

Identification, Isolation, and N-Terminal Sequencing of Style Glycoproteins Associated with Self-Incompatibility in *Nicotiana alata*

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S-Gene-associated glycoproteins (S-glycoproteins) from styles of *Nicotiana alata*, identified by non-equilibrium two-dimensional electrophoresis, were purified by cation exchange fast protein liquid chromatography with yields of 0.5 to 8 micrograms of protein per style, depending on the S-genotype of the plant. The method relies on the highly basic nature of the S-glycoproteins. The elution profiles of the different S-glycoproteins from the fast protein liquid chromatography column were characteristic of each S-glycoprotein, and could be used to establish the S-genotype of plants in outbreeding populations. In all cases, the S-genotype predicted from the style protein profile corresponded to that predicted from DNA gel blot analysis using S-allele-specific DNA probes and to that established by conventional breeding tests. Amino-terminal sequences of five purified S-glycoproteins showed a high degree of homology with the previously published sequences of *N. alata* and *Lycopersicon esculentum* S-glycoproteins.

INTRODUCTION

Flowering plants have evolved a mechanism known as self-incompatibility to promote outbreeding. Self-incompatibility occurs throughout the plant kingdom and is controlled by the S-gene(s) (de Nettancourt, 1977). In plant families in which self-incompatibility has been most extensively studied, the Solanaceae (for example, *Lycopersicon peruvianum* and *Nicotiana alata*) and the Cruciferae (for example, *Brassica oleracea* and *B. campestris*), the S-genes are single locus genes with multiple alleles. The primary gene products of the S-genes in styles are believed to be glycoproteins that segregate with their respective S-alleles. The timing of S-gene expression corresponds to the onset of self-incompatibility during flower maturation. In the style, expression of the S-gene is restricted to tissues which are in contact with the pollen or pollen tube during growth of the pollen toward the ovary (Cornish et al., 1987).

In *N. alata*, S-glycoproteins corresponding to the S₂-, S₃-, and S₆-alleles were previously identified by analysis of style extracts by isoelectric focusing (Bredemeijer and Blaas, 1981). Amino-terminal amino acid sequences of the S₂- and S₆-glycoproteins (Mau et al., 1986) and the complete cDNA sequence for the S₂-glycoprotein (Anderson et al., 1986) have been published.

Here we report a quick and efficient purification method for S-glycoproteins from *N. alata* based on FPLC cation exchange chromatography. The amino-terminal amino acid sequence of the S₁-, S₂-, S₃-, S₆-, and S₇-glycoproteins are also presented.

RESULTS

Two-Dimensional Gel Electrophoresis

Style proteins of plants homozygous for the S-alleles S₁, S₂, S₃, S₆, and S₇ were resolved into similar two-dimensional gel patterns, part of which are presented in Figure 1. Several hundred proteins were resolved, most of which were located in the acidic to neutral range of the gel. Within this range, there was no obvious correlation of particular spots with S-genotype. The basic range of the gels was dominated by a major component, in the range of 28 kD to 34 kD; these components are referred to as the S-glycoproteins. The position of the five S-glycoproteins investigated (arrowheads) in relation to several proteins common to all S-genotypes (arrows) is shown in Figure 1. In relation to these reference proteins, the S₃-glycoprotein has the lowest pI value, S₂- and S₆-glycoproteins have

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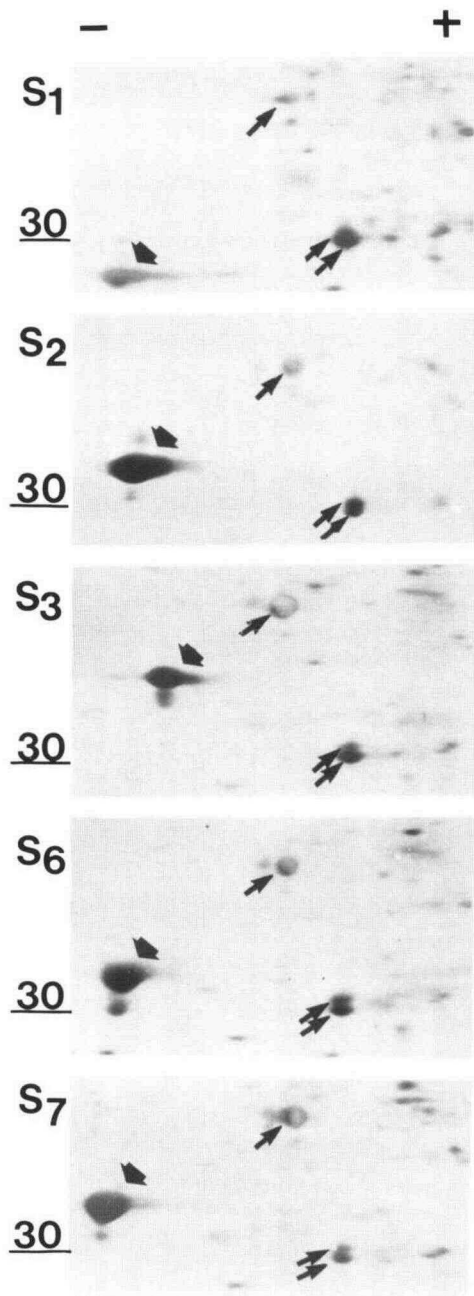


Figure 1. Two-Dimensional Pattern of Style Proteins from Mature Flowers of *N. alata* Homozygous for Particular S-Alleles.

Extracts from styles of plants, homozygous for the S_1 -, S_2 -, S_3 -, S_6 -, and S_7 -alleles, were prepared as described in Methods and separated according to their pI (NEPGHE) in the first dimension and according to their apparent molecular weight (SDS-PAGE) in the second dimension. The part of each gel corresponding to the molecular mass range of approximately 25 kD to 40 kD and to the neutral to basic pH range is shown. Proteins common to all S-genotypes are marked by arrowheads. S-Allele-specific glycoproteins are marked by arrows.

higher, and S_1 - and S_7 -glycoproteins the highest pI values. Due to the non-equilibrium character of the first-dimension separation, exact figures for pI values cannot be given. In relation to the reference proteins, the S_1 -glycoprotein had the lowest, S_2 -, S_6 -, and S_7 -glycoproteins intermediate, and S_3 -glycoprotein the highest apparent molecular weight within the 28 kD to 34 kD range.

Separation of Proteins from Styles of Different S-Genotypes of *N. alata* by FPLC-Cation Exchange Chromatography

The elution profiles of extracts from styles of *N. alata*, homozygous for different S-alleles, are shown in Figure 2. An elution profile of a style extract from *N. tabacum*, a self-compatible species, is also shown for comparison. In each case, peak A represents unbound material. For each of the *N. alata* style extracts, there were two peaks, B and C, which eluted at the same relative positions for each S-genotype. The procedure was not optimized for recovery of peaks B and C; hence, the absolute amounts of these peaks varied in different preparations. Another factor contributing to variation in recovery was the susceptibility of peak B to modification during sample preparation. Nonetheless, the salt concentration required for the elution of peaks B and C (0.1 M, and 0.15 M respectively, Figure 2) was identical in each case.

For each extract of styles homozygous for a particular S-allele, there was a fourth peak, which eluted at a salt concentration (>0.25 M), considerably higher than that required for elution of peaks B and C. The actual concentration required for elution was characteristic of the S-genotype. For example, a peak characteristic of the S_1 -allele was eluted at 0.42 M NaCl (10.5 ml); for S_3 , the concentration was 0.31 M (8.5 ml); for S_6 , the concentration was 0.22 M (6.5 ml); and for S_7 , 0.43 M NaCl (10.6 ml) was required for elution. The peak corresponding to the S_2 -allele differed from the others in that it split into two peaks eluting close together (Figure 2b). The salt concentrations characteristic for elution of these two peaks were 0.35 M and 0.40 M, corresponding to elution volumes of 9.0 ml and 9.6 ml. When the material from either of these peaks was reapplied to the column, it resolved again into the two characteristic peaks in similar proportions. A preparation of S_2 -glycoprotein that had been purified by a method previously published (Anderson et al., 1986), which appeared homogeneous on one-dimensional SDS-PAGE, also split into the two peaks when run on the Mono S column. When probing isolated S-glycoproteins with a polyclonal antibody directed against a synthetic polypeptide [amino acids 29 to 43 of the mature S_2 -glycoprotein, as predicted from cDNA sequence (Anderson et al., 1989)] both S_2 -related glycoproteins, but none of the other four S-glycoproteins tested, reacted strongly in a protein gel blot analysis (not shown).

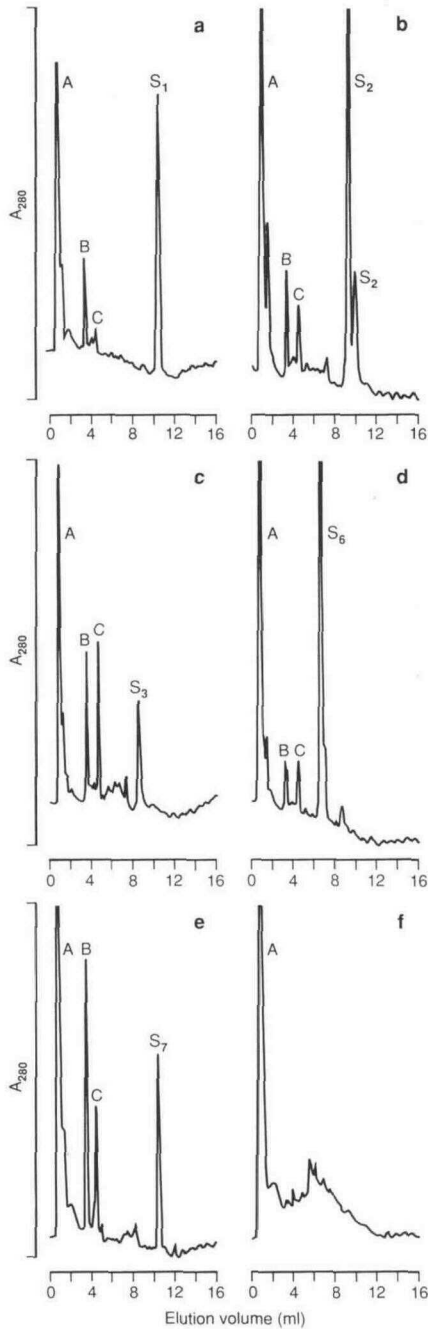


Figure 2. FPLC Elution Profiles of Style Extracts from Mature Flowers of *N. alata* Homozygous for Particular *S*-Alleles.

Extracts from styles of plants, homozygous for the S_1 -, S_2 -, S_3 -, S_6 -, and S_7 -alleles [(a) to (e), respectively], were prepared as described in Methods. Style protein (20 μ g to 50 μ g) was applied to a cation exchange column and eluted with a linear gradient (0 M to 0.5 M NaCl, in sodium acetate buffer 50 mM, pH 5.0; 10 ml) and then isocratically (0.5 M NaCl, in sodium acetate buffer, 50 mM, pH 5.0; 3 ml). An extract from *N. tabacum* (f) was treated in the same way.

The material in the major peaks eluting at NaCl concentrations greater than 0.22 M is characteristic of each *S*-genotype and is referred to as the *S*-glycoprotein. The *S*-glycoproteins were recovered from the peaks and run on SDS-PAGE (Figure 3). The apparent molecular mass was 28 kD for the S_1 -glycoprotein, 32 kD for both S_2 -allele-related protein peaks and for the S_6 - and S_7 -glycoproteins, and 34 kD for the S_3 -glycoprotein. The S_6 -glycoprotein preparations also contained a minor component running at approximately 30 kD, which coeluted with S_6 during FPLC ion exchange chromatography. This material could be separated from the main component by reversed-phase chromatography (not shown).

The elution profiles of *S*-glycoproteins were precisely reproducible. Changing to a different Mono *S* column resulted in slightly different elution positions of the *S*-glycoproteins, but the elution profiles and relative elution positions were comparable with those obtained from the previous column. The profiles obtained using flowers from the same plant in different experiments or flowers from different clonal plants bearing the same *S*-alleles were identical. The elution profiles obtained from style extracts of plants heterozygous for *S*-alleles always showed peaks corresponding exactly to the elution positions for each of the two *S*-glycoproteins. The results of analyses of stylar proteins of plants from an $S_1S_3 \times S_6S_7$ cross are presented in Table 1. There was an exact correspondence between

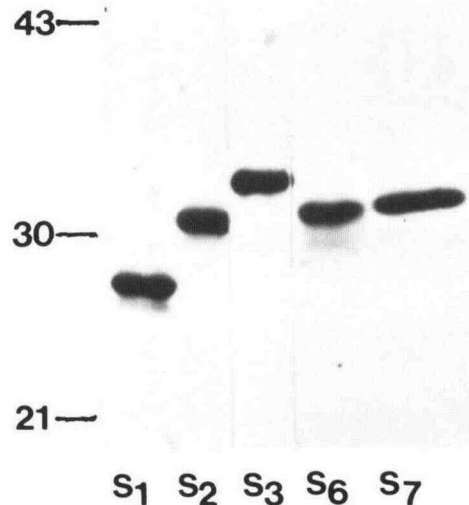


Figure 3. Separation of Isolated *S*-Glycoproteins from Styles of *N. alata* by SDS-PAGE.

A single major component was detected for each *S*-glycoprotein with apparent molecular mass in the range of 28 kD to 34 kD. The S_6 -glycoprotein preparation consistently contained a minor component, 30 kD.

Table 1. S-Glycoproteins Identified in Styles of Progeny of the Cross $S_1S_3 \times S_6S_7$

Plant No.	S-Genotype Identified by Breeding	S-Genotype Identified by RFLPs ^a	S-Glycoproteins Identified by FPLC
1	S_3S_7	S_3S_7	3, 7
3	S_1S_7	S_1S_7	1, 7
5	S_1S_6	S_1S_6	1, 6
6	S_3S_7	S_3S_7	3, 7
7	S_3S_6	S_3S_6	3, 6
8	S_1S_6	S_1S_6	1, 6
9	S_3S_7	S_3S_7	3, 7
15	S_3S_6	S_3S_6	3, 6
16	S_3S_6	S_3S_6	3, 6
17	S_1S_6	ND ^b	1, 6
20	S_3S_7	S_3S_7	3, 7
21	S_3S_7	S_3S_7	3, 7
25	S_1S_7	S_1S_7	1, 7
30	S_3S_7	S_3S_7	3, 7

Assignment of S-genotypes to progeny of the cross $S_1S_3 \times S_6S_7$ was performed by breeding tests, RFLP mapping, and style protein analysis by cation exchange chromatography.

^a Data from Anderson et al. (1989).

^b ND, not determined.

the peak position and S-genotype, as determined by breeding tests, for each of 14 individual heterozygous offspring from an $S_1S_3 \times S_6S_7$ cross described here (Table 1) and for a further 20 heterozygous and homozygous plants from an $S_1S_3 \times S_2S_3$ cross and from S_1S_3 and S_2S_6 selfings (not shown).

The yields of style S-glycoprotein from different alleles varied. The S_2 - and S_6 -glycoproteins were present in extracts at relatively high concentrations, approximately 8 μg to 10 μg of protein per style; however, the S_3 -glycoprotein was less abundant, and only about 0.5 μg to 1 μg of protein per style was recovered. The total amount of protein extracted from styles of *N. tabacum*, a self-compatible plant, was similar to that extracted from *N. alata* styles (Figure 2f), but no proteins were resolved under the conditions which gave good separation for proteins of *N. alata* styles (Figure 2, a to e).

Amino-Terminal Amino Acid Sequence of the Style S-Glycoproteins

Reversed-phase HPLC was used as a final purification step before amino acid sequencing. During reversed-phase HPLC, the S-glycoproteins eluted as single peak fractions at characteristic elution times. The S_1 - and S_7 -glycoproteins were of particular interest, as they were not well separated by cation exchange (Figure 2, a, e) or reversed-phase chromatography (Figure 4). They could only be differentiated with certainty using a combined cation ex-

change and SDS-PAGE analysis. The amino-terminal sequences for the S-glycoproteins purified in this study are shown in Table 2 and for comparison, data published on S_2 - and S_{F11} -glycoproteins (Mau et al., 1986) are also included.

DISCUSSION

Using two-dimensional gel electrophoresis and cation exchange chromatography, we have identified and isolated S-allele-specific glycoproteins for five S-genotypes tested (Figures 1 and 2). During cation exchange chromatography, S-glycoproteins corresponding to a particular S-genotype eluted at characteristic positions that were diagnostic of the S-genotype. These proteins were consistently identified in plants from several populations segregating for the S-alleles. The same plants were tested for their S-genotype, using conventional breeding analysis and restriction fragment length polymorphism (RFLP) analysis of leaf DNA (Bernatzky, Anderson, and Clarke, 1988; Anderson et al., 1989). The strict correlation between assignment of an S-genotype by breeding analysis, RFLP analysis, and the presence of style glycoproteins specific for that genotype suggest that these glycoproteins are, in fact, the style-specific products of the S-gene.

The isolation method described in this paper was, in contrast to the method previously described for the isolation of S_2 -glycoprotein (Anderson et al., 1986), generally applicable, more rapid (1 day compared with 3 days), and gave higher yields [10 μg compared with 2.5 μg of S_2 -

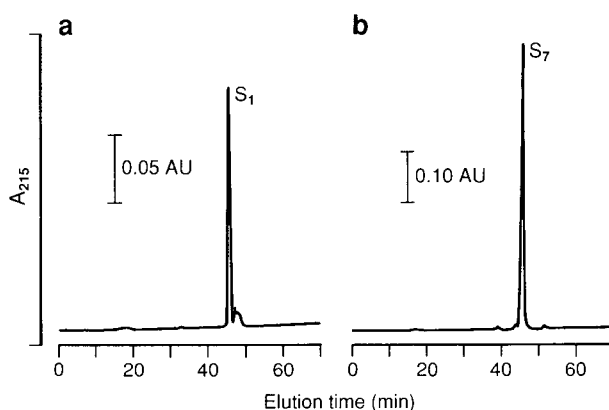


Figure 4. Reversed-Phase HPLC Elution Profiles of S-Glycoproteins.

S-Glycoproteins were purified by cation exchange FPLC as described in Methods. S_1 -glycoprotein [3 μg , (a)] and S_7 -glycoprotein [7 μg , (b)] were loaded onto a reversed-phase column and eluted with a gradient, ranging from 0% to 60% acetonitrile in 0.1% aqueous TFA, as described in Methods.

Table 2. Comparison of Amino-Terminal Sequences of *N. alata* S-Glycoproteins

	1	5	10	15
S ₁	D	F E Y L	Q L V L	T W P A S F
S ₂	A	F E Y M	Q L V L	T W P I T F
S ₃	A	F E Y M	Q L V L	Q W P A A F
S ₆	A	F E Y M	Q L V L	Q W P T A F
S ₇	A	F E Y M	Q L V L	Q W P T A F
S _Z	D	F D Y M	Q L V L	T X P A S F
S _{F11}	D	F E Y L	Q L V L	T W P A S F

Amino acids are identified by their single letter code, with X indicating residues that could not be clearly assigned. Sequences for S₂, S₆, S_Z, and S_{F11} have been published (Mau et al., 1986) and are included for comparison.

glycoprotein per style (genotype S₂S₃). The method took advantage of the fact that the S-glycoproteins are very basic in nature. They were separated from the majority of stylar proteins by fractionation with (NH₄)₂SO₄, followed by resolution from each other and the remaining basic proteins by FPLC cation exchange chromatography. This procedure gave good separation for the S₂-, S₃-, and S₆-glycoproteins. The separation of proteins that eluted close to each other (for example, S₁- and S₇-glycoproteins) could be further improved by adjusting the pH of the buffer system and the slope of the salt elution gradient (data not shown).

Identification of style glycoproteins corresponding to the S₁- and S₇-alleles have not been reported previously. Using isoelectric focusing, Bredemeijer and Blaas (1981) were unable to identify any style protein corresponding to the S₇-allele, and suggested that the S₇-protein might be present in very low amounts and/or stain poorly with Coomassie blue. In our experience, it was not possible to ascribe allele-specific proteins to the S₃-, S₆-, and S₇-alleles on one-dimensional SDS-PAGE; even two-dimensional gel electrophoresis left uncertainties in assigning proteins to the S₂-, S₆-, and S₇-alleles since the respective S-glycoproteins behaved similarly (Figure 1). Only after FPLC cation exchange chromatography were all S-glycoproteins detected as single, allele-specific peaks (Figure 2). S₁- and S₇-glycoproteins were similar with respect to charge and hydrophobicity and eluted in similar positions during both cation exchange and reversed-phase chromatography (Figures 2 and 4); however, they were distinguished by differences in apparent molecular weight (Figure 3).

The S₂-allele differed from the other S-alleles in that two protein peaks were consistently found in all samples prepared from style extracts of plants bearing the S₂-allele. Both peaks could also be resolved in an electrophoretically homogeneous S₂-glycoprotein preparation purified by concanavalin A-affinity chromatography (Anderson et al., 1986). Material from the two peaks ran in identical positions on one-dimensional SDS-PAGE and reacted strongly

with a highly S₂-specific peptide antibody. The finding that rechromatography (up to three times) of either isolated peak under identical conditions resulted in the appearance of the both peaks suggests an equilibrium between two forms, possibly different aggregation states.

The amounts of S-glycoproteins present in style extracts varied considerably for different S-alleles. For example, the yields of S₃-glycoprotein were consistently lower than for the S₂- or S₆-glycoproteins. In a previous study (Bredemeijer and Blaas, 1981), this difference in apparent amounts of S-glycoprotein in the styles was attributed to different solubilities in the extraction buffer and different amino acid compositions of the S-glycoproteins, which can influence the staining intensity on gels. The present study indicates that differences in amounts of S-glycoproteins recovered are likely to be the result of differential expression of S-alleles. The absorbance at 280 nm (Figure 2) is likely to reflect true differences in the amount of S-glycoproteins rather than their amino acid composition, as the S₂-, S₃-, and S₆-glycoproteins all contain approximately equal numbers of aromatic amino acids (S₃: Phe-8, Tyr-8, Trp-4; S₂: Phe-13, Tyr-6, Trp-4; S₆: Phe-9, Tyr-9, Trp-5; Anderson et al., 1989). Further quantitative studies of S-glycoproteins might lead to molecular insights into phenomena described as "pseudocompatibility" as well as "strength of alleles."

The amino-terminal amino acid sequence analysis confirmed and extended the data on S₂-, S₆-, S_Z-, and S_{F11}-glycoproteins from *N. alata* (Mau et al., 1986). The amino-terminal sequences of all S-glycoproteins show a high degree of homology. Fifteen amino-terminal amino acids are identical in S₁- and S_{F11}-glycoproteins, 9 amino-terminal residues are identical in S₂-, S₃-, S₆-, and S₇-glycoproteins, and further matching amino acid sequences of various length occur within the 15 amino-terminal amino acids. The amino-terminal sequences of the S₂-, S₃-, and S₆-glycoproteins match exactly the sequences derived from nucleotide sequences of the corresponding S-allele-specific cDNAs (Anderson et al., 1989).

The described method for rapid purification of S-glycoproteins from *N. alata* has allowed us to use isolated S-glycoproteins for testing biological activity in a pollen growth assay (Jahnen, Lush, and Clarke, 1989) and also to initiate physicochemical and x-ray crystallographic studies of the three-dimensional structure of these molecules.

METHODS

Sources of Defined S-Genotypes of *Nicotiana alata*

Self-incompatible clones of *N. alata* Link and Otto of genotypes S₁S₃ and S₂S₃ were obtained from Dr. Pandey, Palmerston North, New Zealand, and genotype S₆S₇ was from Dr. Bredemeijer, Wageningen, The Netherlands. The plants were bud self-pollinated to obtain homozygous S₁S₁, S₂S₂, S₃S₃, S₆S₆, and S₇S₇

plants. Styles (in this case, style refers to stigma and style) were removed from fully mature flowers (not emasculated) and processed immediately or kept at -70°C until extraction.

Two-Dimensional Gel Electrophoresis

Protein extracts were prepared according to Colas des Francs, Thiellement, and De Vienne (1985) with minor modifications. Twenty styles were ground to a powder in liquid nitrogen using a mortar and pestle. The powder was extracted with 2 ml of a solution containing 0.139 M SDS (4%), 0.72 M β -mercaptoethanol (5%), 0.146 M sucrose (5%), and 20 mg of Polyclar AT. The mixture was heated in boiling water for 3 min and then centrifuged twice (30,000g, 10 min, 4°C). Protein was precipitated by adding pre-cooled acetone (10 mM β -mercaptoethanol, final concentration 80%, -20°C , 1 hr), and the precipitate was collected by centrifugation (30,000g, 20 min, 4°C) and resuspended in 0.5 ml of lysis buffer (O'Farrell, Goodman, and O'Farrell, 1977). Two-dimensional gel electrophoresis was performed according to the non-equilibrium method (NEPHGE) described by O'Farrell, Goodman, and O'Farrell (1977); the second-dimension gels contained 12.5% acrylamide. On the first-dimension tube gels, 100 μg of total protein were loaded, whereas only 50 μg from the S_2S_2 styles were applied since S_2 -glycoprotein was so abundant in crude extracts that it disturbed the separation. NEPHGE was run for 2000 V-hr and the first-dimension rod gels were equilibrated in SDS containing buffer for 30 min. Second-dimension SDS-PAGE was run for an additional 15 min after the bromophenol blue had left the gel. Gels were sequentially stained with Coomassie blue and silver nitrate (Nielsen and Brown, 1984).

Purification of S-Gene-Associated Glycoproteins

Styles were ground to a powder under liquid nitrogen using a mortar and pestle. The powder was extracted with buffer (10 ml per gram of styles, frozen weight; 0.1 M Tris/HCl, pH 7.8, containing 14 mM β -mercaptoethanol and 0.1 gram per gram of tissue Polyclar AT and Dowex 1-X2 Cl). The mixture was stirred for 10 min on ice, filtered through Miracloth (Behring Diagnostics), and centrifuged (20,000g, 20 min, 4°C). The supernatant was adjusted to 60% fractional saturation by adding solid $(\text{NH}_4)_2\text{SO}_4$ and stirred on ice for 30 min. The solution was centrifuged and the $(\text{NH}_4)_2\text{SO}_4$ concentration of the supernatant was increased to 95% fractional saturation. The precipitate was collected by centrifugation and dissolved in 50 mM acetic acid/NaOH buffer, pH 5.0, containing 14 mM β -mercaptoethanol. The solution was desalted on Sephadex G 25 (Pharmacia, Uppsala, Sweden), equilibrated in the same buffer. All sulfate-free fractions were pooled and loaded onto a cation exchange column (Mono S 5/5, Pharmacia) previously equilibrated in acetate buffer. Bound protein was eluted with a salt gradient 0 to 0.5 M NaCl in the same buffer delivered by an FPLC chromatography system (Pharmacia). For analytical runs, 100 μg of style proteins (typically extracted from 3 to 5 styles) were loaded and eluted with 10 ml of buffer, at a flow rate of 1 ml/min. Styles of genotypes S_1S_3 , S_2S_3 , and S_6S_7 were extracted to purify preparative amounts of the respective S-glycoproteins. Up to 10 mg of style proteins (equivalent to 300 styles) were loaded and eluted with 30 ml of buffer at a flow rate of 2 ml/min. Protein elution was monitored at 280 nm and fractions were analyzed using SDS-PAGE (Laemmli, 1970). Gels were

stained sequentially with Coomassie blue and with silver nitrate. Protein was measured according to Bradford (1976) using BSA as a standard.

Protein Gel Blotting

Isolated S-glycoproteins were transferred electrophoretically onto nitrocellulose (BA 83, Schleicher & Schüll) and probed with antibody using the indirect peroxidase technique as described (Jahnen and Hahlbrock, 1988). Primary (sheep) antibody directed against a synthetic S_2 -peptide (Anderson et al., 1989) was affinity-purified and used at 20 $\mu\text{g ml}^{-1}$; secondary, peroxidase-labeled antibody (rabbit anti-sheep IgG, Bio-Rad) was diluted 2000-fold and visualized using the stain, 4-chloronaphthol.

N-Terminal Sequencing of S-Glycoproteins

The S-glycoproteins were further purified by re-running the corresponding peak fractions on the Mono S column and subsequently by reversed-phase HPLC. Chromatography was performed using a Hewlett-Packard model 1090A liquid chromatograph fitted with a model 1040A diode array detector. S-Glycoproteins (typically 100 μg in 1 ml of acetate buffer containing NaCl in concentrations between 0.22 M and 0.43 M) were loaded, at a flow rate of 2 ml/min, onto a Brownlee RP-300 column (4.6 \times 30 mm), which had been previously equilibrated with aqueous 0.1% (v/v) TFA. The column was developed, at a flow rate of 1 ml/min, with a linear 60-min gradient from 0% to 100% B, where secondary solvent B was 60% acetonitrile/40% aqueous 0.09% (v/v) TFA. Automated amino acid sequence analysis of S-glycoproteins was performed using an Applied Biosystems Sequencer (model 477A) as described (Simpson et al., 1987) with minor modifications (Begg and Simpson, 1988).

ACKNOWLEDGMENTS

We would like to acknowledge other members of the group for stimulating discussion and particularly Dr. J.R. Woodward and Dr. A. Bacic for critically evaluating the manuscript. Dr. R. Bernatzky's contribution to the background of this work is gratefully acknowledged. Mr. T. Orpin and Dr. M. Anderson made valuable contributions by providing plants with defined S-genotypes, Mr. B. McGinness and Ms. S. Mau by collecting plant material. This work was supported by a post-doctoral fellowship granted to W.J. by the Deutsche Forschungsgemeinschaft.

Received March 13, 1989.

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