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Valerie Delorme, Jean-Loic Giranton, Yves Hatzfeld, Aline Friry, Philippe Heizmann, et al.. Characterization of the S locus genes, SLG and SRK, of the Brassica S3 haplotype: identification of a membrane-localized protein encoded by the S locus receptor kinase gene. *Plant Journal*, 1995, 7 (3), pp.429-440. 10.1046/j.1365-313x.1995.7030429.x . hal-03086441

HAL Id: hal-03086441

<https://cnrs.hal.science/hal-03086441>

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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 F₂ 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

Received 5 August 1994; revised 7 November 1994; accepted 25 November 1994.

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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

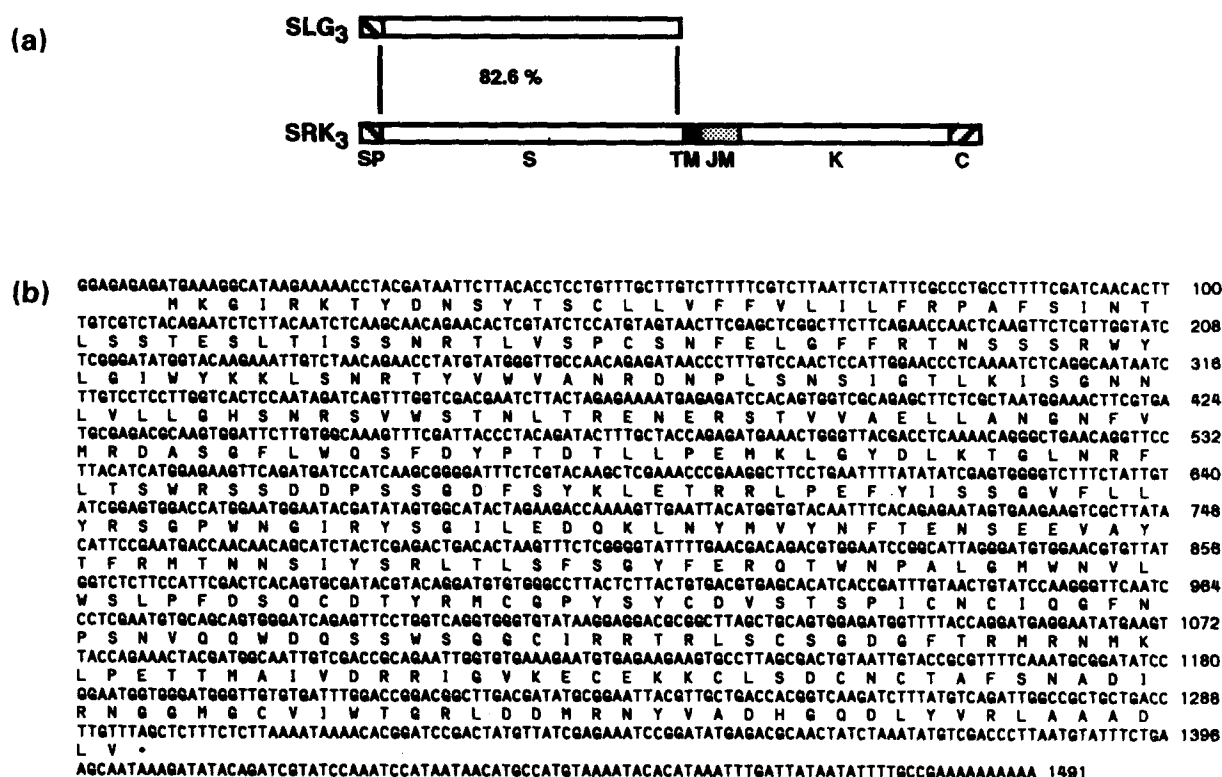


Figure 1. Sequence analysis of the *SLG*₃ and *SRK*₃ genes.

(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.

[illegible]

<u>Box I</u>	<u>Box II</u>	<u>Box III</u>	<u>Box IV</u>
1. <u>1945-1946</u>	2. <u>1947-1948</u>	3. <u>1949-1950</u>	4. <u>1951-1952</u>
5. <u>1953-1954</u>	6. <u>1955-1956</u>	7. <u>1957-1958</u>	8. <u>1959-1960</u>
9. <u>1961-1962</u>	10. <u>1963-1964</u>	11. <u>1965-1966</u>	12. <u>1967-1968</u>
13. <u>1969-1970</u>	14. <u>1971-1972</u>	15. <u>1973-1974</u>	16. <u>1975-1976</u>
17. <u>1977-1978</u>	18. <u>1979-1980</u>	19. <u>1981-1982</u>	20. <u>1983-1984</u>
21. <u>1985-1986</u>	22. <u>1987-1988</u>	23. <u>1989-1990</u>	24. <u>1991-1992</u>
25. <u>1993-1994</u>	26. <u>1995-1996</u>	27. <u>1997-1998</u>	28. <u>1999-2000</u>
29. <u>2001-2002</u>	30. <u>2003-2004</u>	31. <u>2005-2006</u>	32. <u>2007-2008</u>
33. <u>2009-2010</u>	34. <u>2011-2012</u>	35. <u>2013-2014</u>	36. <u>2015-2016</u>
37. <u>2017-2018</u>	38. <u>2019-2020</u>	39. <u>2021-2022</u>	40. <u>2023-2024</u>
41. <u>2025-2026</u>	42. <u>2027-2028</u>	43. <u>2029-2030</u>	44. <u>2031-2032</u>
45. <u>2033-2034</u>	46. <u>2035-2036</u>	47. <u>2037-2038</u>	48. <u>2039-2040</u>
49. <u>2041-2042</u>	50. <u>2043-2044</u>	51. <u>2045-2046</u>	52. <u>2047-2048</u>
53. <u>2049-2050</u>	54. <u>2051-2052</u>	55. <u>2053-2054</u>	56. <u>2055-2056</u>
57. <u>2057-2058</u>	58. <u>2059-2060</u>	59. <u>2061-2062</u>	60. <u>2063-2064</u>
61. <u>2065-2066</u>	62. <u>2067-2068</u>	63. <u>2069-2070</u>	64. <u>2071-2072</u>
65. <u>2073-2074</u>	66. <u>2075-2076</u>	67. <u>2077-2078</u>	68. <u>2079-2080</u>
69. <u>2081-2082</u>	70. <u>2083-2084</u>	71. <u>2085-2086</u>	72. <u>2087-2088</u>
73. <u>2089-2090</u>	74. <u>2091-2092</u>	75. <u>2093-2094</u>	76. <u>2095-2096</u>
77. <u>2097-2098</u>	78. <u>2099-2100</u>	79. <u>2101-2102</u>	80. <u>2103-2104</u>
81. <u>2105-2106</u>	82. <u>2107-2108</u>	83. <u>2109-2110</u>	84. <u>2111-2112</u>
85. <u>2113-2114</u>	86. <u>2115-2116</u>	87. <u>2117-2118</u>	88. <u>2119-2120</u>
89. <u>2121-2122</u>	90. <u>2123-2124</u>	91. <u>2125-2126</u>	92. <u>2127-2128</u>
93. <u>2129-2130</u>	94. <u>2131-2132</u>	95. <u>2133-2134</u>	96. <u>2135-2136</u>
97. <u>2137-2138</u>	98. <u>2139-2140</u>	99. <u>2141-2142</u>	100. <u>2143-2144</u>
101. <u>2145-2146</u>	102. <u>2147-2148</u>	103. <u>2149-2150</u>	104. <u>2151-2152</u>
105. <u>2153-2154</u>	106. <u>2155-2156</u>	107. <u>2157-2158</u>	108. <u>2159-2160</u>
109. <u>2161-2162</u>	110. <u>2163-2164</u>	111. <u>2165-2166</u>	112. <u>2167-2168</u>
113. <u>2169-2170</u>	114. <u>2171-2172</u>	115. <u>2173-2174</u>	116. <u>2175-2176</u>
117. <u>2177-2178</u>	118. <u>2179-2180</u>	119. <u>2181-2182</u>	120. <u>2183-2184</u>
121. <u>2185-2186</u>	122. <u>2187-2188</u>	123. <u>2189-2190</u>	124. <u>2191-2192</u>
125. <u>2193-2194</u>	126. <u>2195-2196</u>	127. <u>2197-2198</u>	128. <u>2199-2200</u>
129. <u>2201-2202</u>	130. <u>2203-2204</u>	131. <u>2205-2206</u>	132. <u>2207-2208</u>
133. <u>2209-2210</u>	134. <u>2211-2212</u>	135. <u>2213-2214</u>	136. <u>2215-2216</u>
137. <u>2217-2218</u>	138. <u>2219-2220</u>	139. <u>2221-2222</u>	140. <u>2223-2224</u>
141. <u>2225-2226</u>	142. <u>2227-2228</u>	143. <u>2229-2230</u>	144. <u>2231-2232</u>
145. <u>2233-2234</u>	146. <u>2235-2236</u>	147. <u>2237-2238</u>	148. <u>2239-2240</u>
149. <u>2241-2242</u>	150. <u>2243-2244</u>	151. <u>2245-2246</u>	152. <u>2247-2248</u>
153. <u>2249-2250</u>	154. <u>2251-2252</u>	155. <u>2253-2254</u>	156. <u>2255-2256</u>
157. <u>2257-2258</u>	158. <u>2259-2260</u>	159. <u>2261-2262</u>	160. <u>2263-2264</u>
161. <u>2</u>			

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 Dzelzalns *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

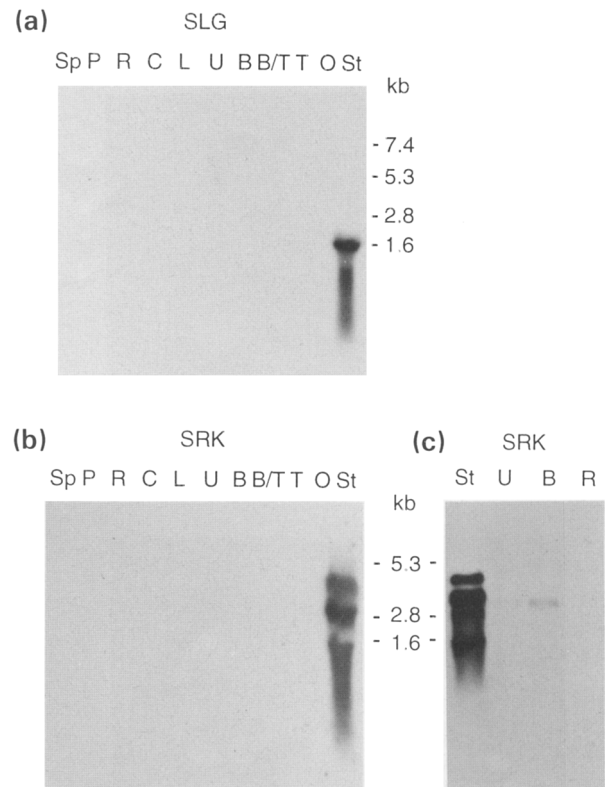


Figure 3. Analysis of expression of the *SLG*₃ and *SRK*₃ genes.

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, 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 procedure, 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

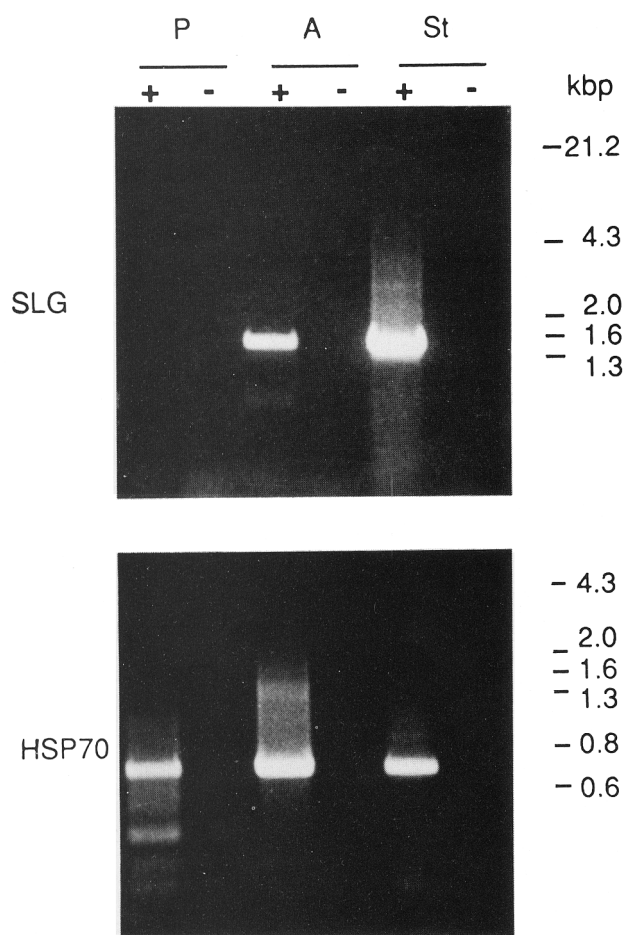


Figure 4. Detection of *SLG₃* transcripts in anthers by reverse transcriptase-PCR.

Poly(A)⁺ RNA was extracted from a single petal (P), anther (A) or stigma (St) of the *S₃/S₃* 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 *SLG₃*-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 *SLG₃* gene is expressed in anthers and that the *SLG₃* mRNA expressed in anthers is not significantly different from that found in the pistil.

Expression of *SLG₃* and *SRK₃* at the protein level

The N-terminal amino acid sequence of the *SLG₃* glycoprotein has been reported (Gaude *et al.*, 1993). An antibody (anti-*SLG₃*N-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-*SLG₃*N-ter detects a triplet of bands of 60.0, 61.5 and 63.5 kDa. A similar pattern

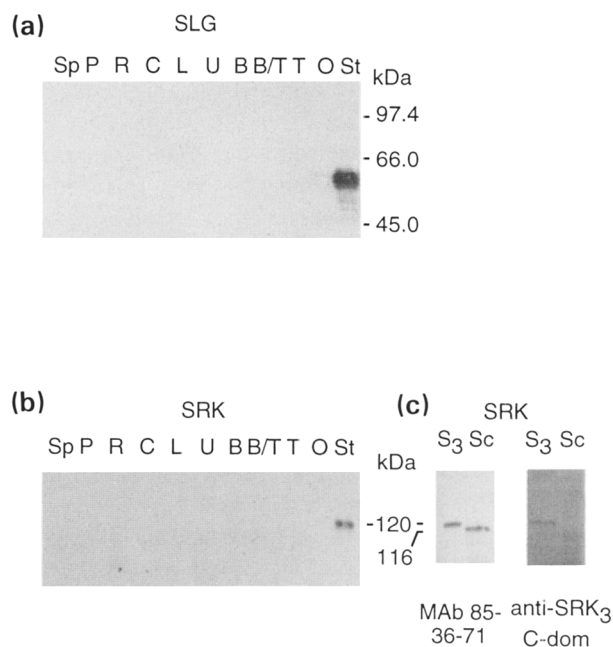


Figure 5. Detection of *SLG₃* and *SRK₃* antigen in proteins extracted from a range of tissues and separated by SDS-PAGE.

(a and b). The primary antibody was either anti-*SLG₃*N-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 *S₃/S₃* homozygous line (*S₃*) and of P57 Sc (Sc) with either Mab 85-36-71 or with the anti-*SRK₃*C-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). *SLG₃* glycoproteins were detected only in stigmas and hence, taking into account the sensitivity of this assay, exhibited the same tissue-specificity as the *SLG₃* transcript (Figure 5).

In order to assay *SRK₃* expression at the protein level it was necessary to have an antibody which recognized *SRK₃*. The first 10 amino acid residues at the N-terminus of the mature *SRK₃* 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 (IYVNTLSSE; 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 *S₃/S₃* line (data not shown). The nature of these latter proteins is unknown. To confirm that the 120 kDa protein corresponded

to SRK₃, a second antibody (anti-SRK₃C-dom) was raised against a synthetic peptide corresponding to part of the C-terminal domain of SRK₃. This antibody was shown to recognize a fusion protein containing the kinase and C-terminal domains of SRK₃ expressed in *E. coli* (data not shown). When used as a probe against total stigma proteins, the anti-SRK₃C-dom antibody also recognized a protein of 120 kDa (Figure 5c) providing strong evidence that the protein detected by these two antibodies is SRK₃.

Based on the gene sequence, SRK₃ 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 SRK₃ protein.

The peptide against which the anti-SRK₃C-dom antibody was raised corresponds to a region of the C-terminal domain of the SRK₃ 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-SRK₃C-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, SRK₂ is predicted to possess the epitope recognized by MAb 85-36-71 whilst SRK₆ is not.

Membrane localization of the SRK₃ 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 SLG₂ 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 SRK₃ 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

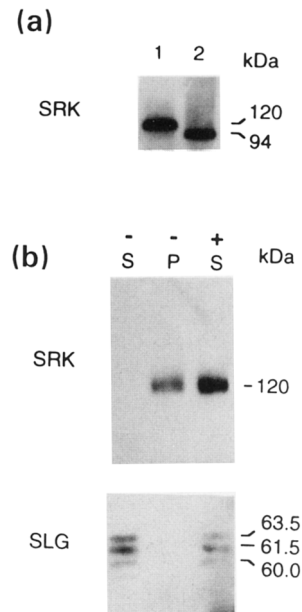


Figure 6. *SRK*₃ encodes a membrane-localized glycoprotein.

(a) Deglycosylation of SRK₃. 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 SRK₃. 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 SRK₃ antigen or anti-SLG₃N-ter (lower panel) to detect SLG₃ antigen. The molecular masses of the SRK₃ and SLG₃ 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-SLG₃N-ter which recognizes the SLG₃ protein. Figure 6(b) shows that SLG₃ 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 SRK₃ 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 SRK₃ is located in the membrane.

Linkage of the SLG₃ and SRK₃ 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 F₂ 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 $EcoRI$ 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 $EcoRI$ -digested DNA of the S_3/S_3 and S_{25}/S_{25} homozygous lines, respectively. The 4.8 kbp $EcoRI$ fragment corresponds to the SLG_3 gene whilst the 7.2 kbp $EcoRI$ 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 $EcoRI$ 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_3 C-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. Similarly, the anti- SLG_3 N-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_3 N-ter (labelled δ and ϵ in figure 7d) correspond to abundant stigma glycoproteins characteristic of the S_3/S_3 line (Gaudé *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

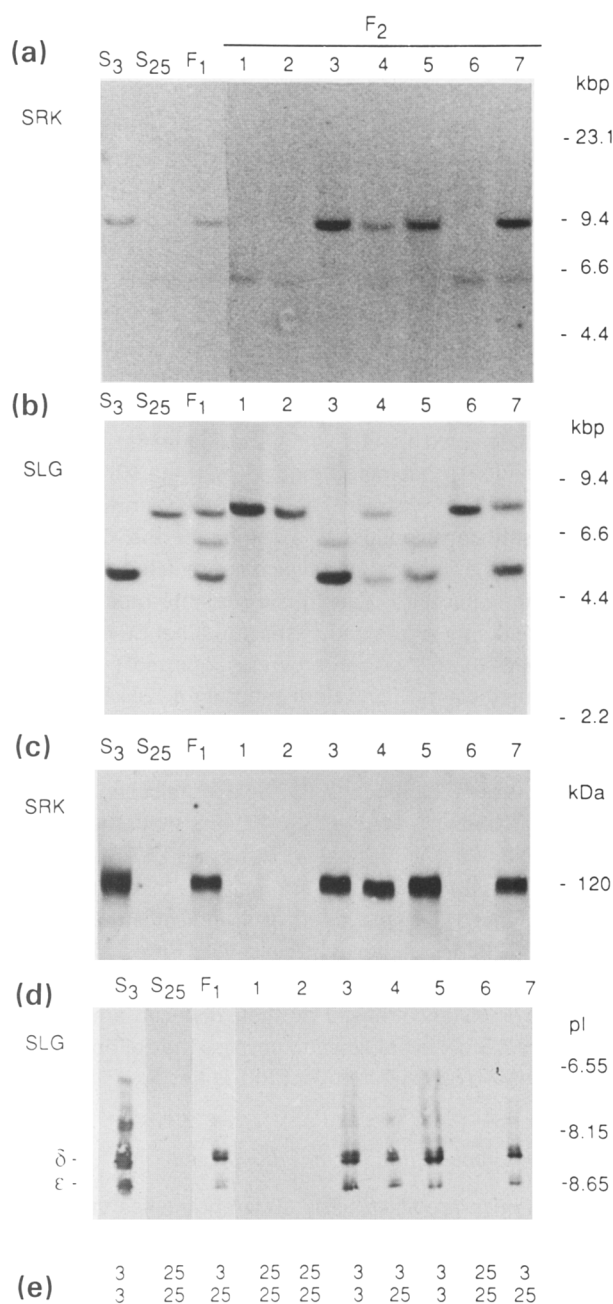


Figure 7. The SRK_3 and SLG_3 genes, and expression of the SRK_3 and SLG_3 proteins, are genetically linked to the S_3 locus.

(a and b) Ten micrograms of genomic DNA were digested with either $EcoRV$ (a) or $EcoRI$ (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_3 N-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 SRK₃ promoter and promoters of other S gene family members

DNA sequence extending 1090 bp upstream of the ATG initiation codon was obtained for the *SRK₃* 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 *SRK₃* and *SRK₆₃* 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 *SLG₁₃* 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 correspond with the limits of the functional promoter.

Transcription of the SLG₃ and SRK₃ genes

At the mRNA level, the *SLG* and *SRK* genes of the *S₃* 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 *SLG₃* 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 *SLG₁₃* gene (Sato *et al.*, 1991). *SLG₃* 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 *SLG₃* anther transcripts indicated that they encode a protein with an identical amino acid sequence to the stigma *SLG₃*. The *SRK₃* 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 *S₆* haplotype (Stein *et al.*, 1991). Using probes corresponding to different domains of the *SRK₆* gene, Stein *et al.* (1991) obtained data indicating that these transcripts result from alternative splicing of the *SRK₆* gene. The conservation of this expression pattern in the *S₃* haplotype indicates that the production of alternative transcripts and their relative abundance may be important to *SRK* function.

The SRK₃ 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 *SRK₃*, 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 *SRK₃* polypeptide and with the presence of nine potential N-glycosylation sites in the *S* domain. Moreover, in an *F₂* population segregating for the *S₃* and *S₂₅* haplotypes, the 120 kDa protein was detected only in stigmas of plants carrying the *SRK₃* gene and expressing the *S₃* phenotype (Figure 7). Taken together, these data provide strong evidence that the 120 kDa protein is an expression product of the *SRK₃* 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 SRK₃ 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 SLG₃ 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'-TCGATCAACACTTTGTTCG-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 *Sau*3A restriction fragments was constructed (Sambrook et al., 1989) in lambda Dash (Stratagene, La Jolla, CA). One million plaques were screened with a mixed probe consisting of the first 1.2 kbp of the SLG_3 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'-AGGGAGATCAAATTTATGTGATTTTACATGG-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 polyadenylation sites were identified by RACE-PCR (Frohman et al., 1988).

PCR cloning of SLG₃ 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'-AGGGAGATCAAATTTATGTGATTTTACATGG-3', which prime at the 5' and 3' ends of the SLG_3 cDNA, respectively. Two degenerate oligonucleotides, 5'-GGGAATT-CGGNGGNGGRGAYTTYGA-3' and 5'-GGGAATTCACNCCNGGYT-GRTRTC-3', were used for PCR amplification of *hsp70* (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 another 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 *EcoRV*-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 NH₄HCO₃, deglycosylated proteins were lyophilized and resuspended in loading buffer for SDS-PAGE electrophoresis.

Antibody production and immunodetection on protein blots

The anti-SLG₃N-ter and anti-SRK₃C-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 SRK₃ kinase domain in *E. coli*

The kinase-encoding domain of *SRK₃* was isolated as a *BclI/BamHI* fragment of the *SRK₃* 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 *SRK₃*. 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 NaH₂PO₄ 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.

Acknowledgements

We thank Dr David J. Ockendon (HRI, Warwick, UK) for supplying us with *S₃/S₃* tester and *S₂₅/S₂₅* *Brassica* lines and for help with determining the SI genotype of the P57 SI line; Dr Martin Trick (John Innes Institute, UK) for the gift of the BS29–2 clone; Dr Elizabeth Bates for the gift of *hsp70*-specific oligonucleotides; Monique Estienne and Richard Blanc for technical assistance and Pierre Audenis for photographic work. T.G. and P.H. are members of the Centre National de la Recherche Scientifique, J.M.C. is a member of the Institut National de la Recherche Agronomique. This work was supported by the EEC BRIDGE programme.

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EMBL Data Library accession numbers X79431 (*SLG₃* cDNA) and X79432 (*SRK₃* gene).