JAPAN. J. GENETICS Vol. 53, No. 1: 27-33 (1978)

STIGMA PROTEINS IN SELF-INCOMPATIBLE *BRASSICA CAMPESTRIS* L. AND SELF-COMPATIBLE RELATIVES, WITH SPECIAL REFERENCE TO S-ALLELE SPECIFICITY

TAKESHI NISHIO AND KOKICHI HINATA

Faculty of Agriculture, Tohoku University, Sendai 980

Received October 12, 1977

Substances which may be the product of S-alleles and by which a stigma may recognize a specific pollen have since long been looked for in the investigations on self-incompatibility in higher plants. Studies on stigma proteins in *Brassica oleracea* L., a sporophytic self-incompatible species, have revealed that S-specific antigens exist (Nasrallah and Wallace 1967). The S-specific antigens are basic proteins (Nasrallah *et al.* 1970), and different S genotypes produce differential glycoproteins which can be distinctly separated by isoelectric focusing (Nishio and Hinata 1977a, 1977b).

B. campestris L. is another sporophytic self-incompatible species whose genomic constitution is different from *B. oleracea*. The present investigation was undertaken to know whether or not S-specific glycoproteins could be found in the stigma of *B. campestris*, as shown in *B. oleracea*.

B. campestris includes one self-compatible variety, *yellow sarson*, which belongs to the same genome type (Olsson 1954; Gonai and Hinata 1971). *B. tournefortii* Gouan is also self-compatible but it has a different genomic constitution (Fukushima and Iwasa 1966). *B. napus* L. is a self-compatible allo-tetraploid species which possesses the genomes of *B. campestris* and *B. oleracea* (U 1935). Isoelectric focusing analysis of the proteins of these self-compatible species was also carried out to obtain information about the specificity of proteins in the self-incompatible species.

MATERIALS AND METHODS

Homozygous strains with respect to S-alleles in *Brassica campestris* L. were raised from a spontaneous population which grows as a field weed in the suburbs of Oguni-machi, Yamagata-ken, Japan. Seeds collected from the population were grown in a glass house and selfed progenies were obtained. S-homozygotes were determined from data about pollen tube penetration in inter- and intra-family pollinations of the progenies. Plants propagated by selfing or by intra S-genotype crossing, were checked again and used as S-homozygotes.

B. campestris L. var. *yellow sarson* (C633, C634 and C636) and *B. tournefortii* Gouan (T162 and T164) were used as monogenomic self-compatible plants (Gonai and Hinata 1971; Hinata, Konno and Mizushima 1974). Both of them have a haploid number of

10 chromosomes. The genome of the former is the same as that of *B. campestris* (Olsson 1954), but the latter is different (Fukushima and Iwasa 1966). All the strains are different in their origins; C633 was sent from Svalöf, Sweden, C634 from U.S.D.A., U.S.A., C636 from Nat. Inst. Agr. Sci., Japan, T164 from I.A.R.I., India and T162 collected at Abu Sir, Egypt by the junior author. Of 2 strains, N108 and N346, in *B. napus* L., a digenomic self-compatible species, the former strain is a cultivar sent from Rech. Agr. l'Exp. Stat., Morocco and the latter is cv. Bronowski sent from Dept. Agr. Res. Stat., Canada.

Isoelectric focusing of stigma proteins was performed by the method described in a previous paper (Nishio and Hinata 1977a) with a slight modification. Twenty five stigmas were homogenized with a mortar and pestle with 0.15 ml of 0.01 M phosphate buffer (pH 7.1). The homogenate was centrifuged for 20 min at 10,000 rpm and the whole supernatant, ca. 0.1 ml, was used as one sample. Acrylamide 7.5 per cent solution containing 0.1 per cent BIS, 2 per cent ampholine (pH 3.5–10.0, LKB Co.), and 5 ppm riboflavin was filled in a glass tube (inner diameter 5 mm) up to 6.5 cm high, and was photopolymerized for 1 h to make a resolving gel. Stigma extract mixed with acrylamide, ampholine and riboflavin, was poured onto the resolving gel and again photopolymerized for 1 h. The tubes were placed in a column acrylamide gel apparatus with 1 M NaOH in the lower vessel (cathode) and 0.02 M phosphate in the upper vessel (anode). Electrophoresis was carried out under constant voltage, 200 V for 3 h at 5°C.

Protein staining: The gels, after electrophoresis, were removed from the tubes, immersed in 0.04 per cent Coomassie Brilliant Blue G250 in 3.5 per cent perchloric acid for one day (Reisner *et al.* 1975), destained in acetic acid/ethanol/water (10:25:65), and then stored in 7 per cent acetic acid.

Periodic acid and Shiff's (PAS) reaction: After isoelectric focusing the gels were stained by the method of Zacharius *et al.* (1969).

Concanavalin A (Con A) reaction: After isoelectric focusing the gels were once dipped in 0.1 M phosphate buffer (pH 7.1), and were immersed in a Con A solution (0.5 mg/ml saline) for one night. After the elution of non-precipitated proteins by saline solution, precipitated bands were stained with Coomassie Blue.

RESULTS

Self-incompatible plants

Isoelectric focusing of stigma extracts in *B. campestris* revealed the presence of numerous bands ranging from pH 5 to pH 9. Band patterns at higher pH region than pH 5.5 showed distinct difference between S-homozygotes as shown in Fig. 1. For example, S_7S_7 had a dense band at pH 6 region, S_8S_8 had two dense bands in between pH 7–8, *etc.* So far, all the S-homozygotes examined were differentiated from each other by the number and the location of their bands. This result that the band patterns can be ascribed to S-homozygotes is similar with that of *B. oleracea* (Nishio and Hinata 1977a).

Variation of the bands among individuals within each S-homozygote was tested using 7 plants of S_3S_3, 7 of S_7S_7, 4 of S_8S_8, 4 of $S_{10}S_{10}$, 5 of $S_{12}S_{12}$. Every plant of the

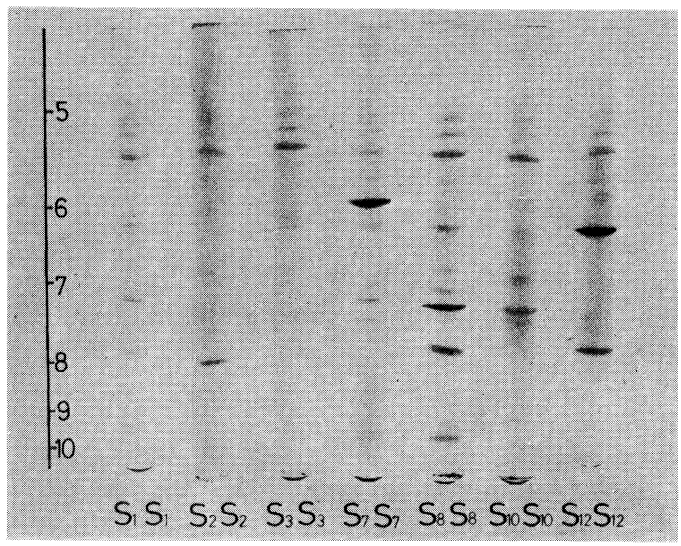

Fig. 1. Electrophoretic separation of proteins from the stigmatic homogenates of different S-homozygotes in *B. campestris* by isoelectric focusing.

same genotype showed the same specific band pattern as illustrated in the figure except S_3S_3, where 4 plants had one more band than the others.

Several F_1 hybrids between these S genotypes were analyzed for the band patterns of their stigma extracts. They were composed of the bands of the both parentals.

Pistils were separated into 3 parts (stigma, style and ovary) and the extracts from the respective parts were examined in the same way. Neither basic nor S genotype specific bands were observed in the styles or in the ovaries. The differential bands were only found in the stigma part.

Some of the differential dense bands were highly positive to PAS reaction as shown in Fig. 2. In the treatment with Con A, white precipitates were observed at the loci of the differential bands. After the elution of non-precipitated bands, the precipitated bands were stained by Coomassie Blue as shown in Fig. 2. The precipitation was inhibited by the presence of 0.1 M α-methyl-D-glucoside. In S_{12} homozygotes, the same bands were stained by both reactions, PAS and Con A. In S_7 and S_8 homozygotes, however, one band was commonly stained, but the other bands showed different reactions to PAS and Con A.

The protein bands from styles and ovaries did not show any PAS reactions so far.

Self-compatible plants

The band patterns of stigma extracts of monogenomic self-compatible plants are shown in Fig. 3. In the high pH region, two dense bands were observed in *yellow sarson* and one band in *B. tournefortii*. No variation was found among strains within each species, so far. These bands were highly PAS positive.

The general band patterns of style or ovary extracts in *yellow sarson* were very alike to those of the respective parts in self-incompatible *campestris*. Such dense bands

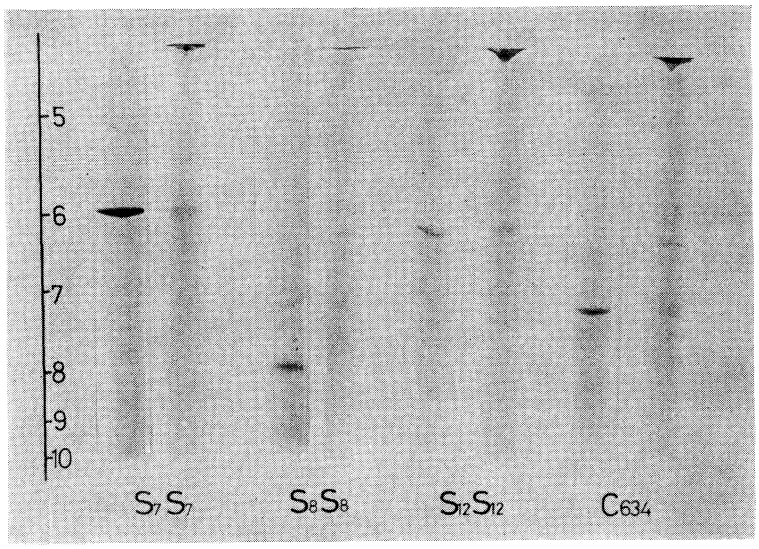

Fig. 2. PAS reaction (left) and Con A reaction (right) of the stigmatic protein bands of S-homozygotes (S_xS_x) in *B. campestris* (self-incompatible) and in var. *yellow sarson* (self-compatible; C634) of the same species.

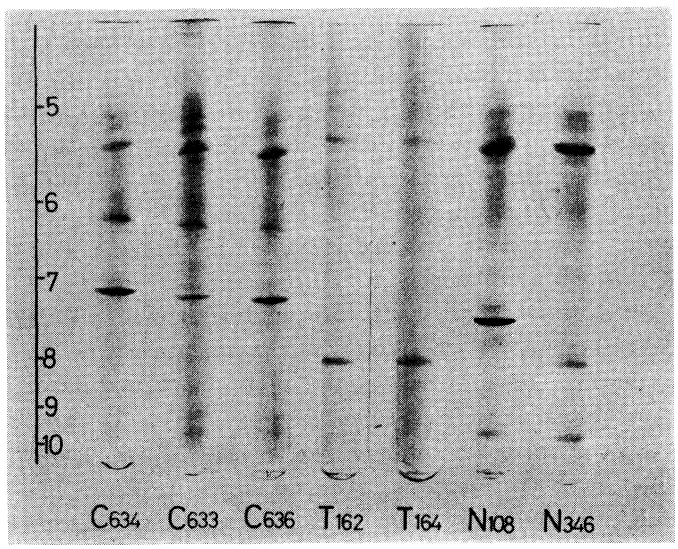

Fig. 3. Electrophoretic separation of the proteins from the stigmatic homogenates of self-compatible species by isoelectric focusing; *B. campestris* var. *yellow sarson* (C634, C633, C636), *B. tournefortii* (T162, T164) and *B. napus* (N108, N346).

as seen in stigmas were not observed in these parts.

The stigma proteins of digenomic self-compatible plants, *B. napus*, were different from those of monogenomic self-compatibles. Numerous weakly stained bands of PAS positive proteins were observed at the basic side of column. Two cultivars tested gave a different band pattern each other.

DISCUSSION

In self-incompatible *Brassica* species, incompatible pollen grains barely germinate on the stigma and when they germinate, their pollen tubes can not grow into the pectin-cellulose layer of papilla cells on the stigma surface, suggesting the importance of the stigma as a recognition site of self-incompatibility (Kanno and Hinata 1969; Dickinson and Lewis 1973). In the previous studies of stigma proteins in *B. oleracea* differential glycoproteins were found among S-homozygotes (Nishio and Hinata 1977a, 1977b). The present results indicate the existence of similar differential glycoproteins in self-incompatible *B. campestris*. The proteins are heritable, and they are found in the stigmas but not in the other parts of a pistil.

B. campestris includes a self-compatible variety, *yellow sarson*. Since the majority of the species are self-incompatible, the self-compatible variety may have been established in the course of diversification of the species (Hinata and Konno 1975). It can be extended to the relations of *B. tournefortii* to *Brassica* genus. As for the stigma extracts of these self-compatible species, one or two protein bands were found at the basic region of the gels. No variation was found so far among strains which were obtained from different sources. The establishment of self-compatible species is probably based on the change of such differential glycoproteins, though certain factors which affect the activity of the proteins in incompatibility response may also participate in this event.

On the other hand, several glycoprotein bands were found in *B. napus* which is self-compatible allotetraploid species between *B. campestris* and *B. oleracea*. The establishment of self-compatibility in polyploid species seems to be much more complicated since it probably based not only on the modifying factors but also on allelic interactions as suggested by Lewis (1947) in terms of competition and dominance in the gametophytic system.

Besides, the analysis of these self-compatible species revealed that they possess glycoproteins in stigmas. It is suggested that the existence of glycoproteins are not always in connection with the expression of self-incompatibility.

Glycoproteins have been noticed as the recognition substances in sexual conjugation of microorganisms (Wiese *et al.* 1972; Crandall *et al.* 1974; Shimoda and Yanagishima 1975). The existence of Con A reactive glycoproteins on papilla surface in *Gladiolus gandavensis* has recently been shown by Knox *et al.* (1976), and they considered that such substances play an important role for the recognition events regulating fertilization in higher plants. The differential glycoproteins analyzed in the present experiment were proved to react with Con A. It can be assumed that the differential glycoproteins play a certain role in recognizing the specificity of pollen of different species as well as the specificity of the S-genotype of pollen in self-incompatible species.

Adding up these informations, it can be postulated about self-incompatible *Brassica* species that the S-allele specificity is ascribable to some of the differential proteins, but not all the differential bands contribute to the recognition events. This postulation might be applicable to all the self-incompatible species in the family Cruciferae, further

to sporophytic self-incompatible species.

The evidence observed here may provide some substantial basis for understanding gene action in self-incompatibility under sporophytic control. Isolation of the S-specific glycoproteins and examination of their physiological role may be one of the prospective approaches for analyzing the mechanisms of self-incompatibility as well as the evolution of self-compatibility.

SUMMARY

The protein band patterns of stigma extracts were differentiated in seven different S-homozygotes of self-incompatible *Brassica campestris* L. by polyacrylamide gel isoelectric focusing. The protein bands which differentiated S-alleles were heritable and appeared in stigmas but neither in styles nor in ovaries. The bands were thought to be glycoproteins in nature since they were PAS positive and precipitated with Con A. Such glycoproteins are postulated to be S-specific proteins.

One or two glycoprotein bands were found in monogenomic self-compatible species (*B. campestris* var. *yellow sarson* and *B. tournefortii*). They were common in every strain within a species so far but different between two species. Digenomic self-compatible *B. napus* had several thin glycoprotein bands.

ACKNOWLEDGMENTS

The authors wish to thank Prof. S. Tsunoda for advice and encouragement. They also thank Prof. C. Gomez-Campo of Universidad Politecnica, Madrid, for reading the manuscript.

LITERATURE CITED

Crandall M., L. M. Lawrence, and R. M. Saunders, 1974 Molecular complementarity of yeast glycoprotein mating factors. Proc. Natl. Acad. Sci. USA **71**: 26-29.

Dickinson, H. G., and D. Lewis, 1973 Cytochemical and ultrastructural differences between intraspecific compatible and incompatible pollinations in *Raphanus*. Proc. R. Soc. London. B. **183**: 21-38.

Fukushima, E., and S. Iwasa, 1966 Genome-analytic studies on *Brassica trilocularis* and *B. tournefortii*. J. Fac. Agr. Kyushu Univ. **13**: 743-748.

Gonai, H., and K. Hinata, 1971 Growth of pistils in relation to phenotypic expression of self-incompatibility in *Brassica*. Japan. J. Breed. **21**: 137-142.

Hinata, K., and N. Konno, 1975 Number of pollen grains in *Brassica* and allied genera. Tohoku J. Agr. Res. **26**: 117-124.

Hinata, K., N. Konno, and U. Mizushima, 1974 Interspecific crossability in the tribe Brassiceae with special reference to the self-incompatibility. Tohoku J. Agr. Res. **25**: 58-66.

Kanno, T., and K. Hinata, 1969 An electron microscopic study of the barrier against pollen tube growth in self-incompatible Cruciferae. Plant and Cell Physiol. **10**: 213-216.

Knox, R. B., A. Clarke, S. Harrison, P. Smith, and J. J. Marchalonis, 1976 Cell recognition in plants: Determinants of the stigma surface and their pollen interactions. Proc. Natl. Acad. Sci. USA **73**: 2788-2792.

Lewis, D., 1947 Competition and dominance of incompatibility alleles in diploid pollen. Heredity **1**: 85–108.

Nasrallah, M. E., J. T. Barber, and D. H. Wallace, 1970 Self-incompatibility proteins in plants: detection, genetics and possible mode of action. Heredity **25**: 23–27.

Nasrallah, M. E., and D. H. Wallace, 1967 Immunogenetics of self-incompatibility in *Brassica oleracea* L. Heredity **22**: 519–527.

Nishio, T., and K. Hinata, 1977a Analysis of S-specific proteins in stigma of *Brassica oleracea* L. by isoelectric focusing. Heredity **38**: 391–396.

Hishio, T., and K. Hinata, 1977b Positive PAS reaction of S-specific proteins in stigma of *Brassica oleracea* L. Incompatibility Newsletter **8**: 31–33.

Olsson, G., 1954 Crosses within the *campestris* group of the genus *Brassica*. Hereditas **40**: 398–418.

Reisner, A. H., P. Nemes, and C. Bucholtz, 1975 The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. Anal. Biochem. **64**: 509–516.

Shimoda, C., and N. Yanagishima, 1975 Mating reaction in *Saccharomyces cerevisiae*. VIII. Mating-type-specific substances responsible for sexual cell agglutination. Antonie van Leeuwenhoek **41**: 521–532.

U, N., 1935 Genomic analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Japan. J. Bot. **7**: 389–452.

Wiese, L., and P. C. Hayward, 1972 On sexual agglutination and mating-type substances in isogamous dioecious chlamydomonas. III. The sensitivity of sex cell contact to various enzymes. Amer. J. Bot., **59**: 530–536.

Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock, 1969 Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**: 148–152.