Theor Appl Genet (1999) 99:885–890

M. Beschorner · V. Heberer · W. Odenbach

Analysis of self-incompatibility interactions in 30 resynthesized *Brassica napus* lines. II. Expression of S-locus glycoproteins (SLGs)

Received: 22 January 1999 / Accepted: 30 January 1999

Abstract Thirty resynthesized *Brassica napus* lines with defined S-allele constitution and the ancestral *B. oleracea* and *B. campestris* lines were used for the analysis of S-locus glycoproteins (SLGs). The aim of this study was to investigate (1) whether the S-specific glycoproteins of the diploid ancestor lines were also expressed in the amphidiploid hybrids and (2) whether the occurrence of SLG bands was correlated with the activity of the respective S-alleles, which had been tested by means of diallele pollination tests in a previous study. Stigma proteins were separated by isoelectric focusing (IEF)-gel electrophoresis, and glycoprotein bands were identified by Western blotting and Con-A/peroxidase reaction. The SLG bands of the *B. campestris* parent could be detected in all 30 resynthesized *B. napus* lines. In contrast, *B. oleracea* SLG bands could only be detected in 12 resynthesized *B. napus* lines. Only *B. napus* lines which carried the dominant *B. oleracea* S-alleles S_8 and S_{29} showed respective SLG bands in all cases. Nine *B. napus* lines showed only glycoprotein bands of the *B. campestris* parent, although the biological functioning of the *B. oleracea* S-alleles was demonstrated by test-pollinations. New SLG bands different from those of the *B. oleracea* and *B. campestris* parents occurred in 16 *B. napus* lines. The expression level of the SLGs in *B. napus* was not correlated with the self-incompatibility phenotype, not only in the case of recessive S-alleles (S_2, S_{15}), but also for dominant alleles (e.g. S_{14}, S_{32}, S_{45}).

Key words *Brassica napus* · Self-incompatibility · S-allele · Glycoprotein

Communicated by H.F. Linskens

M. Beschorner (✉) · V. Heberer · W. Odenbach
Institut für Biologie – Angewandte Genetik –,
Freie Universität Berlin,
Albrecht-Thaer-Weg 6, D-14195 Berlin, Germany

Introduction

Self-incompatibility (SI) has evolved in flowering plants as a mechanism to prevent self-fertilization. In *Brassica*, self-incompatibility is controlled by a single, multiallelic S-locus which contains at least two genes, the S-locus glycoprotein (SLG) gene and the S-locus receptor kinase (SRK) gene (for review see Nasrallah and Nasrallah 1993). The existence of S-specific proteins in stigmas of *B. oleracea* was first pointed out by Nasrallah and Wallace (1967) who used immunological techniques. The isoelectric focusing analysis of Roberts et al. (1979) showed that mature stigmas of *B. oleracea* possess large quantities of a specific glycoprotein not present during earlier stages of development in the bud. The differential bands were found in mature stigmas but not in young stigmas, ovaries and styles. It was postulated that the differential protein bands were S-allele specific. Additional studies of Nishio and Hinata (1977, 1980), Hinata and Nishio (1978, 1980, 1981) and Nasrallah and Nasrallah (1984) revealed that certain glycoproteins co-segregate with S-alleles in S-segregating populations: these glycoproteins, which are believed to participate in the recognition reaction between stigma and pollen, were named S-locus glycoproteins (SLGs). DNA sequences encoding SLGs have been cloned and molecularly characterized in *B. oleracea* by Nasrallah et al. (1985, 1987). A second gene situated at the S-locus, the S-locus-receptor kinase (SRK) gene, was identified and characterized by Stein et al. (1991). Because of the presence of two transcriptional units at the S-locus Boyes and Nasrallah (1993) suggested referring to the S-allele as the S-haplotype. Based on sequence comparison the S-haplotypes have been grouped into two major classes: class I includes haplotypes high in the dominance series and exhibiting a strong incompatibility phenotype; class II consists of the three recessive haplotypes S_2, S_{15} and S_5 which exhibit a weak incompatibility phenotype (Nasrallah et al. 1991).

Two other genes, homologous to the SLG gene but not linked to the S-locus, the S-locus-related (SLR 1 and SLR 2) genes, are also expressed in the stigma (Boyes et

al. 1991). The function of these genes in the self-incompatibility response remains unclear. The fact that SLR genes segregate independently of the S-locus indicates that they are not a determinant of self-incompatibility specificity. However, they may play a supporting role in the functioning of the SLG or the SRK gene in specific pollen recognition (Boyes et al. 1991).

Unlike natural *B. napus*, resynthesized *B. napus* derived from the hybridization of self-incompatible *B. oleracea* and *B. campestris* is self-incompatible. In general, both parental S-loci can contribute to the incompatibility response. In the first part of this investigation the activity of the S-alleles in 30 resynthesized *B. napus* lines was examined by diallele test-pollinations between all resynthesized *B. napus* having one S-allele in common and their respective diploid ancestors (Beschorner et al. 1995). The self-incompatibility phenotype was mainly influenced by the dominance rank of the S-alleles. *B. oleracea* S-alleles high in the dominance series (e.g. S_8, S_{14}, S_{29}) were always active in the resynthesized *B. napus*, whereas recessive alleles (S_2, S_{15}) lost their activity in some test combinations. The *B. campestris* S-alleles were active in most cases, although two alleles were partially inactivated in combination with the recessive *B. oleracea* S-allele S_{15}.

In the study described here we identified the SLG bands of the nine *B. oleracea* and six *B. campestris* parental lines and analysed the occurrence of these SLG bands in the resynthesized *B. napus* lines. The aim of this study was to investigate (1) whether the S-specific glycoproteins of the parental lines were also expressed in the amphidiploid hybrids and (2) whether the differential protein bands were correlated with the results of the diallele test-pollinations (Beschorner et al. 1995).

Materials and methods

Plant material

Nine *Brassica oleracea* lines and six *B. campestris* lines (including one self-compatible line) homozygous for defined S-alleles (Table 1) and 30 lines of resynthesized *B. napus* with different S-allele combinations were used for this investigation. Origin and taxonomy of the diploid ancestors are as per Beschorner et al. (1995). The designation of the *B. napus* lines consists of the *B. oleracea* code followed by the *B. campestris* code (e.g. *B. napus* line RS 1/2 is derived from the cross B.ol. 1 x B. camp. 2).

Isoelectric focusing (IEF)

Sets of 25 buds (approximately 6–10 days before anthesis) and 25 flowers just before anthesis were collected from single plants. Stigmas and styles of buds and open flowers were dissected and stored at –20°C. The samples were homogenized in 50 µl distilled water. After 100 µl dichlorethane was added to the samples they were mixed and centrifuged at 4,000 rpm for 20 min at 4°C. Twenty-microliter aliquots of the supernatant were used for isoelectric focusing on 0.5-mm polyacrylamide gels (gradient pH 3.5–11) at 2,000 V. Total protein patterns were observed after silver staining (Merril et al. 1981). Isoelectric points of the separated proteins were estimated by using the Pharmacia broad pI calibration kit (Pharmacia LKB Biotech, Freiburg, Germany).

Detection of glycoproteins

Glycoprotein bands were detected by the Con-A/peroxidase reaction according to Hawkes (1982) with the following modifications: the proteins separated by IEF were electroblotted onto a NC-membrane (Schleicher and Schuell, 0.45 µm) and treated with 25% ethanol/10% acetic acid (v/v) for 15 min. After washing (5 × 2 min) in TRIS-buffered saline (TBS, 50 m*M* TRIS-HCl, pH 7.4, 150 m*M* NaCl, 150 m*M* KCl) the membrane was washed with boiling 2% SDS/10 m*M* DTT-solution (in TBS) for 2 min. This procedure can increase the sensitivity of detecting glycoproteins by the Con-A-peroxidase reaction (P. Wehling, personal communication). Free binding sites of the NC-membrane were blocked with 0.5% Tween 20 in TBS for 1 h. The incubation of Con A and horseradish-peroxidase was done as described by Hawkes (1982). Bound peroxidase was detected according to Endo (1972).

Table 1 S-allele status of *Brassica oleracea* and *B. campestris* lines used for resynthesis of *B. napus*

Code	S-allele	Classification [a,b]
B. oleracea		
B.ol. 1	S 8	Dominant/-
B.ol. 4	S 2	Recessive/class II
B.ol. 5	S 4	Less dominant/-
B.ol. 7	S 14	Dominant/class I
B.ol. 8	S 15	Recessive/class II
B.ol. 10	S 32	Dominant/-
B.ol. 11	S 45	Dominant/-
B.ol. 13	S 2	Recessive/class II
B.ol. 14	S 29	Dominant/class I
B. campestris		
B. camp. 2	S a	–
B. camp. 3	S b	–
B. camp. 4	S c	–
B. camp. 5	S d	–
B. camp. 6	S e/f	–
B. camp. 7	–	Self-compatible

[a] According to Visser et al. (1982) and Thompson and Taylor (1996)
[b] According to Nasrallah et al. (1991)

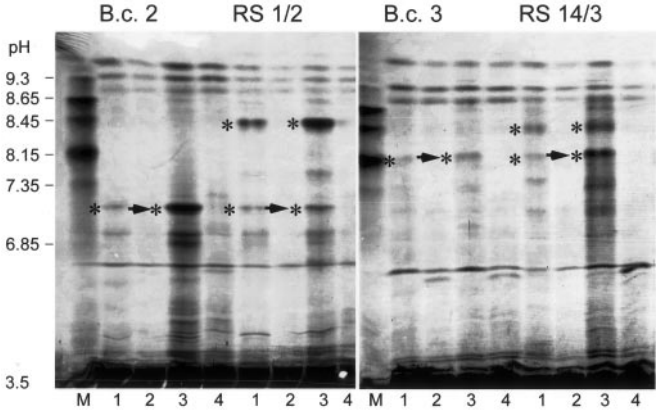

Fig. 1 Separation of stigma and style proteins from two *B. campestris* lines (*B.c. 2*, *B.c. 3*) and two *B. napus* lines carrying the same S-alleles (*RS 1/2*, *RS 14/3*) by analytical IEF (pH gradient 3.5–11). Gel was stained with silve nitrate. *Lanes 1* and *2* Stigma and style, respectively, of buds approximately 6—10 days before anthesis, *lanes 3* and *4* stigma and style, respectively, of flowers just before anthesis, M = pH marker 3–10 (Pharmacia, LKB). Glycoproteins detected by Con-A/peroxidase labelling are marked with an *asterisk*

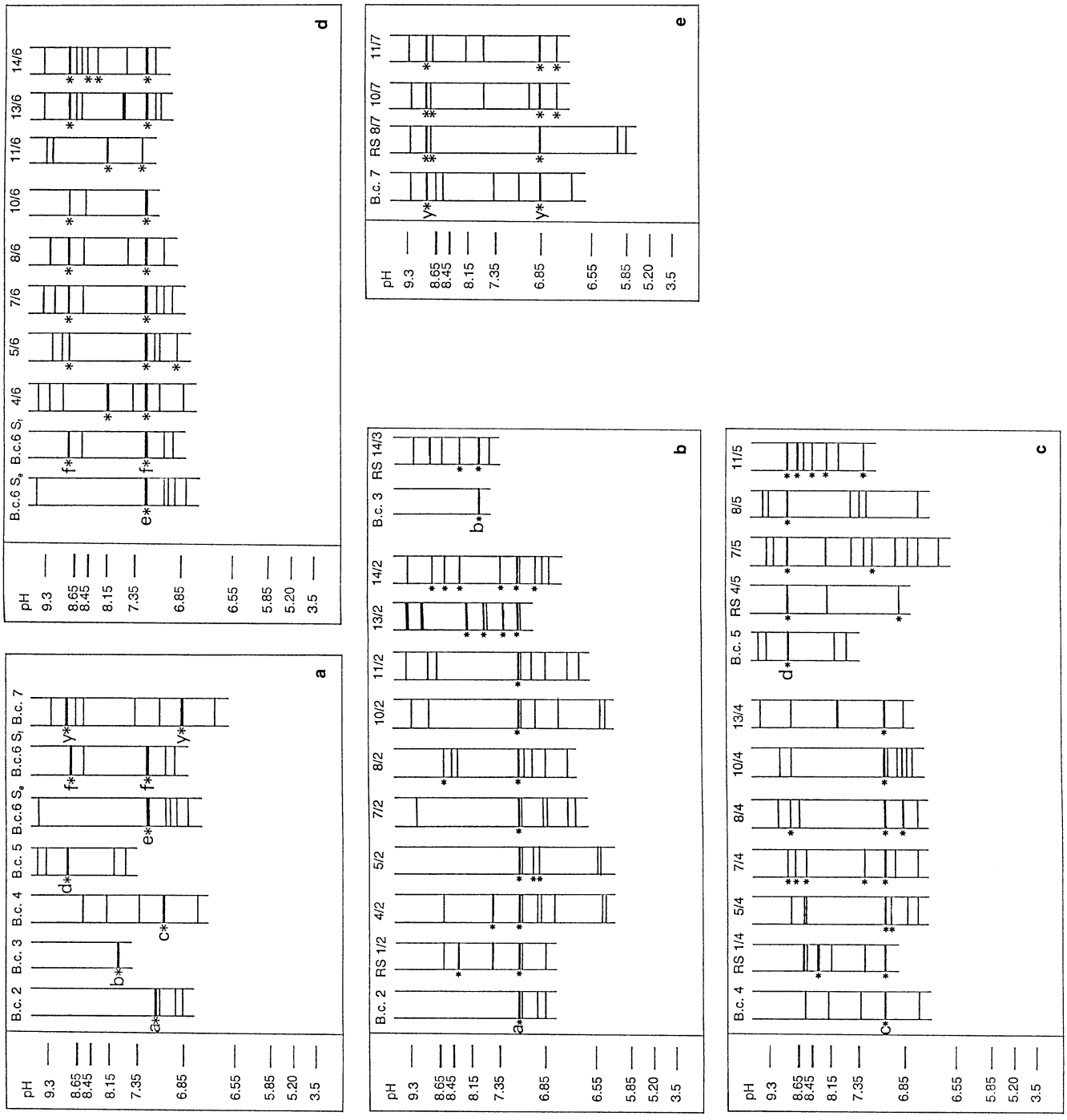

Fig. 2 a–e Patterns of stigma-specific proteins of *B. campestris* lines and resynthesized *B. napus* lines carrying the same S-alleles. Glycoproteins detected by Con-A/peroxidase labelling are marked with an *asterisk*

Identification of S-specific glycoprotein (SLG) bands

Comparing stigma and style samples of buds and open flowers identified protein bands specific for the mature stigma (Fig. 1). Isoelectric points of these proteins were estimated on the silver-stained gel with the help of pI markers. The stigma-specific glycoprotein bands were identified after IEF by Western blotting and detection with Con-A/peroxidase (* in Fig. 1). Single glycoprotein bands could be determined as being S-specific by examining different plants with the same S-allele constitution.

Results

Identification of S-specific glycoprotein bands of *B. campestris*

In *B. campestris*, each S-allele was correlated with one or two glycoprotein bands detectable only in the mature stigma. In Fig. 2a S-specific glycoprotein bands are indicated with the symbol of the respective S-allele. Plants homozygous for the alleles S_e and S_f, selected from the F_2 progeny of the F_1 hybrid cultivar ‘Tokyo King’, could be differentiated through their S-

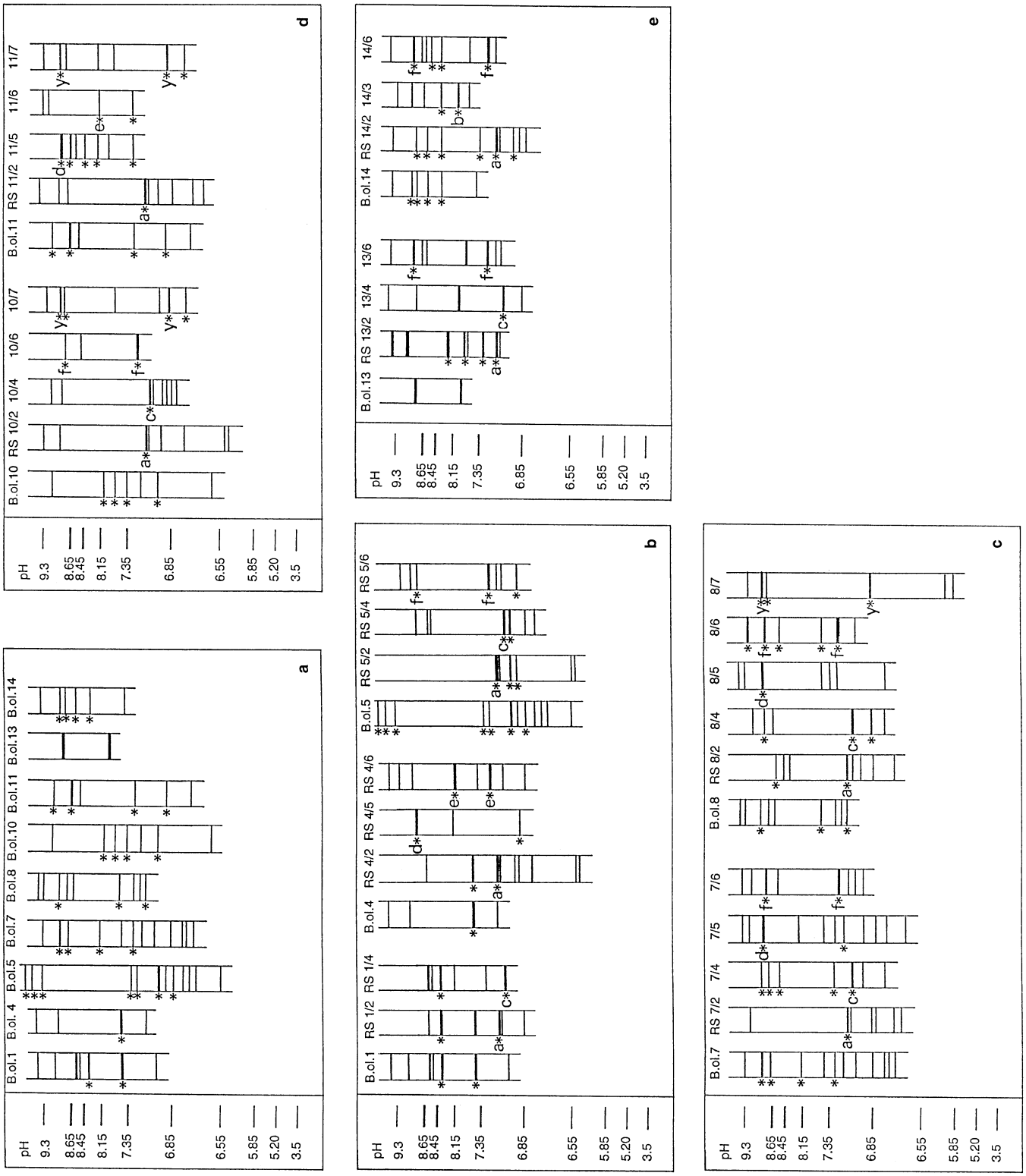

Fig. 3 a—e Patterns of stigma-specific proteins of *B. oleracea* lines and resynthesized *B. napus* lines carrying the same S-alleles. Glycoproteins detected by Con-A/peroxidase labelling are marked with an *asterisk*

specific glycoprotein bands. Whereas the S_e-allele showed a single band at pH 7.2, the S_f-allele could be detected through a secondary band at pH 8.7. The self-compatible cultivar 'Yellow Sarson' (B. camp. 7) showed two stigma-specific glycoprotein bands at pH 6.85 and pH 8.7.

Occurrence of *B. campestris* SLG bands in resynthesized *B. napus*

All resynthesized *B. napus* lines with the *B. campestris* parent 2, 3, 4 or 5 showed the single S-specific glycoprotein band of the *B. campestris* parent (Fig. 2b, c). *B. napus* lines with the *B. campestris* parent 6 (F_1 hybrid cultivar 'Tokyo King') included either the S_e- or the S_f-allele homozygously. The identification of the respective S-alleles was done by means of test pollinations (Beschorner et al. 1995). All lines with the S_f-allele showed the S-specific band at pH 8.7. The lines 4/6 and 11/6, which include the S_e-allele, both showed a new band at pH 8.2 (Fig. 2d). *B. napus* lines which included the self-compatible 'Yellow Sarson' (B. camp. 7) as *B. campestris* parent showed both specific glycoprotein bands unique to this parent (Fig. 2e).

S-specific glycoprotein patterns of *B. oleracea* and their occurrence in the resynthesized *B. napus* lines

Stigma-specific glycoprotein patterns of *B. oleracea* were more complex than the *B. campestris* ones. The S-alleles were correlated with one to seven different glycoprotein bands which were detected only in the mature stigma (Fig. 3a). For *B. oleracea* line 13, which carries the recessive S-allele S_2, no stigma glycoprotein band could be detected, whereas *B. oleracea* line 4, also carrying S_2, showed a SLG band at pH 7.4. Only in the case of the dominant S-alleles S_8 (B.ol. 1) and S_{29} (B.ol. 14) could SLG bands be found in all of the resynthesized *B. napus* lines carrying the respective S-allele (Fig. 3b, e). Nine *B. napus* lines showed no additional glycoprotein band besides the *B. campestris* band. In 16 *B. napus* lines new SLG bands that were different from those of the *B. oleracea* and *B. campestris* parent occurred (Fig. 3b-e).

Discussion

The results of this study demonstrate that there is no correlation between the occurrence of S-locus glycoproteins and the activity of the respective S-alleles in resynthesized *Brassica napus*. In 9 *B. napus* lines only glycoprotein bands of the *B. campestris* parent were detectable, although the biological functioning of the *B. oleracea* S-alleles could be proved (Beschorner et al. 1995). It is possible that a very low (not detectable) amount of S-specific glycoproteins is sufficient to reject pollen of the same S-haplotype. The results from *B. oleracea* line 13, which carries the recessive S-allele S_2, also supports this hypothesis. Although no stigma glycoprotein band could be detected in this line, it was self-incompatible and rejected pollen of line B.ol. 4 which also carries the S_2-allele. As B.ol. 4 possesses a detectable SLG band, it seems to be the different genetic backgrounds of B.ol. 13 and B.ol. 4 which lead to the differences in the expression level of SLGs (see Beschorner et al. 1995 for taxonomical details and origin). However, the self-incompatibility response of both genotypes in the *B. napus* background was very similar (Beschorner et al. 1995). The very low level of SLG proteins of the S_2-haplotype was demonstrated earlier by Nishio and Hinata (1980) who were unable to detect S_2-SLGs by means of isoelectric focusing/ConA staining. Gaude et al. (1995) detected a very low amount of SLG proteins in the stigma papillae of S_2 homozygous plants and correlated this with a low level expression of SLG transcripts. In contrast, a self-compatible line (Sc) and the class II S-haplotypes S_5 and S_{15} showed much stronger immunolabelling of SLGs and high hybridization signals in RNA analysis. Gaude et al. (1995) discuss their results as an indication that the SLG gene may not play a crucial role in the self-incompatibility response or that low amounts of SLG proteins may suffice in the case of certain S-alleles for their function (e.g. the S_2 haplotype).

In resynthesized *B. napus* the interactions between the two ancestral genomes lead to an even more complex situation than in the diploid species. A direct comparison between the S-allele activity of the stigma and the occurrence of SLG proteins was possible in these *B. napus* lines, as the self-incompatibility interactions between the diploid parental lines and the amphidiploid hybrids were tested in diallele test-pollinations in a previous study (Beschorner et al. 1995). The SLG proteins of the *B. campestris* parents were expressed in all cases, in contrast to the complex patterns of the *B. oleracea* SLGs in the resynthesized *B. napus* lines. The newly occurring stigma-specific glycoprotein bands in 16 B. napus lines have to be taken into consideration in this context. New glycoprotein bands occurring in different *B. napus* lines which carry the same *B. campestris* S-allele (e.g. RS 4/6 and 11/6) indicate a *B. campestris* origin in these cases. Further analysis would be necessary to clarify the origin of the newly occurring bands. The 9 *B. napus* lines where only the *B. campestris* SLGs could be detected show that this had obviously no limiting effect on the activity of several *B. oleracea* S-alleles. One example is *B. oleracea* S-allele S_{32} (B.ol. 10), which was fully active in all combinations with self-incompatible *B. campestris* (RS 10/2, 10/4, 10/6) although only the *B. campestris* SLG bands were detected. Only in combination with the self-compatible *B. campestris* line 7 (RS 10/7) was the S_{32}-allele completely inactive in the stigma and weakened in the pollen (Beschorner et al. 1995).

The question that arises is just how the recognition of the *B. oleracea* S-alleles on the stigma surface works in the case of these *B. napus* lines which produce mainly *B. campestris* SLGs and only a very low (not detectable) amount of *B. oleracea* SLGs. One possibility is that although the SLGs must be involved in some way in the process of pollen rejection of the same S-haplotype, they may not play the major role in the signalling pathway. A mutation in the SRK gene, which led to a non-functional protein kinase in *B. napus* (Goring et al. 1993), or the absence of SRK expression in self-compatible mutants

of *B. oleracea* and *B. campestris* (Nasrallah et al. 1994) indicates that the SRK gene plays a key role in the recognition reaction on the stigma surface. On the other hand, crossing experiments with self-compatible and self-incompatible *B. oleracea* lines showed no SLG expression in the self-compatible line and a strong correlation between the self-incompatible phenotype and the occurrence of SLG bands in the segregating populations (Heberer and Beschorner 1995). Therefore, it appears that both the SLG and the SRK gene have to be functional to maintain the self-incompatibility system. The interaction between the SRK and the SLG gene products seems to be the most important issue to clarify.

Acknowledgements We would like to thank Dagmar Frisch, Gudrun Hesse and Anne Miehe for their help in harvesting stigma samples and for their technical assistance. We furthermore wish to thank Dr. Hinrik Junge and Lore Mattiesch for their instruction in IEF electrophoresis, Dr. Peter Wehling for his technical advice on glycoprotein detection, Ralph Underwood for his help with the final preparation of the figures and Dr. Kevin Rozwadowski for his helpful comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

References

Beschorner M, Plümper B, Odenbach W (1995) Analysis of self-incompatibility interactions in 30 resynthesized *Brassica napus* lines. I. Fluorescence microscopic studies. Theor Appl Genet 90: 665–670

Boyes DC, Nasrallah JB (1993) Physical linkage of the SLG and SRK genes at the self-incompatibility locus of *Brassica oleracea*. Mol Gen Genet 236: 369–373

Boyes DC, Chen CH, Tantikanjana T, Esch JJ, Nasrallah JB (1991) Isolation of a second S-locus-related cDNA from *Brassica oleracea*: genetic relationships between the S locus and two related loci. Genetics 127: 221–228

Endo T (1972) Application of the Nadi reaction to rice-peroxidase isozyme stain. Bot Mag 85:147–151

Gaude T, Rougier M, Heizmann P, Ockendon DS, Dumas C (1995) Expression level of the SLG gene is not correlated with the self-incompatible phenotype in the class II haplotypes of *Brassica oleracea*. Plant Mol Biol 27: 1003–1014

Goring DR, Glavin TL, Schafer U, Rothstein SJ (1993) An S receptor kinase gene in self-compatible *Brassica napus* has a 1-bp deletion. Plant Cell 5: 531–539

Hawkes R (1982) Identification of Concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal Biochem 123: 143–146

Heberer V, Beschorner M (1995) Genetic analysis of self-compatible lines of *Brassica oleracea* and *Brassica campestris*. Genes Genomes Chromo 3: 3

Hinata K, Nishio T (1978) S-allele specifity of stigma proteins in *Brassica oleracea* and *Brassica campestris*. Heredity 41: 93–100

Hinata K, Nishio T (1980) Self-incompatibility in Crucifers. In: Tsunoda S, Hinata K, Gomez-Campo C (eds) *Brassica* crops and wild allies. Japan Sci Soc Press, Tokyo, pp 223–234

Hinata K, Nishio T (1981) Con A-Peroxidase method: an improved procedure for staining S-glycoproteins in cellulose-acetate electrofocusing in crucifers. Theor Appl Genet 60: 281–283

Merril CR, Goldman D, Sedman SA, Ebert MH (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1437–1438

Nasrallah JB, Nasrallah ME (1984) Electrophoretic heterogeneity exhibited by the S-allele specific glycoproteins of *Brassica*. Experientia 40: 279–281

Nasrallah JB, Nasrallah ME (1993) Pollen-stigma signaling in the sporophytic self-incompatibility response. Plant Cell 5: 1325–1335

Nasrallah JB, Kao TH, Goldberg ML, Nasrallah ME (1985) A cDNA clone encoding an S-locus-specific glycoprotein from *Brassica oleracea*. Nature 318: 263–267

Nasrallah JB, Kao TH, Chen CH, Goldberg ML, Nasrallah ME (1987) Amino-acid sequence of glycoproteins encoded by three alleles of the S locus of *Brassica oleracea*. Nature 326: 617–619

Nasrallah JB, Nishio T, Nasrallah ME (1991) The self-incompatibility genes of Brassica: expression and use in genetic ablation of floral tissues. Annu Rev Plant Physiol Plant Mol Biol 42: 393–422

Nasrallah JB, Rundle SJ, Nasrallah ME (1994) Genetic evidence for the requirement of the *Brassica* S-locus receptor kinase gene in the self-incompatibility response. Plant J 5: 373—384

Nasrallah ME, Wallace DH (1967) Immunogenetics of self-incompatiblity in *Brassica oleracea* L. Heredity 22: 319–527

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

Nishio T, Hinata K (1980) Rapid detection of S-glycoproteins of self-incompatible crucifers using Con-A-reaction. Euphytica 29: 217–221

Roberts IN, Stead AD, Ockendon DJ, Dickinson, HG (1979) A glycoprotein associated with the acquisition of the self-incompatibility system by maturing stigmas of *Brassica oleracea*. Planta 146: 179–183

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci USA 88: 8816–8820

Thompson KF, Taylor JP (1966) Non-linear dominance relationships between S-alleles. Heredity 21: 345–362

Visser DL, Van Hal JG, Verhoeven WH (1982) Classification of S-alleles by their activity in S-heterozygotes of Brussels sprouts (*Brassica oleracea* var. '*gemmifera*' (DC.) Schultz). Euphytica 31: 603—611