associated with the ovary in *Lycopersicon peruvianum* and *N. alata*, particularly on the placenta surface and between the ovules (Webb and Williams, 1988; Gane, 1994). Analysis of the style AGPs has focused on the glycan moiety and has shown that, like AGPs from many other plant tissues, they consist mainly of carbohydrate (>90%), primarily arabinose (Ara) and galactose (Gal) residues with Gal as a β(1→3, 1→6) galactan and Ara mainly as terminal α-L-arabinofuranosyl residues.

Analysis of the protein moiety of the style AGPs has been restricted to amino acid composition that is typically rich in Hyp/Pro, Ala and Ser/Thr, Asx and Glx. In this article, we report isolation of the protein backbone of a major AGP from styles of *N. alata*. We also report N-terminal amino acid sequencing of the backbone, sequencing of peptides produced by protease digestion, and molecular cloning of a cDNA (AGP*Na*1) encoding this AGP protein backbone.

RESULTS

Isolation of AGPs from *N. alata* Styles

Crossed-electrophoresis of the total style extract ("style" includes both the style and the stigma) resolved a major group of AGPs and a group of poorly resolved, more highly charged AGPs, as shown in Figure 1A. AGPs in the style extract were detected and quantified by the gel diffusion assay with β-glucosyl Yariv reagent. Fractionation of the style extract (~20 to 30 μg of AGPs per style) with ammonium sulfate to 95%

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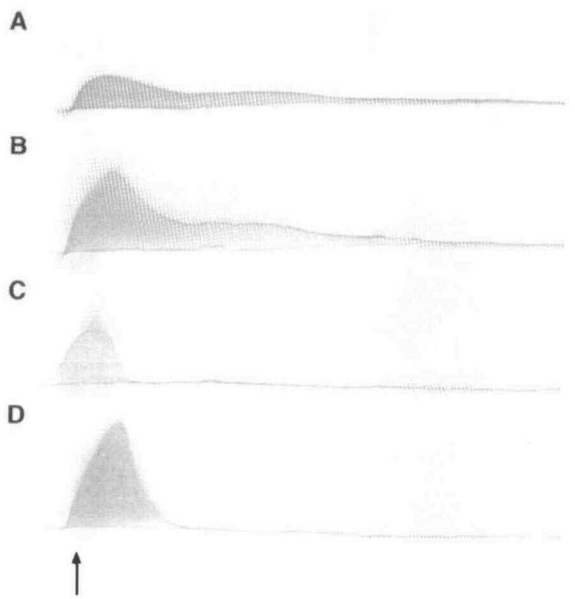

Figure 1. Crossed-Electrophoresis of AGPs from Styles of *N. alata*.

AGPs were first electrophoresed horizontally in a 1% agarose gel, then vertically into a gel containing the β-glucosyl Yariv reagent, as described by van Holst and Clarke (1986). Arrow indicates loading wells.
(A) Crude style extract.
(B) 95% $(NH_4)_2SO_4$ supernatant.
(C) Mono Q–unbound AGP-containing fraction.
(D) Mono Q–bound fraction.

saturation gave no selective loss of AGPs from the supernatant (Figure 1B). Following desalting on a PD-10 column, the 95% $(NH_4)_2SO_4$ supernatant was subjected to Mono Q anion exchange chromatography. As shown in Figure 2A, one group of AGPs did not bind to the column and was essentially neutral (Figure 1C). This group accounted for 10% of the AGPs recovered from the column, as indicated in Table 1, and was not further analyzed. Most of the AGP (90%) bound to the column and was eluted with 0.1 to 0.2 M NaCl. This weakly charged, AGP-containing fraction contained the major group of style AGPs (Figure 1D). The group of more highly charged AGPs (Figure 1A) was not detected in the material eluted after 0.2 M NaCl (Figure 2A). The AGP fractions eluted from the ion exchange column were further purified by Superose 6 gel filtration chromatography under dissociating conditions (6 M urea). A single AGP-containing peak was eluted as shown in Figure 2B. This fraction accounts for ~25% of the AGPs present in the crude style extract (Table 1).

During various stages of the purification procedure, the AGP-containing fractions were examined by SDS-PAGE. Figure 3 shows that the AGPs stained weakly with silver (Figure 3A) but intensely with β-glucosyl Yariv reagent (Figure 3B) and appeared as a high molecular weight smear (M_r > 90,000) in a 10% polyacrylamide gel (Figure 3B). A 32K protein, which is

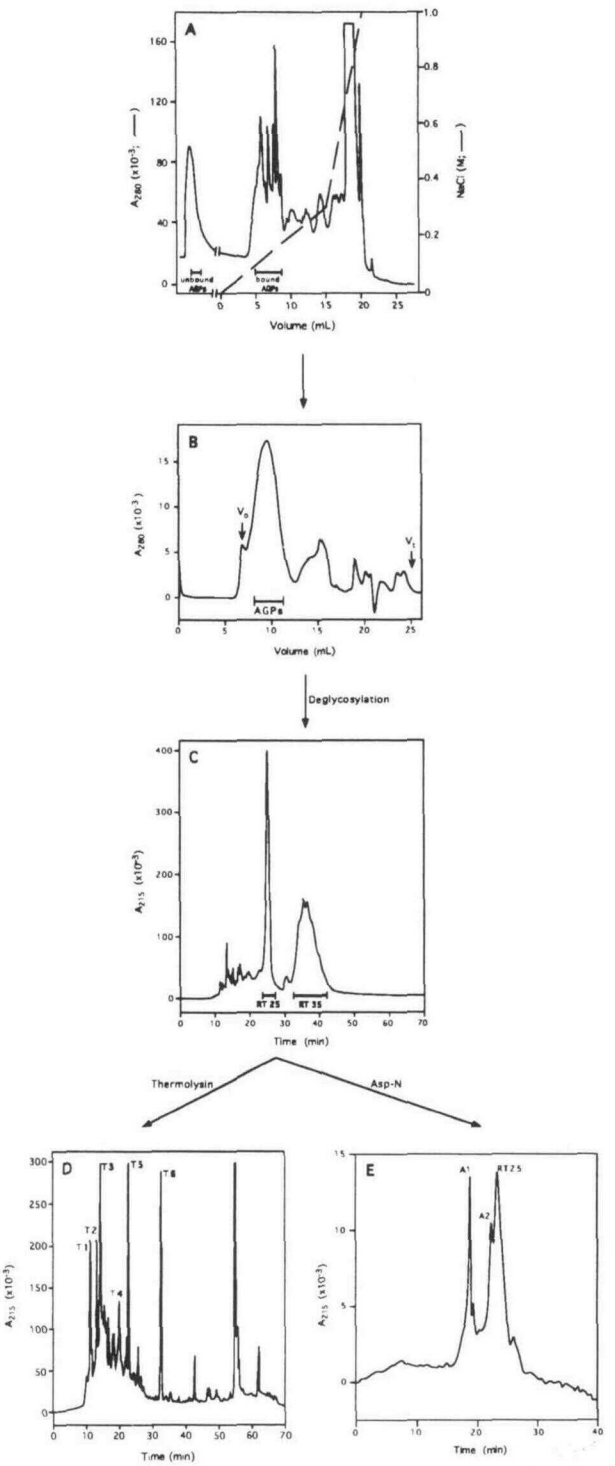

Figure 2. Purification of Native AGPs from Styles of *N. alata*, Separation of Deglycosylated AGP Backbones, and Fragmentation of RT25 AGP Backbone.

Table 1. Recovery of Style AGPs during Purification[a]

Purification Stage	% Recovery
Crude style extract (1000 styles)	100
95% $(NH_4)_2SO_4$ supernatant	68
Mono Q anion exchange chromatography	
Unbound AGPs	5
Bound AGPs	44
Superose 6 gel filtration chromatography	25

[a] After each purification step, the amount of AGPs was quantified by the gel diffusion assay (van Holst and Clarke, 1985), and the recovery was calculated as a percentage of the amount of AGPs present in the crude style extract.

coincident with the style *S*-RNase (product of the *S*-gene controlling self-incompatibility; Anderson et al., 1986; Jahnen et al., 1989), was present during the initial stages of the purification procedure (Figure 3A; lanes 1, 2, 3, and 5) and was only separated from the AGPs after Superose 6 gel filtration in 6 M urea (lanes 4 and 6). After this step, only high molecular mass material was detected. This purified AGP-containing fraction (Figure 2B) was used for all subsequent experiments. By using a combination of monosaccharide and amino acid analyses, the purified AGPs were found to contain primarily carbohydrate (>90% [w/w]), which consisted mainly of Gal (~60%) and Ara (~30%) with mannose, xylose, rhamnose, and glucose as minor components (<10%) as well as protein (<10%) containing high proportions of Ala, Ser, Hyp, Asx, and Glx, as shown in Table 2.

Deglycosylation and Characterization of AGP Protein Backbones

The purified style AGPs (Figure 2B) were deglycosylated using anhydrous hydrogen fluoride, and their protein backbones were

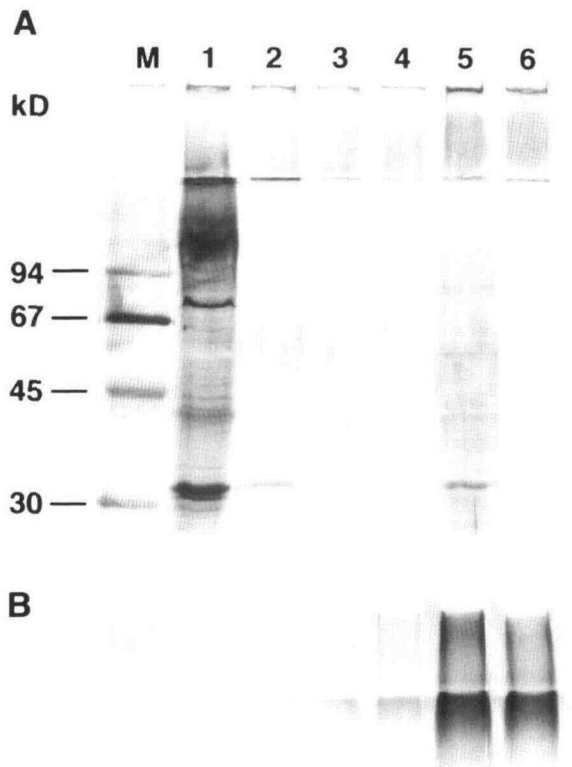

Figure 3. SDS-PAGE Analysis of Style AGP-Containing Fractions at Various Stages of Purification.

Lane 1 contains total style extract (1 μg of AGP); lane 2, 95% $(NH_4)_2SO_4$ supernatant (4 μg of AGP); lane 3, Mono Q–bound AGP-containing fraction (4 μg of AGP); lane 4, Superose 6 AGP-containing fraction (4 μg of AGP); lane 5, same as given for lane 3 but containing 20 μg of AGP; lane 6, same as given for lane 4 but containing 20 μg of AGP. Protein molecular mass markers (M) are shown at left.

(A) SDS-PAGE (10% gel) followed by silver stain.

(B) Stain for AGPs with β-glucosyl Yariv reagent.

Figure 2. (continued).

(A) The 95% $(NH_4)_2SO_4$ supernatant (5 mg AGPs) from the crude style extract were fractionated by a Mono Q anion exchange column with a salt gradient (dashed line). Material eluted from the column was monitored by absorption at 280 nm (solid line). Fractions containing AGPs were identified by the gel diffusion assay and pooled into two fractions, as indicated by the horizontal bars.

(B) The bound AGP-containing fraction (10 mg AGPs) eluted from the anion exchange column was applied to a Superose 6 gel filtration column. AGP-containing fractions were identified using the gel diffusion assay and pooled, as indicated by the horizontal bar. The void volume (V_o) and the total volume (V_t) are indicated.

(C) Deglycosylated AGP backbones were separated by reversed-phase HPLC. Proteins eluted from the column were monitored by absorption at 215 nm. Fractions eluted at retention times of 25 min (RT25) and 35 min (RT35) were pooled separately, as indicated by the horizontal bars.

(D) Thermolysin cleavage products of the RT25 protein backbone were fractionated by reversed-phase HPLC. Individual peptides (T1 to T6) were collected and subjected to amino acid sequencing. Peaks eluted after a retention time of 40 min contained thermolysin and its autolytic products.

(E) Endoproteinase Asp-N cleavage products of the RT25 protein backbone were separated by reversed-phase HPLC. Peptides contained in peak A1 and A2 were subjected to amino acid sequencing. Undigested starting material (RT25) was also detected.

Table 2. Amino Acid Composition of *N. alata* Style AGPs and the Deduced Mature Protein Backbone from the AGP*Na*1 cDNA[a]

	AGP		AGP Protein Backbones[b]			
Amino Acid	Native Protein[c]	Deglycosylated Protein[d]	Unbound Protein	RT35 Protein	RT25 Protein	AGP*Na*1 cDNA[e]
Hyp	7.5	7.6	ND	4.5	18.1	
Pro	3.0	2.9	3.6	3.9	2.7	20.7
Asx	13.0	10.6	8.8	14.9	3.6	2.7
Thr	7.7	6.1	4.0	6.6	6.9	5.4
Ser	6.0	9.6	11.5	5.6	14.8	18.9
Glx	11.2	10.0	13.3	12.2	6.2	2.7
Gly	5.5	7.0	20.7	4.4	9.3	5.4
Ala	15.9	19.3	6.2	10.6	20.1	24.3
Val	3.4	4.3	4.4	3.6	6.3	9.0
1/2 Cys	1.0	0.8	0.8	2.1	ND	0
Met	2.6	2.5	0.9	3.0	1.5	0.9
Ile	4.3	3.2	3.6	4.7	1.7	1.8
Leu	5.9	5.0	7.0	6.4	2.7	3.6
Tyr	3.4	2.1	3.4	3.1	0.5	0
Phe	2.7	2.4	2.2	4.2	1.4	2.7
Trp	ND[f]	ND	ND	ND	ND	0
Lys	3.5	3.4	1.8	5.0	2.3	0.9
His	1.0	1.1	3.2	1.6	1.0	0
Arg	2.2	1.8	4.7	3.4	0.8	0.9

[a] Composition given as mole percentage.
[b] Deglycosylated AGP backbones after reversed-phase HPLC separation.
[c] Superose 6–purified AGPs before HF deglycosylation.
[d] Superose 6–purified AGPs after HF deglycosylation.
[e] Deduced mature protein from AGP*Na*1 cDNA clone.
[f] ND, not detected.

separated by reversed-phase HPLC, as shown in Figure 2C. Three fractions, designated unbound (material that did not bind to the column), RT25, and RT35 (material that eluted at retention times of 25 and 35 min, respectively), were collected. These fractions contained approximately equal amounts of protein as measured by amino acid analysis. The amino acid composition of the deglycosylated AGPs, which is a mixture of the three fractions separated by reversed-phase HPLC, is similar to that of the native AGPs (Table 2). However, distinct differences were apparent in the amino acid compositions of the unbound, RT25, and RT35 fractions. The unbound fraction contains little Hyp but is rich in Gly, Glx, Ser, and Asx. The RT35 fraction is also Hyp poor, but rich in Asx, Glx, and Ala. Together, these two fractions account for most of the Asx and Glx detected in the native and deglycosylated AGPs. The amino acid composition of the material in fraction RT25 is dominated by Hyp (18%), Ala (20%), and Ser (15%) with very little Tyr (<1%). This RT25 protein backbone was selected for further analyses.

Proteolytic Cleavage and Amino Acid Sequencing of the RT25 Protein Backbone

The RT25 protein backbone yielded no N-terminal sequence information, presumably because of N-terminal blockage. However, after treatment with pyroglutamate aminopeptidase, which removes pyroglutamate residue from the N terminus, the sequence Ala-Hyp-Gly was obtained. The RT25 backbone was fragmented by treatment with the endoproteinase thermolysin, and the resulting peptides were fractionated by reversed-phase HPLC. Figure 2D shows that six major peptides,

Table 3. Internal Amino Acid Sequences from the RT25 Protein Backbone

Endoproteinase	Peak No.	Amino Acid Sequences[a]
Thermolysin	T1	Not sequenced
	T2-1[b]	VSAOSQSOSTAA
		IGSAOAGSO
	T2-2	IGSAOAGSOTSSPN
	T3	LASOOAOOTADTOA
	T4	No sequence
	T5	FAOSGGVALPOS
	T6	No sequence
Asp-N	A1	No sequence
	A2	DTOAFAOSGGVAL

[a] O, Hyp. Underlines and dotted lines indicate overlapping sequences.
[b] T2 was separated into several peaks by reversed-phase HPLC; T2-1 contains two peptide sequences.

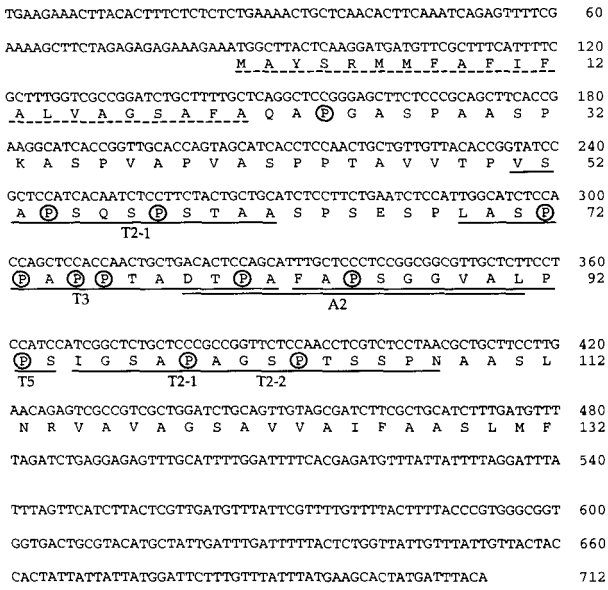

Figure 4. Nucleotide and Deduced Amino Acid Sequences of the AGP*Na*1 cDNA Clone.

The molecular mass of the deduced protein is 12.5 kD. A secretion signal (dashed underline) was predicted by the PSIGNAL program (PC/Gene software; IntelliGenetics) based on the method described by von Heijne (1986). Internal peptide sequences from amino acid sequencing (T2-1, T2-2, T3, T5, and A2) are indicated by solid underlines, and Pro residues (P) known to be hydroxylated from amino acid sequencing are circled. A dash (–) indicates the stop codon. The nucleotide and the predicted amino acid sequences of the AGP*Na*1 cDNA have been submitted to GenBank as accession number U13066.

designated T1 to T6, were collected and subjected to amino acid sequencing. Four sequences were obtained yielding 52 unambiguous amino acid residues, as shown in Table 3. The peptides were rich in Hyp, Ser, and Ala (33 of 52 amino acid residues).

Because peptide T3 (Table 3) contained an Asp residue, endoproteinase Asp-N was used to cleave the RT25 protein backbone. Two major peptides (A1 and A2) were produced, as shown in Figure 2E, suggesting that there is only one Asp residue in the RT25 protein. The cleavage was incomplete, as indicated by the presence of the starting material (RT25 protein; Figure 2E). Peptide sequence information was obtained for A2 (Table 3). The other peptide, A1, yielded no sequence data; this indicated a blocked N terminus. Overlaps were identified between sequences from peptide T3, T5, and A2 (Table 3), which resulted in a contiguous 26-amino acid residue, namely LASOOAOOTADTOAFAOSGGVALPOS.

Cloning of a cDNA Encoding the RT25 AGP Protein Backbone

A gene-specific oligonucleotide (20 nucleotides; see Methods) was designed from one region of the contiguous 26-amino acid sequence TADTOAF. Inosine was used at the third position of the first two codons to reduce the degeneracy of the oligonucleotide to 128. The resulting oligonucleotide contained 55% GC residues. cDNA was synthesized from total style RNA using poly(T) linked to an adapter sequence. Rapid amplification of the cDNA 3′ end (3′ RACE) was performed using the gene-specific primer together with a 3′ primer in the adapter sequence. A polymerase chain reaction (PCR) product of 400 bp was produced. This PCR product was cloned and sequenced. The deduced amino acid sequence from this clone matched the peptide sequence data (Table 3).

The 400-bp PCR product was used to screen a style cDNA library (300,000 plaques). Two cDNA clones were obtained; they differed only in length. Figure 4 shows the sequence of one of the clones, designated AGP*Na*1. The 3′ end of the AGP*Na*1 cDNA is identical to the 400-bp PCR product, except that the PCR product is 20 bp shorter and contains a poly(A) tail. The 712-bp AGP*Na*1 clone encodes a putative protein of 12.5 kD (Figure 4). The derived 132-amino acid sequence includes the sequences obtained from all of the peptides (Table 3). Most of the Pro residues (11 of 13 residues) in the peptide sequences obtained by amino acid sequencing are hydroxylated. A secretion signal peptide was predicted between amino acid residue 1 (Met) and 21 (Ala), as shown in Figures 4 and 5. The deduced N terminus of the mature protein (111 amino acid residues; 10 kD; pI 6.8) is Gln-Ala-Pro-Gly, which matches the N-terminal sequence data. The Pro residue in the N-terminal sequence is also hydroxylated. The amino acid composition of the deduced mature protein and the RT25 protein backbone are in general agreement (Table 2). The C-terminal region of the deduced protein is very hydrophobic (Figure 5)

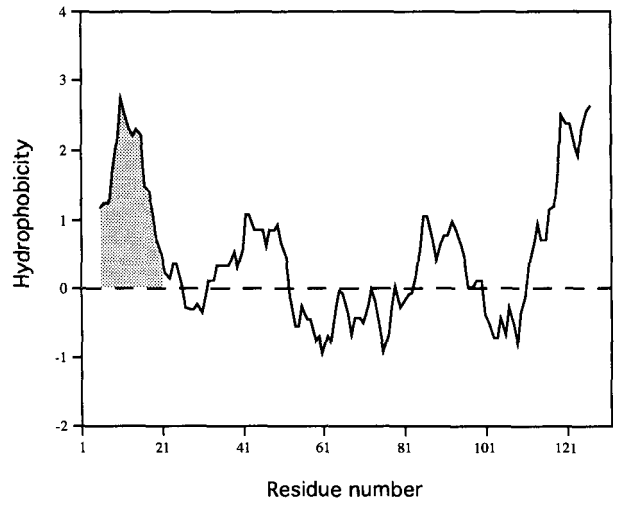

Figure 5. Hydropathy Plot of the Deduced Amino Acid Sequence from the AGP*Na*1 cDNA Clone.

The hydrophobicity of the deduced amino acid sequence was calculated by the SOAP program (PC/Gene software; IntelliGenetics) based on the method of Kyte and Doolittle (1982). The predicted secretion signal is shadowed (von Heijne, 1986).

and predicted to be a transmembrane helix (PC/Gene software, IntelliGenetics, Mountain View, CA).

Expression of the AGP*Na*1 Gene in Plant Tissues

Various tissues from *N. alata* were examined for the expression of the AGP*Na*1 gene. As shown in Figure 6A, mRNA transcripts (700 to 750 nucleotides) of approximately the same length as the cDNA were detected in style, ovary, petal, leaf, and stem at similar levels and a higher level in roots.

Some expression of a transcript that hybridized to the AGP*Na*1 cDNA was detected in the styles of *N. sylvestris* and *N. tabacum*, and at a lower level in *N. glauca* and *L. peruvianum* (Figure 6B). No signal was detected in the leaves of Arabidopsis or rye grass.

DISCUSSION

Isolation of Style AGPs and Individual AGP Protein Backbones

AGPs are currently attracting interest because of their wide distribution in plants and their potential involvement as markers of cellular identity (Pennell et al., 1991) and differentiation (Kreuger and van Holst, 1993). In this study, we separated the AGPs from an extract of styles of *N. alata* (Figure 1A) into several components by anion-exchange chromatography (Figure 2A) and analyzed one of the bound components in detail. It seems likely on the basis of the amino acid analysis of this AGP, and the fact that several AGPs are present in the style AGP preparations, that earlier studies probably dealt with mixtures of closely related AGPs. For example, AGPs isolated by either binding to lectins (Gleeson and Clarke, 1979) or monoclonal antibodies (Andrew and Stone, 1983; Bacic et al., 1988; Gleeson et al., 1989; Gane, 1994) via the glycosyl residues or by precipitation with the β-glucosyl Yariv reagent (Jermyn and Yeow, 1975; Clarke et al., 1978; Jermyn and Guthrie, 1985; Gane, 1994) could contain AGPs with different protein backbones but similar monosaccharide compositions and linkage arrangements.

The style AGP backbone (RT25; Figure 2C) studied had a composition dominated by Hyp, Ser, and Ala, which is thought to be "typical" of AGPs (Showalter, 1993). In contrast, fraction RT35 is dominated by Asx, Glx, and Ala, and the unfractionated AGP both before and after deglycosylation, as expected, had a composition with a high content of Asx, Ser, Glx, Ala, and Hyp. The composition of this mixture of AGPs is similar to that reported for the *Gladiolus* AGPs (Gleeson and Clarke, 1979) and the *N. alata* stigma and style AGPs (Bacic et al., 1988; Gane, 1994).

The major problem in obtaining a cDNA clone encoding an AGP backbone was to obtain a single AGP and then to obtain

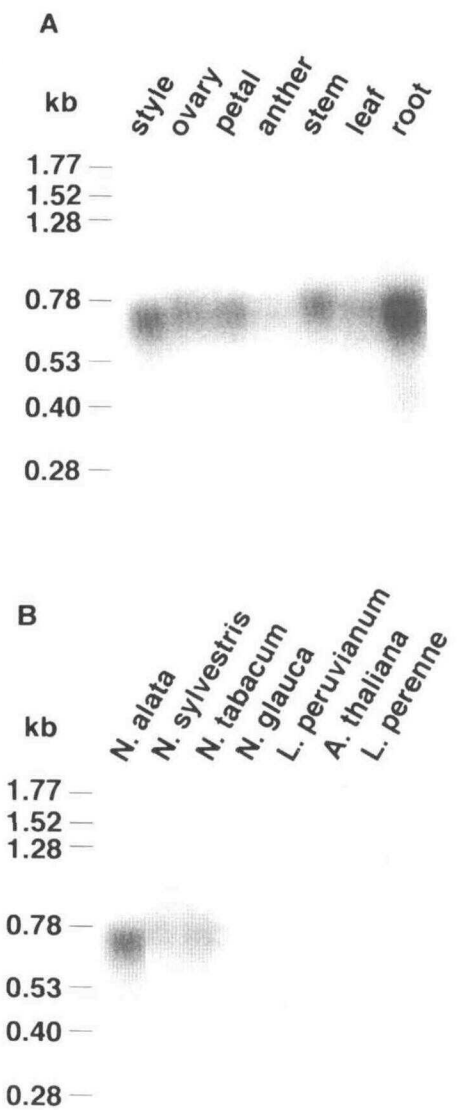

Figure 6. RNA Blot Analysis of Expression of the AGP*Na*1 Gene in *N. alata* and Other Plants.

Total RNA (10 μg per lane) was run in a 2% agarose gel (15% formaldehyde; 40 mM 3-[*N*-morpholino]propanesulfonic acid buffer, pH 7.0) and blotted onto a Hybond-N nylon membrane (Amersham). The AGP*Na*1 cDNA was labeled to 10^8 cpm/μg with ^{32}P-dCTP. Hybridization was performed at 60°C overnight in 0.22 M NaCl, 15 mM NaH_2PO_4, 1.5 mM EDTA, 1% SDS, 1% Blotto, and 4 mg/mL herring sperm DNA. The membrane was washed twice for 10 min at room temperature in 2 × SSC, 1% SDS and twice for 10 min at 60°C in 0.2 × SSC, 1% SDS.

(A) Total RNA was isolated from tissues of *N. alata* (genotype S_6S_6): style, ovary, petal, anther, stem, leaf, and root.

(B) Total RNA was isolated from the styles of *N. alata*, *N. sylvestris*, *N. tabacum*, *N. glauca*, and *Lycopersicon peruvianum* and from the leaves of Arabidopsis and rye grass (*L. perenne*).

useful amino acid sequence data. The five peptides isolated from fragments of the AGP protein backbone together gave 52 amino acid residues (Table 3). However, most of the sequence contained adjacent residues of Hyp, Ser, and Ala for which the codons are highly redundant and GC-rich. These sequences were not useful for cloning. However, the sequence TADTOAF present in the contiguous 26–amino acid sequence, resulting from the overlaps between sequences of T3, T5, and A2 (Table 3), contains two amino acids (Asp and Phe) that are not GC-rich and are encoded by only two codons. This TADTOAF sequence facilitated the design of an oligonucleotide suitable for PCR and the eventual cloning of the AGP*Na*1 cDNA.

Features of the AGP*Na*1 cDNA Clone

The AGP*Na*1 clone (Figure 4) predicts a 132–amino acid protein characterized by hydrophobic stretches at both the N and C termini (Figure 5). The N-terminal hydrophobic sequence corresponds to a signal peptide that would lead to secretion of the encoded protein. This is consistent with the extracellular localization of the style AGPs (Sedgley et al., 1985; Sedgley and Clarke, 1986; Gane, 1994). Modification of the N-terminal residue Gln of the mature protein by intramolecular cyclization to form pyroglutamate is not unusual. This cyclization could be an artifact introduced during purification (e.g., cyclization could occur at low pH during deglycosylation), or it could occur in situ and may protect the N terminus of the AGP backbone from proteolysis.

The C-terminal hydrophobic sequence is predicted to be a transmembrane helix (Figure 5) that may anchor the AGP in the plasma membrane. However, this is unlikely given the extracellular location of AGPs in the style and the fact that RT25 represents one of the major buffer-soluble style AGPs. The hydrophobic C-terminal region could potentially enable the interaction of the AGP with other proteins, such as *S*-RNase that also contains a very hydrophobic sequence (in this case at the N terminus of the mature protein; Anderson et al., 1986). Alternatively, this C-terminal region may be absent from the mature protein, a consequence of proteolytic processing. Studies on the C-terminal sequence of the RT25 protein backbone would resolve whether or not this hydrophobic sequence is present in the secreted AGP.

The central part of the protein contains most of the Hyp, Ala, and Ser residues. Most of the Pro residues within the peptide sequences (Table 3 and Figure 4) are hydroxylated, suggesting that O-glycosylation occurs mostly in the central part of the protein. No potential N-glycosylation sites (Asn-X-Ser/Thr) are present. The abundance of potential O-glycosylation sites is consistent with the high content of carbohydrate (>90% [w/w]). Individual AGPs may differ in the types of saccharide chains and in the number and location of glycosylation sites along the protein backbone.

Potential Interaction of the Style AGP with Other Style Components

Apart from AGPs, styles of *N. alata* also contain other abundant proteins, including the *S*-RNase (Anderson et al., 1986), the 120-kD glycoprotein (Lind et al., 1994), and proteinase inhibitors (Atkinson et al., 1993). Although most proteins of the style extract were removed during the initial ammonium sulfate fractionation (Figure 3, lane 2), a 32K component corresponding to the style *S*-RNase (Anderson et al., 1986) was only removed after gel filtration chromatography under strong dissociating (6 M urea) conditions (Figure 3, lanes 4 and 6). Copurification of the AGPs and the *S*-RNase was also observed when AGPs were purified by alternative procedures (Gane, 1994). It is possible that copurification of the AGPs and the *S*-RNase relates to the abundance of the *S*-RNase in the style and to its strong positive charge (pI >9; Jahnen et al., 1989), which could lead to ionic interaction with the negatively charged AGPs. Both the *S*-RNase (Anderson et al., 1989) and the AGPs (Sedgley et al., 1985; Sedgley and Clarke, 1986) are present extracellularly in the transmitting tract of the style and would be expected to be in physical contact in situ. Whether this binding is of physiological importance is not known.

Style AGP and Other Proline/Hydroxyproline-Rich Proteins

Styles of *N. alata* also contain a major 120-kD glycoprotein with features common to AGPs in that it contains Gal and Ara as the major monosaccharides and Hyp, Ser, and Ala as the major amino acid residues (Lind et al., 1994). This 120-kD glycoprotein differs from the style AGP in that it contains less carbohydrate (35% [w/w]) and that the Gal occurs mainly as terminal Gal and Ara mainly as 2-linked and terminal residues. The glycosyl component of the 120-kD glycoprotein thus has features of both AGPs and extensins (3,6-linked Gal typical of AGPs; 2-linked Ara and terminal Gal typical of extensins). The 120-kD glycoprotein is also basic, as are the extensins, whereas the style AGP is acidic.

As well as AGPs and the 120-kD glycoprotein, styles also express transcripts corresponding to "extensin-like" proteins (Baldwin et al., 1992; Chen et al., 1992; Goldman et al., 1992; Wu et al., 1994) and other proline-rich proteins (Chen et al., 1993; Cheung et al., 1993). Neither the proteins nor their glycosylation patterns corresponding to this last group have been described in detail.

The protein moiety of the style AGP is different from other Hyp/Pro–rich proteins. Extensins characteristically include Ser-Hyp_4 repeats and Tyr residues that may be involved in the intramolecular cross-linking of extensins in the cell wall (Showalter, 1993). The style AGP, like other AGPs, contains no Ser-Hyp_4 repeats and little Tyr. Solanaceous lectins typically have a Cys-rich domain (Allen, 1983; Kieliszewski et al., 1994), whereas AGPs generally contain very little Cys. The

style AGP studied contains no Cys. Chimeric sequences that include domains typical of different classes of proteins have been obtained recently. The maize histidine-rich hydroxyproline-rich glycoprotein (Kieliszewski et al., 1992), for example, contains extensin-like Ser-Hyp_3 repeats and also has "AGP-like" Ala-Hyp repeats. cDNAs from tobacco flower (Wu et al., 1994), on the other hand, encode an extensin-like Pro-rich domain at the N-terminal half of the protein and a "lectin-like" Cys-rich domain at the C-terminal half. The style AGP does not have any of these features.

Other AGP-like peptide sequences have also been reported from *L. multiflorum* (Gleeson et al., 1989), rose (Komalavilas et al., 1991), maize (Kieliszewski et al., 1992) suspension cell culture filtrate, and carrot root (Jermyn and Guthrie, 1985). All of the peptide sequences are composed mainly of Hyp, Ala, and Ser residues but show little sequence identity apart from Ala-Hyp repeats, which are not present in the style AGP studied.

mRNA transcripts hybridizing to AGP*Na*1 cDNA are present in most tissues of *N. alata* and in the styles of related solanaceous species (Figure 6), suggesting a general role of this gene (or closely related genes) in plant development. A cDNA sequence similar to the AGP*Na*1 clone has been obtained from pear cell suspension culture (AGP*Pc*1 cDNA; Chen et al., 1994). Both cDNAs predict protein sequences composed mainly of Pro, Ala, Ser, and Thr, but there is low overall sequence similarity; the only shared motif is at the N terminus. The precise amino acid sequence of AGPs presumably determines the glycosylation sites, but the number of chains and their distribution along the protein backbone are not known for any AGP. Each of these features as well as monosaccharide arrangement within the glycosyl chains may play a role in their physiological functions.

Resolving the precise relationships of the different AGPs in the female sexual tissues and their potential functions, including such events as pollen–pistil interactions and pollen tube nutrition, will depend on having a detailed knowledge of the structures of the individual AGPs. This involves their isolation, cloning and sequencing of their protein backbones, examination of the glycosyl chains and their point(s) of attachment to the protein backbone, and detailed analysis of their location and developmental expression. This study provides a first step by defining the protein backbone for one style AGP of *N. alata.*

METHODS

Plant Material

Nicotiana alata (genotype S_6S_6) and *Nicotiana* and *Lycopersicon* species were maintained under standard glasshouse conditions. Styles (including stigmas) were collected from mature flowers and stored at −70°C before use. Style RNA from *N. sylvestris* and *N. tabacum* were gifts from J. Golz (University of Melbourne, Australia). Leaf RNA from *Arabidopsis thaliana* and *Lolium perenne* were kindly provided by P. Hatfield (University of Wisconsin, Madison) and E.K. Ong (University of Melbourne, Australia).

Isolation of *N. alata* Style AGPs

Styles (~1000) were ground to a fine powder with polyvinylpyrrolidone (1 g) in liquid nitrogen and then extracted with stirring for 30 min with 100 mL of 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, and 14 mM β-mercaptoethanol. The style extract was homogenized in a Waring blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT) at 4°C for 1 min. The homogenate was centrifuged at 10,000*g* for 20 min at 4°C, and the supernatant was collected by filtration through Miracloth (Calbiochem Corp., La Jolla, CA). The pellet was reextracted, and the supernatant was collected. Crossed-electrophoresis of the arabinogalactan-protein (AGP)–containing materials was performed according to van Holst and Clarke (1986).

The pooled supernatants were fractionated with ammonium sulfate in four successive steps to 25, 50, 75, and 95% saturation at 4°C. The supernatant was collected by centrifugation at 10,000*g* for 20 min at 4°C. The 95% $(NH_4)_2SO_4$ supernatant was filtered through 3 MM paper (Whatman), and the volume was reduced to 10 to 20 mL by ultrafiltration through a YM-30 membrane (molecular weight cut off >30 kD; Amicon Inc., Beverly, MA). The concentrated supernatant containing AGPs was then desalted into buffer A (10 mM Tris-HCl buffer, pH 8.0) on a PD-10 column (Pharmacia).

Fractionation of this AGP-containing preparation was performed by anion exchange and gel filtration chromatography on a fast protein liquid chromatography system (Pharmacia). The AGP preparation was loaded onto a Mono Q column (HR 5/5; Pharmacia) equilibrated in buffer A, and the column was washed (three column volumes) in the same buffer. The unbound material was collected. Bound material was eluted by applying a salt gradient (0 to 100% buffer B; 1.0 M NaCl, 10 mM Tris-HCl, pH 8.0, 20 mL at 1 mL/min). The column eluent was monitored by absorption at 280 nm, and the AGP-containing fractions were detected by the gel diffusion assay (see below). The AGP-containing fractions were pooled, desalted, and lyophilized. AGPs that bound to the Mono Q column were redissolved in buffer A (100 μL) and further fractionated on a Superose 6 column (HR 10/30; Pharmacia) developed in 6 M urea, 10 mM Tris-HCl, pH 8.0 (30 mL at 0.2 mL/min). The eluent was monitored by absorption at 280 nm, and the AGP-containing fractions were detected by the gel diffusion assay and pooled.

Detection and Quantification of AGPs

AGPs were detected by a gel diffusion assay using the β-glucosyl Yariv reagent (Yariv et al., 1962, 1967), as described by van Holst and Clarke (1985). Gum arabic (AGP from *Acacia senegal*; Sigma) was used as a standard for the quantification of the AGP.

Deglycosylation and Separation of AGP Protein Backbones

The gel filtration–purified AGPs were desalted (PD-10 column) and dried under vacuum at 40°C overnight in the presence of P_2O_5. Anhydrous methanol (0.2 mL) and anhydrous hydrogen fluoride (HF; 1 mL) were added to the dried AGPs in a dry box under argon. Deglycosylation of the AGPs was performed at 4°C for 2 hr. Distilled water (10 mL) was added to stop the reaction, and the resulting solution was immediately

passed through a PD-10 column equilibrated in 0.1% trifluoroacetic acid (TFA) to remove the HF.

The deglycosylated AGP protein backbones were fractionated by reversed-phase HPLC using an RP-300 column (2.1 × 100 mm; Applied Biosystems, Foster City, CA) equilibrated in 0.1% TFA. The column was washed in 0.1% TFA and the unbound material collected. The column was then eluted with a linear gradient (0 to 60% acetonitrile in 0.1% TFA for 60 min at 100 μL/min) and monitored by absorption at 215 nm. Three fractions, designated unbound, RT25, and RT35, were pooled for further analysis.

Protease Digestion of the RT25 Protein Backbone

Proteolytic digestion of the RT25 protein backbone (2 to 10 μg) was performed at 55°C for 2 hr in 500 μL of 1% ammonium bicarbonate (pH 7.8), 1 mM $CaCl_2$, and 0.01% Tween 20 using the endoproteinase thermolysin (Boehringer Mannheim; 0.2 μg/μg protein). The resulting peptides were separated by reversed-phase HPLC (as described above) and subjected to amino acid sequence analysis.

Endoproteinase Asp-N (Sigma; 0.1 μg/μg protein) digestion was performed at 30°C overnight in 500 μL of 1% ammonium bicarbonate (pH 7.8) and 0.01% Tween 20. The resulting peptides were separated by reversed-phase HPLC and subjected to amino acid sequence analysis.

N-Terminal Deblocking by Pyroglutamate Aminopeptidase

The N terminus of the RT25 protein backbone (1 to 2 μg) was deblocked by treatment with pyroglutamate aminopeptidase (20 μg per 1 nmol of N-terminal amino acid; Boehringer Mannheim) in 100 mM potassium phosphate buffer, pH 8.0, 10 mM EDTA, 5 mM DTT, 5% glycerol at 37°C overnight. Deblocked protein was separated by reversed-phase HPLC, and N-terminal amino acid sequencing was performed.

SDS-PAGE Analysis

SDS-PAGE was performed as described by Laemmli (1970). Proteins were visualized by silver stain (Bio-Rad). AGPs were detected by incubating the gel overnight in 0.2% β-glucosyl Yariv reagent in 1% NaCl after fixation. The gel was then destained in 1% NaCl followed by brief washes in distilled water.

Monosaccharide Analysis

Monosaccharide composition of the style AGPs was analyzed as their trimethylsilyl 1-*O*-methyl glycosides by gas chromatography/mass spectroscopy (Moody et al., 1993) after methanolysis (Chaplain, 1982).

Amino Acid Analysis

AGP preparations (~100 pmol) were dissolved in 100 μL of 6 M HCl containing 0.01% phenol. More HCl (0.5 to 1.0 mL) was added when the carbohydrate content of the sample was higher than 50%. The acid hydrolysis was conducted in vacuo at 110°C for 24 hr. Amino acid analyses were performed on a Beckman (Palo Alto, CA) amino acid analyzer (model 6300) equipped with a Hewlett Packard integrator (model 3390) as described by Simpson et al. (1986).

Amino Acid Sequence Determination

Automated Edman degradation of peptides was performed using either an Applied Biosystems sequencer (model 470A) equipped with PTH-amino acid analyzer (model 120A) or a Beckman sequencer (model LF3400) equipped with a Beckman system Gold HPLC.

Oligonucleotide Design and Synthesis

A gene-specific primer (5′-ACIGCIGA[T/C]AC[T/A/C/G]CC[T/A/C/G]GC-[T/A/C/G]TT) was designed according to one region of the contiguous 26–amino acid sequence TADTOAF. The oligonucleotide was synthesized on an Applied Biosystems DNA synthesizer (model 391).

Rapid Amplification of 3′ End of the cDNA (3′ RACE)

Total RNA was isolated from *N. alata* styles, as described by McClure et al. (1990). cDNA was synthesized from total style RNA (5 μg) in a 20-μL solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphates, 5 μM primer (a 17-mer d[T] stretch linked to a 33-mer adapter sequence), 30 units RNasin, and 50 units avion myeloblastosis virus reverse transcriptase (Promega) at 42°C for 1 hr. cDNA (2 μL) was amplified by polymerase chain reaction (PCR) in a 100-μL solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 30 pmol of the gene-specific primer, 30 pmol of a second primer (20-mer, designed according to the 33-mer adapter sequence), and 2.5 units of *Taq* DNA polymerase (Perkin Elmer-Cetus). Samples were denatured by heating at 96°C for 2 min and then cooled to 80°C before *Taq* DNA polymerase was added. The PCR cycles were run 35 times at 96°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min. The PCR product was cloned and sequenced on a Applied Biosystems DNA sequencer (model 373A).

cDNA Library Screening

A style cDNA library (λZAP II; Stratagene) constructed using mRNA from styles of *N. alata* (S_6S_6) was provided by J. Royo (University of Melbourne, Australia). The cDNA library (300,000 plaque-forming units) was plated out and blotted onto Hybond-N nylon membranes (Amersham) according to the manufacturer's protocol (Stratagene). The PCR product was labeled to 10^8 cpm/μg with ^{32}P-dCTP by random priming. Hybridization was performed at 55°C overnight in 0.22 M NaCl, 15 mM NaH_2PO_4, 1.5 mM EDTA, 1% SDS, 1% Blotto (milk powder), and 4 mg/mL herring sperm DNA (Sigma). The membranes were washed twice for 10 min at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% SDS followed by washing twice for 10 min at 55°C in 0.2 × SSC, 1% SDS. Positive λZAP clones were excised in vivo, and DNA sequences were analyzed as described above. The clone encoding the RT25 protein backbone was designated AGP*Na*1. The nucleotide and deduced protein sequences were analyzed using the PC/Gene software (IntelliGenetics, Mountain View, CA).

RNA Gel Blot Analysis

RNA gel blot analysis was performed as described by Sambrook et al. (1989). Hybridization and washing conditions were the same as

described above, except that the AGP*Na*1 cDNA was used as probe and hybridization was conducted at 60°C.

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