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Characterization of the *S* locus genes, *SLG* and *SRK*, of the *Brassica* S_3 haplotype: identification of a membrane-localized protein encoded by the *S* locus receptor kinase gene

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Summary

The *S* locus, which controls the self-incompatibility response in *Brassica*, has been shown to contain at least two genes. *SLG* encodes a secreted *S* locus glycoprotein whilst *SRK* encodes a putative *S* locus receptor kinase. *SRK* has been shown potentially to encode a functional kinase and genetic evidence indicates that this gene is essential for the self-incompatibility response. Here the characterization of the *SRK* and *SLG* genes of a *Brassica* line homozygous for the S_3 haplotype is described. A 120 kDa glycoprotein was identified in stigmas and several lines of evidence indicated that this protein is encoded by the SRK_3 gene. First, the 120 kDa glycoprotein was recognized by antibodies raised against peptides based on the SRK_3 gene sequence. Secondly, this protein is polymorphic and, in an F_2 population segregating for the S_3 haplotype, was expressed only in plants possessing the S_3 haplotype. Thirdly, the 120 kDa protein was expressed specifically in stigmas. Finally, the 120 kDa protein was only extracted from stigmas in the presence of detergent indicating that it is anchored in the membrane. *SRK* has been predicted to encode a transmembrane glycoprotein based on the deduced amino acid sequence. Located on the membrane, SRK is in a position to interface between an extracellular recognition event between pollen and pistil and an intracellular signal transduction pathway which initiates the self-incompatibility response.

Introduction

Self-incompatibility (SI) in *Brassica* involves a process of specific cell–cell interaction between pollen and the papillar cells of the stigma. The SI response is controlled by a single, highly polymorphic locus, the *S* locus. Normally, when the pollen grain and the papillar cell express the same *S* allele, fertilization is rapidly aborted either because the pollen does not germinate or because the pollen tube does not penetrate the stigma surface. Efforts to understand the molecular mechanism of the rejection reaction have concentrated on the identification of genes linked to the *S* locus. The two genes which have been identified to date encode the *S* locus glycoprotein (SLG; Nasrallah *et al.*, 1987) and a putative *S* locus receptor kinase (SRK; Stein *et al.*, 1991). SLG is an abundant stigma protein which is highly polymorphic in different *S* locus genotypes (Nasrallah *et al.*, 1987) consistent with it playing a role in the SI response. *SLG* gene transcription has also been detected in anthers but at a much lower level than in the stigma (Sato *et al.*, 1991). The *SRK* gene is predicted to encode a transmembrane receptor protein kinase with an extracellular domain (the *S* domain) that is highly similar to SLG, a single transmembrane domain and an intracellular kinase domain. Although the *SRK* gene has not yet been shown to be expressed at the protein level *in vivo*, the kinase domain has been shown to be potentially functional by expression in *Escherichia coli* (Goring and Rothstein, 1992; Stein and Nasrallah, 1993). Like *SLG*, *SRK* appears to be highly polymorphic and is expressed in both pistils and, at a lower level, in anthers. *SLG* and *SRK* are members of a multigene family. Two other members of this family, the *S* locus-related genes *SLR1* and *SLR2*, have also been shown to be expressed specifically in stigmas (Boyes *et al.*, 1991; Lalonde *et al.*, 1989).

Cloning and sequencing of a number of *SLG* alleles has demonstrated a divergence of up to 36% at the amino acid level. *SLG* alleles have been divided into two classes based on the immunoreactivity of their products with an antibody raised against SLG_6 (Kandasamy *et al.*, 1989) and on sequence comparison. Interactions between *S* alleles can involve co-dominance or dominance/recessiveness in the stigma or in the pollen. Class I alleles tend to be dominant and to correspond a strong SI reaction whereas class II alleles tend to be recessive and associated with a weak SI response. Interestingly, when the deduced amino acid sequence of an SLG is compared with that of the *S* domain of SRK from the same *S* haplotype, sequence similarity is generally greater than 84% (Goring and Rothstein, 1992; Goring *et al.*, 1993; Stein *et al.*, 1991). *SLG* and *SRK* genes within an *S* haplotype, therefore, appear to have co-evolved

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despite the generation of a high level of polymorphism between different *S* haplotypes indicating that there may be a functional interaction between the two proteins.

In this study, we have identified the *SLG* and *SRK* genes of the class I, pollen-dominant S_3 haplotype. Antibody probes have been used to show that both SLG and SRK are expressed in the stigma. The *SRK* gene was shown to encode a 120 kDa, membrane-associated glycoprotein. The data presented here are consistent with the proposed role for SRK, perhaps acting in combination with SLG, in coupling pollen–stigma recognition to an intracellular signal transduction pathway involving protein phosphorylation (Stein *et al.*, 1991).

Results

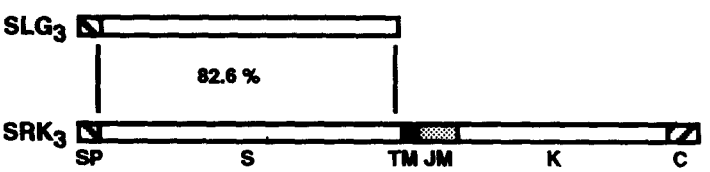
Cloning and sequence analysis of the SLG *and* SRK *genes of the* S_3 *haplotype*

The *B. oleracea* line P57 Si (described in Gaude *et al.*, 1993) has recently been shown to be homozygous for the S_3 haplotype based on two criteria. First, P57 Si has been shown to exhibit an S_3 phenotype in cross-pollinations with tester lines carrying different *S* alleles (Ockendon, personal communication). Secondly, the pattern of SLG isoforms detected by an anti-SLG antibody following separation of stigma proteins by IEF was the same for P57 Si as for an S_3/S_3 tester line (Gaude, unpublished data). For the sake of clarity, P57 Si will be subsequently referred to simply as the S_3/S_3 homozygous line.

Cloning of cDNAs corresponding to the SLG_3 gene based on their hybridization to BS29–2 (SLG_{29} cDNA; Trick and Flavell, 1989) and to an oligonucleotide derived from the N-terminal, amino acid sequence of the SLG_3 protein has been described (Gaude *et al.*, 1993). One of these clones was sequenced (Figure 1) and was shown to be related to *SLG* genes from other *S* haplotypes by comparison with sequences in the database.

A mixed probe consisting of part of this cDNA and part of the SRK_3 *S* domain obtained by PCR amplification from genomic DNA was used to screen a genomic library constructed from DNA of the S_3/S_3 homozygous line. Genomic clones corresponding to SLG_3 and SRK_3 were obtained. PCR

(a)



(b)

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          M  K  G  I  R  K  T  Y  D  N  S  Y  T  S  C  L  L  V  F  F  V  L  I  L  F  R  P  A  F  S  I  N  T
TGTCGTCTACAGAATCTCTTACAATCTCAAGCAACAGAACACTCGTATCTCCATGTAGTAACTTCGAGCTCGGCTTCTTCAGAACCAACTCAAGTTCTCGTTGGTATC  208
L  S  S  T  E  S  L  T  I  S  S  N  R  T  L  V  S  P  C  S  N  F  E  L  G  F  F  R  T  N  S  S  S  R  W  Y
TCGGGATATGGTACAAGAAATTGTCTAACAGAACCTATGTATGGGTTGCCAACAGAGATAACCCTTTGTCCAACTCCATTGGAACCCTCAAAATCTCAGGCAATAATC  316
L  G  I  W  Y  K  K  L  S  N  R  T  Y  V  W  V  A  N  R  D  N  P  L  S  N  S  I  G  T  L  K  I  S  G  N  N
TTGTCCTCCTTGGTCACTCCAATAGATCAGTTTGGTCGACGAATCTTACTAGAGAAAATGAGAGATCCACAGTGGTCGCAGAGCTTCTCGCTAATGGAAACTTCGTGA  424
L  V  L  L  G  H  S  N  R  S  V  W  S  T  N  L  T  R  E  N  E  R  S  T  V  V  A  E  L  L  A  N  G  N  F  V
TGCGAGACGCAAGTGGATTCTTGTGGCAAAGTTTCGATTACCCTACAGATACTTTGCTACCAGAGATGAAACTGGGTTACGACCTCAAAACAGGGCTGAACAGGTTCC  532
M  R  D  A  S  G  F  L  W  Q  S  F  D  Y  P  T  D  T  L  L  P  E  M  K  L  G  Y  D  L  K  T  G  L  N  R  F
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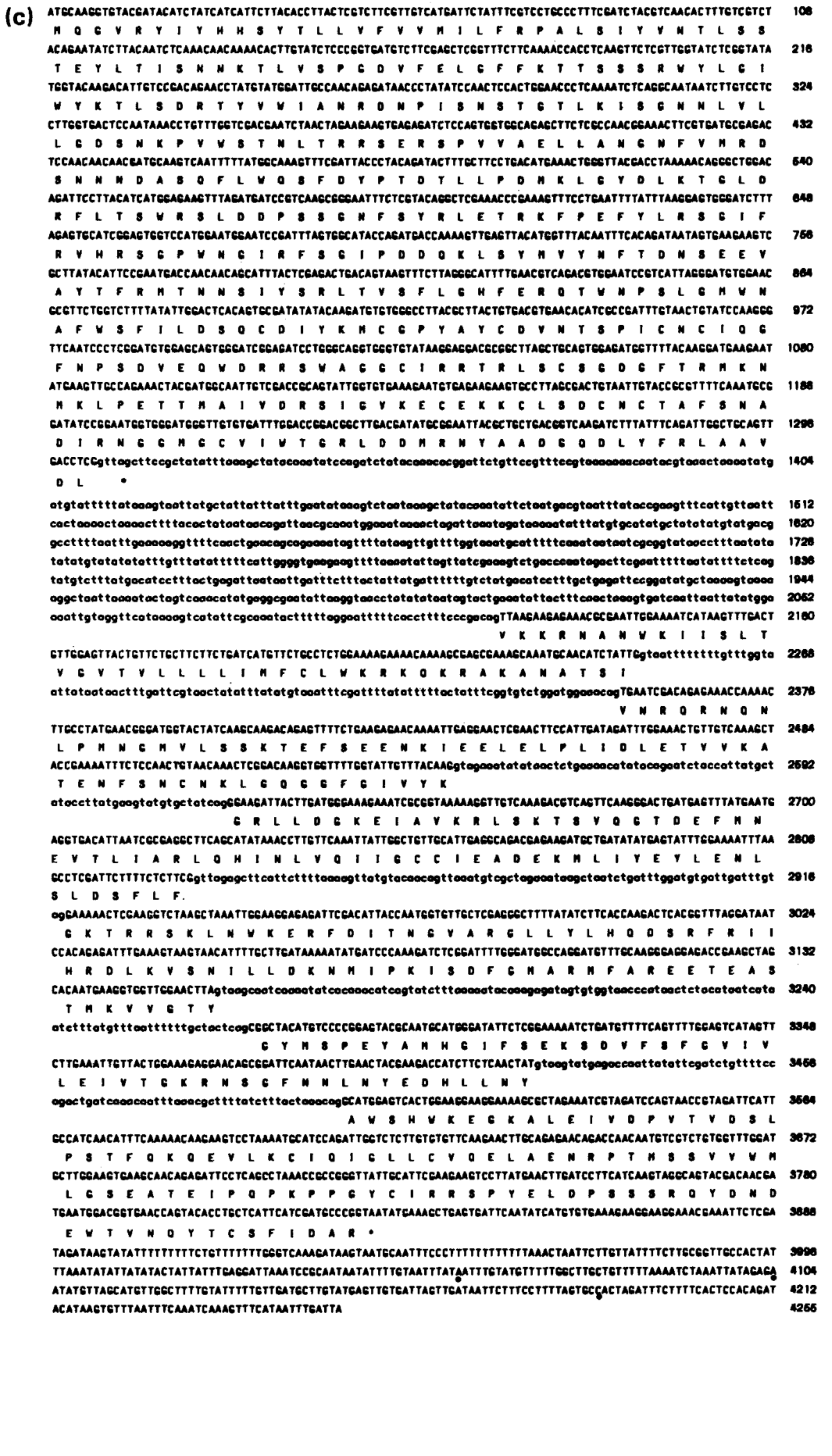
L  T  S  W  R  S  S  D  D  P  S  S  G  D  F  S  Y  K  L  E  T  R  R  L  P  E  F  Y  I  S  S  G  V  F  L  L
ATCGGAGTGGACCATGGAATGGAATACGATATAGTGGCATACTAGAAGACCAAAAGTTGAATTACATGGTGTACAATTTCACAGAGAATAGTGAAGAAGTCGCTTATA  748
Y  R  S  G  P  W  N  G  I  R  Y  S  G  I  L  E  D  Q  K  L  N  Y  M  V  Y  N  F  T  E  N  S  E  E  V  A  Y
CATTCCGAATGACCAACAACAGCATCTACTCGAGACTGACACTAAGTTTCTCGGGGTATTTTGAACGACAGACGTGGAATCCGGCATTAGGGATGTGGAACGTGTTAT  856
T  F  R  M  T  N  N  S  I  Y  S  R  L  T  L  S  F  S  G  Y  F  E  R  Q  T  W  N  P  A  L  G  M  W  N  V  L
GGTCTCTTCCATTCGACTCACAGTGCGATACGTACAGGATGTGTGGGCCTTACTCTTACTGTGACGTGAGCACATCACCGATTTGTAACTGTATCCAAGGGTTCAATC  964
W  S  L  P  F  D  S  Q  C  D  T  Y  R  M  C  G  P  Y  S  Y  C  D  V  S  T  S  P  I  C  N  C  I  Q  G  F  N
CCTCGAATGTGCAGCAGTGGGATCAGAGTTCCTGGTCAGGTGGGTGTATAAGGAGGACGCGGCTTAGCTGCAGTGGAGATGGTTTTACCAGGATGAGGAATATGAAGT 1072
P  S  N  V  Q  Q  W  D  Q  S  S  W  S  G  G  C  I  R  R  T  R  L  S  C  S  G  D  G  F  T  R  M  R  N  M  K
TACCAGAAACTACGATGGCAATTGTCGACCGCAGAATTGGTGTGAAAGAATGTGAGAAGAAGTGCCTTAGCGACTGTAATTGTACCGCGTTTTCAAATGCGGATATCC 1180
L  P  E  T  T  M  A  I  V  D  R  R  I  G  V  K  E  C  E  K  K  C  L  S  D  C  N  C  T  A  F  S  N  A  D  I
GGAATGGTGGGATGGGTTGTGTGATTTGGACCGGACGGCTTGACGATATGCGGAATTACGTTGCTGACCACGGTCAAGATCTTTATGTCAGATTGGCCGCTGCTGACC 1288
R  N  G  G  M  G  C  V  I  W  T  G  R  L  D  D  M  R  N  Y  V  A  D  H  G  Q  D  L  Y  V  R  L  A  A  A  D
TTGTTTAGCTCTTTCTCTTAAAATAAAACACGGATCCGACTATGTTATCGAGAAATCCGGATATGAGACGCAACTATCTAAATATGTCGACCCTTAATGTATTTCTGA 1396
L  V  •
AGCAATAAAGATATACAGATCGTATCCAAATCCATAATAACATGCCATGTAAAATACACATAAATTTGATTATAATATTTTGCCGAAAAAAAAAA 1491
```

Figure 1. Sequence analysis of the SLG_3 and SRK_3 genes.

(a) Schematic representation of the predicted SLG_3 and SRK_3 proteins showing the percentage similarity between *S* domains. SP, signal peptide; S, *S* domain; TM, transmembrane domain; JM, juxtamembrane domain; K, kinase domain; C, C-terminal domain.

(b and c) Nucleotide sequences of the SLG_3 cDNA and the SRK_3 gene. The deduced amino acid sequences of the SLG_3 and SRK_3 proteins are shown below the coding sequences. Introns are indicated by lower case letters. Asterisks indicate termination codons at the end of each coding sequence and a termination codon in the first intron of SRK_3 described in the text. Sequences are numbered from the first base of the ATG initiation codon. SRK_3 polyadenylation sites, identified by RACE-PCR, are indicated with a dot. The EMBL Data Library accession numbers for the SLG_3 cDNA and the SRK_3 gene sequences are X79431 and X79432, respectively.



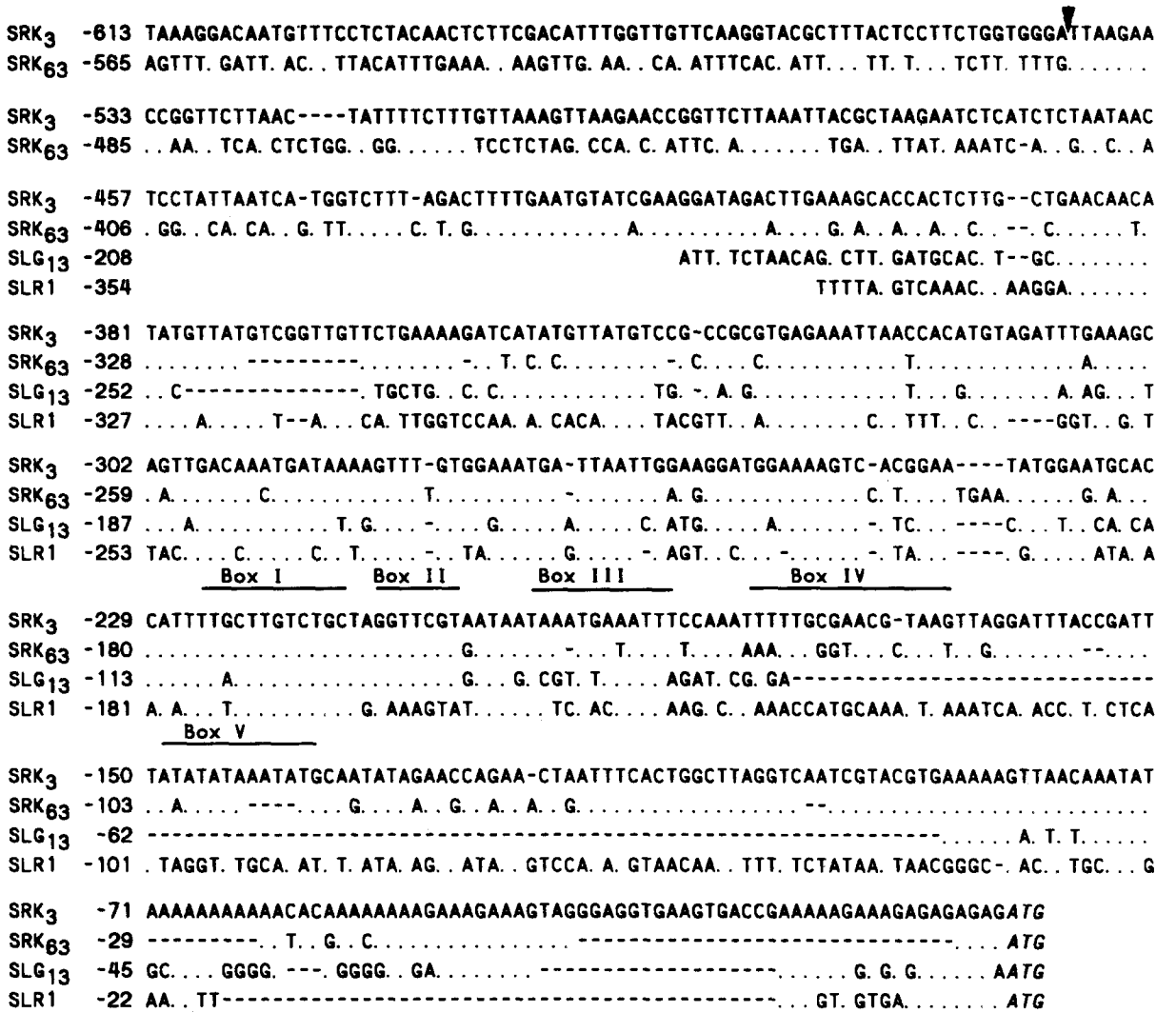


Figure 2. Nucleotide sequence of the SRK_3 promoter aligned with the promoter regions of the SRK_{63}, SLG_{13} and *SLR1* genes.
Dots indicate residues conserved with respect to the SRK_3 promoter, dashes represent gaps introduced to optimize alignment. Sequences are numbered from the first base of the ATG initiation codon which is shown in italics. Regions corresponding to the five boxes identified by Dzelzkalns *et al.* (1993) are underlined. The arrowhead marks the 5′ limit of the region conserved between the promoters of SRK_3 and SRK_{63}. The SLG_{13} and *SLR1* promoter sequences are from Dzelzkalns *et al.* (1993), SRK_{63} promoter: accession number Z18921.

amplification from the SLG_3 cDNA and genomic clones using oligonucleotides which prime from the 5′ and 3′ ends of the cDNA was used to show that the cDNA and genomic clones were co-linear and, therefore, that the SLG_3 gene contains no introns in this region (data not shown).

The SRK_3 gene was subcloned into a plasmid vector and DNA sequence was determined for a region of 5345 bp (Figures 1c and 2). In order to identify the positions of the exons, an SRK_3 cDNA was isolated by PCR amplification from stigma cDNA using oligonucleotides based on the SRK_3 gene sequence (see Experimental procedures). Comparison of the SRK_3 gene and cDNA sequences revealed the same arrangement of seven exons separated by six introns (Figure 1c) as has been reported for the SRK_6 and SRK_2 genes (Stein *et al.*, 1991). The positions of the introns in SRK_3 correspond exactly with those of SRK_6 and the sizes of corresponding introns are very similar. In addition, as has been found for SRK_6 and SRK_2 (Stein *et al.*, 1991), the first intron of the SRK_3 gene contains an in-frame stop codon which could be used to produce a truncated SLG-like protein from an alternative transcript which retains the first intron. Evidence for the existence of such transcripts has been reported previously (Stein *et al.*, 1991). RACE-PCR (rapid amplification of cDNA ends by PCR; Frohman *et al.*, 1988) was used to identify polyadenylation sites at the 3′ end of the SRK_3 gene. Three alternative polyadenylation sites were identified spread over a region of 130 bp. The deduced amino acid sequence of the SRK_3 *S* domain is highly similar to SLG_3 (82.6% similarity, Figure 1a). Comparison with other genes in the database indicated that SLG_3 and SRK_3 are more

similar to class I than to class II *S* locus genes, for example, the deduced amino acid sequence of SLG_3 is 84.6% similar to the class I SLG_6 protein but only 64.4% similar to the class II SLG_2 protein.

The sequence data obtained for the SRK_3 gene included 1090 bp upstream of the ATG initiation codon. This sequence was compared with the promoter region of the SRK_{63} gene and a highly conserved region (74.6% identical) was identified extending 540 bp upstream of the ATG initiation codon (Figure 2). No significant sequence similarity was found upstream of this region. Alignment of the SRK_3 promoter sequence with the promoter regions of the SLG_{13} and an *SLR1* (*S* locus related) gene revealed that the conserved region of the *SRK* promoters includes five conserved 'boxes' identified by Dzelzkalns *et al.* (1993) in the promoters of other members of the *S* gene family (Figure 2). This is consistent with the similar patterns of tissue-specific expression observed for *SLG*, *SLR1* and *SRK* (Hackett *et al.*, 1992; Sato *et al.*, 1991; Stein *et al.*, 1991 and see below)

Transcription of the SLG_3 *and* SRK_3 *genes*

RNA blot analysis was used to look at expression of SLG_3 and SRK_3 transcripts in a number of vegetative and floral tissues. A probe corresponding to the 3′ untranslated region of the SLG_3 gene hybridized to a single band in a Southern blot of genomic DNA (see Figure 7b) indicating that it is gene-specific. This probe detected a 1.6 kb mRNA in stigmas (Figure 3a) but no signal was obtained with anther RNA even if the sensitivity was increased by loading up to 5 μg of poly(A)$^+$ RNA (Figure 3a and data not shown). A 1246 bp *PstI*/*ClaI* fragment from the kinase domain of the SRK_3 cDNA was used to detect SRK_3 expression. This probe also detects a single fragment in genomic DNA digests (see Figure 7a) and is hence gene-specific. When 25 μg of total RNA from a range of different tissues were electrophoresed on a gel and probed, SRK_3 transcripts were detected only in stigmas (Figure 3b). If the sensitivity was increased by loading several micrograms of poly(A)+ RNA, SRK_3 mRNA was also detected in anthers at both the unicellular and bicellular microspore stages, although at much lower abundances than in stigma (Figure 3c). SRK_3 transcripts were not detected in root poly(A)$^+$ RNA indicating that the low levels detected in anthers nonetheless result from an induction of gene expression. The SRK_3 probe detects a major transcript of 3.0 kb in stigma RNA plus slightly less abundant transcripts of 4.1, 2.2 and 1.3 kb. A single transcript of 2.8 kb, slightly shorter than the major transcript in stigmas, was detected in anther RNA (Figure 3c).

Sato *et al.* (1991) have shown by RNA blot and β-glucuronidase reporter gene fusion experiments that the SLG_{13} gene is expressed in anthers. As described above, SLG_3 mRNA was not detected in anthers by RNA blots and

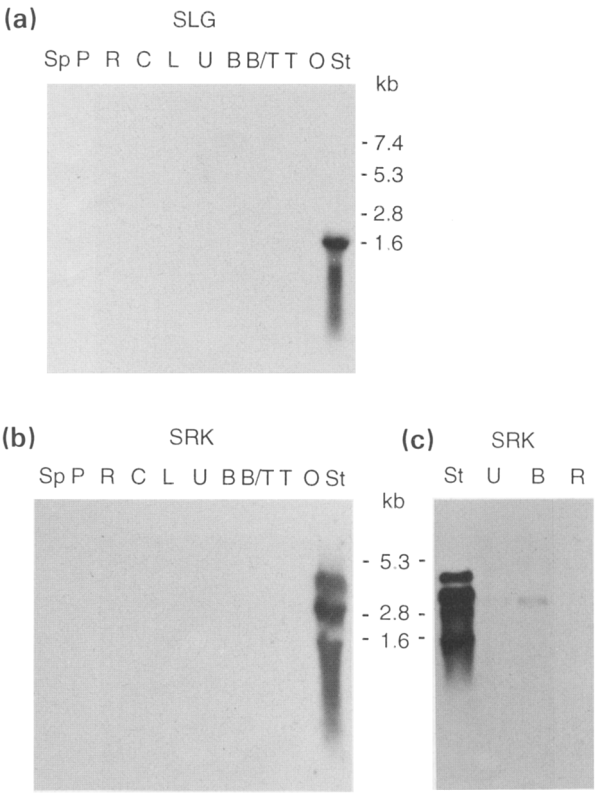


Figure 3. Analysis of expression of the SLG_3 and SRK_3 genes.
RNA blot analysis of SLG_3 (a) and SRK_3 (b and c) transcripts in a range of tissues. Each lane was loaded with either 25 μg of total RNA (a and b) or 2–4 μg of poly(A)$^+$ RNA (c). The gene-specific DNA probes were derived from the 3′ untranslated region of SLG_3 (a) or the kinase-encoding domain of SRK_3 (b and c). The autoradiographs shown in (b and c) were exposed for longer than that in (a). Comparison of the intensity of hybridization indicated that SLG_3 mRNA was at least 10 times more abundant in stigmas than the SRK_3 mRNA. Sp, sepal; P, petal; R, root; C, cotyledon; L, leaf; U,B,B/T,T, anthers containing unicellular, bicellular, intermediate bicellular/tricellular or tricellular microspores respectively; O, ovary; St, stigma. RNA size markers are shown in kilobases (kb).

we were therefore interested to determine whether SLG_3 is expressed in this organ. SLG_3 cDNAs were therefore PCR amplified and cloned from anther mRNA. DNA sequencing of three anther-derived SLG_3 cDNAs allowed the identification of errors introduced during the PCR amplification but otherwise revealed no differences between anther and pistil SLG_3 cDNAs. In order to rule out the possibility that the SLG_3 mRNA detected in anthers was due to a low level of contamination with pistil tissue, mRNA was directly extracted from single organs and the PCR amplification repeated (Figure 4). Using this proceedure, an SLG_3 PCR product was observed following amplification of cDNA from single anthers and single pistils but not from single petals. No amplification occurred if reverse transcriptase was omitted. PCR amplification of *hsp70* sequences was carried out to verify that cDNA had been synthesized in the presence of reverse transcriptase (Figure 4). The degenerate oligonucleotides used are

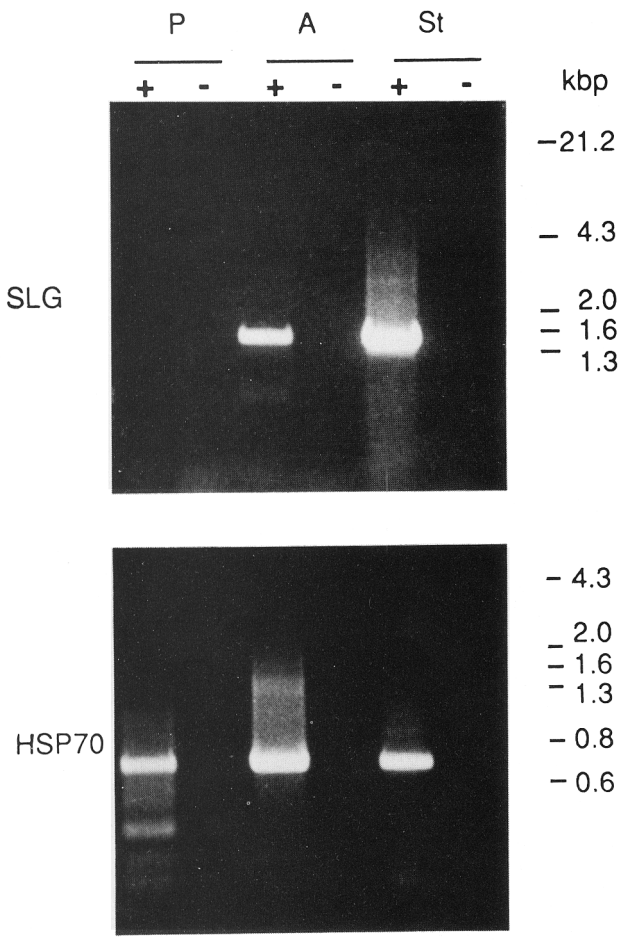


Figure 4. Detection of *SLG_3* transcripts in anthers by reverse transcriptase-PCR.
Poly(A)$^+$ RNA was extracted from a single petal (P), anther (A) or stigma (St) of the S_3/S_3 homozygous line and a reverse transcriptase reaction was carried out in the presence (+) or absence (–) of reverse transcriptase enzyme. PCR amplification was then carried out using either *SLG_3*-specific (upper panel) or 70 kDa heat shock protein (*hsp70*)-specific (lower panel) oligonucleotides. DNA size markers are shown in kilobasepairs (kbp).

predicted to amplify products from both constitutive and heat shock-induced *hsp70* genes. The results of these experiments demonstrate that the *SLG_3* gene is expressed in anthers and that the *SLG_3* mRNA expressed in anthers is not significantly different from that found in the pistil.

Expression of SLG_3 and SRK_3 at the protein level

The N-terminal amino acid sequence of the SLG_3 glycoprotein has been reported (Gaude *et al.*, 1993). An antibody (anti-SLG_3N-ter) was raised against a peptide corresponding to this N-terminal sequence (Gaude, Rougier, Heizmann, Ockendon and Dumas, manuscript in preparation) and used to probe an immunoblot of proteins extracted from a range of tissues and separated by SDS–PAGE. Figure 5(a) shows that antibody anti-SLG_3N-ter detects a triplet of bands of 60.0, 61.5 and 63.5 kDa. A similar pattern

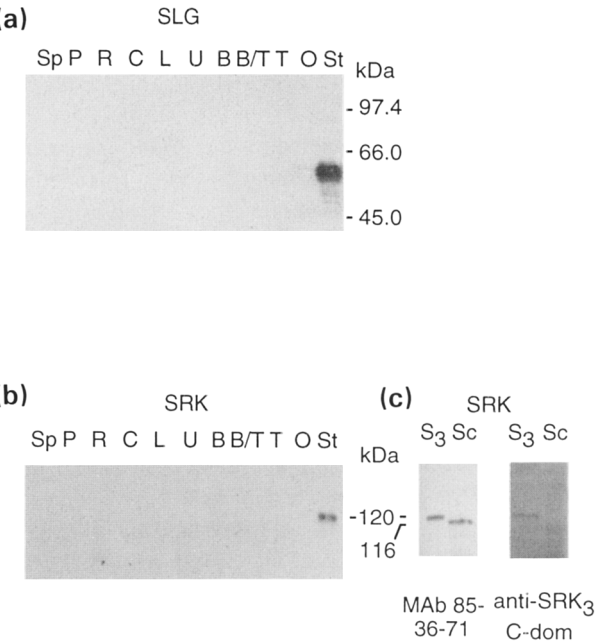


Figure 5. Detection of SLG_3 and SRK_3 antigen in proteins extracted from a range of tissues and separated by SDS–PAGE.
(a and b). The primary antibody was either anti-SLG_3N-ter (a) or Mab 85-36-71 (b). Molecular mass markers are shown in kilodaltons (kDa). The molecular mass of the SRK protein, calculated with reference to molecular mass markers, is shown in kilodaltons (kDa) in (b). Sp, sepal; P, petal; R, root; C, cotyledon; L, leaf; U,B,B/T,T, anthers containing microspores/pollen grains at the unicellular, bicellular, intermediate bicellular/tricellular or tricellular stages, respectively; O, ovary; St, stigma.
(c) Detection of SRK antigen in stigmas of an S_3S_3 homozygous line (S_3) and of P57 Sc (Sc) with either MAb 85–36–71 or with the anti-SRK_3C-dom antibody. Molecular masses of SRK proteins, calculated with reference to molecular mass markers, are shown in kilodaltons (kDa).

has been reported for other *S* haplotypes and the different molecular mass forms have been shown to be the result of different glycoforms of the SLG protein (Isogai *et al.*, 1987; Umbach *et al.*, 1990). SLG_3 glycoproteins were detected only in stigmas and hence, taking into account the sensitivity of this assay, exhibited the same tissue-specificity as the *SLG_3* transcript (Figure 5).

In order to assay *SRK_3* expression at the protein level it was necessary to have an antibody which recognized SRK_3. The first 10 amino acid residues at the N-terminus of the mature SRK_3 protein (IYVNTLSSTE) are predicted to be very similar to the N-terminus of the SLG protein (SLG-Sc) of the self-compatible *B. oleracea* line P57 Sc (IYVNTLSSSE; Gaude *et al.*, 1993). A monoclonal antibody raised against this latter sequence (MAb 85–36–71; Gaude *et al.*, 1993) was shown to recognize a protein of 120 kDa on an immunoblot of stigma proteins separated by SDS–PAGE (Figure 5b and c). In addition to the 120 kDa protein MAb 85–36–71 cross-reacted weakly with two proteins of approximately 62 kDa in stigma extracts of the S_3/S_3 line (data not shown). The nature of these latter proteins is unknown. To confirm that the 120 kDa protein corresponded

to SRK_3, a second antibody (anti-SRK_3C-dom) was raised against a synthetic peptide corresponding to part of the C-terminal domain of SRK_3. This antibody was shown to recognize a fusion protein containing the kinase and C-terminal domains of SRK_3 expressed in *E. coli* (data not shown). When used as a probe against total stigma proteins, the anti-SRK_3C-dom antibody also recognized a protein of 120 kDa (Figure 5c) providing strong evidence that the protein detected by these two antibodies is SRK_3.

Based on the gene sequence, SRK_3 is predicted to possess a polypeptide backbone of 94 kDa which may be modified by glycosylation at nine potential glycosylation sites. Following deglycosylation of stigma proteins, MAb 85–36–71 detected a protein of 94 kDa (Figure 6a), providing further evidence that this antibody detects the SRK_3 protein.

The peptide against which the anti-SRK_3C-dom antibody was raised corresponds to a region of the C-terminal domain of the SRK_3 protein which is predicted to be both highly antigenic and, due to the variable nature of the sequence in this region with respect to other SRK proteins, allele-specific. This prediction was supported by the fact that, using the anti-SRK_3C-dom antibody, a 120 kDa protein was detected in stigma proteins of the S_3/S_3 line used in this study but not in stigma proteins of two lines homozygous for other *S* haplotypes, P57 Sc (Figure 5c) and S_{25}/S_{25} (data not shown). MAb 85–36–71, on the other hand, recognized 120 kDa proteins in S_3/S_3 and P57 Sc stigmas (Figure 5c) but not in S_{25}/S_{25} stigmas (see the parental S_{25} plant in Figure 7c). This latter result indicates that, whilst MAb 853671 does not recognize all allelic forms of the SRK protein, it may be of general use for a subset of *SRK* alleles. For instance, based on their deduced amino acid sequences, SRK_2 is predicted to possess the epitope recognized by MAb 85–36–71 whilst SRK_6 is not.

Membrane localization of the SRK_3 protein

SRK has been predicted to be a membrane-spanning protein based on its deduced amino-acid sequence (Stein *et al.*, 1991). In order to test this hypothesis, we extracted proteins from pistils in a Tris–HCl, NaCl, EDTA buffer either with or without triton X-100 added to 3%. Tantikanjana *et al.* (1993) have used a similar approach to show that an alternative transcript of the SLG_2 gene encodes a membrane-anchored protein. These authors, however, reported the presence of a low level of membrane-anchored SLG_2 in extracts carried out in the absence of detergent and this was ascribed to the presence of membrane vesicles in the extract. A similar problem was initially encountered when extracting the SRK_3 protein. An ultracentrifugation step was therefore included to remove membrane vesicles before separation of the proteins on an SDS polyacrylamide gel. Proteins in the supernatant fractions and in the pellet obtained following centrifugation of the extract in buffer

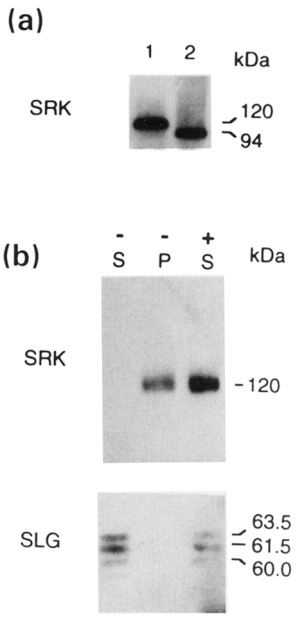


Figure 6. *SRK_3* encodes a membrane-localized glycoprotein.
(a) Deglycosylation of SRK_3. Untreated (lane 1) and deglycosylated (lane 2) stigma proteins were separated by SDS–PAGE and transferred to nitrocellulose. SRK protein was detected with MAb 85–36–71.
(b) Membrane-localization of SRK_3. Stigma proteins were extracted either in the presence (+) or absence (–) of 3% Triton X-100 and separated by SDS–PAGE. The primary antibody was either MAb 85–36–71 (upper panel) to detect SRK_3 antigen or anti-SLG_3N-ter (lower panel) to detect SLG_3 antigen. The molecular masses of the SRK_3 and SLG_3 proteins, calculated with reference to molecular mass markers, are shown in kilodaltons (kDa). S, supernatant; P, pellet following ultracentrifugation.

without added detergent were then separated on an SDS polyacrylamide gel, blotted on to a nitrocellulose filter and probed with Mab 85–36–71. To verify that protein had been extracted under the two extraction conditions, the same immunoblot was reprobed with antibody anti-SLG_3N-ter which recognizes the SLG_3 protein. Figure 6(b) shows that SLG_3 protein was present in the supernatant fraction of extractions carried out both in the presence and in the absence of detergent whereas the 120 kDa protein was detected in the supernatant only when extraction was carried out in the presence of detergent. A small amount of SRK_3 protein was detected in material pelleted from the minus-detergent extract by ultracentrifugation and this was presumed to be protein associated with membrane vesicles. These results indicate that SRK_3 is located in the membrane.

Linkage of the SLG_3 and SRK_3 genes to the S locus

In order to confirm that the SLG_3 and SRK_3 genes described above reside at the *S* locus, gene-specific DNA probes were used to detect restriction fragment length polymorphisms (RFLPs) in an F_2 population of 22 plants segregating for the S_3 and S_{25} haplotypes. Results obtained for the two

parental lines, an F_1 hybrid and for seven representative progeny are illustrated in Figure 7. The *SRK_3* kinase domain probe hybridized to an *Eco*RI fragment of 9.4 kbp which was present only in DNA of plants carrying the *S_3* haplotype. An identical pattern of hybridization was obtained with a probe corresponding to the 3′ untranslated region of *SRK_3* (data not shown). The *SLG_3* 3′ untranslated region probe hybridized to single fragments of 4.8 kbp and 7.2 kbp in *Eco*RI-digested DNA of the *S_3/S_3* and *S_{25}/S_{25}* homozygous lines, respectively. The 4.8 kbp *Eco*RI fragment corresponds to the *SLG_3* gene whilst the 7.2 kbp *Eco*RI fragment presumably corresponds to the *SLG_{25}* gene. In the F_1 and F_2 progeny, the presence or absence of these two fragments corresponded exactly with the presence or absence of the corresponding *S* haplotype. A third *Eco*RI fragment of 5.9 kbp was detected in DNA of some progeny. This fragment was presumed to be a partial digestion product of the *SLG_{25}* gene.

We assayed for expression of the SRK_3 protein in plants of the same F_2 population with the antibodies MAb 85–36–71 (Figure 7c) and anti-SRK_3C-dom (data not shown). With both antibodies, the presence of the 120 kDa protein in stigma extracts corresponded exactly with the presence of the *S_3* haplotype. Similarily, the anti-SLG_3N-ter antibody detected SLG_3 protein only in stigmas of plants shown genetically to carry the *S_3* haplotype (Figure 7d). The two major glycoforms of SLG_3 detected by anti-SLG_3N-ter (labelled δ and ε in figure 7d) correspond to abundant stigma glycoproteins characteristic of the *S_3/S_3* line (Gaude *et al.*, 1993). Taken together, these results demonstrate that both the *SLG_3* and *SRK_3* genes and expression of the SLG_3 and SRK_3 proteins shows tight genetic linkage to the *S_3* locus phenotype.

Discussion

Characterization of the SLG *and* SRK *genes of the* S_3 *haplotype*

We describe here the characterization of the *SLG* and *SRK* genes of the *S_3* self-incompatibility haplotype. DNA sequence analysis has shown that these two genes are most similar to genes of class I, pollen-dominant *S* haplotypes. In addition, the deduced amino acid sequence of the *S* domain of SRK_3 is highly similar to SLG_3 (82.6%; Figure 1a,) a feature typical of *SLG/SRK* gene pairs which originate from the same *S* haplotype. Within each *S* haplotype, the *SLG* and *SRK* genes appear to have evolved concertedly whilst the different *S* haplotypes were diverging with respect to one another (Goring and Rothstein, 1992; Goring *et al.*, 1993; Stein *et al.*, 1991). The general similarity between an SRK *S* domain and the corresponding SLG suggests that these two proteins may function in a similar manner. If this is the case, then as more SLG and SRK sequences become available, it may be possible to

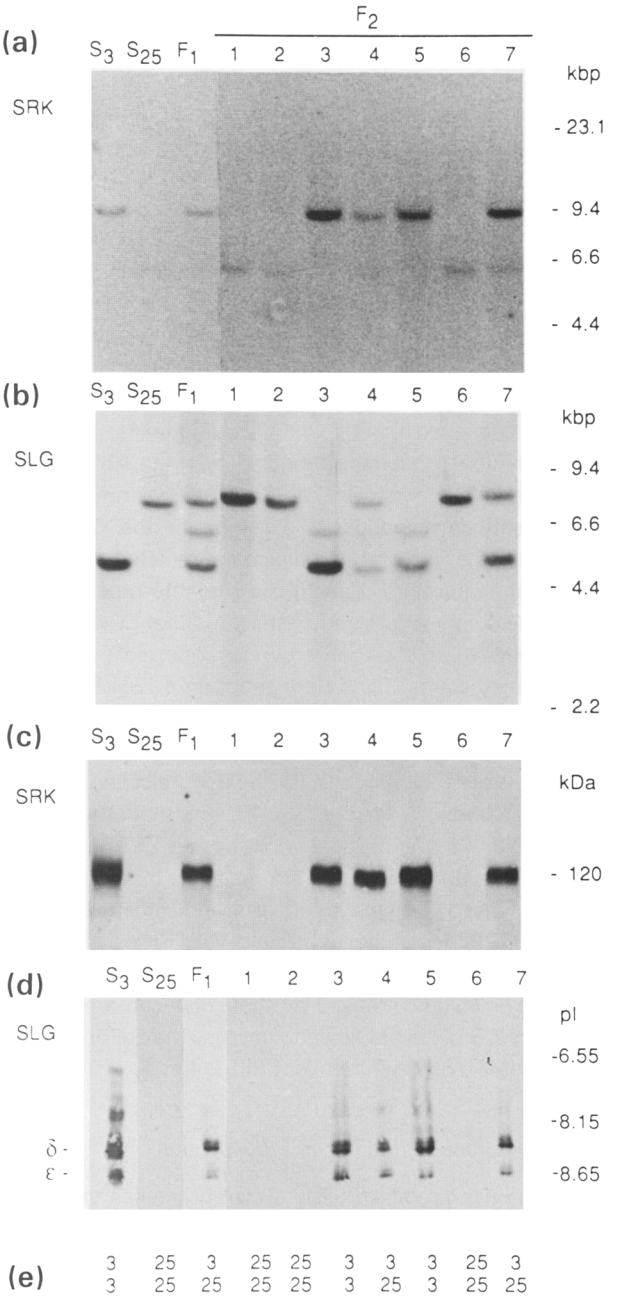


Figure 7. The *SRK_3* and *SLG_3* genes, and expression of the SRK_3 and SLG_3 proteins, are genetically linked to the *S_3* locus.
(a and b) Ten micrograms of genomic DNA were digested with either *Eco*RV (a) or *Eco*RI (b) and separated on 0.8% agarose gels. After transfer to a nylon filter, the blots were hybridized with DNA probes corresponding to either the kinase-encoding domain of *SRK_3* (a) or to the 3′ untranslated region of the *SLG_3* cDNA (b).
(c and d) Detection of SRK_3 (c) and SLG_3 (d) antigen in stigma proteins. For detection of SRK_3 protein, stigma proteins were separated by SDS–PAGE and probed with MAb 85–36–71. For detection of SLG_3 protein, stigma proteins were separated by IEF and probed with the anti-SLG_3N-ter antibody.
(e) *S* locus phenotypes of the plants analysed in (a)–(d) as determined by genetic crosses. The presence of the *S_3* and/or the *S_{25}* haplotype is indicated by 3 and 25 respectively.

identify conserved residues which are potentially important for protein function by comparing pairs of genes from the same *S* haplotype. If, as has been suggested (Nasrallah and Nasrallah, 1993), the recognition event which leads to the self-incompatibility response results from the interaction of SRK with a ligand, it is likely that this interaction involves both a gene-specific component (common to all *S* haplotypes) and a haplotype-specific component. This is the case for an analogous self/non-self-recognition system in *Ustilago maydis* (Gillissen *et al.*, 1992). We have compared the pairs of SLG and SRK sequences of the *S* haplotypes that have been characterized but were unable to identify haplotype-specific motifs at the primary structure (amino acid sequence) level. In particular, neither the variable region between amino acid residues 182 and 275, which has been suggested to have a role in haplotype specificity (Nasrallah *et al.*, 1987), nor the arrangement of potential glycosylation sites show any obvious haplotype-specificity. The identification of haplotype-specific motifs may require determination of the three-dimensional structure of these proteins.

Sequence conservation between the SRK_3 *promoter and promoters of other* S *gene family members*

DNA sequence extending 1090 bp upstream of the ATG initiation codon was obtained for the SRK_3 gene. Part of this sequence was shown to be highly similar to a conserved region identified in the promoters of other members of the *S* gene family. This region is shown in Figure 2 and the five conserved elements, Box I to Box V, identified by Dzelzkalns *et al.* (1993) in *SLG* and *SLR1* promoters are marked. The observation that there is a conservation of structure between the promoters of *SRK* and *SLG* is consistent with the similar tissue-specificity and temporal patterns of expression of the two genes. It should also be borne in mind that, as a result of the similarity between the *SLG* and *SRK* promoters, genes which epistatically control expression of the *SLG* gene may also pleiotrophically affect *SRK* expression. Mutations in genes unlinked to the *S* locus which lead to suppression of the self-incompatibility response (for example, see Nasrallah, 1974) apparently by suppressing SLG expression may therefore exert their effects via modification of either *SLG* and/or *SRK* expression. However, in one case (Nasrallah *et al.*, 1992) such a mutation has been shown to affect *SLG* mRNA abundance without affecting *SRK*.

Comparison of the SRK_3 and SRK_{63} promoters showed that the sequences are conserved upstream as far as −540 bp, after which they diverge. As sufficient upstream sequence data were available only for the two *SRK* promoters, the significance of this observation is unclear, however, it is interesting to note that Dzelzkalns *et al.* (1993) have shown that an SLG_{13} promoter fragment extending −411 bp upstream from the initiation codon functions in an identical manner to a 3.65 kbp promoter fragment. Thus the region conserved between the two *SRK* promoters may corresponds with the limits of the functional promoter.

Transcription of the SLG_3 *and* SRK_3 *genes*

At the mRNA level, the *SLG* and *SRK* genes of the S_3 haplotype are expressed with the same pattern as has been reported for other *S* haplotypes (Goring and Rothstein, 1992; Sato *et al.*, 1991; Stein *et al.*, 1991). Both genes are expressed specifically in stigmas and anthers with mRNA levels significantly higher in stigmas compared with anthers (Figures 3 and 4). We were unable to detect SLG_3 mRNA in anther tissue by an RNA blot procedure indicating that this gene is expressed at a lower level in anthers than has been reported for the SLG_{13} gene (Sato *et al.*, 1991). SLG_3 transcripts were, however, detectable in anthers using a reverse transcriptase-PCR procedure which involved extraction of poly(A)$^+$ RNA from single organs to avoid possible problems of contamination with stigma tissue (Figure 4). Sequencing of cDNAs corresponding to SLG_3 anther transcripts indicated that they encode a protein with an identical amino acid sequence to the stigma SLG_3. The SRK_3 kinase domain probe detected four transcripts in stigma mRNA (Figure 3c) with the same sizes and relative abundances as the transcripts detected in stigmas of an S_6 haplotype (Stein *et al.*, 1991). Using probes corresponding to different domains of the SRK_6 gene, Stein *et al.* (1991) obtained data indicating that these transcripts result from alternative splicing of the SRK_6 gene. The conservation of this expression pattern in the S_3 haplotype indicates that the production of alternative transcripts and their relative abundance may be important to *SRK* function.

The SRK_3 *gene encodes a 120 kDa protein in stigmas*

An immunochemical strategy, based on the use of two antibodies directed, respectively, against the N-terminal and the C-terminal end of SRK_3, allowed the identification of a 120 kDa protein specifically expressed in stigmas (Figure 5). Deglycosylation of the 120 kDa protein reduces its molecular mass to 94 kDa (Figure 6a), consistent with the predicted molecular mass for the SRK_3 polypeptide and with the presence of nine potential N-glycosylation sites in the *S* domain. Moreover, in an F_2 population segregating for the S_3 and S_{25} haplotypes, the 120 kDa protein was detected only in stigmas of plants carrying the SRK_3 gene and expressing the S_3 phenotype (Figure 7). Taken together, these data provide strong evidence that the 120 kDa protein is an expression product of the SRK_3 gene.

The protein detected in extracts of the self-compatible line P57 Sc had a smaller molecular weight than that of

the S_3/S_3 homozygous line (116 kDa compared with 120 kDa; Figure 5c). Expression of this protein correlates with the presence of the P57 Sc *S* haplotype in an F_2 population (Auger and Gaude, unpublished results) indicating that this protein corresponds to the SRK of the P57 Sc line. P57 Sc is a self-compatible line which possesses a class II-type *SLG* gene (Gaude *et al.*, 1993). We are currently investigating the possibility that the smaller molecular weight of the SRK protein of this line is related either to the self-compatible phenotype or to the presence of a class II *S* haplotype.

The SRK_3 protein is localized in the membrane

Based on deduced amino acid sequence, SRK has been predicted to span the plasma membrane. We have shown that the 120 kDa protein is extracted from stigma tissue only in the presence of detergent indicating that this protein is indeed localized in the membrane (Figure 6). Further work will be aimed at determining specifically whether SRK is located in the plasma membrane. In addition, the availability of two antibodies which recognize either the predicted extracellular domain or the predicted intracellular domain will provide a useful tool to confirm the predicted orientation of the SRK protein in the membrane. The observation that SRK is localized in the membrane, taken together with the demonstration that the SRK protein has a protein kinase activity (Goring and Rothstein, 1992; Stein and Nasrallah, 1993), strongly indicates that SRK functions as a membrane-spanning receptor kinase. Recently published genetic evidence that a functional *SRK* gene is essential for the SI response (Nasrallah *et al.*, 1994) supports the hypothesis that the role of this receptor is as a component in the signal transduction pathway leading from recognition of self-pollen by the stigma to inhibition of the fertilization process. These data also support the hypothesis that a number of other genes which exhibit sequence homology to *SRK* also function as receptor kinases. These genes include *ARK1*, *RLK1* and *RLK4* in *Arabidopsis thaliana* and *ZmPK1* in *Zea mays* (Tobias *et al.*, 1992; Walker, 1993; Walker and Zhang,1990), genes which have been shown to be expressed in vegetative tissues and which therefore presumably play a role in processes quite different from that of *SRK* in pollen–pistil recognition.

Experimental procedures

Plant material, genetic crosses and determination of incompatibility phenotype

The *Brassica oleracea* var. acephala homozygous lines P57 Si (S_3/S_3 homozygous) and P57 Sc have been described (Gaude *et al.*, 1993). Seeds of *Brassica oleracea* var. alboglabra homozygous for the S_{25} haplotype were a gift from Dr David J. Ockendon (HRI, Wellesbourne, UK). An F_1 heterozygous plant, the result of a cross between the S_3/S_3 and the S_{25}/S_{25} homozygous line, was self-fertilized to generate F_2 progeny for RFLP analysis. The incompatibility phenotype of the F_2 progeny was determined by self-pollination and by crosses to tester plants as previously described (Gaude *et al.*, 1993).

Cloning of the SLG_3 cDNA

The construction and screening of a cDNA library from mature stigmas of a *B. oleracea* var. *acephala* S_3/S_3 homozygous line (P57 Si) has been described previously (Gaude *et al.*, 1993). The SLG_3 cDNA was one of two clones which hybridized both to a BS29–2 (SLG_{29}) probe and to an oligonucleotide (5′-TCGATCAACACTTTGTCG-3′) based on the N-terminal sequence of the SLG_3 protein (Gaude *et al.*, 1993). The SLG_3 cDNA was sequenced on both strands.

Construction and screening of an S_3/S_3 genomic library

Genomic DNA was extracted (Choumane and Heizmann, 1988) from S_3/S_3 homozygous plants and a genomic library of size-fractionated *Sau3A* restriction fragments was constructed (Sambrook *et al.*, 1989) in lambda Dash (Stratagene, LaJolla, CA). One million plaques were screened with a mixed probe consisting of the first 1.2 kbp of the *SLG3* cDNA and a portion of the S domain of SRK obtained by PCR amplification from genomic DNA of the S_3/S_3 homozygous line. Positive phage were purified by several rounds of plaque purification. Several SLG_3 clones and one SRK_3 clone were obtained. The SLG_3 genomic clone was shown to be co-linear with the SLG_3 cDNA by PCR amplification using two oligonucleotides corresponding to the 5′ and 3′ ends of the SLG_3 cDNA (5′TGGCTCGAGAGATGAAAGGCATAAGAAAAAC-3′ and 5′AGGGAGATCAAATTTATGTGTATTTTACATGG-3′, respectively). A 9 kbp *Sma*I fragment carrying the SRK_3 gene was subcloned into pBluescript-SK$^+$ (Stratagene, La Jolla, CA) and 5345 bp of DNA was sequenced. Exon/intron boundaries within the SRK_3 gene were identified by sequencing an SRK_3 cDNA obtained by PCR amplification with *Pfu* thermostable DNA polymerase (Stratagene, La Jolla, CA) of mature stigma cDNA using two oligonucleotides: 5′-CGACAGAACCTATGTATGGATTGCC-3′ and 5′-GGCGGATCCTAAAAACAGCAAGCCAAAAAC-3′. SRK_3 mRNA polyadenlyation sites were identified by RACE-PCR (Frohman *et al.*, 1988).

PCR cloning of SLG_3 transcripts expressed in anthers

Poly(A)$^+$ RNA was extracted directly (Jakobsen *et al.*, 1990) from a single anther or petal from flowers at the unicellular stage of microspore development or from a single mature stigma and purified by hybridization to magnetic beads carrying oligo(dT)$_{25}$ (Dynal AS, Oslo). A DNase digestion was carried out to remove any contaminating DNA and the poly(A)$^+$ RNA was reverse transcribed (1st strand cDNA synthesis kit; Stratagene, La Jolla, CA). PCR amplification of SLG_3 cDNA was carried out with two specific oligonucleotides, C: 5′-TGGCTCGAGAGATGAAAGGCATAAGAAAAAC-3′ and D: 5′-AGGGAGATCAAATTTATGTGTATTTTACATGG-3′, which prime at the 5′ and 3′ ends of the SLG_3 cDNA, respectively. Two degenerate oligonucleotides, 5′-GGGAATTCGGNGGNGGRGAYTTYGA-3′ and 5′-GGGAATTCACNCCNGGYTGRTTRTC-3′, were used for PCR amplification of *hsp*70 (heat shock protein 70) cDNA. PCR amplification was carried out with AmpliTaq DNA polymerase according to the instructions of the supplier (Perkin–Elmer, NJ) for 38 cycles of 94°C for 1 min denaturation, 55°C for 1 min annealing and 72°C for 4 min extension, followed

by a final extension for 10 min. For cloning, PCR amplification was carried out from anther cDNA as above. The PCR product was treated with Klenow DNA polymerase to create blunt ends and phosphorylated with polynucleotide kinase. The desired product was purified by excision from an agarose gel and digestion of the agarose with Gelase (Epicentre Technologies, Madison, WI). The purified DNA was ligated into *Eco*RV-digested, dephosphorylated pBluescript-SK$^+$ plasmid DNA and positive clones were sequenced on both strands.

DNA sequencing, RNA gel blot analysis and Southern blot analysis

Sequencing was carried out by the dideoxy-nucleotide chain termination method (Sanger *et al.*, 1977) using the Sequenase system (version 2.0; U.S. Biochemicals) and custom-synthesized oligonucleotides. Sequence data were analysed using Lasergene sequence analysis software (DNASTAR, London, UK). For RNA gel blots, total RNA was extracted by the method of Jackson and Larkins (1976). Cotyledons, leaves and roots were harvested from 3-week-old seedlings, all other tissues were from mature, flowering plants. The developmental stage of anthers was determined by fluorescence microscopy observation of DAPI-stained microspores. Poly(A)$^+$ RNA was purified from 100 μg batches of total RNA by hybridization to magnetic beads carrying oligo(dT)$_{25}$ (Dynal AS, Oslo, Norway). RNA was separated on formaldehyde gels and transferred to nylon filters for hybridization. For Southern blots, total genomic DNA was extracted from leaves of *Brassica* plants as described by Choumane and Heizmann (1988). DNA probes were prepared using a random priming DNA labelling kit (Boehringer Mannheim). Hybridizations were carried out under standard conditions and filters were washed at high stringency (Sambrook *et al.*, 1989).

Protein extraction and electrophoretic analysis

Protein extraction, separation of proteins by isoelectric focusing (IEF) or SDS–PAGE, electrotransfer on to nitrocellulose membranes and detection of antigen with antibodies was as described previously (Gaude et al., 1993). For the identification of membrane-localized proteins, protein was extracted from stigmas in TBS buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) with or without added 3% (w/v) Triton X-100. Diluted protein extracts were centrifuged at 100 000 ***g*** for 1 h at 4°C and then concentrated by ultrafiltration on Centricon-30 filters (Amicon, Beverly, USA) and precipitated with cold acetone before loading on an SDS polyacrylamide gel as described (Gaude *et al.*, 1993).

Deglycosylation of proteins

Following extraction of stigma proteins in TBS buffer with 3% (w/v) of Triton X-100 as described above, the protein solution was diluted with 30 volumes of TBS and ultrafiltrated on a centricon 30 filter (Amicon, Beverly, USA) before lyophilization. Protein deglycosylation and subsequent recovery was performed mainly as described by Karp *et al.* (1982). Briefly, 250 μg of lyophilized protein were deglycosylated in 50 μl of TMFS/Anisole (9 vol./1 vol.) on ice for 3 h with magnetic stirring of the reaction mixture. After dialysis against 0.1 M NH_4HCO_3, deglycosylated proteins were lyophilized and resuspended in loading buffer for SDS–PAGE electrophoresis.

Antibody production and immunodetection on protein blots

The anti-SLG$_3$N-ter and anti-SRK$_3$C-dom antibodies were produced by injecting mice or rabbits, respectively, with peptides cross-linked to ovalbumin as described (Gaude *et al.*, 1993). The peptides had the sequences INTLSSTESLY and SSRQYDNDEWT, respectively. Antigen/antibody complexes were visualized using goat anti-mouse antibody or goat anti-rabbit conjugated either to alkaline phosphatase (Promega, Madison, USA) or horseradish peroxidase (Amersham) as secondary antibodies. For alkaline phosphatase development, nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate were used as colour development substrates. For horseradish peroxidase, ECL detection kit reagents (Amersham) were used.

Expression of the SRK$_3$ *kinase domain in* E. coli

The kinase-encoding domain of *SRK$_3$* was isolated as a *Bcl*I/*Bam*HI fragment of the *SRK$_3$* cDNA described above and cloned into the BamHI site of pQE-32 (Qiagen Inc., Chatsworth, CA) to create a fusion protein containing a poly-histidine sequence followed by residues 459–850 of *SRK$_3$*. Expression of the fusion protein in *E. coli* and purification on a nickel-nitrilo-tri-acetic acid (Ni-NTA) resin was essentially as described by the supplier (Qia-expressionist kit, Qiagen Inc., Chatsworth, CA). Briefly, fusion gene expression was induced by addition of 2 mM isopropyl β-thiogalactoside to a 1 l bacterial culture and the cells harvested 2 h 30 min later. Cells were lysed by sonication in 50 mM NaH_2PO_4 pH 8.0, 0.3 M NaCl, 1% Triton X-100, 1 mM PMSF, 10 mM β-mercaptoethanol, centrifuged at 15 000 ***g*** and the supernatant mixed with Ni-NTA resin. After rinsing the resin, bound protein was eluted in the presence of imidazole and the SRK fusion protein detected by immunoblotting.

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