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Purification and N-terminal sequencing of style glycoproteins associated with self-incompatibility in *Petunia hybrida*

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Abstract

We report isolation and N-terminal amino acid sequencing of three style glycoproteins, which segregate with three S (self-incompatibility) alleles of *Petunia hybrida*. The S-glycoproteins were expressed mainly in the upper part of the pistil and showed an increasing concentration during flower development. The glycoproteins were purified by a combination of ConA-Sepharose and cation exchange fast protein liquid chromatography. The amount of S-glycoproteins recovered from style extracts varied from 0.5 to 1.6 μg per style, which was 40–60% of the amount recovered by a simplified analytical method. N-terminal amino acid sequences of S_1-, S_2- and S_3-glycoprotein showed homology within the fifteen amino terminal residues. These amino acid sequences were compared with the previously published sequences of S-glycoproteins from *Nicotiana alata* and *Lycopersicon peruvianum*.

Introduction

In order to maintain a large pool of genetic variability in the population, living organisms have developed several mechanisms to promote outbreeding. Among these, flowering plants have evolved a mechanism known as self-incompatibility. Self-incompatibility systems occur in more than half of all plant families and in most species examined the phenomenon is under the control of a single gene (S-gene) with multiple alleles; fertilization is prevented if the pollen expresses the same S-allele(s) as the pistil (for reviews see [4, 18]).

Petunia hybrida is a member of the family Solanaceae and has a gametophytically controlled self-incompatibility system [12]. Self-pollen tubes are selectively inhibited as they grow through the intercellular matrix of the style, suggesting an interaction between this intercellular material and the growing pollen tubes. Style proteins associated with the S-alleles and probably involved in this recognition process have been characterized in *Prunus avium*, *Nicotiana alata* and *Lycopersicon peruvianum* [14, 8, 15]. *Brassica oleracea* and *B. campestris*, which display sporophytic self-incompatibility, similarly express S-genotype-specific stigma proteins [19, 7]. cDNAs encoding these proteins have been isolated from *N. alata* [1, 2] and *B. oleracea* [16, 17]. All these proteins

are glycoproteins which mainly have basic isoelectric points and relative molecular masses (M_r) of 24000 to 65000.

P. hybrida was one of the first species used in self-incompatibility research. Antigenic style components, corresponding to particular S-alleles, were demonstrated by Linskens [11]. More recently Kamboj and Jackson [9] correlated electrophoretic differences between pistil proteins with the S-genotype of *P. hybrida* plants. Here we report the purification of S-glycoproteins from *P. hybrida* styles, which segregate with particular S-alleles. N-terminal amino acid sequences of S_1-, S_2- and S_3-glycoproteins are also presented.

Materials and methods

Sources of defined S-genotypes of Petunia hybrida

Self-incompatible clones of *Petunia hybrida* were kindly provided from the collection maintained at the Katholieke Universiteit Nijmegen, Netherlands [13] and were crossed manually to generate a collection of plants bearing different combinations of S-alleles. Pure lines were maintained by vegetative propagation. All plants were grown in temperature-controlled glasshouses with supplementary lighting from October to April. Clones used were the following (with the defined S-genotype in parentheses): W43 (S_1S_1), Ka3 (S_2S_2), W166H (S_2S_3) and T2U (S_3S_3) from the collection of Nijmegen; B23M-1, B23M-2, B2X1 and B22 (S_2S_2), B21 (S_1S_2), B31 (S_1S_3) and B23, BX13 and B3X3 (S_2S_3) from our own breedings (see Results). The S-genotypes of these plants were determined by breeding tests.

Mature flowers were harvested daily before anther dehiscence and styles[1] were collected aseptically with the aid of two pairs of tweezers. The styles were processed immediately or frozen by immersion in liquid nitrogen and stored at −20 °C until use.

[1] The term 'style' is used here to indicate only the upper part (ca. 8 mm) of the style, including the stigma, since this segment contained most S-glycoproteins, as is shown later on.

Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were performed on prepacked ultrathin gels with a PhastSystem electrophoresis apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden). For SDS-PAGE 10–15% gradient gels were used, supplied with a stacking zone (discontinuous buffer system [10]) and IEF was run over a pH range from 3 to 9. Selective two-dimensional gel electrophoresis (of the basic proteins only) was done on the same apparatus according to a modified method of Mau *et al.* [15]. Gels were stained with silver nitrate according to the manufacturers' instructions. Molecular weight markers (Pharmacia, low molecular weight kit proteins) included phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α lactalbumin (14.4 kDa). Marker proteins for IEF (IEF-MIX 3.5–9.3, Sigma, St. Louis, MO, USA) ranged from pI 3.55 to 9.3.

Extraction and isolation procedure

Extracts of styles were prepared according to Anderson *et al.* [1]. Frozen styles were ground in liquid nitrogen using a mortar and pestle, and subsequently extracted in buffer (10 ml per gram of styles; 0.1 M Tris-HCl pH 8.5, 0.1 M NaCl, 1 mM $CaCl_2$, 10 mM EDTA, 1 mM DTT), supplemented immediately before use with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% (w/w) insoluble polyvinyl pyrrolidone (Polyclar AT, Sigma). After centrifugation (13000 g, 15 min, 4 °C), the supernatant was passed through a Sephadex G-25 column (2.6 cm × 12.2 cm), equilibrated with 20 mM sodium phosphate buffer pH 7.1, 0.1 M NaCl (PBS). The protein fraction was collected and applied to a Concanavalin A (ConA)-Sepharose (Pharmacia) column (1.3 cm × 6 cm), equilibrated with the same PBS. The column was washed with 10 volumes PBS and bound proteins

were eluted with 0.2 M methyl-α,D-mannoside in PBS. After dialysis against Milli Q water (Millipore, Bedford, MA, USA) at 4 °C, the fraction desorbed from ConA-Sepharose and containing the S-glycoproteins was freeze-dried and dissolved in 20 mM sodium phosphate buffer pH 7.5. The final purification was performed on an FPLC cation exchange column, Mono S (Pharmacia), equilibrated with the same phosphate buffer. Proteins were eluted with a salt gradient of 0 to 0.3 M NaCl in phosphate buffer, flow rate 1 ml/min, and fractions were collected manually. Purity of the S-glycoprotein fractions was monitored by SDS-PAGE. Protein concentration was assayed by the method of Bradford [3] with bovine serum albumin as a standard. For analytical purposes (e.g. determination of the amount of S-glycoprotein per style), twenty pistils (stigma + style, the ovary removed) were extracted in 1 ml of extraction buffer, the homogenates centrifuged (5 min, 10000 *g*) at room temperature and the supernatants equilibrated with 20 mM sodium phosphate buffer pH 7.5 by passage through a Sephadex G-25 column. The samples were applied immediately on Mono S, without prior separation on the affinity matrix.

High-resolution chromatofocusing was performed on a Mono P column (FPLC, Pharmacia) in the range pH 9.4–8.0. Elution conditions were: start buffer 0.025 M diethanolamine-HCl pH 9.4, elution buffer 1.0 ml Pharmalyte 8–10.5, 5.2 ml Polybuffer 96 (Pharmacia) pH 8.0; flow rate 1 ml/min. Fractions (1 ml) were collected automatically and the pH of each fraction was measured.

Amino acid analysis

Isolated proteins were hydrolysed in 5.7 M HCl under vacuum at 110 °C for 22 h, followed by automated analysis according to the method of Spackman *et al.* [21] on a sulphonated polystyrene column (4150 Alpha-amino acid analyzer, LKB, Bromma, Sweden). Cysteine and cystine were analysed as cysteic acid after hydrolysis in 5.7 M HCl in the presence of dimethyl sylphoxide [22]; tryptophan was measured after hydrolysis in 4 M methane sulphonic acid [20], using a borate buffer as third eluent. After reaction with ninhydrine at 130 °C, the separated amino acids were detected photometrically at 570 nm (440 nm for proline). The amount of the amino sugars was determined after a 4 h hydrolysis with 4 M HCl at 100 °C and analysed on the same column by elution with 0.35 M citrate buffer pH 5.28.

N-terminal amino acid sequencing of S-glycoproteins

Automated sequence analysis of purified S-glycoprotein was performed using a 477A Protein Sequencer (Applied Biosystems, Foster City, CA, USA) with on-line detection of the phenylthiohydantoin (PTH) amino acid derivatives by a 120A Analyzer (Applied Biosystems). Proteins were not reduced or alkylated before sequence analysis so that cysteine residues were not detectable.

Results

Cosegregation of style S-glycoproteins with three S-alleles of P. hybrida

From intercrossings between the original homozygous acquisitions S_1S_1, S_2S_2 and S_3S_3, the heterozygotes S_1S_2, S_1S_3 and S_2S_3 (B23) were produced. As the self-incompatibility reaction is not always complete [12], two other S_2S_2 homozygotes (B23M-1 and B23M-2) could be obtained by intercrossing the original S_2S_3 acquisition with a plant selected from a commercial seed sample and carrying the S_2-allele. An accidental weakening of self-incompatibility resulted similarly in a fourth S_2S_2 homozygote (B2X1) by intercrossing the new homozygote B23M-1 with the original homozygote and bud self-pollination of this original acquisition provided another S_2S_2 homozygote (B22). Two other S_2S_3 heterozygotes (BX13 and B3X3) were produced by crossing two of the S_2S_2 homozygotes (B23M-1 and B23M-2) with

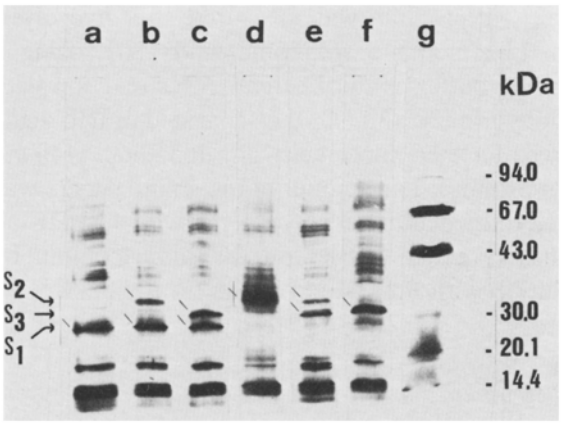

Fig. 1. SDS-PAGE of style extracts from mature flowers of *P. hybrida* homozygous for the S-alleles (lanes a, d and f) and from the heterozygous progeny, resulting from different intercrossings (lanes b, c and e). The position of the S-glycoproteins is indicated by arrows. Lane a, S_1S_1; b, S_2S_2 (Ka3) × S_1S_1; c, S_3S_3 × S_1S_1; d, S_2S_2 (Ka3); e, S_2S_2 (B23M-1) × S_3S_3; f, S_3S_3. Molecular weight markers were run in lane g.

the S_3S_3 acquisition. The exact S-genotypes of these plants were determined by breeding tests. A total of 26 healthy plants were selected from these nine intercrossings and style extracts of these plants were analysed electrophoretically together with the original acquisitions. The most promising plants from each intercrossing – considering the strength of the self-incompatibility reaction, flowering capacity and general health – were vegetatively propagated.

Extracts from styles of mature flowers were prepared and examined by gel electrophoresis. Confirming the observations of Kamboj and Jackson [9], we found differences in the style protein patterns from self-incompatible clones of *P. hybrida*, which correlated with their defined S-genotype. This was apparent on SDS-polyacrylamide gels (Fig. 1) and in selective two-dimensional gels (not shown); detection of these low molecular weight (27–33 kDa), basic proteins on the gels was relatively easy because of their relative abundance in the extracts. The progeny resulting from different intercrossings showed genetic segregation of the putative S-glycoproteins with the three S-alleles involved. The protein patterns from styles homozygous for the S-alleles revealed only one component which was specific for a particular S-allele, whereas two components were always present in extracts from heterozygous plants (Fig. 1). The observed one-to-one relationship between S-allele and S-glycoprotein was found for all 30 plants examined, but the relative amount of these proteins in style extracts varied for different plants.

Some physical characteristics of S-glycoproteins

The S-glycoproteins differed slightly in molecular weight (S_1 27000, S_2 33000, S_3 30000, as determined by SDS-PAGE) and in isoelectric point (S_1 8.7, S_2 8.9, S_3 9.3, using previously purified S-glycoproteins). The pI values obtained were slightly higher than the values reported previously [9], using IEF and two-dimensional gel electrophoresis. The S-glycoproteins were detected in the female reproductive tissues of the plant only and were not found in extracts from leaves, roots, corolla, calyx and pollen grains, nor in extracts from pistils of a self-compatible cultivar (not shown).

Distribution of S-glycoproteins in the pistil and expression in different stages of flower development

Isoelectric focusing (pH 3–9) was used to demonstrate both distribution and developmentally regulated expression of S-glycoproteins in the female reproductive tissues. The different S-glycoproteins focused almost at the same position on these gels (close to the cathode), but they were better separated from the other proteins in the extracts (as compared to SDS-PAGE), since most of these were in the acidic to neutral region of the gel.

Extracts were prepared from segments, cut at different positions along the length of the pistil, and run on an IEF gel. Comparison of the protein patterns revealed a heterogenous distribution of the S-glycoproteins in the female tissues (Fig. 2). A relatively high concentration was present in the stigma and in the uppermost part of the style;

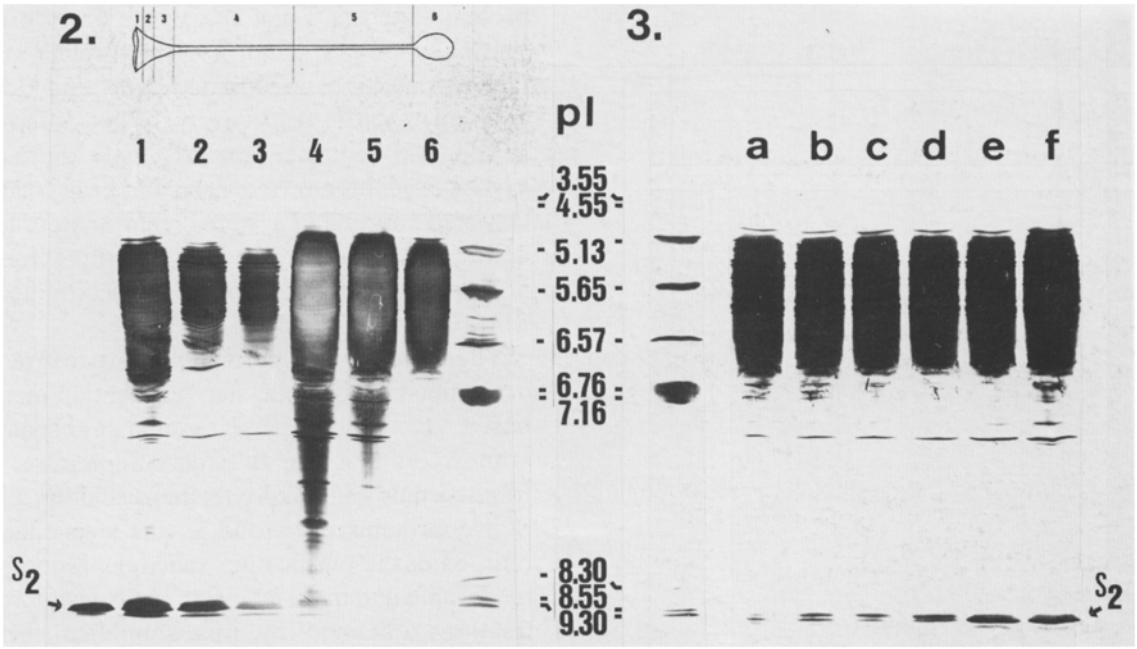

Fig. 2. Distribution of S-glycoproteins in pistils of *P. hybrida*. IEF (pH 3–9) of extracts from segments of mature pistils (S_2S_2, clone B23M-1), prepared by cutting four pistils with a razor blade into six segments as indicated in the upper panel. In the first lane purified S_2-glycoprotein was run; the last lane contained pI marker proteins. The first two 1 mm pistil segments included the stigma, the third (2 mm), fourth and fifth (each 12 mm) were style sections, and the sixth comprised the ovary (only one ovary was taken for preparing the extract). The S_2-glycoprotein focused in the high pI region of the IEF gel, as indicated.

Fig. 3. Expression of style S-glycoproteins from *P. hybrida* (S_2S_2, clone B23M-1, cf. Fig. 2) in different stages of flower development, as shown by IEF (pH 3–9). Lanes a–d, flower buds of 2, 3, 4 and 5 cm length respectively; lane e, flowers just before anthesis; lane f, open flowers. In the first lane pI marker proteins were run. The position of the S_2-glycoprotein is indicated. Extracts were prepared from three styles. The same pattern was obtained when extracts, containing equal amounts of total protein, were applied to the gel.

decreasing amounts were found in the lower parts of the style and in the ovary extract only a faint band was detected. The same distribution pattern was obtained for different S-genotypes (not shown).

Examination of style extracts from flowers at successive developmental stages revealed an increase in S-glycoprotein concentration during flower maturation (Fig. 3). The same pattern was found whether the comparison was made on a per style or per total protein basis. Analytical FPLC analyses (see below) showed that style extracts of flowers just before anthesis contained 67% of the S-glycoprotein content of style extracts from fully opened flowers (comparing the peak areas); 37% and 23% of the maximum value was found in flower buds 5 and 4 cm long respectively. Style extracts of earlier stages of flower development contained less than 6% of the S-glycoprotein content of open flowers.

Purification of three S-glycoproteins from styles of P. hybrida

Style S-glycoproteins corresponding to the S_1-, S_2- and S_3-alleles were purified using a combination of affinity and FPLC cation exchange chromatography. The affinity matrix ConA-Sepharose retained about one fifth of the total protein content in the extracts, including the S-glycoproteins. On SDS-PAGE, no protein

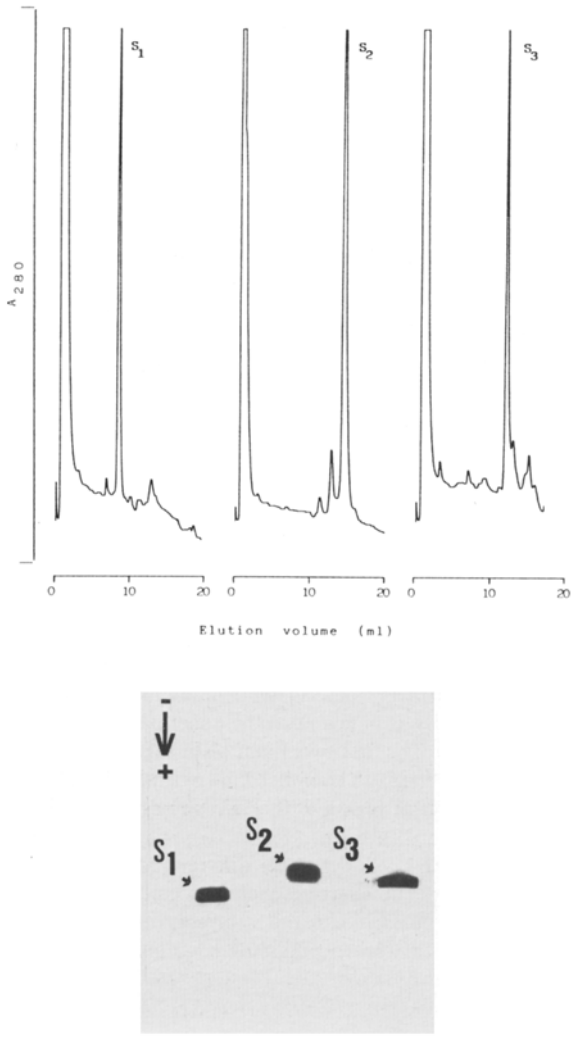

Fig. 4. Purification of style S-glycoproteins from mature flowers of *P. hybrida*. A. (top). FPLC elution profiles of style extracts, homozygous for the S-alleles. Extracts were prepared from 20 styles of plants, homozygous for the S_1-, S_2-(B23M-1) and S_3- allele and run on a cation exchange column; a linear gradient (0 to 0.3 M NaCl in 20 ml) was applied to the column, starting after 2 min. The three S-glycoproteins eluted at characteristic positions as indicated. B. (bottom). SDS-PAGE of three purified S-glycoproteins, isolated from styles of plants, homozygous for the S-alleles (cf. 4A). The position of the S-glycoproteins is indicated by arrows.

bands which could be attributed to any of the S-glycoproteins were apparent in the unbound fraction. Figure 4A shows the cation exchange elution profiles of style extracts from mature flowers homozygous for different S-alleles. S_1-glycoprotein eluted at a salt concentration of 0.10 M NaCl (8.6 ml), S_2- and S_3-glycoproteins at salt concentrations of 0.20 M (15.0 ml) and 0.16 M (12.3 ml) NaCl respectively. For N-terminal amino acid sequence analysis, only the homozygous S-genotypes were used and peak fractions obtained on FPLC were rechromatographed under the same conditions. The resulting S-glycoprotein fractions were pure, as determined by SDS-PAGE (Fig. 4B).

The yield of the purification procedure was determined using a one-step purification method, based on FPLC cation exchange chromatography. As the use of ConA-Sepharose contributed only marginally to the resolution of the S-glycoproteins on Mono S, this step could be omitted in the purification schedule, rendering it more rapid and more efficient. The S-glycoprotein fractions obtained by this simplified method contained less than 5% of contaminating material. The elution profiles of different S-glycoproteins were the same by using the extended or the simplified method. Style extracts from flowers heterozygous for S-alleles always showed two major peaks, eluting at positions characteristic for each of the S-glycoproteins. The yield for the extended purification procedure was 40 to 60% of the yield for the one-step method, depending on the particular S-glycoprotein isolated.

For different self-incompatible clones, the amount of S-glycoproteins recovered from style extracts varied. Up to 4 μg of S-glycoprotein per style were recovered from flowers of clone B23M-1 (S_2S_2). For eight other clones examined, between 1.2 and 3.5 μg per style were isolated; the original acquisitions from Nijmegen generally contained less S-glycoproteins per style than the plants resulting from our own breeding.

Amino acid composition and N-terminal sequence of S-glycoproteins

Table 1 shows the results of the amino acid analyses on purified S_2- and S_3-glycoproteins (the S_1-glycoprotein was not investigated). Both proteins were rich in threonine and lysine and rela-

Table 1. Amino acid composition (mol %) of S-glycoproteins from *P. hybrida* styles.

	S_2	S_3
Asx	10.41	9.17
Thr	8.90	9.25
Ser	5.22	3.70
Glx	8.25	10.32
Pro	5.96	5.84
Gly	5.68	4.22
Ala	5.21	4.91
Cys	5.34	5.10
Val	4.02	3.08
Met	1.64	1.68
Ile	4.47	4.16
Leu	7.59	9.08
Tyr	3.64	4.44
Phe	5.43	5.14
His	2.16	2.91
Lys	8.59	9.20
Arg	4.42	5.06
Trp	1.01	nd
GlcNAc[a]	2.06	1.71
GalNAc[b]	0.00	0.00

[a] N-acetyl glucosamine; [b] N-acetyl galactosamine.

tively poor in valine and methionine. A remarkable high cysteine/half cystine content was noticed, corresponding to an estimated number of 9–11 residues per protein. Generally, the relative amount of most amino acids was very similar for both proteins, with only minor differences. N-acetyl glucosamine was found in equivalent amounts in both proteins (approx. 2 mol%), while N-acetyl galactosamine was absent in both.

The N-terminal amino acid sequence analyses on S_1-, S_2- and S_3-glycoproteins allowed determination of about twenty residues per protein. Yields at each cycle of the Edman degradation are shown in Fig. 5A. During the sixteenth step no PTH amino acid signal was detected for all three proteins. The N-terminal sequences of the three glycoproteins are shown in Fig. 5B. A considerable level of similarity at the N-terminal region of these style proteins was found. Nine of the first fifteen positions yielded identical residues for the three proteins. An invariant stretch of seven residues was present from position 6 to 12. At the other positions, except for the N-terminus, always two of the three residues could be aligned. Moreover, all but one of the variations observed in the first 15 positions could be explained on the DNA level by single base pair substitutions (two such substitutions would be needed at position 4). No indication of a potential glycosylation site was found in the sequence determined.

Discussion

This paper describes isolation and partial chemical analysis of three style S-glycoproteins, associated with self-incompatibility in *P. hybrida*. In addition, segregation, distribution in the pistil and development of S-glycoproteins were shown. The purification procedure took advantage of both the glycosylation and basic nature of these proteins. The amino acid sequence of the first 20 N-terminal residues revealed a high degree of similarity between the S_1-, S_2- and S_3-glycoproteins. The N-terminal sequences of the *P. hybrida* S-glycoproteins are also homologous with the N-terminal S-glycoprotein sequences already reported for two other solanaceous plants, *N. alata* and *L. peruvianum*, both of which express gametophytic self-incompatibility.

On crossing different *P. hybrida* clones with defined S-genotypes, it was found that S-gene-associated glycoproteins from styles cosegregated exactly with self-incompatibility behaviour, as determined by breeding. These S-glycoproteins were major style components (6 to 18% of the total protein content) with an M_r of 27000–33000. The pIs determined by chromatofocusing (8.7, 8.9 and 9.3) were slightly higher than the pI values published by Kamboj and Jackson (8.3, 8.5 and 8.7 [9]), as determined by gel electrophoresis. The association between S-glycoproteins and self-incompatibility was further supported by the main location of these proteins at the site of pollen tube arrest (upper part of the pistil) and by their increasing expression during flower development (self-incompatibility is only expressed in the later stages of flower maturation [6]). The same patterns of spatial and tem-

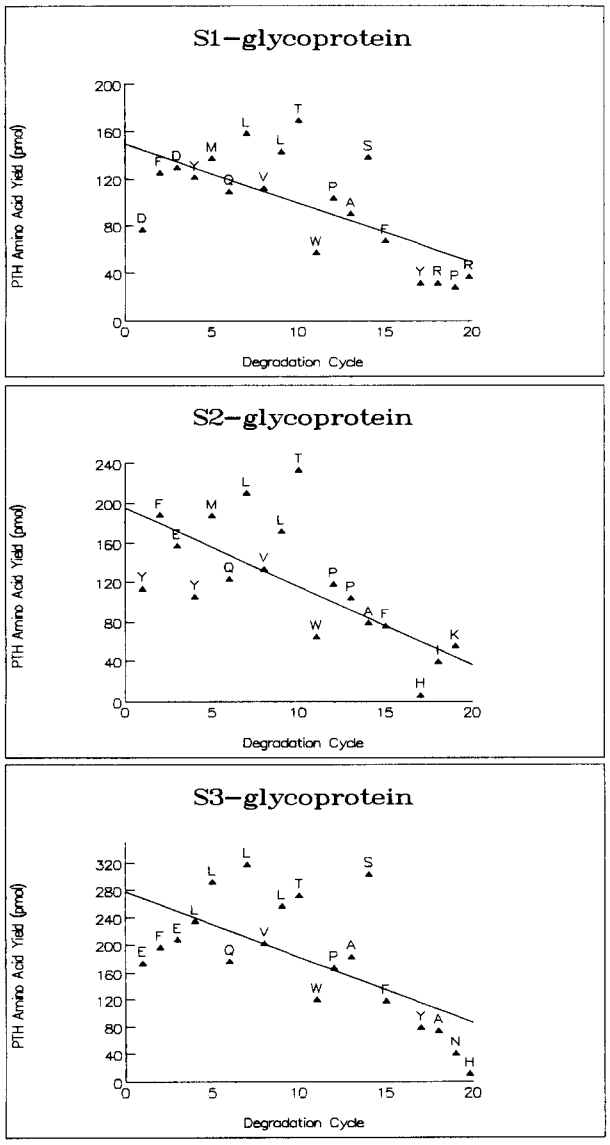

Fig. 5. N-terminal sequence analysis of S-glycoproteins from *P. hybrida* styles. Purified S_1- (ca. 150 pmol), S_2- (ca. 200 pmol) and S_3-glycoprotein (ca. 280 pmol) were sequenced without prior cysteine alkylation. A. (previous page). Yields of PTH amino acids for the three isolated S-glycoproteins. No PTH amino acid was detected at cycle 16 (all three S-glycoproteins) and at cycle 20 (S_2-glycoprotein). The combined repetitive yields for the three sequence analyses, as calculated in the data analysis from the sequenator, were 93% (S_1), 83% (S_2) and 90% (S_3). B. (this page). Comparison of the pI, M_r and N-terminal amino acid sequences of style S-glycoproteins from *P. hybrida* (this work), with those of the S-allele-associated proteins from styles of *N. alata* and *L. peruvianum*. Only the 15 amino-terminal residues were identified for these two other solanaceous plants and have been published previously [8, 15]. The sequences of the S-glycoproteins marked with an asterisk were deduced from the corresponding cDNAs, prepared by Anderson et al. [1 and 2]. Amino acids are identified by their single-letter code, with X indicating residues which could not be clearly assigned. The slashes (/) at position 19 and 20 in the S_2 and S_6 *N. alata* sequences indicate gaps which were included in the nucleic acid sequences of the cDNA clones to maximize similarity. Residues which are identified in at least two of the three S-glycoproteins from *P. hybrida* are boxed with dotted lines, while those which are identical in all twelve proteins are boxed with full lines.

		pI	Mr	1				5				10				15					20
P. hybrida																					
S_1		8.7	27 000	D	F	D	Y	M	Q	L	V	L	T	W	P	A	S	F	X	Y	R P R
S_2		8.9	33 000	Y	F	E	Y	M	Q	L	V	L	T	W	P	P	A	F	X	H	I K X
S_3		9.3	30 000	E	F	E	L	L	Q	L	V	L	T	W	P	A	S	F	X	Y	A N H
N. alata																					
S_1				D	F	E	Y	L	Q	L	V	L	T	W	P	A	S	F			
S_2	*	>9.5	32 000	A	F	E	Y	M	Q	L	V	L	T	W	P	I	T	F	C	R	I / /
S_3	*			A	F	E	Y	M	Q	L	V	L	Q	W	P	A	A	F	C	H	T T P
S_6	*	>9.5	31 000	A	F	E	Y	M	Q	L	V	L	Q	W	P	T	A	F	C	H	T / /
S_7				A	F	E	Y	M	Q	L	V	L	Q	W	P	T	A	F			
S_2		9.0	30 000	D	F	D	Y	M	Q	L	V	L	T	X	P	A	S	F			
S_{f11}		9.5	27 000	D	F	E	Y	L	Q	L	V	L	T	W	P	A	S	F			
L. peruvianum																					
S_1		7.5	28 000	Y	F	E	Y	L	Q	L	V	L	Q	X	P	T	T	F			
S_3		>9.5	28 000	D	F	D	Y	L	Q	L	V	L	Q	X	P	R	S	F			

poral distribution of S-gene-associated glycoproteins were previously described for *N. alata* and *L. peruvianum* [1, 5, 15]. Present investigations focus on biological activity testing of the S-glycoproteins against pollen growth.

Purification procedure of S-glycoproteins combined specificity of ConA-Sepharose with resolution of cation exchange fast protein liquid chromatography. The elution profiles of S-glycoproteins on FPLC were highly reproducible. Besides the peak of the void volume, chromatograms contained only one or two major peaks, depending on whether extracts from styles homozygous or heterozygous for the S-alleles were applied to the column. Omitting the pre-purification step on ConA-Sepharose resulted in recovery of higher yields without considerably affecting resolution on Mono S (since most proteins eluted in the void volume). A similar approach was very recently used to isolate style S-glycoproteins in *N. alata* [8], using a Mono S column at lower pH; in this case, $(NH_4)_2SO_4$ precipitation was used as a pre-purification step.

The amounts of S-glycoproteins in style extracts varied for different self-incompatible clones examined. Whether these differences were allele-specific, as has been reported for *N. alata* [8], was not clear; yield of S_2-glycoprotein, for example, was much lower than for S_3-glycoprotein in the heterozygous acquisition S_2S_3 (W166H), but the opposite was seen in clone B23, bearing the same S-genotype.

The N-terminal amino acid sequences of three S-glycoproteins from *P. hybrida* styles show similarities at least for the first fifteen residues. Comparing these sequences with those of previously published sequences of S-glycoproteins from *N. alata* and *L. peruvianum* [2, 8, 15] reveals that most of the identical residues in *P. hybrida* are conserved in the other two plant species (Fig. 5B). Matching amino acid sequences of different length occur within the fifteen amino-terminal residues. At position 4, all sequences but one (S_3-glycoprotein in *P. hybrida*) reveal a tyrosine residue. At position 16 no amino acid could be assigned during sequencing. The same position in the predicted amino acid sequences of S_2-, S_3- and S_6-glycoproteins from *N. alata* (as deduced from the corresponding cDNAs [2]) is occupied by a cysteine residue; since our sequence analyses were not preceded by a reduction or alkylation step, the sixteenth residue in our sequences may well be a cysteine. Our evidence supports the hypothesis that all these proteins are different allelic products of a highly conserved gene, which is thought to be the S-gene. Three alleles of this gene have been cloned and sequenced in *N. alata*. Comparison of these sequences shows that defined regions of homology, including the N-terminal sequence, are separated by regions of considerable diversity [2]. So far, there is no indication that the intrafamiliar similarity we found here for the N-terminal amino acid sequence, extends to other families and perhaps to all species. No such similarity exists, however, between gametophytically and sporophytically regulated systems [7].

The style S-glycoproteins may recognize growing pollen tubes bearing the same S-genotype. Up to now, no corresponding S-gene-associated components have been found in pollen. Consequently, there is no evidence for any underlying mechanism by which the pollen inhibition might be established. In the near future efforts should be made to complement the present investigations on S-glycoproteins from the female partner by identifying the presumed S-allele-specific pollen tube component. The purification and partial characterization of the style S-glycoproteins were a prerequisite for such research.

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