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K. Okazaki · M. Kusaba · D. J. Ockendon · T. Nishio

Characterization of *S* tester lines in *Brassica oleracea*: polymorphism of restriction fragment length of *SLG* homologues and isoelectric points of *S*-locus glycoproteins

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Abstract Forty three *S* tester lines of *Brassica oleracea* were characterized using DNA and protein gel-blotting analyses. DNA gel-blot analysis of *Hin*dIII-digested genomic DNA with class-I and class-II *SLG* probes revealed that 40 lines could be classified as class-I *S* haplotypes while three lines could be classified as class-II *S* haplotypes. The band patterns in the *S* tester lines were highly polymorphic. Although the *S* tester lines typically showed two bands corresponding to *SLG* and *SRK* in the analysis with the class-I *SLG* probe, only one band was observed in the S^{24} homozygote. This band was identified as *SRK*, suggesting that this haplotype has no class-I *SLG* band. In the analysis using the class-II *SLG* probe, one plant yielded a different band pattern from the known class-II haplotypes, S^2, S^5 and S^{15}. Unexpectedly, this plant was reciprocally cross-incompatible with the S^2 haplotype. Therefore, it was designated as $S^{2\text{-}b}$. We found an S^{13} haplotype having a restriction fragment length polymorphism different from that of the S^{13} homozygotes of the *S* tester line. These findings indicate that *S* homozygous lines with the same *S* specificity do not necessarily show the same band pattern in the DNA gel-blot analysis. Soluble stigma proteins of 32 *S* homozygotes were separated by isoelectric focusing and detected using anti-S^{22}SLG antiserum. *S* haplotype-specific bands were detected in 27 *S* homozygotes but not in five *S* homozygotes, including the S^{24} homozygote. This is consistent with the observation that the S^{24} haplotype had no SLG band.

Key words *Brassica oleracea* · *S* haplotype · Self-incompatibility · RFLP · IEF

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K. Okazaki[1] · M. Kusaba · T. Nishio[2] (✉)
Institute of Radiation Breeding, NIAR, MAFF, Ohmiya
P.O. Box 3, Naka-gun, Ibaraki, 319-15, Japan

D. J. Ockendon[3]
Horticultural Research International, Wellesbourne,
Warwick CV35 9EF, UK

Present addresses:
[1] Faculty of agriculture, Niigata University, Niigata,
950-2181, Japan
[2] Faculty of agriculture, Tohoku University, Sendai,
981-8555, Japan
Fax: + 81 22 717 8654
E-mail: nishio@bios.tohoku.ac.jp
[3] 7, Talbot Road, Stratford-on-Avon, Warwick, CV37 6SU, UK

Introduction

Self-incompatibility in the Brassicaceae is regulated by multiple alleles on a single locus, *S* (Bateman 1955). Soluble polymorphic glycoproteins specific to the *S* alleles were identified in *Brassica oleracea* L. (Nasrallah et al. 1970; Hinata and Nishio 1978). These glycoproteins, designated as *S*-locus glycoproteins (SLGs), were found to be localized in the papillar cells of stigmas (Kandasamy et al. 1989) and were differentiated by their isoelectric points (pI) (Nishio and Hinata 1977). cDNA clones and the genes of SLGs have been isolated and characterized (Nasrallah et al. 1985, 1987, 1988; Dwyer et al. 1989, 1991). As a homologue of the SLG gene, a gene of the transmembrane receptor kinase that has an extracellular domain highly similar to SLGs was identified at the *S* locus (Stein et al. 1991). It was designated as the *S*-locus receptor kinase gene (*SRK*). DNA gel-blot analysis using pulsed-field gel electrophoresis has confirmed that *SLG* and *SRK* are physically linked at the *S* locus (Boyes and Nasrallah 1993). On the other hand, two *SLG*-related genes that are not linked to the *S* locus have been reported: *SLR1* (Lalonde et al. 1989) and *SLR2* (Boyes et al. 1991). The DNA sequence of *SLR2* shares more than 90% identity with SLG^2 and the *S* domain of SRK^2 (Boyes et al. 1991).

Comparisons of *SLG* sequences from different *S* haplotypes revealed extensive sequence polymorphism (Nasrallah et al. 1987; Trick and Flavell 1989; Scutt and Croy 1992), and *SLG* alleles were divided into two classes, class I and class II (Chen and Nasrallah 1990). The amino-acid sequence divergence among class-I SLGs ranged from 2.5 to 20%, while that between class-I and class-II SLGs was about 30% (Kusaba et al. 1997). Based on sequence diversity, it is possible to discriminate between the class-I and class-II *S* haplotypes by DNA gel-blot analysis (Chen and Nasrallah 1990). When a class-I *SLG* probe is used, two or more bands corresponding to *SLG* and *SRK* can be detected in class-I *S* haplotypes whereas no band is detectable in class-II *S* haplotypes. When a class-II *SLG* probe is used, one band corresponding to *SLR2* can be detected in the class-I *S* haplotypes and three or more bands corresponding to SLG, *SRK* and *SLR2* are detectable in the class-II *S* haplotypes. An analysis with the polymerase chain reaction (PCR) using *SLG* class-specific primers can also distinguish between the class-I and the class-II *S* haplotypes, but in several *S* haplotypes, *SLG* alleles were not amplified with these primers (Nishio et al. 1996).

In molecular genetic studies of self-incompatibility, designation of the *S* haplotype is indispensable. DNA gel-blot analysis is reported to be useful as a method for the identification of *S* haplotypes (Nasrallah et al. 1985). Fifty *S* haplotypes have been identified by conventional test-crossing in *B. oleracea* (Ockendon 1974). The *S* homozygous lines of this collection are called *S* tester lines and are used as standard references to identify the *S* haplotype. Although the *S* tester lines are widely used in the study of self-incompatibility in *B. oleracea*, few of them have been well-characterized. It is not clear whether *S* homologues having high homology with *SLG* and *SRK* exist in the genomes of all the *S* tester lines, whether all the tester lines have different hybridization patterns, and whether the same haplotypes isolated from different accessions have the same hybridization patterns.

In this study, we analyzed 43 *S* tester lines of *B. oleracea* using DNA gel-blot analysis with the *SLG* probes and 32 lines using protein gel-blot analysis of soluble stigma protein with anti-*SLG* antiserum. In addition, we conducted DNA gel-blot analysis of commercial cultivars of cabbage and broccoli to compare their DNA polymorphism with that of the *S* tester lines.

Materials and methods

Plant material

Forty three *S* homozygous inbred lines (*S* tester lines) of *B. oleracea* were kindly provided by Dr. Astley (HRI Wellesbourne, UK). F_1 hybrid cultivars of *B. oleracea* var. *capitata* and var. *italica*, which were heterozygotes for the class-I and class-II *S* haplotypes, were also included in the study. In these cultivars, *S* haplotypes were identified by reciprocal pollination experiments using the *S* tester lines.

DNA-blot analysis

Genomic DNA was extracted from young leaf tissue by the CTAB method (Murray and Thompson 1980). Aliquots of the DNA (2–6 μg) were digested to completion with *Hin*dIII and electrophoresed on an 0.8% agarose gel. The gel was blotted onto a Hybond N membrane (Amersham International). The membrane was pre-hybridized and then hybridized at 42°C in a solution containing 50% (v/v) formamide, 5 × SSC, 2% blocking reagent (Boehringer Mannheim), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine, 100 μg/ml of denatured sonicated salmon sperm DNA. Digoxigenin-labelled DNA probes were prepared by PCR as follows: probes specific to class-I and -II *SLG*s were amplified from plasmid clones of S^{12} *SLG* (Kusaba et al. 1997) and S^{5} *SLG* (kindly provided by Prof. J. B. Nasrallah), respectively, using a pair of primers: 5′-GGTTACGACCTCAAAACAGG-3′ (bp 517–536 of S^{6}*SLG*) and 5′-TCCGGTCCAAATCACACAAC-3′ (bp 1211–1230 of S^{6}*SLG*). About 1 ng of plasmid DNA was mixed with the pair of primers, 50 pmol together with, 2.5 μl of 10 × buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 2 μl of a dig-dNTP mixture (Boehringer Mannheim), 0.1 μl of *Taq* DNA polymerase (Takara *Taq* 0.5 units), and 17.9 μl of distilled water for a final volume of 25 μl. The PCR was conducted with a thermal cycler (Perkin Elmer 9600) for 30 cycles at each of the following conditions: 1 min at 93°C, 2 min at 55°C, and 3 min at 72°C. The *SRK*-specific probe was prepared by PCR-amplification from a cosmid clone harboring the *SRK* gene of S^{12} (Nishio et al., unpublished) by using the primer pair of PK1, 5′-CTGCTGATCATGTTCTGC-CTCTGG-3′ and PK4, 5′-CAATCCCAAAATCCGAGATCT-3′ (Nishio et al. 1997). Filters were washed twice at room temperature in 2 × SSC, 0.1% SDS for 5 min, and twice in 0.1 × SSC, 0.1% SDS at 68°C for 15 min and then incubated with an anti-digoxigenin alkaline phosphatase conjugate. The detection of DNA was carried out by chemiluminescence using AMPPD or CSPD (Tropix, Bedford, Mass. USA) according to the manufacturer's manual.

Protein blotting

Stigmas were collected from buds 1 day before anthesis and stored at − 20°C. Protein was extracted from 30 stigmas with 0.15 ml of 0.02 M phosphate buffer, pH 7.0. After centrifugation, glycerol was added to the supernatant at a final concentration of 5%, and 10–20 μl of the supernatant was subjected to non-equilibrium pH gradient electrophoresis. The samples were applied to the acidic end of a gel containing 8.4% (w/v) acrylamide (acrylamide : bis-acrylamide = 29 : 1), 7.2% (v/v) Ampholine pH 3–10 (Pharmacia), 2.4% (v/v) Pharmalyte pH 9–11 (Pharmacia), 0.033% TEMED, 0.023% ammonium persulphate and 5.3% glycerol. The anode and cathode solutions were 0.01 M H_3PO_4 and 0.02 M NaOH, respectively. Electrophoresis was performed at 4°C for 0.5 h at 100 V, then at 200 V for the following 4 h, and finally at 300 V for 1 h. After electrophoresis, the separated proteins were electroblotted onto a PVDF membrane in 0.7% acetic acid. After blocking with 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS) containing 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl, the PVDF membrane was incubated for 60 min with anti-S^{22} SLG antiserum (kindly provided by Prof. M.E. Nasrallah) diluted to 1 : 2000. Goat anti-rabbit IgG AP conjugate (Sigma) diluted to 1 : 3000 was used as the secondary antibody. After washing for 15 min × 2 with TBS, the PVDF membrane was stained by an enzymatic reaction using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Estimation of pI

The pI values of the SLGs were measured by isoelectric focusing and also estimated from their deduced amino acid sequences (Kusaba et al. 1997; Nishio et al., in preparation) by using GENETYX software, ver. 9.0 (Software Development, Tokyo).

Results

In the DNA gel-blot analysis, using *Hin*dIII-digested genomic DNA of the *S* tester lines, 0–4 strongly hybridizing bands were detected with the class-I *SLG* probe: one fragment in three *S* homozygotes, two in 23 *S* homozygotes, three in 12 *S* homozygotes and four in 2 *S* homozygotes (Fig. 1). The class-I *SLG* probe did not hybridize with the S^2, S^5, S^{15}, nor with one plant of the S^{23}, homozygotes. The lines which showed only one fragment were S^6, S^{24} and S^{65}. The S^6 and S^{65} homozygotes showed two bands in *Eco*RI digestion (data not shown), but the S^{24} homozygote showed only one strongly hybridizing band in the digestion with *Hin*dIII, *Eco*RI or *Xba*I (Fig. 2). In the S^{12}, S^{39}, S^{46}, S^{51}, S^{52}, S^{58}, S^{62}, S^{63}, S^{64} and S^{65} tester lines, one of the bands detected with the class-I *SLG* probe was also detected with the kinase-specific probe of S^{12}*SRK* (Fig. 3). The only band detected with the class-I *SLG* probe in the S^{24} homozygote also hybridized with the kinase-specific probe (Fig. 2), suggesting that this band corresponds to the *SRK* of the S^{24} haplotype.

Re-probing with the class-II *SLG* probe revealed *S* haplotype-specific bands in S^2, S^5, and S^{15} homozygotes, and non-*S*-specific bands having a size polymorphism in the *S* tester lines (Fig. 4). The non-*S*-specific bands were inferred to be of the *SLR2* gene, which exhibits a high degree of sequence similarity to the class-II *SLG* (Boyes et al. 1991). In class-I *S* haplotypes, the class-II *SLG* probe yielded one or two bands. Polymorphism of the DNA fragment length of the *SLR2* gene has been reported (Tantikanjana et al. 1996). The *S* tester lines have been maintained by sib-crossing. Therefore, the plants showing two bands were inferred to be heterozygotes of *SLR2*. In the S^{23} homozygote line, one plant showed class-II-specific bands, which were different from those of the known class-II haplotypes, S^2, S^5, and S^{15}. Unexpectedly, this plant was reciprocally cross-incompatible with the S^2 homozygote (data not shown), indicating that it has an S^2 haplotype. The other plants in the S^{23} line had class I-specific bands but no class II-specific bands, suggesting that the seeds of S^{23} homozygotes had been contaminated by S^2 homozygotes. Thus, the class-II haplotype found in S^{23} was designated as $S^{2\text{-}b}$. This

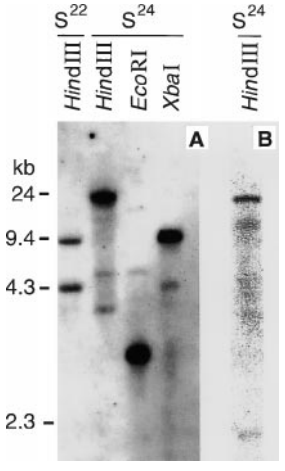
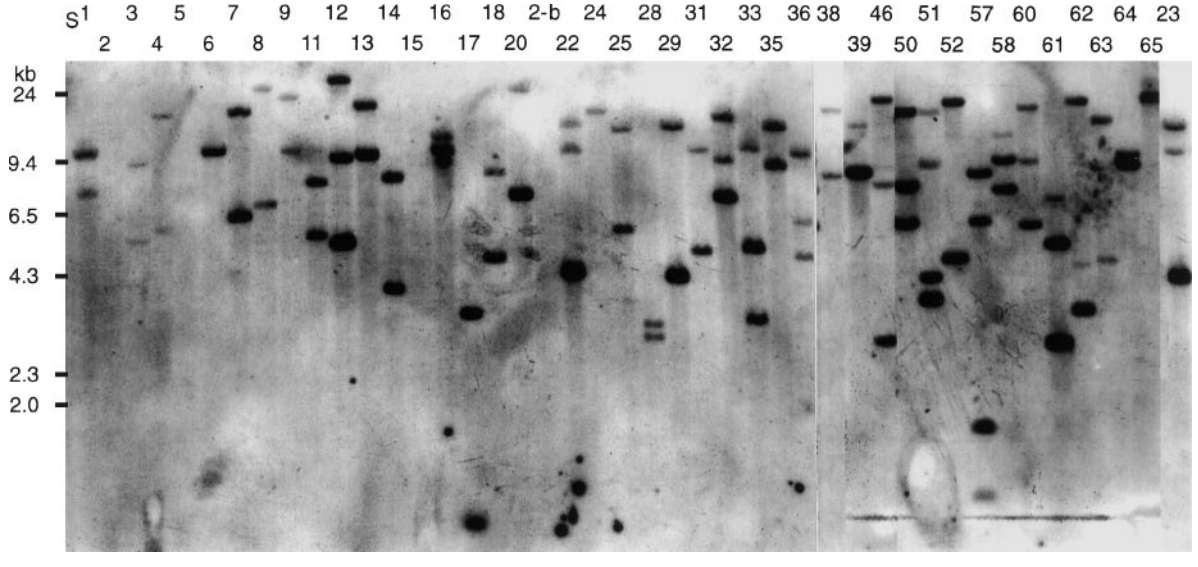


Fig. 2 DNA-blot analysis of genomic DNA isolated from S^{22} (control) and S^{24} homozygotes. Restriction enzymes used to digest the DNA are indicated above each lane. The DNA blot was hybridized with the class-I *SLG* probe (*A*) and the kinase-specific probe (*B*)

Fig. 1 DNA-blot analysis of *Hin*dIII-digested genomic DNA isolated from the *S* tester lines of *B. oleracea*. The blot was hybridized with the class-I *SLG* probe. To each lane, 2–6 μg of DNA was loaded. Molecular-weight markers are indicated on the left



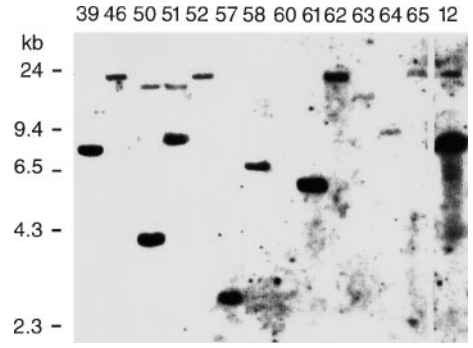


Fig. 3 DNA-blot analysis of *Hin*dIII-digested genomic DNA isolated from the *S* tester lines of *B. oleracea*. The blot was hybridized with the kinase-specific probe

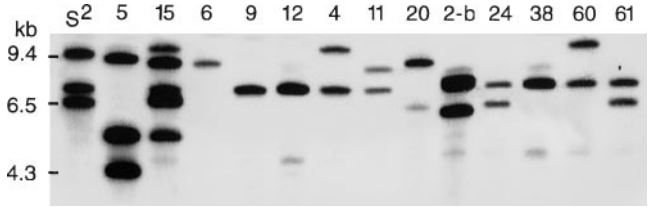


Fig. 4 DNA-blot analysis of *Hin*dIII-digested genomic DNA isolated from the *S* tester lines using the class-II *SLG* probe

means that there are at least two types of S^2 haplotype showing different band patterns in the DNA gel-blot analysis.

To compare band patterns of the lines from different accessions having the same *S* specificity, DNA-blot analysis with the class-I *SLG* probe was conducted using the *S* tester lines and commercial F_1 hybrid cultivars of cabbage (S^6) and broccoli (S^{13}, S^{18}, S^{39}). These cultivars are heterozygotes of class-I and class-II haplotypes (data not shown). In S^6, S^{18}, S^{39} haplotypes, the same band patterns were observed between the *S* tester lines and the F_1 hybrid cultivars. On the other hand, the S^{13} haplotype of broccoli showed a band pattern different from that of the *S* tester line (Fig. 5).

Stigma extracts of 32 *S* homozygotes were analyzed by protein gel blots with the anti-S^{22} antiserum. *S* haplotype-specific bands were detected in 27 *S* homozygotes but not in S^4, S^{18}, S^{24}, S^{60} and S^{61} (Fig. 6). Non-*S*-specific bands were found in the neutral zone. Some *S* homozygotes showed several bands specific to *S* haplotypes, sometimes showing *S*-specific broad bands, probably due to variation in the carbohydrate moiety. The pI values of SLGs in the 27 *S* tester lines ranged from pI 5.0 to 10.0. SLGs of 18 *S* tester lines had a pI of 7.0–10.0 and those of nine *S* tester lines had a pI of 5.0–6.9. The pI values estimated from electrophoretic mobility were highly correlated with those estimated from the deduced amino-acid sequences of SLGs. In highly basic SLGs, however, the pI values

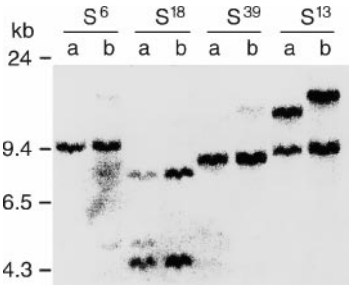


Fig. 5 DNA-blot analysis of *Hin*dIII-digested genomic DNA isolated from the *S* tester lines (*a*) and F_1 hybrid cultivars of broccoli (*b*) using the class-I *SLG* probe

estimated from the sequences were lower than those estimated from the electrophoretic mobility.

Discussion

DNA gel-blot analysis with the class-I *SLG* probe revealed *S*-specific bands in all the *S* tester lines except for S^2, S^5, and S^{15}. The hybridization with the class-II *SLG* probe revealed *S*-specific bands in the known class-II S^2, S^5 and S^{15} homozygotes, and a new band pattern in the $S^{2\text{-}b}$ homozygote. These observations suggest that the 43 *S* haplotypes can be divided into two classes: 40 *S* tester lines belong to the class-I *S* haplotype while 3 *S* tester lines and $S^{2\text{-}b}$ belong to class II. We did not find any *S* haplotype which was neither class-I nor class II. This report confirms that *B. oleracea* has fewer class-II *S* haplotypes, perhaps only three, compared with *B. campestris*, which has six class-II *S* haplotypes out of the 24 analyzed (Nishio et al. 1996), and *Raphanus sativus*, which has five class-II *S* haplotypes out of the 18 analyzed (Sakamoto et al. 1998).

Most commonly, two *Hin*dIII fragments were detected with the class-I SLG probe. However, more than two bands were detected in some *S* haplotypes. This may be due to internal *Hin*dIII restriction sites within the sequences detected by the probe. In fact, *SLG* alleles of S^{17}, S^{33} and S^{57}, as well as *SRK* of S^{33}, had an internal *Hin*dIII restriction site within the sequences identified by the probe (Nishio et al., in preparation). In S^{12}, S^{32}, S^{36}, S^{46}, S^{50}, and S^{51}, however, no *Hin*dIII restriction site was found within *SLG*s and the *S* domains of *SRK*s. In these lines, a sequence highly homologous with a class-I *SLG* may exist in the genome together with *SLG* and *SRK*.

In the S^{23} homozygous line, we found one plant, $S^{2\text{-}b}$, to be a contaminant. It has a different band pattern from that of the S^2 tester line, although they were reciprocally cross-incompatible. This indicates that different hybridization patterns exist in the same *S* homozygotes. In addition, the S^{13} haplotype of the *S* tester lines had a different band pattern from that of the S^{13} haplotype of an F_1 hybrid cultivar of broccoli. These RFLPs in the same *S* haplotypes would be due to

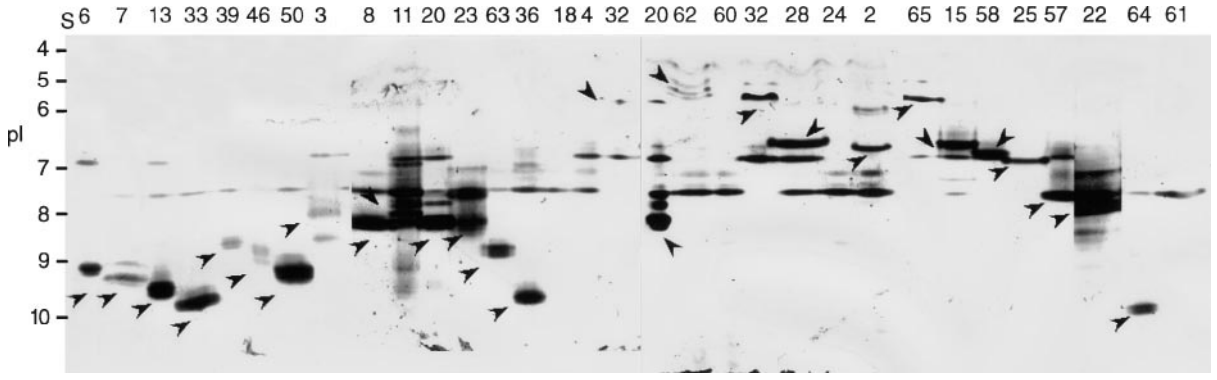


Fig. 6 Protein-blot analysis of stigma proteins from the *S* tester lines. Stigma proteins were separated by non-equilibrium pH gradient electrophoresis and detected by immunoblotting with an anti-S^{22}SLG antiserum. Major SLGs are marked with *arrowheads*. Standard pIs are on the left

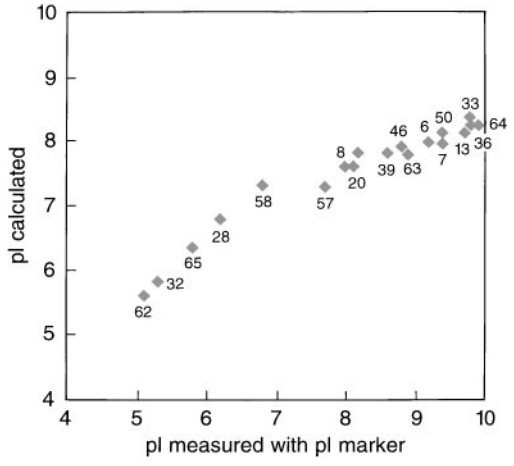


Fig. 7 Relationships between the pI value measured in IEF and the one calculated from the deduced amino-acid sequences of *SLG*s

a mutation in either the coding region or the flanking region of *SLG* and/or *SRK*. These observations in S^2 and S^{13} haplotypes indicate that *S* homozygous lines with the same *S* haplotype do not necessarily show the same band pattern in DNA gel-blot analysis.

Only one restriction fragment was detected in the DNA gel-blot analysis of the S^{24} homozygote with the class-I *SLG* probe in digestion with *Hin*dIII, *Eco*RI, or *Xba*I. This DNA fragment was considered to be of *SRK* origin since it was also detected with the kinase-specific probe. There is little possibility for the existence of *SLG* and *SRK* in one restriction fragment because the *Eco*RI-fragment was only 3 kb. Consistent with our observation, a *B. napus* line into which the S^{24} haplotype of *B. oleracea* was introduced by crossing showed only one band in the DNA-blot analysis of a *Hin*dIII digestion using a class-I *SLG* probe (Goring et al. 1993). The *SLG* gene might have been deleted in the S^{24} haplotype, although the self-incompatibility phenotype of the S^{24} homozygote was normal (data not shown).

In the protein-blot analysis, we preliminarily conducted both non-equilibrium pH-gradient electrophoresis and horizontal thin-layer IEF. In the horizontal thin-layer IEF, some basic *S*-specific bands reached the end of the cathode (data not shown). In the non-equilibrium pH-gradient electrophoresis, *S* haplotype-specific bands (SLGs) were well separated in the basic zone. SLGs were also mainly observed in the basic zone in the IEF analysis (Nishio and Hinata 1977). In the present experiment, SLGs exhibited extensive polymorphism and were observed not only in the basic zone but also in the acidic one. Although it is not possible to accurately measure the pI of SLGs in non-equilibrium pH-gradient electrophoresis, the pI values estimated from the electrophoretic mobility were highly correlated with those calculated from the deduced amino acid sequences of SLGs.

The immunoblot analysis of *S*-locus-related (SLR) proteins showed that SLR1 and SLR2 have pI values of 7.0 and 7.5, respectively, and a lower content of SLR2 than that of SLR1 (Tantikanjana et al. 1996). In the present experiment, the anti-SLG antiserum cross-reacted with non-*S*-specific proteins as well as with the class-I and -II SLGs. It is considered that the non-*S*-specific bands are SLR1 or SLR2, but we were unable to distinguish them.

*SLG*s were not observed in S^4, S^{18}, S^{24}, S^{60} and S^{61} homozygotes, and there were large differences in the amount of SLGs between strong bands and weak bands in our experiment. The immunoreactivity of the S^{22}SLG antiserum to all SLGs may be questionable. However, wide cross-reactivity to class-I and -II SLGs, as well as to the SLR proteins, suggests that the S^{22} SLG antiserum is appropriate for the detection of SLGs. An absence or variation in the amount of SLG has been observed in *Brassica* (Hinata and Nishio 1978; Nou et al. 1993) and in *R. sativus* (Okazaki and Hinata 1984). In addition, Gaude et al. (1995) showed that the expression level of the *SLG* gene was not correlated with the strength of self-incompatibility in the class-II *S* haplotypes of *B. oleracea*. These results suggest that some *S* haplotypes may not need SLG for

self-incompatibility. Alternatively, it is possible that a very small amount of SLG in the stigmas is sufficient for the self-incompatibility reaction. However, we found that the S^{24} haplotype harbors no class-I *SLG* in its genome, suggesting that SLG is not essential to self-incompatibility, at least in this haplotype.

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