**Variation of S-alleles and S-glycoproteins in a
naturalized population of self-incompatible
Brassica campestris L.**

Ill Sup NOU¹, Masao WATANABE¹, Akira ISOGAI², Hideyuki SHIOZAWA²,
Akinori SUZUKI² and Kokichi HINATA^{1,*}

¹*Faculty of Agriculture, Tohoku University, Sendai 981, Japan*

²*Department of Agricultural Chemistry, The University of Tokyo,
Bunkyo-Ku, Tokyo 113, Japan*

(Received 13 February 1991)

ABSTRACT

Variation of S-alleles and S-glycoproteins associated with self-incompatibility was studied in a naturalized population of *Brassica campestris* growing in Oguni-machi, Japan. Of 58 plants collected from the population, 45 were self-incompatible and 8 were self-compatible. Of 32 families investigated on their selfed progenies, 30 families showed segregation fitting to one locus S-allele model of sporophytic self-incompatibility. From cross-pollination experiments between 35 S-homozygotes so far isolated, 16 different S-alleles were identified, and the number of S-alleles involved in this population was estimated to be 20-30. S-glycoproteins corresponding to each S-allele were determined by immunoblotting with polyclonal antibody against S^g-glycoprotein. Concanavalin A and Coomassie Blue stainings were also applied to determining corresponding S-glycoproteins, but were not so clear as the antibody cross reaction. It is pointed out that a stigma involves a number of proteins with different pI points, which are cross-reactive with anti-S-glycoprotein-antiserum. Many of these proteins are heritable in correlation with major S-glycoproteins. Since the content of these proteins was variable, we tentatively classified major and minor S-glycoproteins, and assumed that these S-glycoproteins were controlled by S-like DNA sequences with closely linked S-locus. Beside these S-glycoproteins, presence of heterozygote specific proteins was also pointed out, suggesting occurrence of post-transcriptional modification of these proteins. The pI values of major S-glycoproteins ranged from 5.0-9.0 and those at 7.0-9.0 were frequent.

1. INTRODUCTION

The self-incompatibility system of *Brassica campestris* L. is under the sporophytic control of multiple allele, S, on a single locus (see review, de Nettancourt, 1977). Studies on stigma proteins suggest that there are S-allele specific glycoproteins termed as S-glycoproteins in stigma (Nasrallah, 1967, 1970, 1972; Nishio and Hinata, 1977, 1978; Hinata and Nishio, 1978, 1982). S-glycoproteins

* Corresponding author.

are heritable in correlation with *S*-alleles and they are found only in mature stigma. Molecular structures have been analyzed on some *S*-glycoproteins and DNA regions coding them, and their amino acid sequences have high homology with a putative protein kinase (Walker and Zhang, 1990). However, the function of *S*-glycoproteins in the recognition reaction is not yet clear.

As for stigma proteins, Isogai et al. (1988) revealed the presence of NS-glycoprotein, which is independent of *S*-alleles, but has very similar amino acid sequence to *S*-glycoproteins. Since then, similar phenomena have been reported for cDNA in *B. oleracea* by Lalonde et al. (1989) and Umbach (1990), who named this gene *SLR1*, and *BS29-1* and *BS63-1* for *B. oleracea* (Trick and Flavell, 1989; Trick, 1990). These studies suggest that *Brassica* plants produce a group of proteins which have similar structure with *S*-glycoproteins in stigma.

The analyses of *S*- and NS-glycoproteins have been carried out on a few plants whose *S*-alleles had already been characterized. On the other hand, the number of *S*-alleles in the wild population have been estimated to be about 20 to 50 (Bateman, 1954; Sampson, 1974; Ockendon, 1974; Stevens and Kay, 1989). In order to have a wide scope on the *S*-alleles and *S*-glycoproteins, we planned to examine the variation of these characters in a population which grow in wild or in near wild situation, since they have received no artificial selection on their self-incompatibility. The present investigation was carried out on a naturalized population in Oguni-machi, Japan.

2. MATERIALS AND METHODS

Plant materials

We took up *B. campestris* as a material, because this species has *S*⁸-glycoproteins characterized by us (Hinata et al., 1982), and involves many important cultivars. It is believed that this species has been introduced from old continent to the Japanese Islands 1500–1800 years ago, and in this sense, we have no real wild population of this species in Japan. There is, however, a naturalized population which grows on the land in fallow since many hundred years ago in a suburb of Oguni-machi, Yamagata Pref., Japan, and has received no artificial selection on self-incompatibility so far. Fifty eight plants were sampled at random from this population in May 1988.

Experiments were conducted in Tohoku University, Sendai. In general, the material plants were raised in pots twice a year, winter-spring and summer-autumn, in a greenhouse without heating system. In summer-autumn cultivation, 9°C vernalization treatment was given to young seedlings for 30 days. Selfed seeds were obtained through bud-pollination for each plant.

Estimation of S-genotypes

S-genotype of each plant in a family was estimated from self- and cross-

compatibility in intra-family diallel pollinations. Compatibility was determined by the observation of pollen tube penetration to stigma through fluorescence microscopy after basic aniline blue staining.

S-glycoproteins of each plant were analyzed by the combination of isoelectric focusing (IEF) and immunoblot analysis with anti-S⁸-glycoprotein-antiserum, and the S-glycoprotein pattern was compared with the S-genotypes estimated by the diallel pollination method. In some other cases, the IEF-immunoblot analysis was performed to determine S-glycoprotein pattern at first, and then intra-family diallel pollinations were carried out between plants to confirm the genotypes. In this diallel pollination, two heterozygotes and two of each homozygotes were provided for the test.

Anti-S⁸-glycoprotein-antiserum (S⁸-antiserum)

S⁸-glycoprotein was isolated as described in the previous papers (Isogai et al., 1987; Takayama et al., 1987). For immunization, 340 µg S⁸-glycoprotein was dissolved in 6 ml of PBS (pH 7.0). Eight-week-old female mice (Balb/c) were injected intraperitoneally with 75 µg of S-glycoprotein each. Freund's complete adjuvant was used in the first and second injections. After three days from the third injection, the serum was collected.

Electrophoresis of stigma proteins

Stigmas from fresh or stored samples at -20°C were homogenized with 50 mM Tris buffered saline at pH 7.5 in an Eppendorf tube fitted with a plastic-stick, and the homogenate was centrifuged at 10,000×g for 20 min. Isoelectric focusing (IEF) was carried out according to the instructions of LKB. In general, 13 µl (protein concentration was about 1.9 µg/µl) of the supernatant was applied to each IEF applicator piece. The gel 0.5 mm in thickness was used with pH 3.0–10.0 Pharmalyte. Pre-electrophoresis was conducted under constant power of 7.2 W for 30 min at 4°C. After sample application, electrophoresis was performed under constant power of 14.4 W for 2 hr.

SDS-PAGE was carried out with vertical slab gel of 10% polyacrylamide, with a stacking gel of 4.5% polyacrylamide according to Laemmli (1970). Extracts were treated with SDS and 2-mercaptoethanol whose final concentrations were 2% and 5%, respectively. Molecular weight marker was LMW (LKB), and constant current, 25 mA, was used for electrophoresis.

Immunoblotting

After IEF or SDS-PAGE, the proteins in the acrylamide gel were transferred to GVHP membrane (Millipore) by Nova Blot (LKB) according to the manual of LKB. The membrane sheet was immersed in a solution containing 0.9% NaCl, 0.02% NaN₃, 50 mM Tris-buffered saline (TBS) for a minute and blocked with TBS containing 0.5% skim milk (SM) at room temperature for 6 hr or more. The sheet

was transferred to a fresh TBS-SM solution containing the S⁸-antiserum (1:2,000 dilution) at room temperature for 6 hr. Subsequently, it was washed twice with TBS containing 0.1% Nonidet P-40 for 10 min and once again with TBS without the Nonidet for 10 min. Thereafter, the sheet was treated with the second antibody (goat anti-mouse IgG conjugated with alkaline phosphatase, 1:6,000 dilution), and the reactive bands were detected with 0.1 M AP 9.5 solution (0.1 M Tris HCl, 0.1 M NaCl, 5 mM MgCl₂) containing 0.033% nitro blue tetrazolium and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt.

Staining with Concanavalin A (Con A)-peroxidase.

After IEF-PAGE, the proteins were transferred to the membrane sheet, and the sheet was immersed in a 50 mM TBS solution (pH 7.4) containing 200 mM NaCl and 0.02% NaN₃. Blocking of the sheet was conducted with SM in the same manner as immunoblotting. The sheet was once treated with 0.05% (w/v) Con A for 6 hr, and after blocking, it was treated with 0.05% (w/v) peroxidase at room temperature for 6 hr. Peroxidase activity was detected by the reaction with 0.5% 3-3-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-saline, pH 7.4.

Staining with Coomassie Brilliant Blue

Staining procedure by Allen et al. (1976) was applied. But the electrofocused gel was fixed with 20% trichloroacetic acid (TCA) for 1 hr, and then fixed again with 40% MtOH and 10% acetic acid for 30 min. The gel was washed twice with a destaining solution (25% EtOH and 8% acetic acid) to remove Pharmalyte, and then stained with 0.115% Coomassie Brilliant Blue G-250.

Staining with Silver nitrate

The staining procedure by Yuen et al. (1982) was applied with a little modification. After the fixation of protein with TCA, the gel was washed twice with distilled water for 10 min each. Then, the gel was sensitized with 12.5% glutaraldehyde solution for 30 min, washed with distilled water for 2 hr and treated with 5 ppm dithiothreitol solution for 30 min to prevent oxidation of silver nitrate.

3. RESULTS

Isolation of S-alleles

Out of 58 plants sampled from a population in Oguni, 45 plants were highly self-incompatible, 8 were self-compatible and the others were intermediate between them (Table 1).

Selfed seeds were obtained from the self-incompatible plants, and 32 families were subjected to intra-family diallel pollination experiments to determine *S* homo- and heterozygotes. An example of intra-family diallel pollination is pre-

Table 1. Frequency of self-compatible and incompatible plants in the Oguni population

Self-compatibility*	No. of plants	Percent
Self-incompatible	45	77.6
Intermediate	5	8.6
Self-compatible	8	13.8
Total	58	100.0

* Penetrating pollen tubes were less than 5 (self-incompatible), 5–20 (intermediate), and more than 20 (self-compatible).

a

Female	1	6	9	11	Male								S-geno- types	Band	
					2	3	4	5	10	7	8			A	B
1	-	-	-	-	-	-	-	-	-	+	+		S ^a S ^a	+	-
6	-	-	-	-	-	-	-	-	-	+	+			+	-
9	-	-	-	-	-	-	-	-	-	+	+			+	-
11	-	-	-	-	-	-	-	-	-	+	+			+	-
2	-	-	-	+-	-	-	-	-	-	-	-		S ^a S ^b	+	+
3	-	-	-	-	-	-	-	-	-	-	-			+	+
4	-	-	-	-	-	-	-	-	-	-	-			+	+
5	-	-	-	-	-	-	-	-	-	-	-			+	+
10	-	-	-	-	-	-	-	-	-	-	-			+	+
7	+	+	+	+	-	-	-	-	-	-	-		S ^b S ^b	-	+
8	+	+	+	+	-	-	-	-	-	-	-			-	+

b

Fig. 1. An example of segregation of S-alleles (a) and IEF-immunoblot profile of stigma proteins of each plant (b) in family no. 38. The bands indicated as A are specific to S^a and three bands as B are specific to S^b. Symbols in (a) are as follows; +: more than 20 pollen tubes penetrating. +-: 5–20 pollen tubes penetrating. -: less than 5 or no pollen tubes penetrating. The presence (+) or absence (-) of the specific bands is also indicated.

sented in Fig. 1, where plants showing the same pollination response are grouped together, and three groups are identified corresponding to two homozygotes, $S^a S^a$, $S^b S^b$, and heterozygotes $S^a S^b$. Segregation of S -allele was observed in 30 families out of 32 analyzed (Table 2), and Chi square analysis showed that the observed number has fitted well to the expected segregation ratio (1:2:1) in all families. No segregation of incompatibility was observed in family no. 21, indicating S -homozygosity of the parental plant. In another family no. 50, several self-compatible individuals appeared suggesting participation of some self-

Table 2. S -allele segregation, interaction and S-glycoproteins in a family

Family	Segregated types			P	Allelic interaction		S-glycoproteins	
	$S^a S^a$	$S^a S^b$	$S^b S^b$		Pollen	Stigma	S^a	S^b
1	3	8	4	>.9	$S^a = S^b$	$S^a = S^b$	+	+
2	3	8	3	>.8	=	=	+	+
3	3	10	2	>.3	=	=	+	+
5	4	6	1	>.3	=	=	+	+
7	4	7	3	>.3	>	=	+	+
9	4	7	1	>.3	=	=	+	+
11	2	9	1	>.2	=	=	+	+
12	6	4	2	>.1	=	=	+	+
17	6	6	1	>.1	=	=	+	-
18	2	6	5	>.3	>	=	+	-
19	2	6	3	>.8	=	=	+	+
23	2	6	5	>.5	>	=	+	+
25	3	4	5	>.3	=	=	+	+
27	2	7	0	>.1	=	=	+	-
28	2	6	2	>.8	=	=	+	+
29	1	8	1	>.1	=	=	nt*	nt
33	4	5	2	>.5	=	=	+	+
36	3	8	0	>.1	=	=	+	-
37	2	7	2	>.5	=	=	+	+
38	4	5	2	>.5	=	=	+	+
39	4	4	2	>.5	=	=	+	+
41	2	5	3	>.9	=	=	+	+
42	1	5	1	>.5	=	=	+	-
44	3	6	2	>.8	=	=	+	+
49	4	5	3	>.7	=	=	+	+
51	2	4	5	>.2	=	<	+	+
54	4	4	3	>.5	<	=	+	+
56	2	7	1	>.3	=	=	+	+
57	3	9	1	>.2	=	=	+	+
58	4	6	3	>.8	=	=	+	+

* nt: not tested

compatible factors. Dominant interaction between S-alleles was observed in four cases (13%) at the pollen side and only in one case (3%) at the stigma side (Table 2).

Selfed progenies were raised from each S-homozygote, and the identity of S-alleles were determined by diallel pollination between them. Thirty five S-homozygotes, so far analyzed, were comprised of 16 different S-alleles (Fig. 2).

Male	7	9	21	33	38	51	1	57	7	38	9	42	11	12	1	19	37	2	25	44	12	36	39	23	51	54	17	18	44	58	11	19	23	25	49
Female	5	1	14	5	7	6	1	13	6	6	14	7	6	1	6	2	7	1	10	5	2	8	9	3	5	6	7	13	6	5	12	5	11	9	8
7 × 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
9 × 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
21 × 14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
33 × 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
38 × 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
51 × 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1 × 13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
57 × 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
7 × 38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
9 × 42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
11 × 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1 × 19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
37 × 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
2 × 25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
44 × 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
36 × 39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
23 × 51	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
54 × 17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
18 × 44	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
58 × 11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
11 × 19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
23 × 25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
25 × 49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

Fig. 2. Results of diallel pollination between 35 S-homozygotes indicating that they are composed of 16 different S-alleles.

S-glycoproteins in stigmas

Segregation of IEF-immunoblot positive proteins in stigma is illustrated by the data of family no. 38 (Fig. 1). A basic thick band and several more acidic narrow bands were assigned to S^a , and a few more acidic bands to S^b . Among basic bands, a thick band is termed as the major S-glycoprotein band of S^a and other thin bands as the minor bands of S^a , tentatively. Thus, a number of bands were assigned to one S-allele that was determined by diallel pollination.

Another case was found in family no. 2, where plant 1, 9 & 15 were classified to be $S^a S^a$, 2, 3 & 6 were $S^b S^b$, and the other plants were heterozygotes. These S-genotypes well corresponded to the classification by zymograms shown in Fig. 3. Besides the S-glycoproteins segregating in both homozygotes, there was a band which appeared only in the heterozygotes. These heterozygote specific bands (arrow) were found in two cases out of the 30 families analyzed.

Of 62 S-alleles being tested, S-glycoproteins were assigned to 54 S-alleles by

Fig. 3. IEF-immunoblot profile of stigma proteins of each plant in family no. 2, showing that a new band (arrow) appears in the heterozygotes (plant 4, 5, 7, 8, 10, 11, 13 & 14). Plant 1, 9 & 15, and 2, 3 & 6 are two *S*-homozygotes, *S^aS^a* and *S^bS^b*, respectively.

IEF-immunoblot. Fig. 4a shows the IEF-immunoblot profile of 35 *S*-homozygotes. The identical *S*-alleles determined by the pollination experiment (Fig. 2) had the same *S*-glycoproteins as shown by arrows. The same *S*-alleles had the same *S*-glycoproteins, but the content was variable according to strains. Some strains had many antiserum positive bands other than *S*-glycoproteins, but others were not. Besides these major and minor *S*-glycoprotein bands, several faintly stained bands were recognized sometimes and these were not co-segregated with *S*-glycoproteins.

When stigma proteins were stained with Con A-peroxidase, a dark band was found at the equivalent position with the *S*-glycoprotein assigned by the immunoblot analysis (data not shown), and this was considered to be *S*-glycoprotein. No bands were detected in 7 *S*-homozygotes (1·6, 9·14, 19·5, 36·8, 44·5, 51·5 and 58·5), although immunoblot analysis revealed *S*-glycoproteins in them. There were some darkly stainable bands other than *S*-glycoproteins, and the number of these bands were different among individuals.

In the case of Coomassie Brilliant Blue staining, some bands were ascribed to the *S*-glycoproteins (Fig. 4b). *S*-glycoproteins were not stained in very similar strains as in the case of Con A-peroxidase; ie. 1·6, 9·14, 36·8, 44·5, 44·6, 51·5 and 58·5. A band (open arrow) at about pI 6 was observed in almost all strains, and one or a few bands other than *S*-glycoprotein were also observed in some strains. By Silver staining a number of bands were detected, but it was difficult to determine any specific bands to *S* (data not shown).

Fig. 4. IEF profiles of stigma proteins stained with immunoblot (a) and Coomassie Brilliant Blue (b) of 35 *S*-homozygotes which are comprised of 16 different *S* alleles as shown in Fig. 2.

The pI value of the major S-glycoproteins for 16 *S*-alleles ranged from pI 5.0 to 9.0, and the most frequent value was between pI 7.0–9.0 (Table 3).

SDS-PAGE-immunoblot analysis of the stigma proteins of 10 *S*-homozygotes is shown in Fig. 5. A band (arrow) found at about 46 KD in every *S*-homozygotes was considered to be a non-glycosylated S-glycoprotein, because this band was not observed by Con A-peroxidase staining. A few bands at 50 to 60 KD were the S-glycoproteins glycosylated by different numbers of residues. It was not known

Table 3. Frequency distribution of pI value of major S-glycoproteins in 16 different *S*-alleles

pI range	Number of alleles
5.0–5.9	4
6.0–6.9	1
7.0–7.9	6
8.0–8.9	5
Total	16

why a *S*-homozygote 1·6 only contained many low molecular S-glycoproteins. The bands of *S*-homozygotes 2·2 and 23·3 were always faintly stained; the former *S*-allele was codominant to that of 2·1, but the latter *S*-allele was recessive to that of 23·11 at pollen side. Thus, the *S*-allele with low yield of S-glycoproteins was not always recessive.

Fig. 5. SDS-PAGE immunoblot analysis of stigma proteins for 10 different *S*-genotypes, comparing their molecular weight.

4. DISCUSSION

Self-compatibility was observed in 13.3 percent plants of the population. Bateman (1954) found that a few self-compatible plants were included in a mostly self-incompatible population of *Iberis amara*. Genetic analysis of self-compatibility of this population is now in progress.

One locus *S*-multiple-allele model can be applied in this species, so far as the small size segregation experiments are concerned. The *S*-allele identity test by diallel pollination revealed that 35 *S*-homozygous plants in this population were

composed of 16 independent *S*-alleles. Total number of *S*-alleles in this population was estimated to be 22 by the application of the formula by O'Donnell and Lawrence (1984). The direct application of this formula, however, may bring about a certain bias, because the *S*-allele analysis has not been completed with all sampled plants yet. Although it is very difficult to estimate the bias, we may have an image that the number of *S*-alleles in this population ranges from 20 to 30. This value is similar to 22 *S*-alleles in *Iberis amara* (Bateman, 1954) and 25 to 34 in *Raphanus raphanistrum* (Sampson, 1967), but fewer than 52 in *Sinapis arvensis* (Stevens and Kay, 1989).

In the present analysis, dominance interactions were found in 4 combinations (13%) in pollen and one (3%) in stigma out of the 30 heterozygotes studied. Although we did not test all the combinations of *S*-alleles, the frequencies were lower than those of *Raphanus raphanistrum* (Sampson, 1964) and *Sinapis arvensis* (Stevens and Kay, 1989).

S-allele specific glycoproteins were assigned to 54 *S*-alleles by IEF-immunoblot analysis where the cross-reaction of anti-S⁸-glycoprotein-antiserum was applied. *S*-alleles and S-antigen-stainable bands were completely co-segregated. No stainable bands were assigned to a few *S*-alleles, probably due to low content of S-glycoproteins to be detected. Wide variation of the quantity of S-glycoproteins in a stigma was observed when they were stained by Coomassie Blue and Silver-staining. Of the four methods examined, the most sensitive one was the immunoblot by using cross reaction of antiserum.

The stigma of an *S*-homozygote produced many bands having different pI points and were tentatively considered to be composed of major and minor bands. These bands may have very similar structure and similar molecular weight ranging between 50–60 KD. Some of the minor bands were newly observed in heterozygous plants and assumed to be made by post-transcriptional modification. Many of them, however, were heritable in correlation with the major band. Probably, they are controlled by some genomic DNA sequences which is located very near to the *S*-locus, since Scutt et al. (1990) and Dwyer et al. (1989) reported the presence of several DNA sequences that hybridize with SLSG in Southern analysis and, therefore, *S*-locus is a multigene family. High heritable correlation between major and minor S-glycoproteins pointed out in this study also suggest highly complex nature of *S*-alleles, in spite of its simple inheritance in the segregation study of individuals. Studies of these glycoproteins are expected to give us details about the function and variation of S-glycoproteins.

Beside these densely stainable proteins, there were faintly stained NS-glycoproteins which were independent from S-glycoproteins but have very similar structure in view of oligosaccharides and amino acid sequences (Isogai et al., 1988). We could raise an antiserum to this protein and detect four different isoforms of it. This will be detailed elsewhere.

The present study was supported by a Grant-in-Aid for Special Research on Priority Areas (no. 0166002, Cellular and Molecular Basis for Reproductive Processes in Plants) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- Allen, R. C., Spicer, S. S. and Zehr, D. (1976). Concanavalin A-horse radish peroxidase bridge staining of α -1 glycoproteins separated by isoelectric focusing on polyacrylamide gel. *J. Histochem. Cytochem.* **24**, 908-914.
- Bateman, A. J. (1954). Self-incompatibility system in Angiosperms. II. *Iberis amara*. *Heredity* **8**, 305-332.
- de Nettancourt, D. (1977). "Incompatibility in Angiosperms", Springer-Verlag, Berlin-Heidelberg-New York, pp. 230.
- Dwyer, K. G., Chao, A., Cheng, B., Chen, C. H. and Nasrallah, J.B. (1989). The *Brassica* self-incompatibility multigene family. *Genome* **31**, 969-972.
- Hinata, K. and Nishio, T. (1978). S-allele specificity of stigma proteins in *Brassica oleracea* and *B. campestris*. *Heredity* **41**, 93-100.
- Hinata, K., Nishio, T. and Kimura, J. (1982). Comparative studies on S-glycoproteins purified from different S-genotypes in self-incompatible *Brassica* species. II. Immunological specificities. *Genetics* **100**, 649-657.
- Isogai, A., Takayama, S., Shiozawa, H., Tsukamoto, C., Kanbara, T., Hinata, K. Okazaki, K. and Suzuki, A. (1988). Existence of a common glycoprotein homologous to S-glycoproteins in two self-incompatible homozygotes of *Brassica campestris*. *Plant Cell Physiol.* **29**, 1331-1336.
- Isogai, A., Takayama, S., Tsukamoto, C., Ueda, Y., Shiozawa, H., Hinata, K., Okazaki, K. and Suzuki, A. (1987). S-locus-specific glycoproteins associated with self-incompatibility in *Brassica campestris*. *Plant Cell Physiol.* **28**, 1279-1291.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lalonde, B. A., Nasrallah, M. E., Dwyer, K. G., Chen, C. H., Barlow, B. and Nasrallah, J. B. (1989). A highly conserved *Brassica* gene with homology to the S-locus-specific glycoprotein structural gene. *Plant Cell* **1**, 249-258.
- Nasrallah, M. E. and Wallace, D. H. (1967). Immunogenetics of self-incompatibility in *Brassica oleracea* L. *Heredity* **22**, 519-527.
- Nasrallah, M. E., Barber, J. T. and Wallace, D. H. (1970). Self-incompatibility proteins in plants: detection, genetics and possible mode of action. *Heredity* **25**, 23-27.
- Nasrallah, M. E., Wallace, D. H. and Savo, R. M. (1972). Genotypes, protein phenotype relationships in self-incompatibility of *Brassica*. *Genet. Res.* **20**, 151-160.
- Nishio, T. and Hinata, K. (1977). Analysis of S-specific proteins in stigma of *Brassica oleracea* L. by isoelectric focusing. *Heredity* **38**, 391-396.
- Nishio, T. and Hinata, K. (1978). Stigma proteins in self-incompatible *Brassica campestris* L. and self-compatible relatives, with special reference to S-allele specificity. *Jpn. J. Genet.* **53**, 27-33.
- Ockendon, D. J. (1974). Distribution of self-incompatibility alleles and breeding structure of open-pollinated cultivars of Brussel sprouts. *Heredity* **33**, 159-171.
- O'Donnel, S. and Lawrence, M. J. (1984). The population genetics of the self-incompatibility polymorphism in *Papaver rhoeas*. IV. The estimation of the number of alleles in a population. *Heredity* **53**, 495-507.
- Sampson, D. R. (1964). A one-locus self-incompatibility system in *Raphanus raphanistrum*. *Can. J. Genet. Cytol.* **6**, 435-445.
- Sampson, D. R. (1967). Frequency and distribution of self-incompatibility alleles in *Raphanus*

- raphanistrum*. *Genetics* **56**, 241–251.
- Sampson, D. R. (1974). Equilibrium frequencies of sporophytic self-incompatibility alleles. *Can. J. Genet. Cytol.* **16**, 611–618.
- Scutt, C. P., Gates, P. J., Gatehouse, J. A. Boulter, D. and Croy, R. R. D. (1990). A cDNA encoding an S-locus specific glycoprotein from *Brassica oleracea* plants containing the S^5 self-incompatibility allele. *Mol. Gen. Genet.* **220**, 409–413.
- Stevens, J. P. and Kay, Q. O. N. (1989). The number, dominance relationships and frequencies of self-incompatibility alleles in a natural population of *Sinapis arvensis* L. in South Wales. *Heredity* **62**, 199–205.
- Takayama, S., Isogai, A., Tsukamoto, C., Ueda, Y., Hinata, K., Okazaki, K. and Suzuki, A. (1987). Sequence of S-glycoproteins, products of the *Brassica campestris* self-incompatibility locus. *Nature* **326**, 102–105.
- Trick, M. and Flavell, R. B. (1989). A homozygous S genotype of *Brassica oleracea* expresses two S-like genes. *Mol. Gen. Genet.* **218**, 112–117.
- Trick, M. (1990). Genomic sequence of a *Brassica* S locus-related gene. *Plant Mol. Biol.* **15**, 203–205.
- Umbach, A. L., Lalonde, B. A., Kandasamy, M. K., Nasrallah, J. B. and Nasrallah, M. E. (1990). Immunodetection of protein glyco forms encoded by two independent genes of the self-incompatibility multigene family of *Brassica*. *Plant Physiol.* **93**, 739–747.
- Walker, J. C. and Zhang, R. (1990). Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. *Nature* **345**, 743–746.
- Yuen, K. C. L., Johnson, T. K., Denell, R. E. and Consigli, R. A. (1982). A silver staining technique for detecting minute quantities of proteins on nitrocellulose paper: Retention of antigenicity of stained proteins. *Anal. Biochem.* **126**, 398–402.