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Self-incompatibility alleles control a low molecular weight, basic protein in pistils of *Petunia hybrida*

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Summary. Proteins extracted from the pistils of several clones of *Petunia hybrida* carrying differing pairs of S alleles were examined by gel electrophoresis. The major protein of pistils, a basic glycoprotein of relatively low molecular weight, showed properties which varied in a simple manner with the S genotype. For each S allele we were able to assign a specific molecular weight (ranging from 27,000 to 33,000) and isoelectric point (in the range 8.3 to 8.7) for this putative S protein. Pistils homozygous at the S locus showed only one major protein on two-dimensional gel electrophoresis, while pistils from plants heterozygous at the S locus showed two. No evidence was obtained for the presence of this putative S protein in pollen extracts.

Key words: Self-incompatibility – *Petunia hybrida* – S alleles – Pistil proteins

Introduction

Self-incompatibility in *Petunia hybrida* is of the gametophytic type, controlled by a single S locus (Linskens 1975). S allele specific antigens have been detected in pistil extracts of *Petunia hybrida* (Linskens 1960), while Gilissen (1978) has shown that the S allele products are present in the style before pollination. This is in agreement with the observations of Lewis (1952) who concluded that the pistil incompatibility substance is preformed in *Oenothera organensis*, which also shows gametophytic self-incompatibility. Certain stylar proteins have been correlated with S genotype in *Nicotiana alata* (Bredemeijer and Blaas 1981) and *Prunus avium* (Mau *et al.* 1982), both species exhibiting gametophytic incompatibility. We have therefore examined the pistil proteins of several clones of *Petunia hybrida* carrying different pairs of S alleles. We find a simple relationship between each S allele and the properties of the major pistil protein.

Materials and methods

Pollen and pistils were harvested from the collection of *Petunia hybrida* clones maintained by Prof. Dr. H. F. Linskens at the Katholieke Universiteit, Nijmegen, Netherlands (Linskens and Straub 1978). Plant material was dried over P_2O_5 before shipment by air to South Australia. Clones used and lettering adopted for Fig. 1 are as follows (S alleles in brackets); a – W43 (S_1S_1); b – Ka3D (S_2S_2); c – T_2UD (S_3S_3); d – Ka3O (S_3S_3); e – Ka3 (S_4S_4); f – KaD3 (S_4S_4); g – W166H (S_2S_3); h – W166K (S_1S_2); i – T_2U (S_3S_3).

Pistil and pollen extracts were prepared by first freezing the plant material in liquid nitrogen in a precooled mortar, covering the plant material with 10 mM Tris-HCl, pH 7.5 and after all had frozen, allowing the mixture to gradually thaw while grinding vigorously with the pestle.

For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), an equal amount of pistil or pollen extract and SDS buffer solution (0.125 M Tris-HCl, pH 6.8–4% SDS–20% glycerol–10% mercaptoethanol–0.01% bromophenol blue) were mixed, the samples heated at 100 °C for 3 min (Kamboj et al. 1984) and centrifuged prior to loading on the gel. Twelve per cent polyacrylamide slab gels with the modified Laemmli discontinuous buffer system were used (Laemmli 1970; Laemmli and Favre 1973).

Two-dimensional slab gel electrophoresis was carried out using a modification of the methods of O'Farrel (1975), and of Iborra and Buchler (1976) as described by Hallenbeck et al. (1982). The samples for isoelectric focussing were prepared by adjusting the extracts to final concentration of 6.7 M urea–2% ampholines (LKB, pH range 3.5 to 10.0)–2% triton X100–3% mercaptoethanol. The final mixture was centrifuged (Eppendorf) for 5 min before loading onto the gel. First dimension isoelectric focussing was carried with slab gels containing 4.5% acrylamide–8.4 M urea–2% ampholines (pH range 3.5 to 10.0). SDS-PAGE in the second dimension was

performed as described above. After migration, the gels were placed directly into staining solution (0.1% Coomassie Blue R-250–45% ethanol–10% acetic acid) and stained overnight. The gels were destained with a solution containing 45% ethanol–10% acetic acid. For glycoprotein staining PAS (periodic acid – Schiffs reagent) was used (Segrest and Jackson 1972).

Molecular weight markers used for SDS-PAGE were purchased from Pharmacia (low molecular weight kit proteins). These standard proteins were: phosphorylase b (94,000 daltons); albumin (67,000 daltons); ovalbumin (43,000 daltons); carbonic anhydrase (30,000 daltons); trypsin inhibitor (20,100 daltons); α lactalbumin (14,400 daltons).

Results

1 Effect of S alleles on pistil protein patterns obtained by SDS-PAGE

The proteins of pistil extracts were examined for molecular weight differences among the dissociated, denatured components by SDS-PAGE. Nine clones of *Petunia hybrida* with varying pairs of S alleles when analysed in this way gave the protein patterns shown in Fig. 1. It was apparent from this pattern that there was a simple relationship between the S alleles carried by the pistil and the molecular weight of the major protein in the extract which migrated in the region of the 30,000 dalton marker protein. By inspection we were able to assign a specific molecular weight for each putative "S protein", varying from 27,000 daltons for S_1, up to 33,000 daltons for S_2, the values for S_2 and S_4 lying in between (Table 1). Where the pistil was homozygous for an S allele, only one major protein band was seen, while two major bands appeared for extracts from pistils heterozygous at the S locus, as expected. As can be seen in Fig. 1, we did find some variation in the intensity of several minor bands between the clones, but none of these showed the clearcut differences or consistent relationship with the S genotype that was evident for the major protein in the 30,000 dalton region.

Table 1. Estimated molecular weight (M.W.) and isoelectric point (pI) of major pistil proteins assigned to S alleles in *Petunia hybrida*

S allele	M.W. $\times 10^{-3}$	pI
S_1	27	8.3
S_2	33	8.5
S_3	30	8.7
S_4	31	8.6

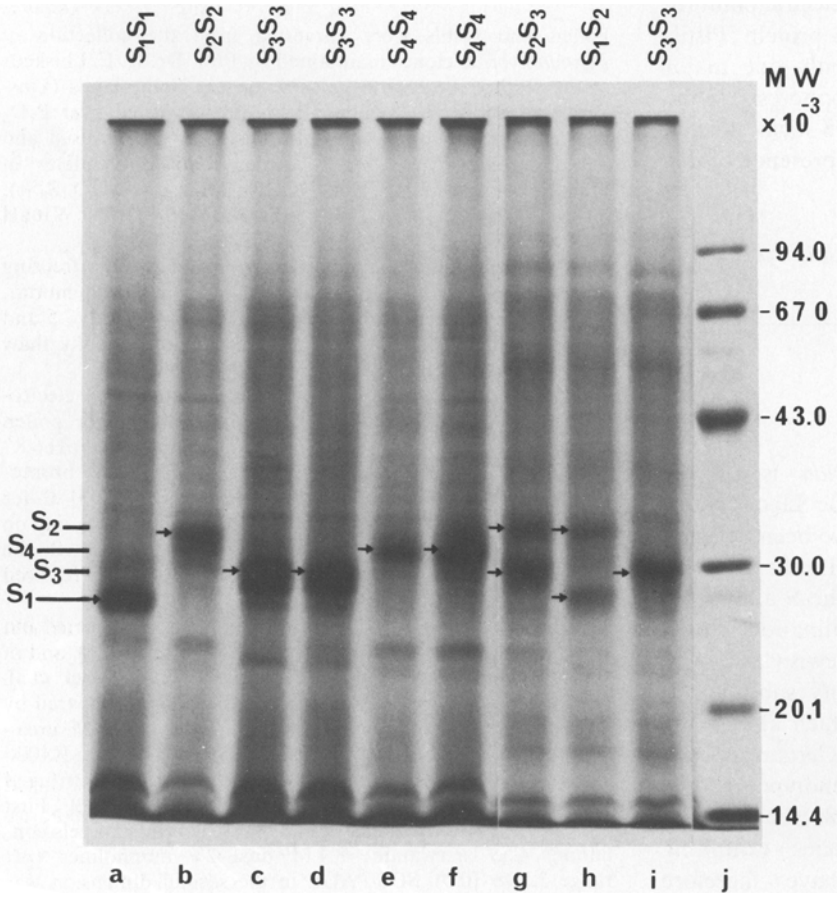

Fig. 1. SDS-PAGE of pistil proteins from clones (listed in "Materials and methods") of *Petunia hybrida*. The position of major proteins assigned to each S allele is indicated by arrows. Molecular weight markers were run in *lane j*

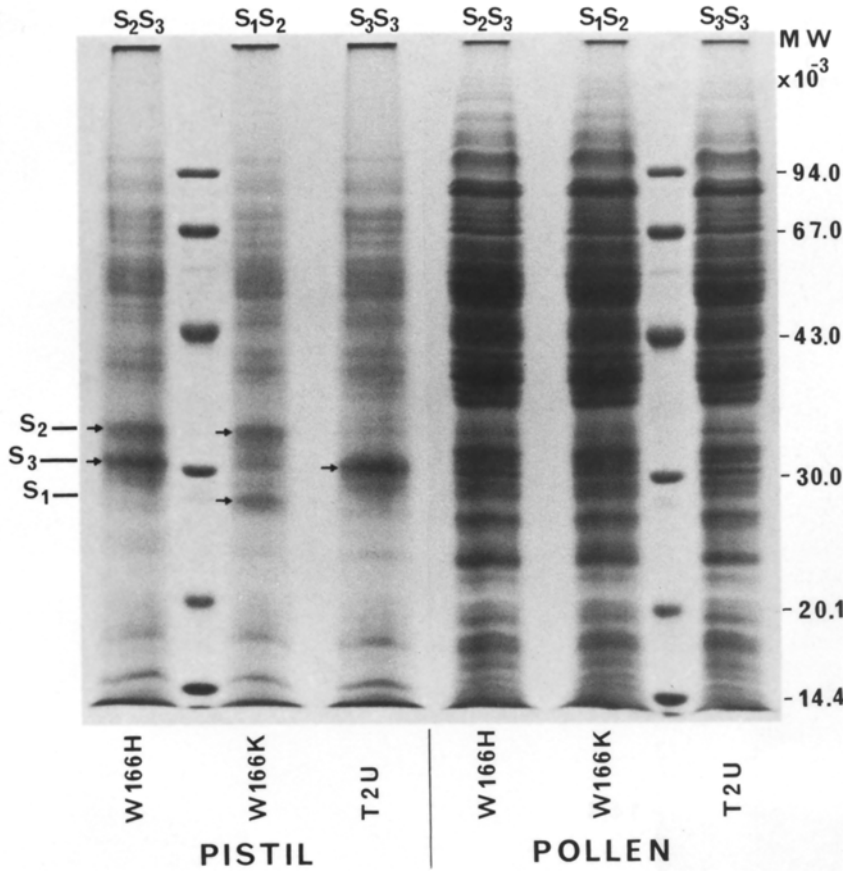

Fig. 2. Comparison of pistil and pollen proteins by SDS-PAGE. Molecular weight markers were run in the *unmarked lanes*

2 Comparison between pistil and pollen proteins

Proteins in extracts of pistils and pollen from two heterozygous clones (bearing S_2S_3 and S_1S_2 alleles) and one homozygous (S_3S_3) clone were compared by SDS-PAGE. The results, after staining the gels with Coomassie Blue, are shown in Fig. 2. It can be seen that while the major protein of pistils varied according to the S genotype as noted above, no such simple relationship is apparent with the protein patterns of pollen extracts. The major proteins of pollen have a much higher molecular weight than the pistil counterpart and do not vary markedly according to the S alleles carried. Due to the multiplicity of protein bands obtained with pollen extracts, we cannot say whether or not the "S protein" seen in pistil extracts is present in pollen. We can say that it is not a major protein in pollen, and that no other correlations with S genotype is evident from our SDS-PAGE pollen protein patterns.

A similar gel to that shown in Fig. 2 was stained with periodic acid – Schiffs reagent instead of Coomassie Blue, in order to reveal glycoproteins. As shown in Fig. 3, the major "S protein" of pistils stains as a glycoprotein; there is little evidence for any other glycoproteins in pistil extracts. In contrast, pollen extracts show many glycoprotein bands, but none however in the "S protein" 30,000 dalton region, and none showing any type of correlation with the S genotype.

3 Isoelectric focussing and two-dimensional gel electrophoresis of pistil proteins

Pistil extracts (four homozygous and two heterozygous at the S locus) were first subjected to isoelectric focussing in one direction, and then to separation by molecular weight in another, to achieve a two-dimensional separation of proteins. The spreading of minor proteins in the pistil extracts by this means results in the "S protein", by virtue of its abundance, being the only protein visible on the gel after staining with Coomassie Blue (Fig. 4). A simple correlation with S genotype is again evident, and specific isoelectric points can be assigned to each "S protein", all around the region of 8.5. Specific isoelectric points calculated from Fig. 4 and from a one-dimensional isoelectric focussing gel are listed in Table 1, they range from a pI of 8.3 for S_1 protein to 8.7 for S_3 protein.

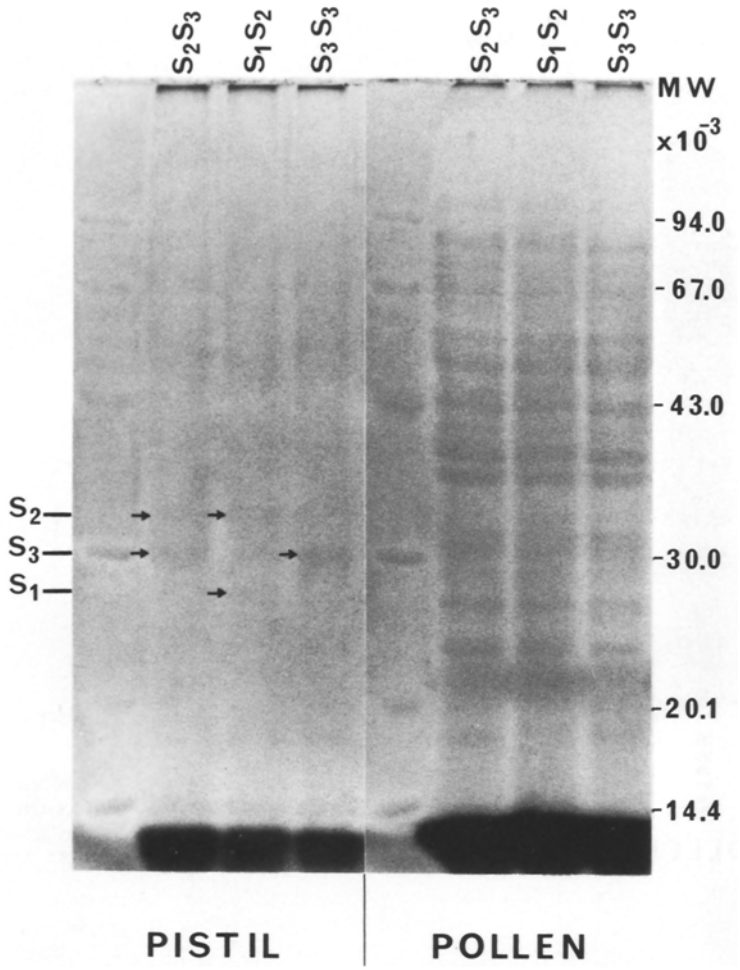

Fig. 3. Schiffs stain for glycoprotein of pistil and pollen after SDS-PAGE. Molecular weight markers were run in *unmarked lanes*

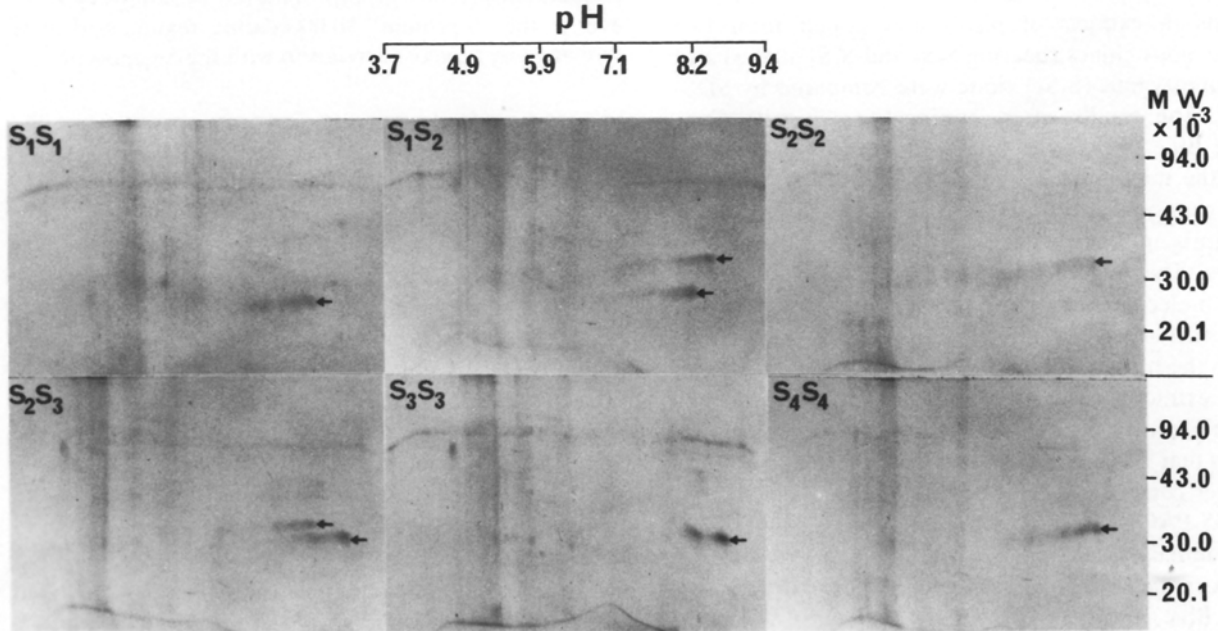

Fig. 4. Two dimensional gel electrophoresis of pistil extracts from clones *W43* (S_1S_1). *W166K* (S_1S_2), *Ka3D* (S_2S_2), *W166H* (S_2S_3). T_2U (S_3S_3) and *KaD3* (S_4S_4)

Discussion

There has been an indication from the few gametophytic incompatibility systems so far studied, that the S genotype correlates with a protein of relatively high isoelectric point. Thus the S_3S_4 genotype of *Prunus avium* styles has yielded an S antigen with a pI of 8.8 (Mau et al. 1982), and *Nicotiana alata* styles with S_2 alleles showed an "S-specific protein" with the same pI (Bredemeijer and Blaas 1981). Moreover the S antigen of *Prunus avium* was shown to have a molecular weight of 37,000–39,000 (Mau et al. 1982). These parameters are remarkably close to those found here for *Petunia hybrida* (Table 1). In addition we have an indication that the "S protein", like the S antigen of *Prunus avium,* is a glycoprotein. Although the S antigen of *Prunus avium* is not the major protein of pistils, for *Nicotiana alata* S_2 genotypes the "S-specific protein" is indeed the major protein of stylar extracts, as we have shown here for all "S proteins" of *Petunia hybrida.*

A feature of the *Petunia hybrida* system described herein is that each of the proteins assigned to the S_1, S_2, S_3 and S_4 alleles, while being clearly separable on a molecular weight and pI basis, nevertheless, appear to belong to one particular class of proteins with a low molecular weight and high pI. This is perhaps not unexpected, if they are indeed gene products of four different alleles at the one S locus. In this respect, the *Petunia hybrida* system appears to differ from that described for *Nicotiana alata* (Bredemeijer and Blaas 1981), where only one of the "S specific proteins" (corresponding to S_2) was the major protein and exhibited an high pI. Other genotypes in *Nicotiana alata* were characterized by minor protein bands.

We have no evidence so far that the pistil "S proteins" so clearly shown for each of the four alleles in *Petunia hybrida,* occur in pollen as well. Bredemeijer and Blaas (1981) were also unable to comment on this from their investigations with *Nicotiana alata,* for similar reasons. However, Linskens (1960), working with the same clones used here, was able to demonstrate serological interactions with pollen consistent with the presence of pistil S antigen in pollen extracts. If the S antigen of Linskens (1960) corresponds with our "S protein", then the concentration of the latter in pollen must be considerably lower than in pistils.

There is no conclusive proof as yet that the "S proteins" are in fact S gene products and not merely the products of genetic backgrounds. However, when taken together with the indications from other gametophytic systems so far studied, it does seem that there is a strong possibility that they are, and that it may soon be possible to test the molecular models put forward for example by Linskens (1975); Lewis (1965); Heslop-Harrison (1983) or by Dumas et al. (1984), to explain the mechanism of self-incompatibility and how it relates to other biochemical events taking place during the interaction betwen style and pollen (Jackson et al. 1983).

Acknowledgement. The authors dedicate this work to Prof. Dr. H. F. Linskens who retires from his post in Nijmegen in 1986, after leading the way over many years in unravelling the mechanism of self-incompatibility.

References

Bredemeijer GMM, Blaas J (1981) S-specific proteins in styles of self-incompatible *Nicotiana alata.* Theor Appl Genet 59:185–190

Dumas C, Knox RB, Gaude T (1984) Pollen-pistil recognition: new concepts from electron microscopy and cytochemistry. Int Rev Cytol 90:239–272

Gilissen LJW (1978) Effects of X-rays on seed setting and pollen tube growth in self-incompatible *Petunia.* Incompatibility Newslett 10:15–19

Hallenback PC, Meyer CM, Vignais PM (1982) Regulation of nitrogenase in the photosynthetic bacterium *Rhodopseudomonas capsulata* as studied by two-dimensional gel electrophoresis. J Bacteriol 151:1612–1616

Heslop-Harrison J (1983) Self-incompatibility: phenomenology and physiology. Proc R Soc Lond, Ser B 218: 371–395

Iborra F, Buchler JM (1976) Protein subunit mapping. A sensitive high resolution method. Anal Biochem 74:503–511

Jackson JF, Kamboj RK, Linskens HF (1983) Localization of phytic acid in the floral structure of *Petunia hybrida* and relation to the incompatibility genes. Theor Appl Genet 64:259–262

Kamboj RK, Linskens HF, Jackson JF (1984) Energy-driven protein release from germinating pollen of *Petunia hybrida.* Ann Bot 54:647–652

Laemmli WK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Laemmli WK, Favre M (1973) Maturation of the head of bacteriophage T4. J Mol Biol 80:575–599

Lewis D (1952) Serological reactions of pollen incompatibility substances. Proc R Soc Lond, Ser B 140:127–135

Lewis D (1965) A protein dimer hypothesis on incompatibility. Genet Today 3:657–663

Linskens HF (1960) Zur Frage der Entstehung der Abwehrkörper bei der Inkompatibilitätsreaktion von Petunia III. Mitteilung. Serologische Teste mit Leitgewebs- und Pollenextrakten. Z Bot 48:126–135

Linskens HF (1975) Incompatibility in Petunia. Proc R Soc Lond, Ser B 188:299–3311

Linskens HF, Straub J (1978) A mutant collection of Petunia. Incompatibility Newslett 10:123–131

Mau SL, Raff J, Clarke AE (1982) Isolation and partial characterization of components of *Prunus avium* L. styles, including an antigenic glycoprotein associated with a self-incompatibility genotype. Planta 156:506–516

O'Farrell PH (1975) High resolution two dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Segrest JP, Jackson RL (1972) Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Methods Enzymol 28:54–63