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Identification of pistil-specific proteins associated with three self-incompatibility alleles in *Solanum chacoense*

B. Xu[1], P. Grun[1,2], A. Kheyr-Pour[3,*], and T.-H. Kao[3]
[1] Department of Horticulture, [2] Department of Biology, [3] Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802, USA

Summary. Pistil proteins associated with three different S-alleles of the self-incompatibility locus (S locus) in *Solanum chacoense* have been identified which cosegregated with their respective S alleles in a series of genetic crosses involving six *S. chacoense* plants, their F_1 progeny, and backcrosses. The molecular weights of these three S-allele-associated proteins, designated S_1, S_2, and S_3, were 29 kDa, 30 kDa, and 31 kDa, respectively. They were all basic proteins with a similar pI of approximately 8.6. They have been found only in the stigma and style of the pistil where their maximum synthesis was reached at one day before anthesis. Their rate of synthesis in both self- and cross-pollinated pistils was the same as that in the unpollinated pistil until 2 days after pollination.

Key words: Pistil – S-allele-associated proteins – Self-incompatibility – *Solanum chacoense*.

Introduction

In many flowering plant species, self-fertilization is blocked by a natural outbreeding mechanism termed self-incompatibility, which acts via an interaction between pollen, or pollen tube, and pistil to prevent pollen germination or normal pollen tube growth (Heslop-Harrison 1975, 1978; Nettancourt 1977; Harris et al. 1984).

Pistil S proteins have been identified in several plant species, including *Brassica oleracea* (Nasrallah et al. 1970; Nishio and Hinata 1977), *Brassica campestris* (Nishio and Hinata 1978; Takayama et al. 1987), *Nicotiana alata* (Bredemeijer and Blaas 1981; Kheyr-Pour and Pernes 1986), *Petunia hybrida* (Kamboj and Jackson 1986), and *Lycopersicon peruvianum* (Mau et al. 1986). All but *Brassica* display gametophytic self-incompatibility. The identification of these proteins has led to sequencing of the S proteins of *Brassica campestris* (Takayama et al. 1987), and cDNA clones coding for the S proteins of *Brassica oleracea* (Nasrallah et al. 1985b, 1987) and *Nicotiana alata* (Anderson et al. 1986, 1989). There is no similarity between the amino acid sequences of the six *Brassica* S proteins and the three *Nicotiana* S proteins, suggesting that the sporophytic and gametophytic self-incompatibility systems might have evolved separately.

Self-incompatibility in *Solanum* has been determined to be gametophytically controlled (reviewed by Nettancourt 1977), as it is in other genera of the Solanaceae such as *Nicotiana, Petunia,* and *Lycopersicon.* However, various *Solanum* species differ in the number of controlling genes. Pal and Pushkarnath (1942) found that self-incompatibility in *S. chacoense* and *S. jamesii* is controlled by a single locus. Investigation by Pandey (1962) showed that self-incompatibility in *S. pinnatisectum* is controlled by two loci. The same mechanism was found in *S. phureja* and *S. stenotomum* (Abdalla and Hermsen 1971).

In this paper, the identification of three different S alleles and their associated proteins of *S. chacoense* is described. These proteins exhibit many features typical of S proteins found in other species.

Materials and methods

Plant material

Seeds of *S. chacoense* obtained from crosses between PI 133619 and PI 133664 were provided by Dr. R.E. Hanneman, Jr. (USDA Potato Introduction Station, Sturgeon Bay, Wis.). Six plants, designated GKR-8, -13, -18, -32, -40, and -54, were used for analysis of the self-incompatibility alleles (S alleles). These plants were first self-pollinated to confirm their self-incompatibility. A series of diallele crosses were then made to determine the number of different alleles present in the six

* On sabbatical leave from Laboratoire de Genetique et Physiologie du Developpement des Plantes, C.N.R.S., F-91190 Gif-sur-Yvette, France

Offprint requests to: T.-H. Kao

plants. At least five flowers were used for each cross. These crosses were judged to be compatible or incompatible by two criteria: presence or absence of fruit setting and extent of pollen tube growth. Pollen tube growth was monitored using fluorescence microscopy after staining the pistil with decolorized aniline blue (Martin 1959).

Electrophoretic analysis of pistil proteins

Plant tissues were ground with a micropestle in distilled water (1 mg tissue/ml) and centrifuged at 14,000 rpm in an Eppendorf microfuge for 20 min. The supernatant was subjected to either sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF). SDS-PAGE was carried out according to Laemmli (Laemmli 1970; Laemmli and Favre 1973). Samples were prepared by mixing the crude extract with an equal volume of sample buffer (0.0625 *M* TRIS-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 3-mercaptoethanol, 0.001% bromophenol blue) and then boiling for 5 min. The electrophoresis buffer was 0.025 *M* TRIS-HCl, pH 8.3, 0.192 *M* glycine, and 0.1% SDS. Electrophoresis was carried out on vertical 12.5% polyacrylamide gels containing 0.375 *M* TRIS-HCl, pH 8.8, and 0.1% SDS, at a constant current of 5 mA until the dye front reached the bottom of the gels. Molecular weight markers (Sigma SDS-7) contained a mixture of bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (21,000), and lactalbumin (14,000). IEF was carried out according to the LKB user manual. The crude extracts were separated on 5% polyacrylamide gels with a pH gradient of pH 3.5–10 or 7–11. The staining solution for both SDS-PAGE and IEF contained 0.5% Coomassie Blue R-250, 8% acetic acid, and 25% ethanol. After at least 5 h of staining, gels were destained in a solution of 8% acetic acid and 25% ethanol.

Metabolic labeling of pistil proteins with $[^{35}S]$methionine

Plant tissues were collected and placed in the wells of microtiter plates. Labeling with $[^{35}S]$methionine was carried out essentially according to Nasrallah (Nasrallah et al. 1985a). Labeling was allowed to proceed for 24 h with 10 μCi of $[^{35}S]$methionine per style, and then stopped by washing the plant tissue thoroughly with distilled water. Total protein was extracted and electrophoresed as described above. The gel was treated with ENHANCE (New England Nuclear), dried in a gel drier and exposed to Kodak X-Omat film. Throughout this paper, the term "style" refers to the segment of the pistil above the ovary, thus including the stigma.

Results

Identification of S-allele-associated proteins in pistils of S. chacoense

Diallele crosses were carried out among the six *S. chacoense* plants in order to determine the S genotype of each plant and the number of different S-alleles represented in these plants. The results are summarized in Table 1. Each plant was self-incompatible, and the six plants could be classified into three incompatibility groups: GKR-13, -18, and -40 belonged to one group, GKR-8 and GKR-32 to another, GKR-54 to a third. Plants within each group were cross-incompatible, i.e., no fruit setting was observed upon reciprocal cross-pollinations, and fluorescence microscopy revealed arrest of pollen tube growth in the upper segment of the style. Occasionally, one or two fruits were found, but contained very few or no seeds. Plants of different groups were reciprocally cross-compatible; over half the flowers pollinated set berries that contained more than 30 seeds.

To identify the S-allele-associated pistil proteins, total protein was extracted from pistils of six GKR plants and analyzed on SDS-polyacrylamide gels. Comparison of the protein band patterns of these plants revealed that three proteins, designated S_1, S_2, and S_3, were good candidates to be the S proteins (Fig. 1). Each plant contained only two of these three proteins in a manner consistent with the incompatibility group classification determined initially by the diallele crosses. Specifically, GKR-13, -18, and -40 belonged to one incompatibility group and all had S_1 and S_2 proteins, but not S_3. GKR-8 and GKR-32 belonged to another incompatibility group and had S_2 and S_3 proteins, but not S_1. GKR-54, in the third incompatibility group, had S_1 and S_3 proteins, but not S_2. Thus, the genotype of each incompatibility group can be denoted on the basis of which S proteins it contains. Although the three S proteins dif-

Table 1. Diallele crosses among six *Solanum chacoense* plants

♀ \ ♂	GKR-13	GKR-18	GKR-40	GKR-8	GKR-32	GKR-54
GKR-13	0/10[a]	0/13	0/10	4/5	14/14	15/22
GKR-18	2/8	0/10	0/10	5/6	5/5	3/5
GKR-40	0/10	0/10	0/10	3/5	4/6	3/5
GKR-8	4/6	3/6	3/6	0/7	1/8	4/6
GKR-32	17/17	3/6	4/5	0/10	0/7	15/15
GKR-54	11/15	3/3	4/9	5/5	12/12	0/10

[a] Results shown as number of fruits set over number of flowers pollinated. Intra-group incompatible crosses are indicated within blocks

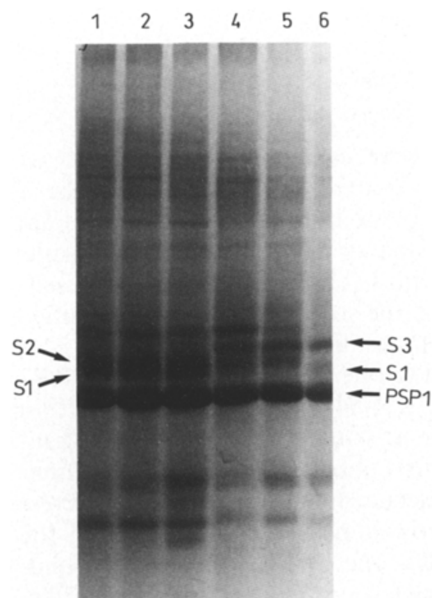

Fig. 1. SDS-polyacrylamide gel electrophoresis of stylar extracts of three *S. chacoense* self-incompatible genotypes. Three S-allele-associated proteins, S_1, S_2, and S_3, and one common abundant protein, PSP_1, are indicated by *arrows*. *Lanes 1–3*, GKR-13, GKR-40, and GKR-18 (S_1S_2); *lanes 4 and 5*, GKR-8 and GKR-32 (S_2S_3); *lane 6*, GKR-54 (S_1S_3)

fered in molecular weight, 29 kDa for S_1, 30 kDa for S_2, and 31 kDa for S_3, they had similar pI of approximately 8.6 as determined by isoelectric focusing gels (results not shown). A very abundant protein having a molecular weight of 26 kDa appeared in all three genotypes (Fig. 1). This protein was designated PSP_1, since it was found to be pistil-specific (see below).

Further, to confirm the correlation of these three proteins with their respective S alleles, F_1 plants obtained from crosses between plants in different incompatibility groups were backcrossed to their respective parents. The outcome of these crosses is summarized in Table 2. Since each pair of parents from different incompatibility groups shared one common S-allele, theoretically half of the F_1 progeny should be compatible with both parents, while the other half should be compatible only with their female parent. For example, the genotype of F_1 progeny from crosses between S_2S_3 (female) and S_1S_2 (male) is either S_1S_2 or S_1S_3. The S_1S_2 progeny were compatible with the female parent S_2S_3, but incompatible with the male parent S_1S_2. The S_1S_3 progeny were compatible with both

Table 2. Berry set from backcrosses of F_1 hybrids and their parents. (Four F_1 plants which failed to set berries in any crosses have been omitted)

Parents Female × male	Backcross			
	F_1 used as female		Parent used as male	
	Plant no.	Genotype[a]		
			GKR-32 S_2S_3	GKR-40 S_1S_2
GKR-32 × GKR-40	1	S_1S_2	6/7[b]	0/7
S_2S_3 S_1S_2	2	S_1S_3	8/12	6/7
	3	S_1S_3	3/7	4/5
			GKR-40 S_1S_2	GKR-54 S_1S_3
GKR-40 × GKR-54	1	S_1S_3	1/3	0/6
S_1S_2 S_1S_2	2	S_1S_3	13/14	0/12
	3	S_1S_3	12/12	0/11
			GKR-54 S_1S_3	GKR-40 S_1S_2
GKR-54 × GKR-40	1	S_1S_2	11/13	0/12
S_1S_3 S_1S_2	2	S_1S_2	5/5	0/6
	3	S_1S_2	1/4	0/3
	4	S_1S_2	11/14	0/22
			GKR-54 S_1S_3	GKR-32 S_2S_3
GKR-54 × GKR-32	1	S_2S_3	8/9	0/10
S_1S_3 S_2S_3	2	S_1S_2	5/7	8/21
	3	S_1S_2	2/13	1/16

[a] Genotypes as determined by protein patterns on SDS-polyacrylamide gels
[b] Berries set/flowers pollinated

Table 3. Diallele crosses among F_1 plants of GKR-13 (S_1S_2) × GKR-54 (S_1S_3)

Genotype[a]	♀ \ ♂	HAG-5	HAG-6	HAG-7	HAG-8	HAG-9	HAG-10
S_1S_3	HAG-5	7/10[b]	11/12	5/5	5/5	4/5	5/5
S_1S_3	HAG-6	0/8	0/10	5/5	5/9	4/4	5/5
S_2S_3	HAG-7	4/5	6/7	0/10	0/6	0/5	0/5
S_2S_3	HAG-8	4/6	6/6	0/5	0/10	0/5	0/6
S_2S_3	HAG-9	2/8	4/7	0/8	0/5	0/10	0/10
S_2S_3	HAG-10	5/7	7/7	0/7	0/8	0/5	0/10

[a] Determined by S protein patterns on SDS-polyacrylamide gels
[b] Results shown as number of fruits set/number of flowers pollinated

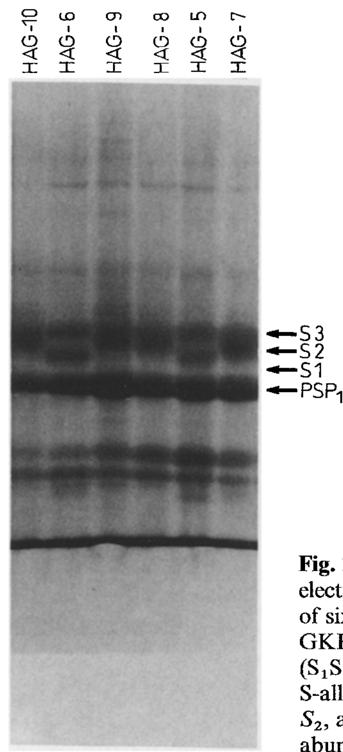

Fig. 2. SDS-polyacrylamide gel electrophoresis of stylar proteins of six plants from cross between GKR-13 (S_1S_2) and GKR-54 (S_1S_3). *Arrows* point to the three S-allele-associated proteins, S_1, S_2, and S_3, and one common abundant protein PSP_1

parents. In most cases, the outcome of the back-crosses was what was predicted based on the genotypes of the F_1 plants as determined from the S-protein patterns. That is, when the S genotypes of the F_1 progeny were the same as those of the parents with which they were crossed, as occurred in 9 examples in Table 2, the crosses all failed to set berries. Seed set occurred in 17 examples where the S genotypes of the F_1 progeny differed from those of the parents. There were, in addition, 4 F_1 plants that failed to set berries in any crosses, and were judged to be female sterile (results not included in Table 2).

The cosegregation of these three proteins with their respective S alleles was also tested by diallele crosses between six F_1 plants obtained from crosses between GKR-13 (genotype S_1S_2) as female and GKR-54 (genotype S_1S_3) as male. The S genotypes of these F_1 plants, designated HAG-5, -6, -7, -8, -9 and -10, were determined by SDS-PAGE patterns of their total pistil protein (Fig. 2). These plants contained either S_1 and S_3 proteins, or S_2 and S_3 proteins, as expected from the S genotypes of their parents. Each of the four plants with S_2S_3 genotype, HAG-7, -8, -9, and -10, was reciprocally incompatible with the others, and reciprocally compatible with HAG-5 and HAG-6, both of which had S_1S_3 genotype (Table 3). Plant HAG-5 was of particular interest because it is self-compatible. Moreover, its pistil did not reject pollen of HAG-6, which has the same genotype, though its pollen was rejected by the pistil of HAG-6. This incompatibility behavior is reminiscent of classic style-part self-compatible mutations observed in several other species (reviewed by Nettancourt 1977). The PSP_1 protein again appeared in all the genotypes of the F_1 progeny (Fig. 2).

Temporal and tissue specific expression of S-allele-associated proteins

The rate of S-protein synthesis during the course of flower development was studied by metabolic labeling of total stylar protein with [^{35}S]methionine. Styles were collected from buds of the GKR-32 plants at 3 days, 2 days, and 1 day before flower opening, and from just opened flowers. An equal number of styles was used for the in vivo labeling experiment, as descirbed in Materials and methods. The results of gel electrophoretic analysis of the labeled stylar proteins are shown in Fig. 3. The synthesis of both S_2 and S_3 proteins started at a low level at 3 days before flower opening, increased subsequently, and reached a maximum level at 1 day before flower opening. This same temporal profile and tissue specificity was shown by PSP_1.

Similar in vivo labeling experiments were carried out on ovary, petal, anther tissues of freshly

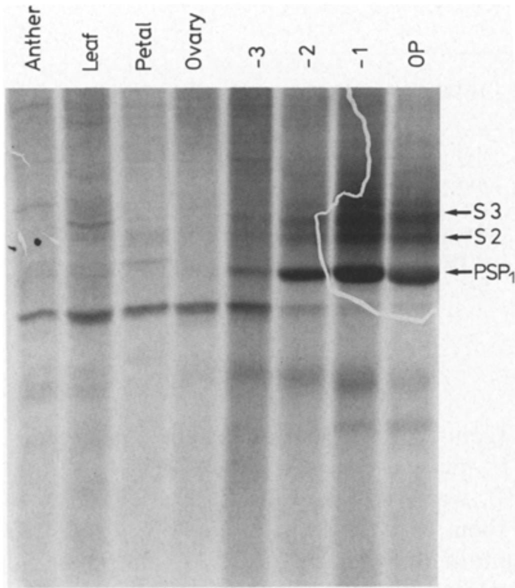

Fig. 3. Autoradiographic pattern of SDS-polyacrylamide gel electrophoresis of proteins synthesized in the presence of [^{35}S]methionine by styles at different developmental stages and by other tissues of GKR-32. The numbers *-3*, *-2*, and *-1* indicate styles from flower buds at 3 days, 2 days, and 1 day before flower opening; *OP* indicates styles from freshly opened flowers. *Anther, petal*, and *ovary* tissues were from freshly opened flowers

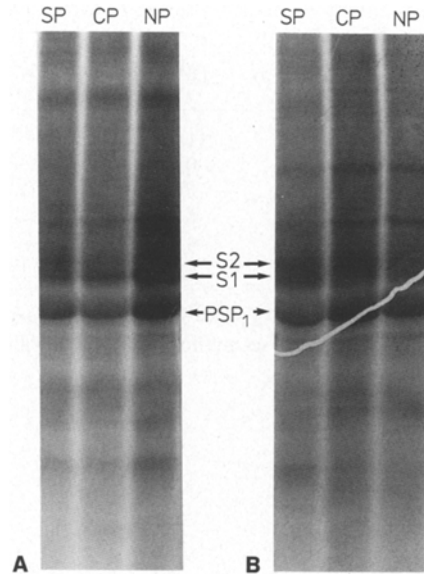

Fig. 4A, B. Comparison of S_1 and S_2 protein synthesis in self-pollinated (*SP*), cross-pollinated (*CP*), and non-pollinated (*NP*) styles of GKR-13. Styles were excised 1 day (*A*) or 2 days (*B*) after pollination, and metabolically labeled with [^{35}S]methionine. Labeled proteins were extracted and electrophoresed on a SDS-polyacrylamide gel and visualized by autoradiography

opened flowers, and leaf tissue (Fig. 3). The S_2 and S_3 proteins were not detectable in any of these tissues.

Effect of pollination on the synthesis of S-allele-associated proteins

Examination of pollen tube growth in vivo by fluorescence microscopy revealed that it took 2 days for pollen tubes resulting from cross-pollination to reach the ovules. Pollen tubes resulting from self-pollination grew more slowly and did not reach the bottom of the style, most being stopped in the upper third or middle of the style. To test whether the level of pistil S-protein synthesis is affected by either self- or cross-pollination, freshly opened flowers were self- or cross-pollinated. Equal numbers of styles were collected at 1 and 2 days following pollination, and metabolically labeled with [^{35}S]methionine. Total stylar protein was extracted and analyzed by SDS-PAGE. As a control, some of the emasculated flowers were left unpollinated and their styles were collected and treated the same way. There was no detectable difference in the levels of the S_2 or S_3 protein synthesized in self-, cross-, or unpollinated styles one to two days after pollination (Fig. 4). Thus, neither cross- nor self-pollination changed the amount of the proteins present.

Homozygous S_2S_2 plant obtained by bud selfing

In many species displaying either sporophytic or gametophytic self-incompatibility, plants homozygous for a single S allele can be obtained by forced selfing at the immature bud stage of flower development. This is possible because S proteins are produced in very small amounts in immature buds. Bud pollinations were attempted on GKR-13, GKR-32, and GKR-54 plants to obtain plants homozygous for the S_1, S_2, or S_3 allele. A number of selfings were carried out, 32 on GKR-13, 36 on GKR-32, and 35 on GKR-54, but very few set berries. Most of the seeds obtained from these berries failed to germinate. Only three plants were obtained, two from selfing of GKR-32, and one from selfing of GKR-54. Backcrosses of these three plants to their respective parents suggested that one of them, a selfed progeny plant from GKR-32, was a homozygote because its pistil was compatible with pollen of its parent. This homozygote, designated GZF-2, grew and flowered considerably more slowly than its heterozygous sibling GZF-1. As expected, GZF-2 only contained the S_2 protein band, while GZF-1 contained both S_2 and S_3 protein bands as did its parent GKR-32 (Fig. 5).

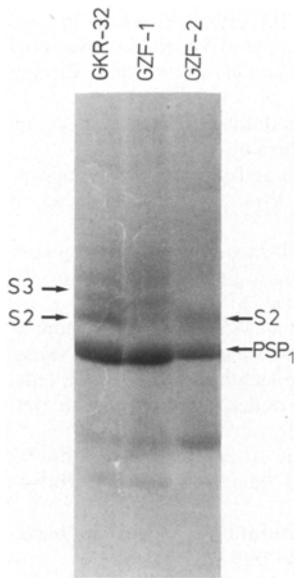

Fig. 5. SDS-polyacrylamide gel electrophoretic pattern of S-allele-associated proteins of *GKR-32* (S_2S_3) and its two selfed progeny *GZF-1* (a heterozygote, S_2S_3) and *GZF-2* (a homozygote, S_2S_2)

Discussion

Pistil S-allele-associated proteins of the self-incompatibility system have previously been identified in a number of species, based on the cosegregation of these proteins with their respective S alleles in genetic crosses. Although direct evidence that these S proteins are involved in self-incompatibility reaction in vivo is still lacking, many lines of evidence suggest correlation between these proteins and self-incompatibility behavior (Nasrallah and Nasrallah 1986). Recently, a purified pistil S-protein of *Nicotiana alata* was shown to inhibit pollen tube growth in vitro in an allele-specific manner (Harris et al. 1989). In this study, we identifed pistil-specific proteins associated with three different S alleles of *S. chacoense,* a species displaying gametophytic self-incompatibility.

The six *Solanum* plants used here were raised from seeds obtained from crosses between two parent plants. Thus, either three or four different S alleles were expected to be present in these six plants, depending on whether their parents shared a common allele. Three incompatibility groups were found based on results of diallele crosses among these six plants, indicating the presence of three different S-alleles (Table 1). We designated the three alleles S_1, S_2, and S_3, and the genotypes of the three incompatibility groups were S_1S_2, S_2S_3 and S_1S_3, respectively. The associated pistil proteins of these three alleles were identified by comparison of SDS-polyacrylamide gel electrophoretic patterns of total pistil protein from these six plants (Fig. 1). The presence or absence of these three proteins in each plant correlated with the genotype determined by diallele crosses. That is, the plants in each incompatibility group contained the same two, but not the third of these three proteins.

Two sets of genetic crosses were carried out to test whether these three proteins cosegregated with their respective S alleles in the F_1 hybrids. One set was between the F_1 hybrids and their respective parents and the other was a set of diallele crosses among the F_1 hybrids. The S genotype of each F_1 plant was first determined by identification of the two S proteins it contained. This information was then used to predict the outcome of each backcross or diallele cross involving each particular F_1 hybrid. In the majority of cases where compatible crosses were predicted, seed setting was observed (Tables 2, 3). In all the cases where incompatible crosses were expected, only one notable exception was found, which involved a self-compatible mutant HAG-5 (Tables 2, 3; see also below).

Further support for the cosegregation of the identified S proteins with the S alleles came from the study of two selfed progeny, GZF-1 and GZF-2, of GKR-32 (S_2S_3 genotype). Backcross results revealed that GZF-1 was a heterozygote and GZF-2 was a homozygote for the S allele. Consistent with this finding was the fact that GZF-1 contained both S_2 and S_3 proteins, while GZF-2 contained only the S_2 protein (Fig. 5).

Results from the above genetic crosses demonstrated that S_1, S_2, and S_3 proteins were the products of either the S alleles, or genes closely linked to the S alleles. Furthermore, these three S proteins of *S. chacoense* also exhibited features characteristic of S proteins so far identified in other species. First, they were found only in the style by Coomassie blue staining, but not detected in anther, petal, ovary, or leaf tissue (Fig. 3). (While S proteins were not detected in the ovary of *N. alata* by Coomassie blue staining of the ovarian proteins, it was recently discovered that, using immunogold localization and in situ hybridization, the S protein and the mRNA for S protein are actually present in the epidermis of the placenta and other surface of the ovules (Cornish et al. 1987; Anderson et al. 1989). Second, their synthesis during flower development was temporally regulated (Fig. 3), being lowest in immature buds and highest at 1 day prior to anthesis (Nasrallah et al. 1985a; Mau et al. 1986).

Many of the S proteins reported so far from *Brassica, Petunia, Nicotiana* or *Lycopersicon* constitute the most abundant protein in the pistil. However, the most abundant pistil protein in these three genotypes of *S. chacoense,* was a 26-kDa protein, designated PSP_1, which did not appear to cos-

egregate with any of the three S alleles (Figs. 1, 2, 5). It is not known whether PSP_1 plays a role in the self-incompatibility reaction. It was, however, pistil-specific, and its synthesis followed a temporal profile similar to that of the S-allele-associated proteins (Fig. 3).

The self-compatible mutant, HAG-5, identified from among the F_1 progeny of crosses between GKR-13 and GKR-54, is worth further study. Its incompatibility behavior suggests that the mutation affects only the style, and not pollen activity in the incompatibility interaction (Table 3). This mutant appeared to contain normal levels of S_1 and S_3 proteins when compared with the levels of those proteins in HAG-6, a self-incompatible F_1 plant with the same genotype (Fig. 2). On SDS-polyacrylamide gels, the mobility of the S_1 and S_3 proteins of this mutant and HAG-6 also appeared to be indistinguishable (Fig. 2). Thus, the mutation did not seem to have occurred in the S-allele genes. Genetic studies need to be carried out to determine whether this mutation is linked to the S locus. If so, this self-compatible mutant might be a style-part mutant of the S locus, similar to those which have been observed in several gametophytic self-incompatible species during mutagenesis studies (Nettancourt et al. 1971).

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