SPOROPHYTIC SELF-INCOMPATIBILITY
(Nasrallah and Nasrallah, 1993; Matton et al. 1994; Dodds et al. 1997; Franklin-Tong 2002a and b; Nasrallah 2002)

- Pollen expresses both paternal parent alleles at S locus, even though it only carries one allele. Co-dominant and/or dominant allelic interactions occur that determine the ultimate phenotype of the stigma and pollen interaction.
- Pollen rejection occurs when the same S allele is active in both stigma and pollen.
- Growth arrest occurs on the stigma, either before pollen germination or before the pollen tube can penetrate the wall of the papillar cells.
- Transfer of incompatible pollen to a compatible stigma results in germination, so the effects of the SI reaction are reversible.
- Developmentally regulated in stigma – bud pollination day before pollination – no SI.

1. Alleles can manifest dominance, independence, interaction, or mutual weakening
   Two types of S alleles
   Class I: high activity–strong dominance – average of 0-10 pollen tubes/self-pollination.
   Class II: low activity–weak/leaky incompatibility reaction – 10-30 pollen tubes/self-pollination.

   Thus, Class I are *dominant* over Class II, but within a class, alleles are usually codominant

2. Compatibility is more complex than in gametophytic systems because it depends on the nature of the dominance in both the male and female parents.

Model system: *Brassica*

Three component have been characterized at the molecular level.

(Nasrallah group)

A. SLG
   1. S-locus glycoprotein.
   2. Secreted to exterior of papillar cell.
   3. A soluble cell wall-localized protein.
   4. A role in the full manifestation of incompatibility (will be discussed later).

B. SRK
   1. S-locus receptor kinase.
   2. Transmembrane protein (single transmembrane domain)
   3. N-terminus is similar to SLG and extracellular

4. C-terminus is a serine/threonine kinase and cytoplasmic
5. SRK is the female determinant of SI–it interacts directly with the pollen S-locus product to initiate the SI reaction (Takasaki et al., 2000).
6. The extracellular domain of SRK is similar to SLG
7. Class I SLG/SRK allele sequences are more similar to each other (80% identity at amino acid level) than they are to Class II alleles (only 70%) and vice versa.

Similarities between SLG and SRK

1. SRK has a region very similar to SLG (~90% amino acid conservation within a haplotype).
2. SLG likely derived from a duplication of SRK (probably before the diversification of S-alleles).
3. Generally, more similarity between the SLG-like domain of SRK and SLG within a haplotype than between SLG alleles in different haplotypes

C. SCR

1. S-locus cysteine-rich protein (*Brassica oleraciea*).
2. Pollen determinant of SI isolated by the Nasrallah group(Schopfer et al., 1999).
3. Small, secreted protein (~8.5 kDa)
4. Alleles have highly conserved NH_2-termini, highly divergent and only 7 highly conserved cysteine residues and one glycie residue are found to be conserved among 22 SCR sequences.
5. Cysteine residues possibly give the protein its 3-dimensional structure via disulfide bridges; loops between the Cys residues, which are highly divergent, would give the protein its specificity.
6. Takayama et al. (2000) cloned and characterized *SP11* (S locus protein 11 of *B. rapa*), which is identical to SCR.

Female determinant(s) of sporophytic self-incompatibilty:

Although the female determinant SRK was cloned for some time, real experimental evidence supporting its function came from the work of Takasaki et al. (2000). Earlier transgenic experiments were failed due to sense suppression resulting breakdown of self-incompatibility. This Japanese group took the advantage of sequence diversity between two classes of S alleles or S haplotypes. For example, SRKs of class I is more diverse to SRKs of class II S haplotypes than those of the class I haplotypes.

1. Developed transgenic $S^{60}S^{60}$ (class II type) lines carrying SRK^{28} (class I type) and $S^{52}S^{60}$-lines carrying SLG^{28}. S^{52} is class I type though.
2. Used SLG28 promoter and SRK28 cDNA to reduce the sense suppression.
3. Only 3/17 transgenic plants carrying SRK^{28} showed only 32-35% of that in normal SRK^{28} heterozygote – low level of expression. Rest 14 did show S28 transcripts.
4. They used these three transgenic plants to show that transgene S28 specifically reject S28 pollen, not S43, S45 and S52 from homozygous plants.
5. One out of 4 transgenic $S^{52}S^{60}$ plants carrying SLG^{28} was producing a high level of the SLG28 protein.

6. *SLG*^{28}transgene did not reject S28 pollen indicating no role in pollen rejection.
7. However, the rejection of the S28 pollen by transgene *SRK*^{28} was significantly enhanced (about 5 folds) in the presence of transgene *SLG*^{28}.
8. Conclusion: SRK is the female determinant and SLG is necessary to enhance the activity of SRK.

Male determinant of the sporophytic self-incompatibilty:

(Schopfer et al. 1999 and Takayama et al. 2000)

Two groups, Nasrallah , Cornell University and Isogai, Nara, Japan, independently cloned the male determinant of the sporophytic self-incompatibilty. However, direct interaction between the male and female determinants came from recent work by the same two groups.

Schopfer et al. (1999)

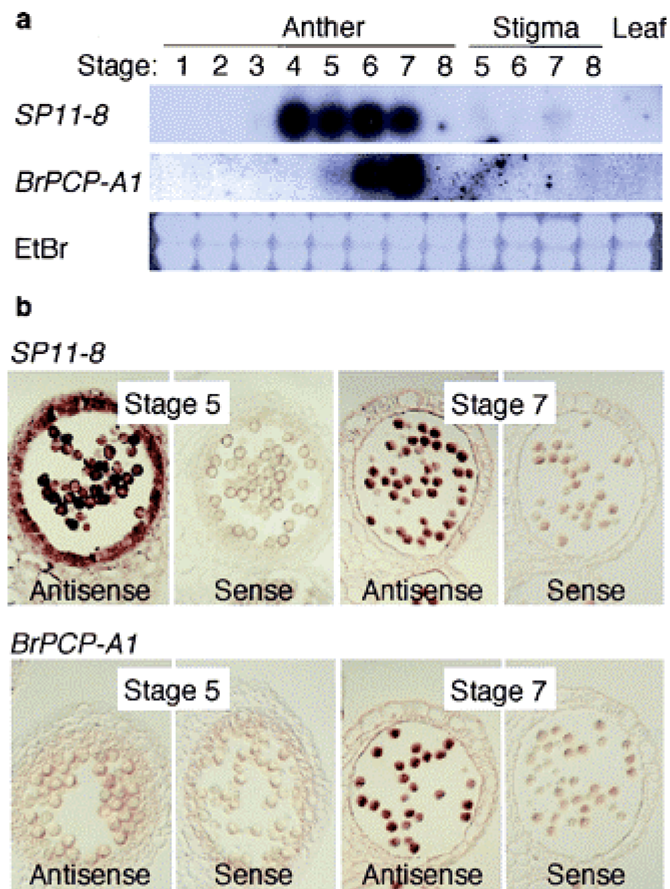
1. Schopfer et al. (1999) identified the S locus cysteine-rich protein (*SCR*) gene from sequence analysis of a 13 kb DNA fragment from the 65 kb DNA fragment that contains both SLK8 and SLG8 genes in Brassica campestris.
2. Cloned cDNAs from the cauliflower (*Brassica oleracea*) S6 and S13 and termed SCR6 and SCR13. Co-segregate with SLG6/SRK6 and SLG13/SRL13 gene pairs as well as S6 and S13 specificities.
3. Expression of this gene is in anthers and haploid microspores – indicating postmeiotically active. Most likely is expressed in tapetum cells also.
4. Homozygous *m1600* mutant (from gama-irradiation) failed to show *S13* transcripts.
5. Transgenic (S2S2) carrying the SCR6 cDNA fused to the SCR8 promoter rejected pollen from *S6S6* homozygotes.
6. Sequence analyses indicate a huge divergence among SCR alleles-conservation limited to 11 amino acids, of which 8 are cysteine involved in 3D structure formation through sulfhydril bonds.
7. Conclusion: The SCR protein is the male determinant of the sporophytic self-incompatibilty.

Takayama et al. 2000

1. Earlier (Suzuki et al. 1999) a potential candidate (*SP11*: S locus protein 11) for the pollen determinant was isolated from the S9 haplotype of *B. capmpestris*.
2. Northern blot analysis of RNA samples showed that the gene is expressed in anthers. In situ hybridization confirmed that the expression of the gene occurs in the tapetum cell layer (Figure 4 of Takayama et al. 2000).
3. Recombinant SP11-9 protein (S9 haplotype), when used to coat the S9 papillar cells, inhibited the hydration of S8 pollen significantly. But the inhibitory affect of the recombinant protein was not observed against S9 pollen when applied to S8 papillar cells.

4. Conclusions: SCR and SP11 are the same protein that determines the specificity from the male side. It is expressed in the tapetum cell layer allowing to accumulate on the pollen grains.

Fig. 4. Expression of *SP11*. (*a*) Northern blot analysis. Total RNA from anther, pistil, and leaf tissues was hybridized with an *SP11-8* (coding region) probe and a *BcPCP-A1* (coding region) probe. The stage numbers correspond to different bud sizes, with 1 = 0-1 mm, 2 = 1-2 mm, 3 = 2-3 mm, 4 = 3-4 mm, 5 = 4-5 mm, 6 = 5-7 mm, 7 = 7-10 mm, and 8 = open flower. EtBr, ethidium bromide staining of the gel before blotting. (*b*) *In situ* hybridization. Anther sections derived from flower buds of stages 5 and 7 were hybridized with *SP11-8* and *BcPCP-A1* antisense riboprobes and their sense riboprobes (negative control).



Localization of the male determinant on pollen coat and in the tapetum cell layer:

Shiba et al. 2001

1. Applied a promoter – GUS fusion (GUS is a bacterial gene that produces blue color when suitable substrate is provided) technique to show that SP11 gene is transcribed in the tapetum cells and pollen grains (Figure 2 of Shiba et al. 2001).
2. Immunoelectron microscopy showed clearly that S8-SP11 protein is localized to the surface of the transgenic S52S60 line carrying the *S8-SP11* gene (Figure 6 of Shiba et al. 2001).
3. Conclusion: SP11/SCR is produced in both tapetum cells and pollen grains and is localized in the pollen coat. Therefore, sporophytic determination of the male determinant is possible.

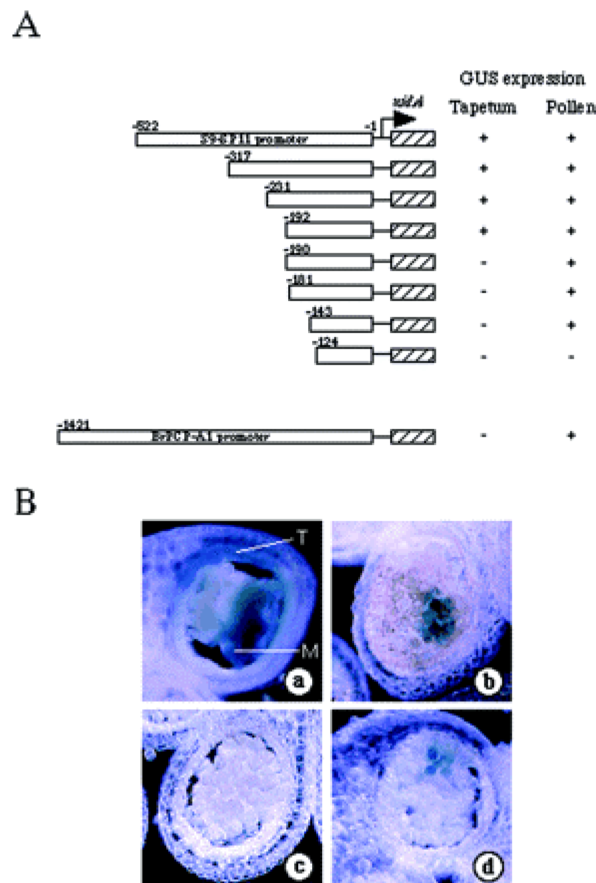


Figure 2. Deletion analyses of the *SP11* promoter region. A, *SP11* promoter deletion constructs and summary of GUS staining results. Numbers denote the 5' most positions of the truncated promoters relative to the translation initiation codon (ATG) of the S_9-*SP11* gene. The GUS expression of each promoter construct in the tapetum and pollen is represented by + (positive) or − (negative). B, Representative GUS staining results of

transient promoter-*gus* fusion analyses. Cross-sections of anthers that had been bombarded with 317-bp *SP11* promoter-*gus* (a), 143-bp *SP11* promoter-*gus* (b), 124-bp *SP11* promoter-*gus* (c), and 1,421-bp *BrPCP-A1* promoter-*gus* (d). T and M represent tapetum and microspore, respectively.

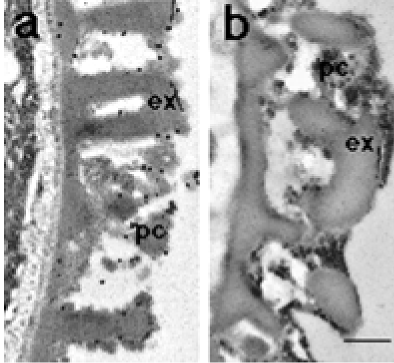


Figure 6. Immunoelectron microscopy of pollen grains. Immunogold localization of pollen grains treated with the anti-S_8-SP11 antibody and 20-nm gold-conjugated anti-rabbit IgG. a, T66 line ($S_{52}S_{60}$/ S_8-*SP11*); b, wild-type plant. ex, Exine; pc, pollen coating. Scale bar = 1 µm.

Interaction of the male and female determinants in the development of the self-incompatibility

(Kachroo et al. 2001; Takayama et al 2001)

Recent work in the Nasrallah and Isogai labs revealed evidence suggesting direct interaction between the male and female determinants of self-incompatibility.

Kachroo et al. 2001

1. Nasrallah group studied the SRK and SCR interaction by generating recombinant tagged proteins. Each protein was tagged with a known peptide, for which antibody is commercially available.
2. Only ectodomain of SRK6 (eSRK6) was tagged with FLAG epitop tag and SCRs of the S6 and S13 haplotypes were tagged with myc-His6 tag.
3. Recombinant SCRs were biologically active (pollen growth bioassay).
4. SRK and SCR interact *in vitro*. “Pull down assay”: eSRK6 was immobilized on FLAG-affinity agarose and SCR6-myc-His6 and SCR13-myc-His6 were hybridized in separate experiments. SCR6 bound strongly to eSRK6, whereas SCR13 bound poorly to eSRK6.
5. eSRK6 also bound to endogenous SCR6 homodimer protein.
6. Reverse experiments: eSRK6 bound to tagged SCR6. Again immobilized tagged SCR6 bound to endogenous SRK6 not SRK13. Likewise SCR13 immobilized on Ni-agarose bound to endogenous SRK13 but not SRK6.
7. SLG bound poorly to SCR6.

8. Conclusion: *In vitro* SRK and SCR interact directly, without the need of an additional factor.

Takayama et al. 2001

1. SP11 (same as SCR) was isolated by the Japanese group (Takayama et al. 2001) and they apply a little different rout to come to the same conclusion. They, however, provide additional evidence suggesting that SRK and SLG together form a high affinity receptor complex for the male determinant S8-SP11.
2. Prepared the anti-S8-Sp11 antibody and use it in isolating S8-SP11 protein. Protein was identified (based on the analysis of the antibody bound proteins in MALDI-TOF-Mass Spectrometry) from S8 haplotype but not from S9 haplotype.
3. Chemically synthesized the S8-SP11 protein and subjected to mild oxidation. The oxidized form had all 8 cyteine residues involved in intermolecular disulfide bonding (Figure 1b). The MS spectrum of this molecule was similar to one obtained from the native molecule (Figure 1a).
4. Also oxidized a recombinant S9-SP11 molecule.
5. Both SP11 molecules inhibited the penetration of compatible pollen tubes in an S-haplotype-specific and dose dependent manner (Figure 1c).
6. Synthesized S8-SP11 that was labeled with ^{125}I or non-radioactive iodine.
7. ^{125}I S8-SP11 specifically bound to microsome prepared from stigma of S8 homozygotes not to that of S9 homozygotes.
8. Cross linking study: to identify the receptor complex ^{125}I S8-SP11 protein was cross linked to two protein band specifically to S8 haplotype microsomes not S9 microsomes. Cross-linking was diminished in presence of S8-P11 protein (Figure 4 a and b).
9. The cross linked protein was purified and shown that those two molecules were composed of SRK8 and SLG8 (Figure 4 c).
10. Triton X-100 (mild detergent, non-ionic) instead of SDS (strong detergent) and use of the SRK-C antibody (C-terminal specific, cannot cross react with SLG) pulled down both SRK and SLG indicating close association between SRK and SLG (Figure 4 d).
11. Soluble SLG addition did not change the cross-linking pattern confirming that soluble form has no affinity to SRK (Figure 4 e).
12. Addition of S8-SP11 not S9-SP11 to microsomal preparation from stigma resulted autophosphorylation of SRK8 (Figure 4 f).
13. Conclusion: SP11 bind to SRK and SLG directly participates with SRK in forming the high affinity receptor complex. Following binding SP11 induces the autophosphorylation of SRK and self-incompatibility response in the papilla cells.

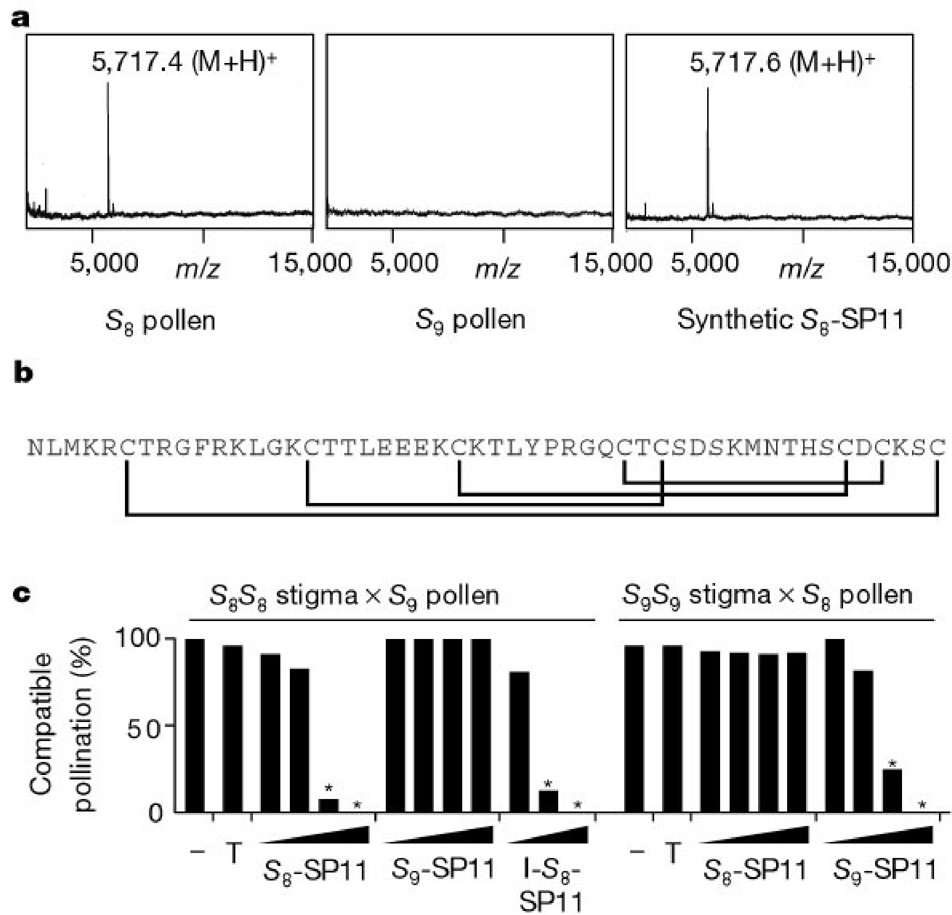


Figure 1 Molecular characterization of S_8-SP11. **a**, MALDI-TOF-MS spectra of proteins immunoprecipitated from pollen extracts of S_8 and S_9 haplotypes, and synthetic S_8-SP11. **b**, Primary structure of S_8-SP11. The four intramolecular disulphide linkages are indicated by connecting lines. **c**, Pollination bioassay for SP11 proteins. Effect of pretreatments of stigma with S_8-SP11 or S_9-SP11 (each at 0.5, 5, 50 or 500 fmol per stigma) or non-radioactive iodinated S_8-SP11 (I-S_8-SP11, at 5, 50 or 500 fmol per stigma). -, no treatment; T, 0.05% Tween-20-treated control. Data represent the percentage of compatible pollination (n = 11–28 experiments; asterisk, $P < 0.05$ with Fisher's exact probability test versus the Tween-20-treated control).

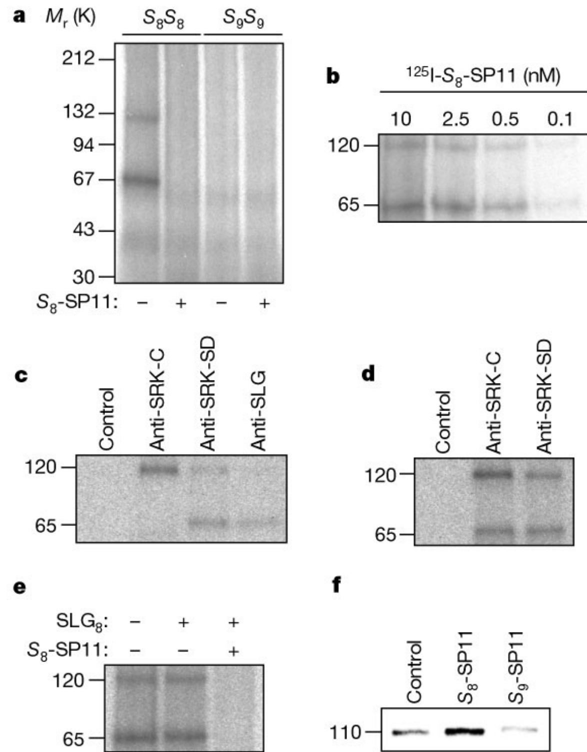


Figure 4 Interaction of S_8-SP11 with SRK_8–SLG_8 receptor complex. **a**, Chemical crosslinking of ^{125}I-labelled S_8-SP11 to stigma microsomal membranes of S_8 and S_9 homozygotes. Microsomal membranes were subjected to crosslinking in a solution containing ^{125}I-labelled S_8-SP11 (10 nM) in the absence or presence of S_8-SP11 (1 μM) as competitor. **b**, Crosslinking patterns at various concentrations of ^{125}I-labelled S_8-SP11. **c**, Immunoprecipitation of crosslinked microsomal proteins that had been solubilized with SDS. IgG fraction of pre-immune serum (Control), a polyclonal peptide antibody against the SRK_8 C terminus (Anti-SRK-C), a polyclonal antibody against the SRK_8 *S* domain (Anti-SRK-SD), and a monoclonal antibody for SLG (Anti-SLG) were used for immunoprecipitation. **d**, Immunoprecipitation of crosslinked microsomal proteins that had been solubilized with Triton X-100. **e**, Crosslinking in the absence or the presence of 100 nM of SLG_8 and S_8-SP11. **f**, Induction of autophosphorylation of SRK_8 by S_8-SP11 and S_9-SP11 (both at 10 nM).

Manifestation of dominance and recessiveness in sporophytic self-incompatibility

A. Male determinants (SP11):

Shiba et al. (2002) recently have shown that the dominance/recessive relationship in the male determinant *SP11* is manifested at the RNA level.

1. Earlier male determinant *SP11/SCR* was cloned from both *B. rapa* and *B. oleracea*. It was cloned from many class I type haplotypes (pollen dominant).

2. Shiba et al. (2002) reported the cloning of *SP11* from pollen-recessive haplotype (clas II).
3. In this paper they have shown that the transcripts of class II type SP11s are suppressed by dominant SP11 alleles in heterozygotes (Figure 6 of Shiba et al. 2002). Expressed only at an early stage of anther development when tapetum cells are intact. In class I type SP11 the expression continued in tapetum cells for later stages also, and especially in the pollen grains.
4. In situ hybridization analysis indicated clearly that the expression class II type of SP11 only expressed in an early developmental stage (stage 5) (Figure 7 of Shiba et al. 2002). In heterozygous condition the transcripts of the class II type SP11 allele was not detectable (Fig 7 E and F). Only transcripts of the dominant allele (class I type) was detected in the heterzygotes (Fig. 7 G and H). Note the gametophytic expression (pollen grains) of the dominant allele.
5. Mechanism is not known. But it is clear that the expression patterns are distinct between the two classes; with more active expression for the class I type alleles.

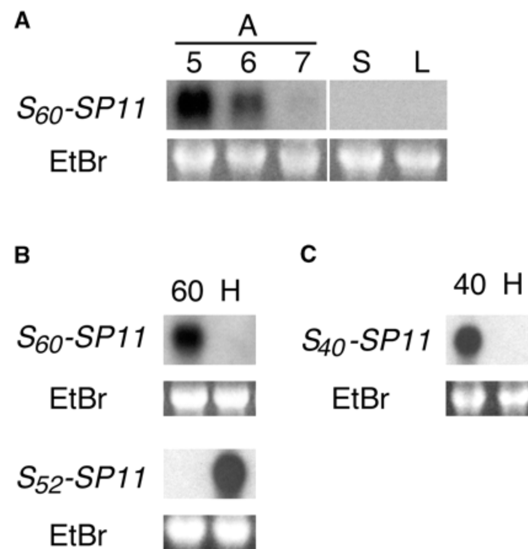


Figure 6. RNA Gel Blot Analyses of the Class II *SP11*s.

(A) RNA gel blot analysis of S_{60}-*SP11* in an $S_{60}S_{60}$-homozygote of *B. rapa*. The total RNAs of anther (A), stigma (S), and leaf (L) were used. Numbers represent developmental stages of anther classified by bud sizes, where 5 = 4 to 5 mm, 6 = 5 to 7 mm, and 7 = 7 to 10 mm in length (Takayama et al., 2000a).

(B) RNA gel blot analysis of S_{60}-*SP11* and S_{52}-*SP11* in an $S_{60}S_{60}$-homozygote (60) and an $S_{52}S_{60}$-heterozygote (H) of *B. rapa*. The same amount of total RNA of anthers (mixture or stages 5 to 7) was loaded in each lane. Similar results were obtained on three independent $S_{60}S_{60}$-homozygotes and $S_{52}S_{60}$-heterozygotes, and the results of a representative experiment are shown.

(C) RNA gel blot analysis of S_{40}-*SP11* in an $S_{40}S_{40}$-homozygote (40) and an $S_{35}S_{40}$-heterozygote (H) of *B. rapa*. The same amount of total RNA of anthers (stages 5 to 7, mixture) was loaded in each lane. The blot shown is representative of two independent experiments.

The bottom gel of each blot shows ethidium bromide (EtBr)–stained rRNA bands.

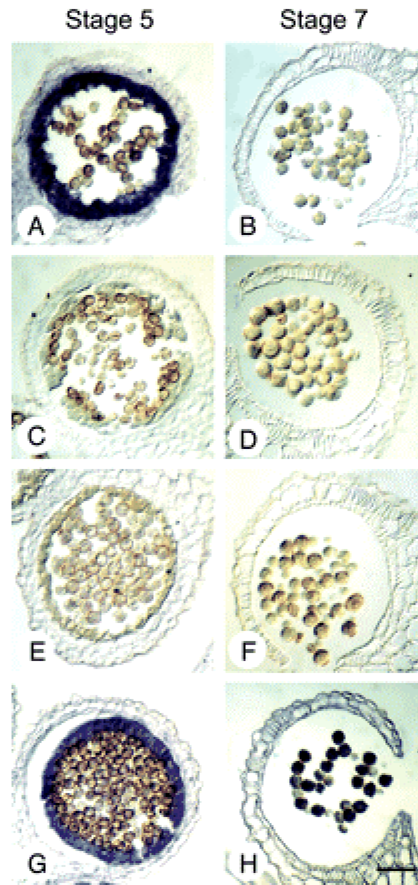


Figure 7. Analysis of S_{60}- and S_{52}-*SP11* Expression in Anther Using in Situ Hybridization. Anther sections (stages 5 and 7) of an $S_{60}S_{60}$-homozygote or an $S_{52}S_{60}$-heterozygote were hybridized with S_{60}-*SP11* or S_{52}-*SP11* antisense riboprobes or their sense riboprobes.

(A) and **(B)** $S_{60}S_{60}$-homozygote hybridized with an antisense S_{60}-*SP11* probe.
(C) and **(D)** $S_{60}S_{60}$-homozygote hybridized with a sense S_{60}-*SP11* probe.
(E) and **(F)** $S_{52}S_{60}$-heterozygote hybridized with an antisense S_{60}-*SP11* probe.
(G) and **(H)** $S_{52}S_{60}$-heterozygote hybridized with an antisense S_{52}-*SP11* probe.
Bar in **(H)** = 50 µm for **(A)** to **(H)**.

B. Female determinants (SRK):

Hatakeyama et al. 2001:

1. Transgene SRK^{28} was analyzed in different haplotypes carrying different SRK genes. They observed that the transgene SRK^{28}affected only the stigma but not pollen phenotype.
2. Studied the possible mechanism of dominance relationship in the expression of the female determinant. S28 is dominant over S43. The amount of transgene transcript S28 is much less in transgenic plants carrying S43S43 haplotype.

(Figure 3 a of Hatakeyama et al. 2001). But transcripts of S43 is not reduced in presence of the transgene S28 (Fig 3 b) as we did observe for SP11 (pollen determinant).

3. These transgenic plants were self compatible because of the dominance of S28 over S43.
4. Transgenic plants (S52S52) carrying the transgene S28 were self incompatible, because of the co-dominance relationship between S28 and S52.
5. Transcripts are not important in determining the dominance relationship of the female determinant.
6. Most likely dominance relationship is determined at the protein level.

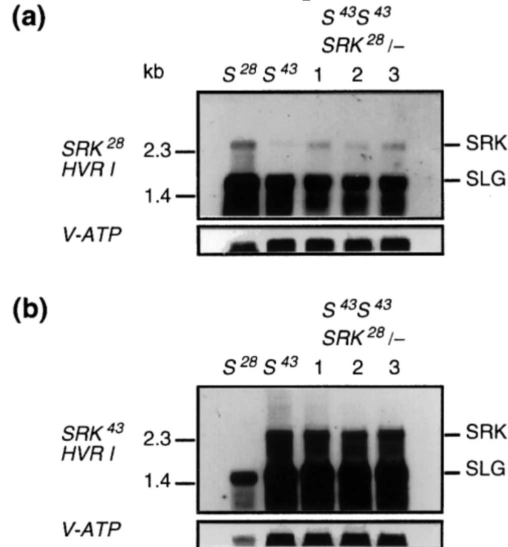


Figure 3. RNA gel-blot analysis of S^{43} homozygous plants carrying the SRK^{28} transgene.

Poly(A)+ RNA isolated from an S^{28} homozygote (S^{28}), an S^{43} homozygote (S^{43}), and three individual S^{43} homozygotes carrying the SRK^{28} transgene (1, 2 and 3) was probed with HVRI of SRK^{28}(a) and SRK^{43}(b) , respectively. Both blots were reprobed with *V-ATP*.