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SRK, the Stigma-Specific S Locus Receptor Kinase of Brassica, Is Targeted to the Plasma Membrane in Transgenic Tobacco

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The *S* locus receptor kinase (*SRK*) gene is one of two *S* locus genes required for the self-incompatibility response in Brassica. We have identified the product of the SRK_6 gene in *B. oleracea* stigmas and have shown that it has characteristics of an integral membrane protein. When expressed in transgenic tobacco, SRK_6 is glycosylated and targeted to the plasma membrane. These results provide definitive biochemical evidence for the existence in plants of a plasma membrane–localized transmembrane protein kinase with a known cell–cell recognition function. The timing of *SRK* expression in stigmas follows a time course similar to that previously described for another *S* locus–linked gene, the *S* locus glycoprotein (*SLG*) gene, and correlates with the ability of stigmas to mount a self-incompatibility response. Based on SRK_6 promoter studies, the site of gene expression overlaps with that of *SLG* and exhibits predominant expression in the stigmatic papillar cells. Although reporter gene studies indicated that the *SRK* promoter was active in pollen, SRK protein was not detected in pollen, suggesting that SRK functions as a cell surface receptor exclusively in the papillar cells of the stigma.

INTRODUCTION

Many plant species with hermaphroditic flowers are able to limit self-fertilization by a mechanism called self-incompatibility (SI). In Brassica, the SI response represents the culmination of cell-to-cell recognition and signal transduction events that occur soon after pollen makes contact with the papillar cells of the stigma surface. Specificity of this response is controlled by the *S* locus complex, a highly polymorphic cluster of genes that are organized into distinct haplotypes (Boyes and Nasrallah, 1993, 1995). In general, SI is elicited when parents of a cross carry an identical *S* locus haplotype, resulting in the disruption of pollen germination and tube growth at the stigma surface. Depending on their combination in heterozygotes, *S* haplotypes may be codominant or show dominant/recessive interactions. For example, class I haplotypes, such as S_6, confer a strong SI reaction and are dominant to the weaker class II haplotypes, such as S_2 (Nasrallah et al., 1991).

At least some of the genes within the *S* locus complex are thought to encode recognition molecules that act at the pollen–papillar cell interface. Among these are the *S* locus glycoprotein (*SLG*) gene and the *S* receptor kinase (*SRK*) gene, two stigma-expressed genes that are required for phenotypic expression of SI (Toriyama et al., 1991a; Nasrallah et al., 1992, 1994a; Goring et al., 1993). *SLG* encodes an abundant glycoprotein that accumulates in the cell wall of papillar cells (Nasrallah et al., 1985b; Kandasamy et al., 1989). Based on its sequence, we had predicted *SRK* to encode a plasma membrane receptor protein kinase having an extracellular domain that is highly similar to SLG, a single pass transmembrane domain, and a cytoplasmic kinase domain (Stein, et al., 1991). When expressed in *Escherichia coli*, the kinase domain is capable of autophosphorylating on serine and threonine residues (Goring and Rothstein, 1992; Stein and Nasrallah, 1993).

Both SLG and SRK have the potential to function as recognition molecules by virtue of their extensive polymorphism (Nasrallah et al., 1987; Stein et al., 1991). Greatest amino acid divergence, up to 33%, is observed between alleles derived from class I and class II haplotypes (Chen and Nasrallah, 1990; Stein et al., 1991), and this divergence has allowed the development of class I–specific antibodies to SLG (Kandasamy et al., 1989). Of great interest is the finding that despite their extensive divergence between haplotypes, SLG and the SLG-like domain of SRK share a high degree of sequence identity within a given haplotype, showing as little as 10% divergence (Stein et al., 1991; Goring and Rothstein, 1992; Delorme et al., 1995). Because of this apparent concerted evolution of the *SLG/SRK* gene pair within each haplotype, we had suggested that the protein products of these genes interact functionally, perhaps as components of a receptor system involving binding of a common ligand (Nasrallah et al., 1994b). The validity of such a

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hypothesis rests in part on whether SRK is in fact localized to the plasma membrane and is expressed within the same cells as SLG.

In this study, we focus primarily on *B. oleracea* SRK_6, the *SRK* allele contained within the S_6 haplotype (a class I haplotype) that was previously cloned and sequenced (Stein et al., 1991). We show that stigmatic SRK_6 is an integral membrane protein, consistent with sequence-based predictions. Moreover, when expressed in transgenic tobacco leaves, SRK_6 is targeted to the plasma membrane. We also show by RNA and protein blot analyses that *SRK* and *SLG* are coordinately regulated genes, displaying similar temporal patterns of expression. The *SRK* promoter contains functional elements similar to that of *SLG* and drives reporter gene expression in papillar cells, where *SLG* is also known to be expressed. Finally, despite evidence that both of these genes are transcribed at low levels within male reproductive organs (Sato et al., 1991; Stein et al., 1991; Goring and Rothstein, 1992), we found no evidence of SLG or SRK proteins in pollen grains. These findings support the hypothesis that SRK functions as a plasma membrane–localized receptor and, along with SLG, acts principally as a female determinant of SI recognition.

RESULTS

Identification of SRK in Stigmas of Brassica

Previously, we raised monoclonal antibody MAb/H8 against purified SLG_6 (Kandasamy et al., 1989). Because the SLG-like domain of SRK_6 has 89% amino acid sequence identity with SLG_6 (Stein et al., 1991), we hypothesized that MAb/H8 would also recognize SRK_6 and possibly other class I SRK proteins as well. To test this hypothesis, we expressed the SLG-like domain of SRK_6 as a glutathione *S*-transferase (GST) fusion protein in *E. coli* and subjected the affinity-purified protein to immunoblotting. In Figure 1A, the protein blot stained with Coomassie blue shows that a GST–SRK_6 fusion protein of the expected size was produced and that this band was absent in *E. coli* harboring the parental pGEX vector. In the accompanying blot subjected to immunostaining, MAb/H8 detected the GST–SRK_6 fusion protein but did not detect GST in the negative control lane (Figure 1A). In addition to the full-length fusion protein, several smaller immunoreactive proteins were also detected with MAb/H8 (Figure 1A). These bands, which are absent from control extracts that lack GST–SRK_6 and therefore do not represent endogenous bacterial proteins, are proteolytic derivatives of the GST–SRK_6 fusion protein, resulting from the commonly observed instability of recombinant proteins in *E. coli*. The cross-reactivity of the GST–SRK_6 fusion protein with MAb/H8 indicates that MAb/H8 does indeed bind to SRK_6 and establishes the utility of this antibody in studies of SRK.

To study the native SRK protein in Brassica stigmas, we used F_2 populations segregating for the S_6 haplotype. One such population was derived from a cross between an S_6S_6 homozygote and an S_2S_2 homozygote. Among 32 plants in this population, eight were S_6S_6 homozygotes, 18 were S_2S_6 heterozygotes, and six were S_2S_2 homozygotes, as determined by pollination tests and DNA gel blot analysis (see Methods).

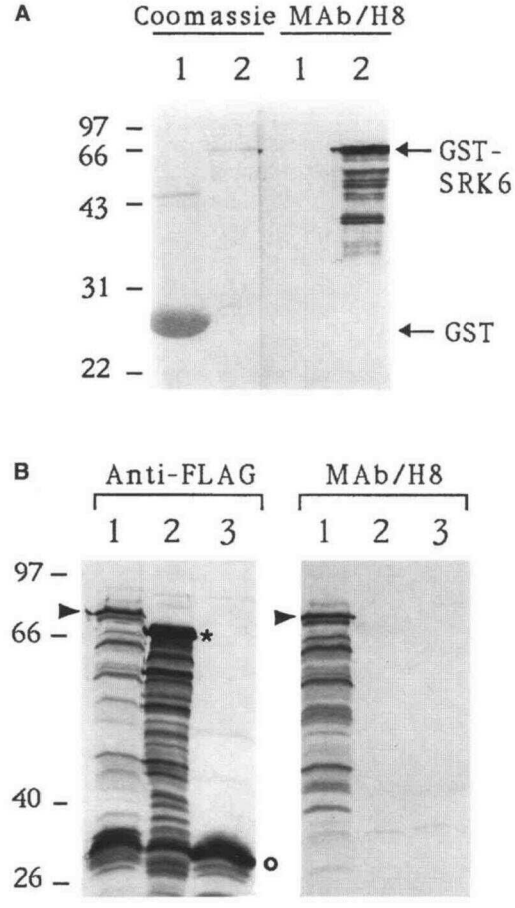

Figure 1. Immunodetection of the SLG-like Domain of SRK_6 and SRK_2.

(A) GST (lanes 1) and a GST–SRK_6 fusion protein (lanes 2) expressed in bacteria were affinity purified with glutathione–agarose beads. Proteins were fractionated by SDS-PAGE, blotted onto PVDF membrane, either stained with Coomassie blue or probed with MAb/H8, and developed using chromogenic substrates.

(B) Total extracts of bacteria expressing GST–FLAG–SRK_6 (lanes 1), GST–FLAG–SRK_2 (lanes 2), or GST–FLAG (lanes 3) were subjected to SDS-PAGE, blotted onto PVDF membrane, probed with anti-FLAG antibody (see Methods) or MAb/H8, and developed using chemiluminescent substrates. The bands corresponding to the full-length fusion proteins are indicated by an arrowhead for SRK_6 and an asterisk for SRK_2. The GST–FLAG protein encoded by the modified pGEX vector is indicated by an open circle.

The immunoreactive bands that migrate faster than the full-length SRK fusion proteins are breakdown products. Molecular mass standards shown at left are given in kilodaltons.

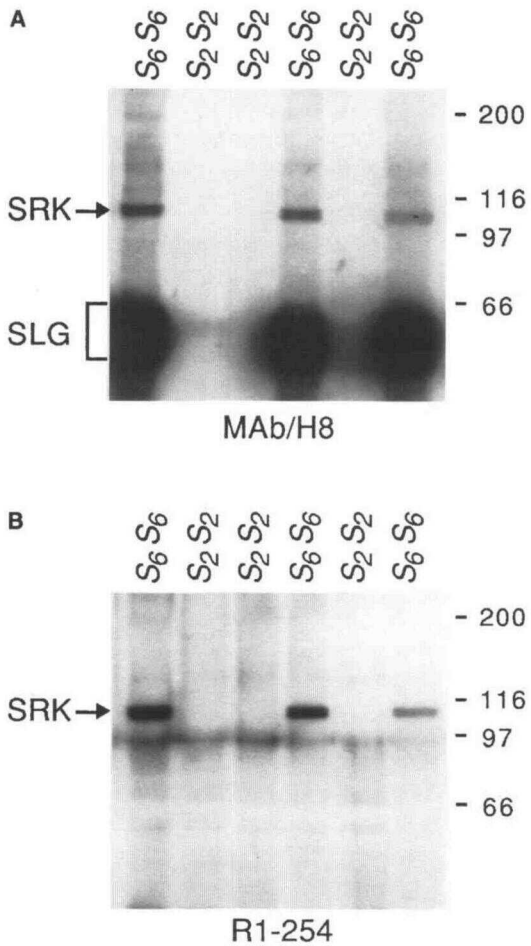

Figure 2. Detection of Stigmatic SRK_6 in Brassica with Two Antibodies and Demonstration of Linkage to the *S* Locus Complex.

Stigma microsomal membrane proteins (20 μg per lane) were prepared from individuals in an F_2 population segregating for the S_6 and S_2 haplotypes. The *S* locus genotype of each plant is indicated above each lane.

(A) Immunoblot probed with MAb/H8.

(B) The same blot was stripped and reprobed with affinity-purified R1-254, a polyclonal antiserum raised against a peptide from the C-terminal domain of SRK_6.

The blots in **(A)** and **(B)** were developed with chemiluminescent substrates. SRK_6 is detected by both antibodies as a band of ~108 kD. The band of ~94 kD evident in the blot probed with the R1-254 rabbit antiserum was detected in all lanes, regardless of genotype, and is attributed to nonspecific reactivity. Numbers at right indicate molecular mass markers in kilodaltons.

S_2 is a class II haplotype whose *SRK* and *SLG* alleles are highly diverged from SRK_6 and SLG_6 (Stein et al., 1991), and MAb/H8 cross-reacts with neither SLG_2, as previously shown (Kandasamy et al., 1989), nor the S domain of SRK_2, as demonstrated by immunological analysis of the GST–SRK_2 fusion protein (Figure 1B). The S_2 haplotype therefore served as a negative control in these studies.

Figure 2A shows an immunoblot of stigma proteins extracted from six representative F_2 individuals. SRK_6 was identified tentatively as an immunoreactive band of 108 kD, a size consistent with a predicted SRK_6 polypeptide of 92 kD having multiple N-linked glycosylation sites (Stein et al., 1991). In addition to this 108-kD band, MAb/H8 detected multiple bands of 55 to 65 kD (Figure 2A) previously identified as SLG_6 glycoforms (Umbach et al., 1990). Because SLG is highly abundant in stigmas, representing up to 5% of the total synthesized protein (Nasrallah et al., 1985a), its detection by the chemiluminescence method used here resulted in a highly intense and diffuse signal. The additional minor immunoreactive bands that migrated more slowly than did the 116-kD molecular mass marker (Figure 2A) appear to be artifacts and probably resulted from oxidative cross-linking of SLG during tissue homogenization. Their detection was increased when antioxidants and polyvinylpyrrolidone were excluded from the homogenization buffer (data not shown).

By testing the entire F_2 population, the eight S_6S_6 and 18 S_6S_2 plants were found to exhibit identical immunoblot patterns in which both the 108-kD protein and the 55- to 65-kD SLG_6 glycoforms were evident. In contrast, all six S_2S_2 individuals lacked the 108-kD and 55- to 65-kD immunoreactive proteins. This perfect cosegregation of the 108-kD protein with the S_6 haplotype and with SLG_6 demonstrates linkage to the *S* locus complex.

To confirm the identity of the 108-kD band as SRK_6, we tested whether this protein binds to R1-254, an antiserum raised against a peptide sequence within the C-terminal domain of SRK_6 and previously shown to recognize a bacterially expressed SRK_6 fusion protein containing only the kinase and C-terminal domains (Stein and Nasrallah, 1993). The peptide sequence used to raise antiserum R1-254 intentionally was chosen to be specific to SRK_6, having <36% sequence identity with SRK_2 (Stein et al., 1991). The blot in Figure 2A was stripped and reacted with affinity-purified R1-254 antibodies. The results, shown in Figure 2B, demonstrate that R1-254 recognized a 108-kD protein identical to the one detected with MAb/H8. This protein was detected in all plants bearing the S_6 haplotype but was absent in the S_2S_2 individuals (Figure 2B).

Cosegregation of the 108-kD protein with the S_6 haplotype was also demonstrated in a second F_2 population segregating for the S_6 and S_{f1} haplotypes. The S_{f1} haplotype, which specifies a self-fertile phenotype in S_{f1} homozygotes, is a nonfunctional haplotype that carries a null allele of *SRK* in which the promoter and a sizable portion of the coding sequence are deleted (Nasrallah et al., 1994a). S_{f1} does, however, possess an intact and expressed allele of *SLG*, which is classified as a class I allele (Nasrallah et al., 1994a). We examined 18 individuals in an F_2 population derived from crossing an S_6S_6 parent with an $S_{f1}S_{f1}$ parent. In this population, 14 individuals (six S_6S_6 homozygotes and eight S_6S_{f1} heterozygotes) were self-incompatible, whereas four individuals exhibited a

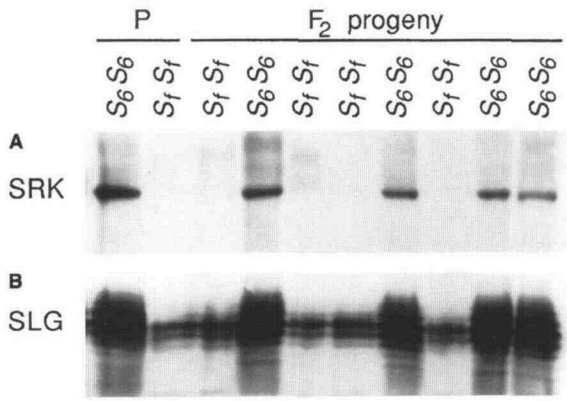

Figure 3. Absence of SRK in a Self-Fertile Mutant.

Stigma microsomal membrane proteins (20 μg per lane) were prepared from F_2 progeny that were segregating for the S_6 haplotype and the self-fertile S_{f1} haplotype, which carries a null *SRK* allele. The homozygous parental lines from which the cross was derived are shown in the two leftmost lanes. The immunoblot was probed with MAb/H8 and then cut in half.

(A) Detection of SRK. The upper half of the blot was developed with chemiluminescent substrates.

(B) Detection of SLG. The lower half was developed with chromogenic substrates.

self-fertile phenotype and were homozygous for S_{f1} (see Methods).

Figure 3 shows the results obtained when stigma extracts from the F_2 plants were subjected to immunoblot analysis with MAb/H8. SLG glycoforms were detected in all F_2 plants irrespective of their genotype. The SLG signal was most intense in S_6S_6 homozygotes and least intense in $S_{f1}S_{f1}$ homozygotes (Figure 3B). S_6S_{f1} heterozygotes (data not shown) produced an SLG pattern identical to that of S_6S_6 homozygotes, because SLG_{f1} migrates to a position in the gel similar to that of SLG_6 and is masked by the more intense SLG_6 signal. The reduced intensity of the SLG_{f1} signal relative to SLG_6 was not due to uneven loading of the gel, because the amounts of total protein in each lane were equalized. Rather, the reduced intensity must reflect either an actual lower level of SLG in S_{f1} stigmas or a lower avidity of MAb/H8 for SLG_{f1}. In any event, no immunoreactive band of 108 kD or similar size was detected in stigma extracts of the four self-fertile $S_{f1}S_{f1}$ F_2 plants, even when the protein blots were developed with chemiluminescent substrates (Figure 3A) and subjected to prolonged exposures to x-ray film. However, a 108-kD protein was identified in all of the remaining 14 plants, which were self-incompatible and carried the S_6 haplotype (Figure 3A).

Because plants homozygous for the S_{f1} haplotype can produce SLG but not SRK, this result serves not only to confirm the identity of the 108-kD protein as SRK_6, but also to demonstrate that this MAb/H8 cross-reactive protein is not an alternative product of the *SLG* gene. Based on the above findings showing genetic linkage of the 108-kD protein to the *S* locus complex, its cross-reactivity with antibodies directed against both the SLG-like domain and C-terminal domain, and the absence of a similar protein in a strain carrying a null *SRK* allele, we conclude that the 108-kD protein is the product of the SRK_6 gene.

Stigmatic SRK Is an Integral Membrane Protein

Based on the sequence of several different alleles, *SRK* is predicted to encode a single-pass transmembrane protein that is targeted to the plasma membrane (Stein et al., 1991; Goring and Rothstein, 1992). Recently, Delorme et al. (1995) also described this structure for the SRK_3 allele and showed that extraction of SRK_3 protein from stigmas required the presence of a detergent, suggesting that SRK is associated with a membrane component of the cell. However, the nature of this membrane association and the identity of the membrane component involved were not defined.

As a first step in our characterization of SRK_6, we prepared stigma microsomal membranes by differential centrifugation and compared this fraction with the soluble protein fraction by immunoblotting with MAb/H8. As shown in Figure 4, SRK_6 was detected in the microsome fraction (lane 2) but was ab-

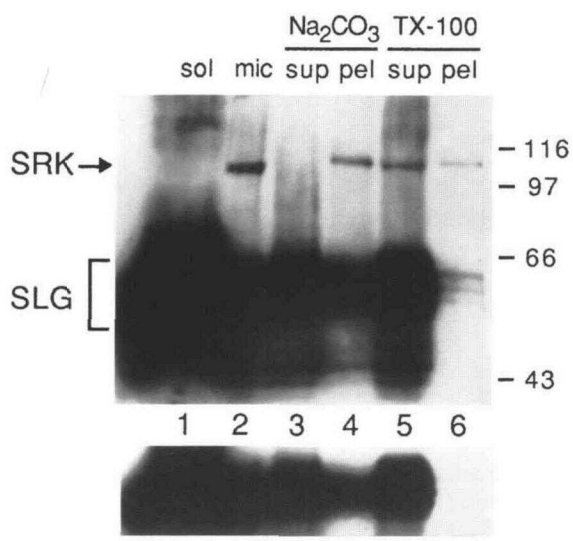

Figure 4. Membrane Association of SRK_6 in Brassica Stigmas.

Stigmas homozygous for the S_6 haplotype were fractionated into soluble (sol; lane 1) and microsomal (mic; lane 2) membrane fractions. Microsomal membranes were treated with either Na_2CO_3 or Triton X-100 and centrifuged to give a Na_2CO_3-soluble supernatant (sup; lane 3), Na_2CO_3-insoluble pellet (pel; lane 4), and Triton X-100–soluble (lane 5) and Triton X-100–insoluble (lane 6) fractions. Equal proportions based on volume were loaded in each lane. The blot was probed with MAb/H8 and developed by chemiluminescence. At bottom, a shorter exposure of the SLG-containing section of the blot is shown. Molecular mass markers are indicated at right in kilodaltons.

sent from the soluble protein fraction (lane 1), consistent with the prediction that SRK is bound to the membrane.

To test the nature of this interaction, we compared the ability of sodium carbonate and detergent treatments to solubilize membrane-bound SRK_6. Sodium carbonate has been shown to convert closed membrane vesicles into open membrane sheets, thereby releasing proteins held in the lumen (Fujiki et al., 1982). Sodium carbonate also strips away peripheral membrane proteins while leaving integral membrane proteins embedded in the membrane. On the other hand, detergents generally are required to solubilize integral membrane proteins but also will solubilize peripheral membrane proteins indirectly. In this study, proteins were deemed soluble if they remained in the supernatant after treated membranes were centrifuged at 100,000*g* or greater for 1 hr (Penefsky and Tzagoloff, 1971).

When microsomal membranes were washed with sodium carbonate, virtually all of the SRK_6 protein remained associated with the pelleted membranes (Figure 4, lanes 3 and 4). However, almost complete solubilization of SRK_6 was achieved by treating the microsomal membranes with Triton X-100 (Figure 4, lanes 5 and 6). These solubility characteristics are consistent with SRK being an integral rather than peripheral membrane protein and contrast markedly with the solubility characteristics of SLG. Most of SLG_6 was detected in the soluble fraction (Figure 4, lane 1), as expected for this cell wall–localized secreted glycoprotein, but a significant amount of SLG_6 was detected also in the microsomal membrane fraction (Figure 4, lane 2). Unlike SRK_6, most of this membrane-associated SLG_6 was released into the supernatant after treatment with sodium carbonate (Figure 4, lanes 3 and 4).

SRK Encodes an Integral Plasma Membrane-Bound Glycoprotein

Further biochemical localization of SRK at the subcellular level was restricted by the limited quantities of stigmatic tissue available. To provide an abundant source of SRK protein, the SRK_6 gene was expressed in transgenic tobacco leaves by fusing its coding region to the cauliflower mosaic virus (CaMV) 35S promoter. Tobacco has been used frequently as a heterologous plant system for the study of protein targeting, production of correctly targeted proteins, and determination of the subcellular localization of proteins (reviewed in Frommer and Ninnemann, 1995). In addition, we had shown previously that Brassica SLG is properly glycosylated and targeted to the extracellular matrix in tobacco either by cells of the transmitting tract when expressed under the control of the SLG promoter (Kandasamy et al., 1990; Moore and Nasrallah, 1990) or by tobacco cell cultures when expressed under the control of the CaMV 35S promoter (Perl-Treves et al., 1993).

Forty-six independent transgenic lines were screened for the presence of *SRK* RNA, and those showing high relative levels of transcript were selected for further study. To test for the presence of the SRK protein, whole extracts of leaf proteins were analyzed by immunoblotting with MAb/H8. Figure 5A shows six independent transgenic lines, each of which expressed a protein that reacted with the antibody. This protein was not detected in untransformed leaf material or in material that was transformed with a binary vector lacking *SRK* coding sequences and was thus encoded by the SRK_6 transgene. As in Brassica stigmas, tobacco-expressed SRK_6 has an apparent molecular mass of 108 kD.

To determine the subcellular location of SRK_6 in transgenic tobacco, we purified plasma membranes away from intracellular membranes, using the aqueous two-phase partitioning method (Larsson, 1985). Membrane purity was confirmed by assaying for marker enzymes immunologically, as shown in Figure 5B, and biochemically, as shown in Table 1. The 100-kD plasma membrane H^+-ATPase was enriched in the plasma membrane fraction (Figure 5B), and this was reflected in a >4.5-fold enrichment of vanadate-sensitive ATPase activity (Table 1). Purity of this plasma membrane fraction was confirmed by the absence of markers for the tonoplast (Figure 5B), endoplasmic reticulum, Golgi apparatus, and mitochondria (Table 1). SRK_6 was assayed in soluble, crude microsome, intracellular membrane, and plasma membrane fractions by immunoblotting. As shown in Figure 5C, SRK_6 was associated with microsomal membranes and copurified with the plasma membrane; it was largely depleted from intracellular membranes and could not be detected in the lane containing soluble proteins.

Tobacco-expressed SRK_6 has solubility characteristics similar to the native stigmatic protein. As shown in Figure 5D, treatment of microsomal membranes containing SRK_6 (lane 1) with sodium carbonate resulted in little or no solubilization of SRK_6 (lane 2): all of the detectable SRK_6 remained bound to the pelleted membranes (lane 3). In contrast, treatment of membranes with RIPA, a buffer that contains a mixture of ionic and nonionic detergents (see Methods), resulted in the release of most of the bound SRK_6 (lane 4), although trace amounts of the protein still were detected in the pelleted insoluble material (lane 5). Thus, tobacco-expressed SRK_6 appears to be embedded in the membrane.

The SRK_6 sequence contains seven potential N-linked glycosylation sites within its SLG-like domain, several of which are conserved in the SLG_6 sequence (Stein et al., 1991). We tested whether tobacco-expressed SRK_6 is glycosylated by assessing its ability to bind the lectin concanavalin A (ConA). ConA–Sepharose beads were incubated with detergent-solubilized microsomal membranes containing SRK_6, and bound proteins were analyzed by immunoblotting. As shown in Figure 5D, SRK_6 was detected in the ConA-bound fraction (lane 6), consistent with the presence of glycan chains covalently linked to the SRK_6 polypeptide. These results agree with those of Delorme et al. (1995), who showed by different methods that SRK_3 is glycosylated.

Coordinate Regulation of SRK and SLG Expression

We and others have shown that transcription of *SRK* and *SLG* is confined to reproductive organs, predominantly the stigma

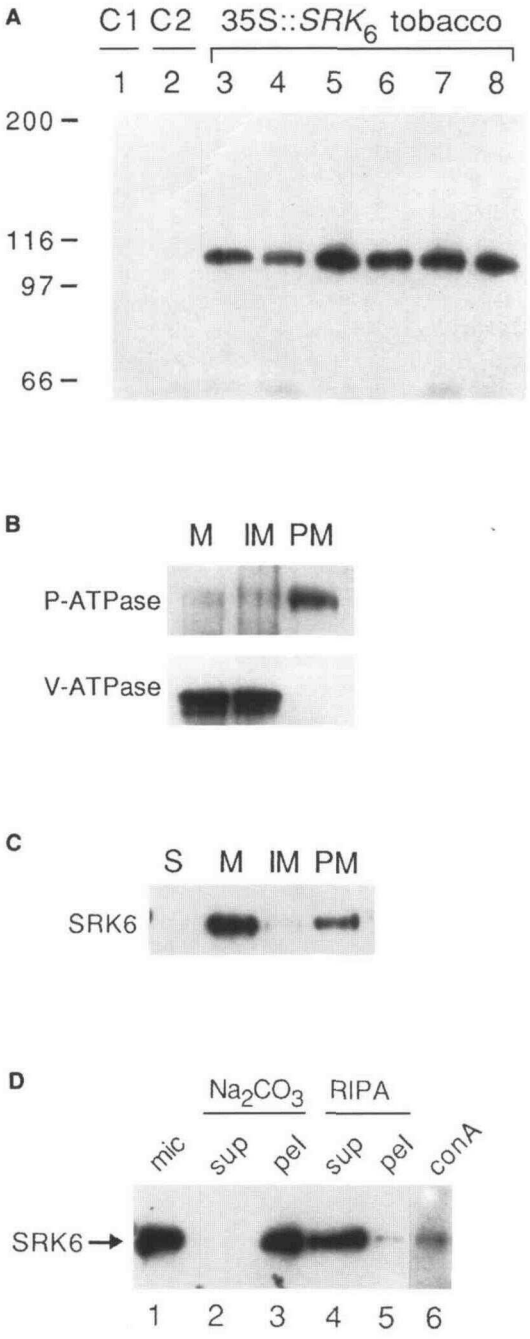

Figure 5. Tobacco-Expressed SRK_6 Is Bound to the Plasma Membrane.

(A) Tobacco proteins (100 μg/lane) from leaf whole-cell extracts were subjected to immunoblot analysis with MAb/H8 and chemiluminescence. Lanes 1 and 2 are negative controls that contain, respectively, protein extracts of untransformed plant material and transgenic plant material transformed with a binary vector that lacks *SRK* coding sequences. Lanes 3 to 8 contain extracts of six independent transgenic tobacco lines, each harboring the SRK_6 coding region under control of the CaMV 35S promoter. Molecular mass markers are indicated at left in kilodaltons.

Table 1. Marker Enzyme Analysis of Microsomes, and Upper and Lower Phases of Phase-Partitioned Membranes from CaMV 35S::SRK_6–Transgenic Tobacco Leaves

Marker (Organelle)[a]	Specific Activity[b] (μmol mg^{-1} min^{-1}) M	L	U	Enrichment (U/L)
VO_4-sensitive ATPase (PM)	0.074	0.046	0.214	4.65
NADH cyt-*c* reductase (ER)	0.063	0.054	0	0
UDPase (Golgi)	0.028	0.043	0.006	0.140
Cyt-*c* oxidase (Mt)	0.068	0.070	0.005	0.071

[a] PM, plasma membrane; cyt, cytochrome; ER, endoplasmic reticulum; Mt, mitochondria.

[b] M, total microsomes; L, lower phase containing intracellular membranes; U, upper phase enriched in plasma membrane.

and, to a lesser extent, the anther (Nasrallah et al., 1985b; Sato et al., 1991; Stein et al., 1991; Goring and Rothstein, 1992; Kandasamy et al., 1993). We investigated whether these two genes exhibit qualitatively similar spatial and temporal expression patterns. Evidence that *SRK* and *SLG* are subject to common transcriptional control mechanisms was found in a comparison of their promoter sequences. As shown in Figure 6, the alignment of two *SRK* alleles, SRK_6 and SRK_2, with the promoter sequence of SLG_{13} revealed a conserved region of 140 bp (*−86 to −227 of* SLG_{13}), in which 74% of the nucleotides were identical among the three sequences. Within this shared domain were located five sequence motifs, designated boxes I through V, which are highly conserved among *SLG* promoters and have been shown to function as *cis*-acting regulatory elements that direct pistil- and anther-specific expression (Dzelzkalns et al., 1993). These motifs also were identified recently in the SRK_3 allele (Delorme et al., 1995).

(B) and **(C)** Transgenic leaf material harboring the CaMV 35S::SRK_6 construct was fractionated into soluble (lane S in **[C]**) and microsomal membrane (lane M) fractions. Microsomal membranes were fractionated further by the aqueous two-phase partitioning method to give fractions enriched in intracellular membranes (lane IM) and plasma membranes (lane PM). Proteins (12 μg per lane) were immunoblotted and probed with antibodies raised against the indicated marker proteins **(B)** or with MAb/H8 **(C)** and then developed with chemiluminescent substrates. P-ATPase, plasma membrane H^+-ATPase; V-ATPase, vacuolar H^+-ATPase.

(D) Microsomal membranes prepared from transgenic tobacco were untreated (lane 1), washed with Na_2CO_3 (lanes 2 and 3), or washed with RIPA buffer (lanes 4 and 5). Membrane washes were centrifuged, giving soluble fractions in the supernatant (lanes 2 and 4) and insoluble fractions in the pellet (lanes 3 and 5). Equal proportions based on volume were loaded in each lane. Proteins in RIPA-solubilized microsomal membranes were bound to ConA–Sepharose beads (conA) and analyzed in lane 6. The blots were probed with MAb/H8 and developed by chemiluminescence. mic, microsomal fraction; sup, supernatant; pel, pellet.

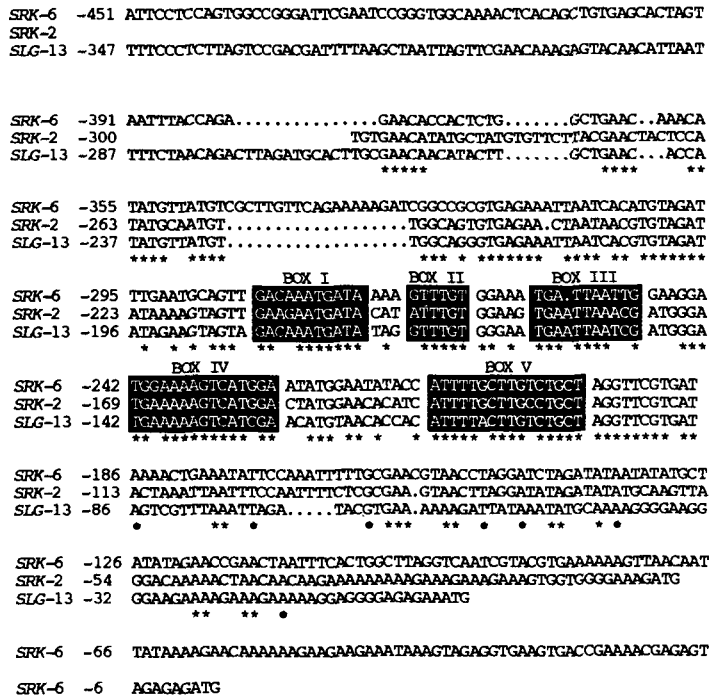

Figure 6. *SRK* Promoter Analysis.

The SLG_{13}, SRK_2, and SRK_6 promoter sequences were aligned using the program PILEUP. Final adjustments were made by eye. Dots represent gaps introduced to optimize the alignment. Asterisks indicate nucleotides that are conserved in all three sequences. Boxes I through V delineate highly conserved sequence motifs that were identified previously in a comparison of *SLG* and *SLR1* promoter sequences (Dzelzkalns et al., 1993). The sequences are numbered from the translation initiation codon of each gene.

To investigate the hypothesis that the expression patterns of *SRK* and *SLG* overlap spatially and temporally, we examined the expression of *SRK* in detail. First, we monitored *SRK* RNA in pistils collected from successive stages of maturation and compared this developmental profile with that observed for *SLG*. The maturation of Brassica and other cruciferous flowers may be defined in terms of the number of days until flower opening or anthesis (stage 0). Stigmas from young buds at 5, 4, 3, and 2 days before anthesis (stages −5, −4, −3, and −2) are self-fertile but become self-incompatible at 1 day before anthesis (stage −1). In the RNA blot hybridization shown in Figure 7A, *SRK* transcripts were detected first at the −3 stage, increased during stigma maturation and reached a maximum at the −1 stage, and declined in open flowers. This course of developmental regulation paralleled that observed for *SLG* (Figure 7B; Nasrallah et al., 1985b). In contrast to the dynamic patterns of expression displayed by *SRK* and *SLG*, actin transcripts varied little during stigma maturation (Figure 7C).

To investigate the temporal regulation of SRK protein, we prepared stigma microsomal membranes from successive stages of floral development and subjected them to immunoblot analysis with MAb/H8. Two strains from two different Brassica species were used for this study: a *B. oleracea* strain homozygous for the S_6 haplotype and a *B. campestris* strain homozygous for the S_8 haplotype. Figure 8A shows that SRK_6 protein levels are regulated tightly during flower maturation in *B. oleracea*. Consistent with the results of RNA gel blot analysis, SRK_6 protein was detected first at 3 days before flowering (stage −3), increased during maturation to maximal levels at stage −1, and declined in open flowers. The levels of SRK were regulated also during stigma maturation in *B. campestris*, as shown in Figure 8B. However, in contrast to SRK_6, the *B. campestris* SRK_8 protein was not detected until the −1 stage when it reached maximal levels, and relatively high levels of SRK_8 were maintained in open flowers.

These differences in the details of SRK regulation in the two strains likely are due to the fact that flower maturation in *B. campestris* is compressed into a shorter time frame relative to *B. oleracea*, in keeping with the overall smaller stature, more rapid development, and shorter life cycle of members of this species. An additional observation made in the course of this developmental analysis concerns the minor immunoreactive bands that migrate slower than SRK. These bands, which were noted earlier as being due to oxidative cross-linking of SLG, appeared to increase in intensity with stigma maturation and were evident particularly in mature *B. campestris* stigmas (stage 0; Figure 8B). This increase may be related to an increased level of SLG and of other factors that promote cross-linking upon flower maturation.

To identify the cells in which *SRK* is expressed, we monitored *SRK* promoter activity by using a reporter gene in transgenic plants. The construct, SRK_6::*uidA*, consisted of

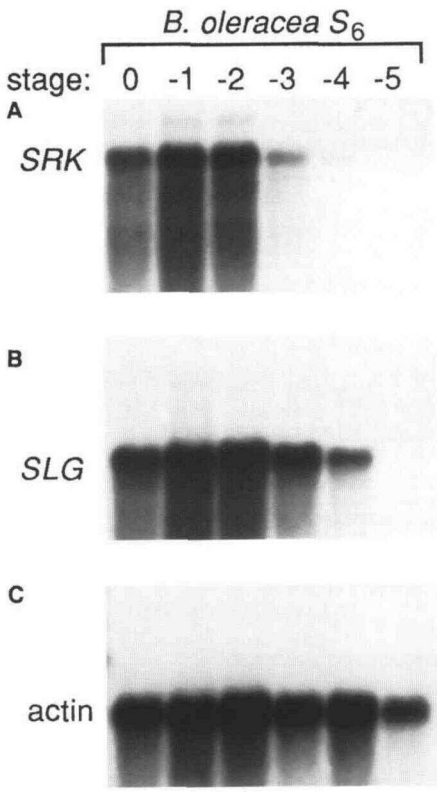

Figure 7. *Temporal Regulation of SLG and SRK RNA in Maturing Brassica Stigmas.*

Poly(A)$^+$ RNA (2 μg per lane) was isolated from developing stigmas of *B. oleracea* S_6 homozygotes. Flower stages ranging from −5 (5 days before flowering) to 0 (open flowers) are indicated above each lane. The blot was sequentially hybridized with ^{32}P-labeled probes that detect *SRK* (top panel; exposed 22 hr), *SLG* (middle panel; exposed 5 hr), and actin (bottom panel; exposed 20 hr) transcripts.

452 bp of the SRK_6 promoter (shown in Figure 6) fused to the *E. coli uidA* gene, which encodes β-glucuronidase (GUS). The chimeric gene was introduced into tobacco and Arabidopsis by Agrobacterium-mediated transformation. These heterologous hosts were chosen because they have previously served as model systems for studying the *SLG* promoter in similar studies (Thorsness et al., 1991; Toriyama et al., 1991b; Dzelzkalns et al., 1993).

Using the SRK_6::*uidA* construct, eight independent tobacco transformants were obtained, and their characterization is summarized in Table 2. Histochemical GUS assays of control untransformed plants and transgenic plants were performed, as described in Methods, on vegetative tissues and on floral tissues isolated from a variety of developmental stages. As shown in Figure 9, no GUS staining was observed in any tissues of nontransformed control plants, including stigmas (Figure 9A) and pollen (Figure 9C). All eight of the SRK_6::*uidA* tobacco transformants expressed GUS activity in the stigma (Table 2). Blue staining was observed throughout the stigma, including the papillar cells and subepidermal secretory cells (Figure 9A). In six out of eight transgenic lines, GUS activity was also detected in pollen (Figure 9B). In transgenic lines carrying a single integration of the transgene, the proportion of blue-staining pollen grains approached 50% (Table 2). In transformant 7, which was predicted to carry T-DNA integrations at two loci, 75% of the pollen grains stained blue.

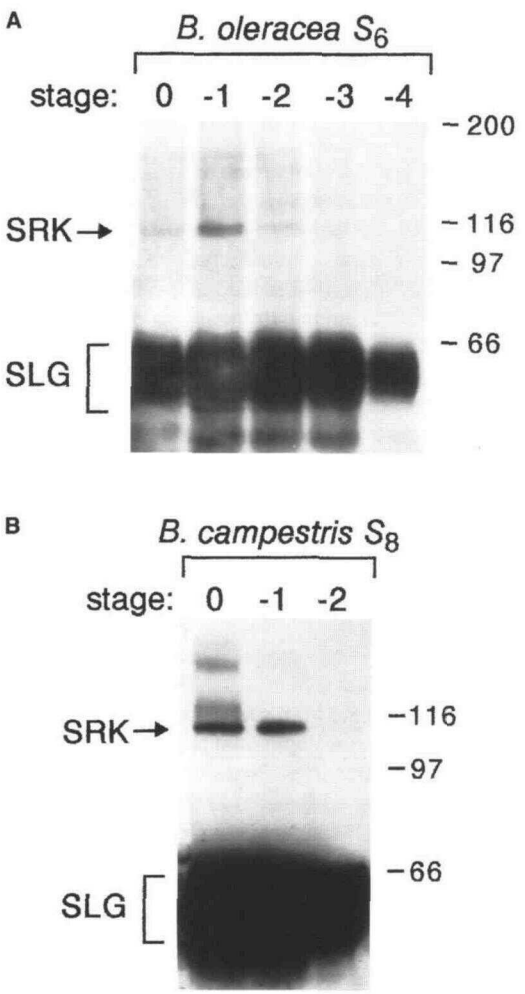

Figure 8. *Temporal Regulation of SRK Protein in Maturing Stigmas.*

(A) Stigma microsomal membranes (20 μg per lane) were prepared from *B. oleracea* S_6 homozygotes. Flower stages ranging from −4 (4 days before flowering) to 0 (open flowers) are indicated above each lane.
(B) Stigma microsomal membranes (20 μg per lane) were prepared from *B. campestris* S_8 homozygotes. Flower stages ranging from −2 (2 days before flowering) to 0 (open flowers) are indicated above each lane.
The blots were probed with MAb/H8 and developed with chemiluminescent substrates. Numbers at right indicate molecular mass markers in kilodaltons.

Table 2. Analysis of SRK_6::*uidA*–Transformed Tobacco

Plant No.	T_2 Plants (Km^r:Km^s)[a]	No. of Loci[b]	GUS Activity[c] Pistil	Pollen (%)
2	86:24	1	+	617/1358 (45)
3	240:78	1	+	– (0)
4	100:37	1	+	368/847 (43)
5	91:25	1	+	340/710 (45)
6	80:33	1	+	98/324 (30)
7	149:10	2	+	696/923 (75)
8	101:32	1	+	– (0)
10	157:61	1	+	196/472 (42)

[a] Km^r, kanamycin resistance; Km^s, kanamycin sensitivity.
[b] Number of loci determined from Km^r:Km^s ratios: one locus is indicated by a 3:1 ratio; two loci are indicated by a 15:1 ratio (each significant at the 0.05 level based on χ^2 tests).
[c] (–) indicates no GUS activity; (+) indicates positive GUS activity. Pollen grains isolated from one to three flowers of each primary transformant were assayed; the number of blue-staining pollen grains over the total number scored in at least 20 consecutive microscopic fields is given.

This segregation of the GUS-positive phenotype in pollen indicates that the SRK_6 promoter was active in the haploid pollen grain and thus directed gametophytic, rather than sporophytic, expression. This conclusion is consistent with the observed developmental regulation of SRK_6::*uidA* in developing microspores: GUS staining was not detected in premeiotic microspore mother cells and was detected only in microspores following their maturation to the binucleate stage (data not shown). The transient nature of *SRK* promoter activity in pollen was underscored by the lack of detectable GUS staining in the tubes of pollen germinated in vitro (data not shown). No GUS activity was detected in vegetative tissues or in other floral organs of transgenic plants, including anthers, filaments, sepals, petals, and ovaries.

Transformation of Arabidopsis with the SRK_6::*uidA* construct resulted in 17 independent transgenic lines, as summarized in Table 3. As in tobacco, GUS activity in transgenic Arabidopsis was restricted to stigma tissues and pollen. Fourteen transformants exhibited GUS expression in stigmas (Table 3). Blue staining was localized to the stigmatic papillae and subepidermal tissue (Figure 9D). In some transformants, GUS

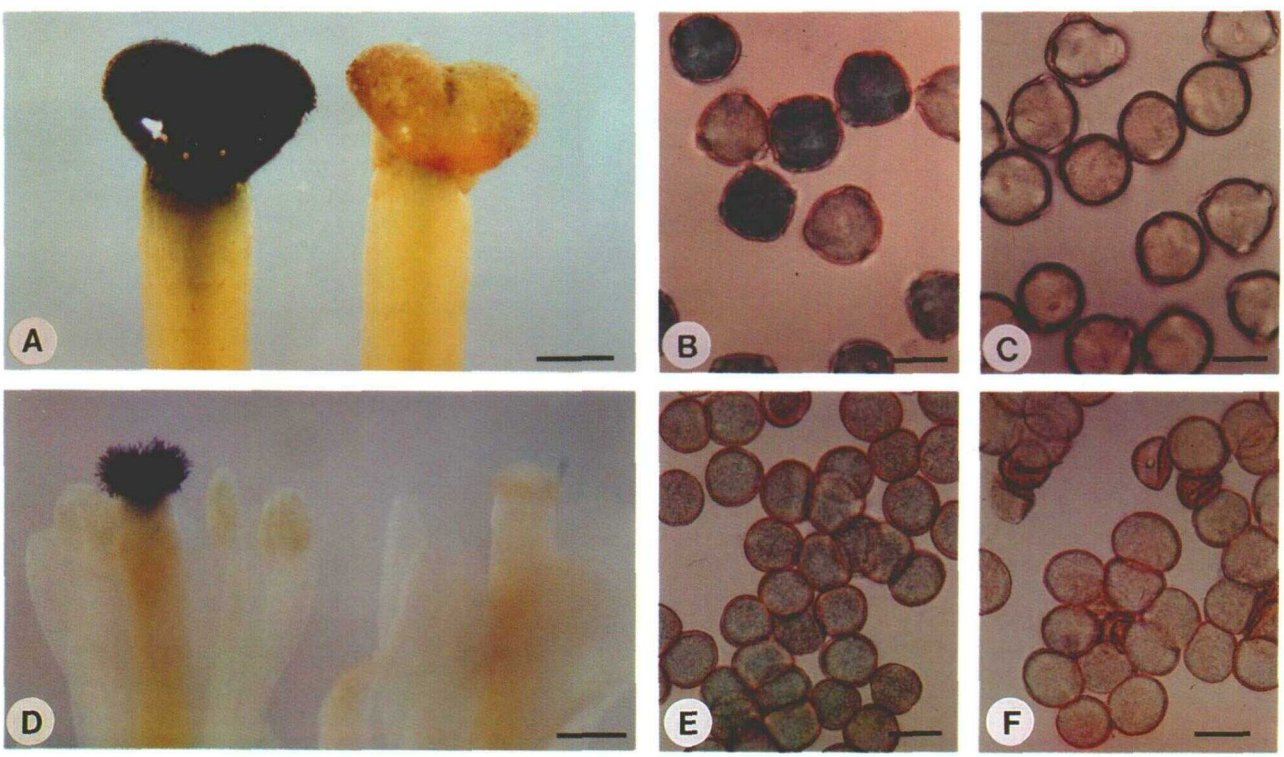

Figure 9. Histochemical Localization of GUS Activity Conferred by the SRK_6 Promoter in Flowers of Transgenic Tobacco and Arabidopsis.

Tissues were assayed using the chromogenic substrate X-Gluc. Blue staining indicates GUS activity.
(A) Stigma and upper style of tobacco transformed with SRK_6::*uidA* (left) and an untransformed control (right). Bar = 1 mm.
(B) SRK_6 promoter activity in tobacco pollen. Bar = 25 μm.
(C) Histochemically stained pollen from untransformed tobacco. Bar = 25 μm.
(D) Mature flower of Arabidopsis transformed with SRK_6::*uidA* (left) and an untransformed control (right). Bar = 0.2 mm.
(E) Pollen grains of transgenic Arabidopsis. Bar = 25 μm.
(F) Pollen grains of untransformed Arabidopsis. Bar = 25 μm.

Table 3. Analysis of SRK_6::*uidA*–Transformed Arabidopsis

Line No.	Km^r:Km^s[a]	No. of Loci[b]	T_2 Stigma	GUS Activity[c] Pollen	(%)	Generation
1	18:5	1	–	ND		
2	23:14	1	+	–		T_2
3	24:15	1	–	ND		
5	19:9	1	+	–		T_2
6	39:0	>2	+	–		T_2
8	27:1	2	+	–		T_2
9	98:22	1	+	+	(100%)	T_3,T_4
10	159:11	2	+	+	(100%)	T_3
11	9:0	2	+	–		T_2
13	104:26	1	+	–		T_3
14	13:4	1	–	–		T_2
15	72:0	>2	+	–		T_3
16	100:36	1	+	+	(100%)	T_3
18	85:19	1	+	–		T_3
19	55:15	1	+	+	(100%)	T_3
23	5:2	1	+	51/179	(32%)	T_2
24	2:69	ND	+	ND		

[a] Km^r, kanamycin resistance; Km^s, kanamycin sensitivity.

[b] Number of loci determined from Km^r:Km^s ratios: one locus is indicated by a 3:1 ratio; two loci are indicated by a 15:1 ratio (each significant at the 0.05 level based on χ^2 tests). Ratios that are statistically greater than 15:1 were assigned >2 loci. ND, number of loci could not be determined based on the data.

[c] (–) indicates no GUS activity; (+) indicates GUS activity. Stigma activity was evaluated in the T_2 generation and in subsequent generations as indicated. Pollen activity was evaluated only in the generations indicated. Pollen GUS staining was scored in at least 10 consecutive microscopic fields. The number of blue-staining pollen grains over the total number scored is given only when <100% staining was observed. ND, not determined.

activity was detected also within the transmitting tissue of the style, with the intensity of blue staining decreasing toward the ovary. In the maturing inflorescence, GUS activity in the stigma was detected first in buds at 1 day before flower opening, reached a maximum in open flowers, and persisted until flower senescence. Five of the transgenic Arabidopsis lines also exhibited GUS activity in pollen (Table 3 and Figure 9E). Segregation of the GUS-positive pollen phenotype was observed in transformant 23, which was tested in the T_2 generation, indicating gametophytic expression of the transgene (Table 3). Plants tested in the T_3 and T_4 generations were true breeding for kanamycin resistance, and as expected for plants homozygous for the transgene, 100% of the pollen grains produced by these plants were positive for GUS activity. No GUS activity was detected in the pistil (Figure 9D) or pollen (Figure 9F) of untransformed control plants.

SRK and SLG Proteins Are Absent in Pollen

In light of the above results showing *SRK* promoter activity in pollen and similar findings obtained with the *SLG* promoter (Sato et al., 1991; Thorsness et al., 1991, 1993; Toriyama et al., 1991b; Dzelzkalns et al., 1993; Kandasamy et al., 1993), it was of interest to determine whether SRK and SLG proteins could be detected in pollen by using the highly sensitive chemiluminescent detection system. We therefore subjected pollen extracts, as well as leaf extracts that were not expected to contain SRK and SLG, to immunoblot analysis with MAb/H8. Figure 10 shows that, although both SRK and SLG were detected easily in stigmas after a short exposure to film, no bands were detected in either pollen or leaf extracts, despite the fact that the pollen and leaf lanes contained nearly eight times more total protein than the stigma lanes. Upon longer exposure to film, some bands were detected in the pollen lanes. However, these bands did not comigrate with SRK and SLG and apparently were due to nonspecific reactivity with the secondary antibody used in the assay: they were observed in the absence of primary antibody and were present in pollen from S_2 as well as S_6 homozygotes. Microsomal membrane preparations and extraction with RIPA, SDS, and Triton X-100 similarly have failed to reveal SLG or SRK proteins in pollen (data not shown).

DISCUSSION

As a putative receptor protein kinase, SRK has a postulated role in both the recognition and signal transduction phases of SI. Genetic evidence for this role includes linkage to the *S* locus and the identification of mutant alleles that are associated with self-fertility; structural and biochemical evidence include the high degree of allelic polymorphism in the SLG-like domain and the demonstrated catalytic activity of the kinase domain. The present study adds to this body of evidence by showing that SRK is expressed in the papillar cell, the site at which the SI reaction is manifested, and that SRK is an in-

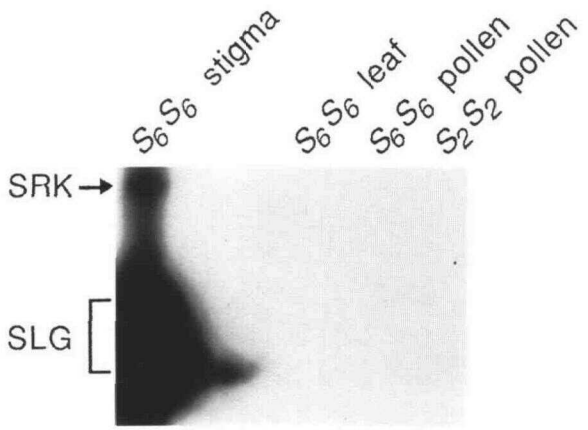

Figure 10. Distribution of SRK and SLG Proteins in Brassica Tissues.

Protein extracts of stigma (11 μg), leaf (85 μg), and pollen (85 μg) from the indicated *S* genotypes were immunoblotted, probed with the MAb/H8 antibody, and developed with chemiluminescent substrates.

tegral plasma membrane glycoprotein, placing it at the cell surface, where it would be available for mediating cell-to-cell signaling events.

SRK_6 Is a 108-kD Integral Plasma Membrane Glycoprotein

Immunological and genetic criteria were used to identify SRK_6 as a 108-kD protein in *B. oleracea* stigmas. The polypeptide was recognized by two different antibodies, one that binds the N-terminal domain of SRK_6 and a second that binds the C-terminal domain (Figure 2). Genetic evidence that the 108-kD stigma protein is encoded by SRK_6 derives from the finding that, among F_2 plants segregating for the S_6 haplotype and the highly diverged class II S_2 haplotype, this protein was detected only in plants carrying the SRK_6 gene (Figure 2). Additionally, a protein of similar size was detected in self-incompatible plants carrying other class I haplotypes, such as *B. campestris* S_8S_8 plants (Figure 8B), but not in $S_{f1}S_{f1}$ plants (Figure 3), which have a null allele of *SRK* but do express immunoreactive SLG proteins. Corroborating this evidence, SRK_6 sequences placed under the control of the CaMV 35S promoter direct the synthesis of a 108-kD protein in leaves of transgenic tobacco (Figure 5A).

The 108-kD SRK_6 protein cofractionates with stigma microsomal membranes, indicating its association with stigma membranes. Our procedure for isolating microsomes included a centrifugation step to remove cell debris and large organelles and a washing step to remove residual soluble proteins. Although the resulting cell fraction would include the plasma membrane, the bulk of membranes would be derived from the tonoplast, endoplasmic reticulum, and Golgi apparatus, thus preventing a precise determination of the subcellular location of SRK_6. We therefore subjected SRK_6-expressing tobacco leaves to aqueous two-phase partitioning, which yielded well-purified plasma membranes (Table 1 and Figure 5B).

Localization of SRK_6 to plasma membranes was indicated clearly by its enrichment in the plasma membrane fraction relative to the fraction containing intracellular membranes (Figure 5C). In Brassica stigmas (Figure 4) and transgenic tobacco (Figure 5D), solubilization studies demonstrated that membrane-bound SRK_6 exhibited properties of an integral membrane protein, in agreement with sequence data showing that SRK_6 possesses a hydrophobic region located between the SLG-like and kinase domains that conforms to the requirements of a single-pass transmembrane domain (Stein et al., 1991).

Tobacco-expressed SRK_6 also was shown to bind ConA, a lectin that recognizes the α-D-mannose moiety of glycoproteins. The SRK_6 sequence contains seven potential N-linked glycosylation sites within its SLG-like domain, four of which are conserved with at least four different alleles of SLG (Stein et al., 1991). Moreover, at least some of these sites are known to be glycosylated in SLG (Takayama et al., 1986, 1987). The observed glycosylation of SRK_6 (see also Delorme et al., 1995) provides experimental evidence that the SLG-like domain was translocated into the lumen of the endoplasmic reticulum during its synthesis, as was originally proposed based on the presence of a putative signal sequence at the N terminus of the polypeptide (Stein et al., 1991). In light of our results demonstrating that SRK is embedded in the plasma membrane, the SLG-like domain would be exposed to the exterior face of the cell, whereas the kinase domain would be located cytoplasmically. However, this topology remains to be verified by direct experimental methods.

In contrast to SRK, SLG is a soluble glycoprotein that accumulates in the papillar cell wall (Kandasamy et al., 1989). Accordingly, in fractionated stigma extracts, most of the SLG_6 glycoprotein was present in the soluble fraction, but we also identified a membrane-associated pool of SLG_6 (Figure 4). Although at least one *SLG* allele has been shown to produce an alternative transcript that encodes a membrane-bound isoform (Tantikanjana et al., 1993), the SLG_6 allele is not predicted to encode such a form. Therefore, we hypothesize that the microsomal membrane pool of SLG_6 represents protein in transit through the secretory pathway. In agreement with this hypothesis, we found that the microsome-associated SLG_6 was solubilized in the presence of sodium carbonate (Figure 4), consistent with a location of this SLG pool within the lumen of membrane vesicles or a peripheral interaction with the membrane. Additional support for this hypothesis can be found in immunocytochemical localization studies of SLG in the papillar cell (Kandasamy et al., 1989). In these studies, a fraction of antigens recognized by MAb/H8 and likely to be SLG because of its high abundance relative to SRK was localized to the rough endoplasmic reticulum and Golgi apparatus.

Coordinate Regulation of SRK and SLG in the Stigma

The conservation of *cis*-acting regulatory elements in *SLG* and *SRK* suggests that these genes (Figure 6) are subject to common transcriptional control mechanisms that, in effect, would coordinately regulate their expression. In support of this hypothesis, we showed that *SRK* and *SLG* have similar spatial and temporal patterns of expression by analyzing levels of RNA and protein and by monitoring SRK_6 promoter activity in transgenic plants. In a previous study, we had estimated that in the stigma, *SLG* transcripts are 140 to 180 times more abundant than *SRK* transcripts (Stein et al., 1991), and an abundance of SLG far in excess of SRK is obvious in the immunoblots displayed in Figures 4 and 10. Yet, despite these quantitative differences, the steady state transcript levels of the two genes increased coordinately during maturation and reached a maximum at 1 day before flower opening, a timing that coincides with the ability of stigmas to distinguish between self- and non-self-pollen (Figure 7). The levels of SLG and SRK protein followed a similar time course. However, whereas maximal SLG levels are attained in open flowers (Nasrallah et al., 1985a), the level of SRK was observed to decline at this stage in at least one genotype (Figure 8). This decline, which may be

genotype specific, is not understood but may reflect differences in the relative strengths of the SI response in different genotypes.

The *SRK* and *SLG* genes overlap in their expression patterns not only temporally but also spatially. The reporter gene studies described in this article demonstrate conclusively that the SRK_6 promoter is active primarily in the papillar cells of the stigma, as shown previously for the SLG_{13} promoter in transgenic tobacco (Thorsness et al., 1991; Dzelzkalns et al., 1993), Arabidopsis (Toriyama et al., 1991b), and Brassica (Sato et al., 1991). In addition to this primary site of activity, the SRK_6 promoter also was active in the subepidermal cells of the stigma in Arabidopsis and tobacco and the transmitting tissue of the style in Arabidopsis but not in tobacco. In this respect, the SRK_6 promoter differs from the SLG_{13} promoter, which was found to be active in the subepidermal cells and transmitting tissue of tobacco but not Arabidopsis. However, the activity of the SRK_6 promoter in subepidermal cells and transmitting tissue of the Arabidopsis pistil does coincide with a similar activity observed for the SLG_{13} promoter in *B. oleracea* (Sato et al., 1991).

Because different transgenic hosts impose somewhat different patterns of activity on the SRK_6 and SLG_{13} promoters at secondary sites of activity, it is not clear to what extent promoter activity at these sites reflects promoter activity in the native plant. However, no such variability has been observed in the primary site of activity for either the *SRK* or *SLG* promoter. Indeed, reporter studies have been predictive of the primary site of expression of the *SLG* gene in Brassica papillar cells, as determined by in situ hybridization (Nasrallah et al., 1988) and immunolocalization studies (Kandasamy et al., 1989). We therefore conclude that *SLG* and *SRK* are transcribed simultaneously in the papillar cells of the stigma.

SLG and *SRK* Expression in Anthers and Pollen

Because genetic models of SI based on single-locus control historically have predicted the expression of a common *S* gene in both male and female tissues, much attention has focused on demonstrating the expression of *SLG* and *SRK* in anthers and pollen. In Brassica, expression is expected to occur either in the sporophytic tissue of the anther tapetum or in premeiotic microspores, because the recognition phenotype of pollen is determined by the diploid genome of the parent plant. Conventional RNA blot hybridization techniques have revealed the presence of *SLG* and *SRK* transcripts in microspores and/or anthers (Sato et al., 1991; Stein et al., 1991). However, these transcripts are rare, and several studies have resorted to amplification by the polymerase chain reaction (PCR) to detect expression in anthers and pollen (Guilluy et al., 1991; Goring and Rothstein, 1992; Delorme et al., 1995).

Consistent with the presence of *SRK* transcripts in male tissues, we found that the SRK_6 promoter directed expression of the GUS reporter in pollen grains of tobacco and Arabidopsis (Figure 9). Similarly, the SLG_{13} promoter had been shown to direct expression of the GUS reporter as well as the diphtheria subunit A toxin in cells of the anther and/or developing pollen grains in transgenic tobacco (Thorsness et al., 1991), Arabidopsis (Toriyama et al., 1991b; Thorsness et al., 1993), and Brassica (Sato et al., 1991; Kandasamy et al., 1993). However, we found no evidence of SRK_6 or SLG_6 proteins in pollen or anther extracts even when we used several different extraction protocols and the highly sensitive chemiluminescent immunodetection method (Figure 10). It thus appears that, although the SRK_6 and SLG_6 promoters are active transcriptionally in cells of the anther, additional regulatory mechanisms that affect either transcript stability, protein translation, or protein stability must function to prevent the accumulation of the SRK_6 or SLG_6 proteins to detectable levels in these cells.

Implications for the Mechanism of SI in Brassica and Receptor-Mediated Signaling in Plants

The lack of SLG and SRK proteins in male reproductive tissues refutes models of SI based on the homophilic interaction of *S* locus products and the notion of control by a single *S* gene. However, control of SI by a single genetic locus is still valid, given the potential for additional recognition genes within the physical and genetic limits of the *S* locus complex (Boyes and Nasrallah, 1993). In particular, we anticipate the presence of a pollen-expressed determinant of recognition that would act as a haplotype-specific ligand of SRK and SLG. A recently described gene, the *S* locus anther (*SLA*) gene, possesses several features expected of a recognition determinant, including linkage to the *S* locus, haplotype-specific polymorphism, and anther-specific expression (Boyes and Nasrallah, 1995). It remains to be determined whether the polypeptides of 7.5 and 10 kD potentially encoded by *SLA* transcripts are expressed on the pollen surface and are capable of interacting with SRK and SLG.

The simultaneous expression of SRK and SLG in the papillar cells of the stigma supports the hypothesis that they act together as determinants of the SI response in the stigma, perhaps by binding to a common ligand via their highly related S domains. Models of SI based on competition between SRK and SLG for ligand binding are inconsistent with genetic evidence that expression of both proteins is required for pollen recognition. We propose therefore that SLG functions cooperatively with SRK. The presence of a thick cell wall in plants may represent an obstacle for plasma membrane–based signaling. A soluble molecule like SLG theoretically could solve this problem by shuttling ligand from the outer cell wall and presenting it to the plasma membrane–localized SRK. Such a mechanism may be based on a higher affinity of the ligand for SRK than for SLG. These possibilities remain open questions.

Also to be resolved are exactly how cooperativity in SLG and SRK function is achieved and whether there is a direct interaction between these two proteins. The different levels of SLG and SRK that accumulate in papillar cells might imply that the

mechanism of action of SLG and SRK as female determinants of SI requires a high local molar excess of SLG relative to SRK rather than stoichiometric amounts of the two proteins. However, it is important to note that SLG is distributed throughout the volume of the cell wall, whereas SRK is restricted to the area of the plasma membrane. Based on a value of 615 nm for the thickness of the papillar cell wall (Kandasamy et al., 1989), we calculated that an excess of SLG over SRK of two orders of magnitude would be required to give equal local molar concentrations of protein.

SRK belongs to a family of related genes that includes in maize the *Zea mays* protein kinase gene *ZmPK1* (Walker and Zhang, 1990) and in Arabidopsis the Arabidopsis receptor kinase genes *ARK1*, *ARK2*, and *ARK3* (Tobias et al., 1992; Dwyer et al., 1994) and the receptor-like kinase genes *RLK1* and *RLK4* (Walker, 1993). These genes are expressed in a variety of vegetative tissues, but they all share structural features in common with *SRK*. Based on our results, which provide conclusive experimental evidence that the product of a member of this family is an integral plasma membrane protein, it may be inferred that proteins similar to SRK exist in the plasma membranes of vegetative plant cells, where they presumably fulfill cell surface signaling functions unrelated to pollination. SRK would have been recruited from such proteins for the specialized function of cell–cell signaling and the recognition of self during reproduction.

METHODS

Plant Material

Brassica oleracea inbred lines bearing S_6, S_2, and S_{f1} haplotypes and the *B. campestris* S_8 inbred line have been described previously (Nasrallah et al., 1987, 1994a; Chen and Nasrallah, 1990; Toriyama et al., 1991a). Homozygous parental lines were crossed, and the resulting F_1 hybrids were selfed by bud pollination to generate F_2 progeny. *S* locus genotypes within segregating populations were determined by pollination to tester lines. Genotype assignments also were made on the basis of restriction fragment length polymorphism analysis using allele-specific *S* locus receptor kinase (*SRK*) and *S* locus glycoprotein (*SLG*) probes (Boyes and Nasrallah, 1993) and on the basis of allele-specific charge polymorphisms displayed by the SLG protein when subjected to isoelectric focusing and immunoblotting (Nasrallah and Nasrallah, 1984).

DNA Cloning, Sequence Analysis, and RNA Gel Blot Hybridization

Isolation of genomic clones for SRK_6 and SRK_2 was described by Stein et al. (1991). Dideoxy sequencing was accomplished using the Sequenase Kit (United States Biochemical). Sequence alignment was performed with the GCG version 6.0 software package (University of Wisconsin Biotechnology Center, Madison).

Isolation of poly(A)$^+$ RNA and gel blot hybridization with ^{32}P-labeled probes was performed as described by Stein et al. (1991).

Construction of a *uidA* Reporter Gene Fusion, Transformation of Arabidopsis and Tobacco, and Histochemical Analysis of β-Glucuronidase Activity

To construct an SRK_6 promoter::*uidA* chimeric gene, a 452-bp fragment upstream of the protein coding region was amplified by the polymerase chain reaction (PCR) and cloned into vector pCR1000 (Invitrogen, San Diego, CA). The integrity of the amplified sequence was confirmed by dideoxy sequencing and subsequently inserted into the the Ti binary vector pBI101 (Jefferson et al., 1987) upstream of the *uidA* coding region. The resulting construct was introduced into *Agrobacterium tumefaciens* pCIB524/A136 (derived from helper plasmid pEJA101; Hood et al., 1986).

Nicotiana tabacum cv Petit Havana was transformed by the method of Horsch et al. (1988). *Arabidopsis thaliana* strain C24 was transformed by the method of Valvekens et al. (1988). Kanamycin-resistant tobacco and Arabidopsis plants resulting from independent transformation events were transferred to soil and grown to maturity in a greenhouse. DNA gel blot analysis confirmed the presence of the transgene in all primary regenerants. Subsequent generations derived by selfing were germinated from seed on Murashige and Skoog medium (Murashige and Skoog, 1962) containing either 300 μg/mL kanamycin (tobacco) or 25 μg/mL kanamycin (Arabidopsis) and grown in a 25°C growth chamber with a 16-hr light/8-hr dark cycle. To assess antibiotic resistance, the number of green kanamycin-resistant seedlings and bleached kanamycin-sensitive seedlings were scored 2 to 4 weeks after sowing.

Histochemical localization of β-glucuronidase (GUS) activity in vegetative and floral organs was performed by using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), as described previously (Thorsness et al., 1991; Toriyama et al., 1991b). Pollen and isolated microspores were assayed with 2 mM X-Gluc, 0.1 M $NaPO_4$, pH 7.0, 0.1% (v/v) Triton X-100, without vacuum infiltration, and were observed without ethanol destaining. Constant mixing of the pollen was required during incubation with X-Gluc to avoid false-positive staining. The nuclear stages of the grains were assessed by staining with 4′, 6-diamidino-2-phenylindole and fluorescence microscopy, as detailed by Thorsness et al. (1991). Pollen was isolated from Arabidopsis by rinsing whole mature flowers with buffer and pipetting off the suspended pollen grains. To avoid possible contaminating GUS activity from stigmas, the pollen was washed four times with 1 mL of buffer. As an additional control, the pollen from nontransformed plants was assayed in buffer that was used previously in the first rinse of transgenic flowers.

Expression of *SRK* in *Escherichia coli*

A 1.2-kb fragment consisting of the *SLG* homologous domain of SRK_6 exclusive of the signal peptide was amplified by PCR, ligated into the pCR1000 vector, and subsequently inserted into the bacterial expression vector pGEX-3X (Pharmacia, Piscataway, NJ). The resulting plasmid encoded a 73-kD glutathione *S*-transferase (GST) fusion protein, which could be affinity purified from JM109 cells using glutathione–agarose beads (Smith and Johnson, 1988). Purified fusion proteins were visualized following transfer to Immobilon PVDF membranes (Millipore, Bedford, MA) by staining with Coomassie Brilliant Blue R 250 or by probing with MAb/H8.

The *SLG* homologous domain of SRK_6 also was cloned into a modified pGEX vector containing a FLAG epitope at the 3′ end of the GST coding region. A similar construct was generated by using the *SLG* homologous domain of SRK_2. The resulting fusion proteins were transferred to PVDF membranes and probed with the commercially

available monoclonal M2 anti-FLAG antibody (International Biotechnologies, New Haven, CT) or with MAb/H8.

Because immunostaining repeatedly revealed the presence of proteolytic derivatives of the full-length fusion proteins for all three GST–SRK constructs, we prepared bacterial extracts in the presence of a spectrum of protease inhibitors. This treatment did not prevent degradation of the fusion protein, indicating that the proteolytic products occurred not as a consequence of cell disruption and protein extraction but rather as a result of in vivo instability, a commonly observed feature of recombinant proteins produced in *E. coli*.

Construction of an SRK_6 Plant Expression Vector and Selection of Transgenic Tobacco Lines Expressing SRK Protein

To construct a cauliflower mosaic virus (CaMV) 35S promoter::SRK_6 chimeric gene, we used the pCT37 vector (Tobias, 1995), a Ti binary vector derived from pBIN19 (Bevan, 1984) and containing a duplicated CaMV 35S promoter (Kay et al., 1987) and a *nos* terminator between the T-DNA borders. Because only partial SRK_6 cDNA clones were available (Stein et al., 1991), we had to reconstitute the full-length coding sequence. This was accomplished in a series of cloning and PCR steps involving the ligation of sequences from different cDNA clones and extension of the cDNA sequence to include the ATG initiation codon. The SRK_6 genomic clone was used as template for PCR. After verifying the fidelity of PCRs by sequence analysis, the reconstituted SRK_6 full-length clone, which included three nucleotides upstream of the SRK_6 start codon, was inserted between the CaMV 35S promoter and the *nos* terminator within pCT37.

Leaves of greenhouse-grown primary transformants were screened for SRK transcript 3 weeks after transfer to soil by using the rapid screening method described by Verwoerd et al. (1989). Those individuals expressing relatively high levels of SRK_6 RNA were screened further for the expression of SRK_6 protein in primary transformants and in their kanamycin-resistant T_2 progeny. Leaf segments were ground to a powder in liquid nitrogen, extracted with hot SDS-PAGE sample buffer (2% [w/v] SDS, 100 mM DTT, 80 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 5 mg/mL bromphenol blue), and analyzed by immunoblotting. Because highest SRK_6 protein levels were observed in leaves of 1- to 2-month-old, growth chamber–grown seedlings (Murshige and Skoog medium; 50 μg/mL kanamycin), all subsequent experiments used plant material grown in this manner.

Preparation of Tobacco Membrane Fractions

Unless otherwise specified, all steps were carried out at 4°C and all buffers contained the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/mL), leupeptin (10 μg/mL), and pepstatin A (1 μg/mL). Tobacco leaves (15 g fresh weight) were homogenized with a polytron in buffer (2 mL/g fresh weight) containing 30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM ascorbate, 20% (v/v) glycerol. The homogenate was filtered through one layer of Miracloth (Calbiochem, LaJolla, CA), and the resulting filtrate was centrifuged at 10,000*g* (9000 rpm, SM 24 rotor; Beckman, Fullerton, CA) for 15 min. The supernatant was centrifuged at 100,000*g* (33,200 rpm, 50.2 Ti rotor; Beckman) for 1 hr. Soluble protein retained in the supernatant was stored in liquid nitrogen.

The microsomal membrane pellet was fractionated by the aqueous two-phase partitioning method described by Larsson (1985). Phase separations were carried out in a series of 5-g phase systems that contained 6.2% (w/w) dextran T500 and 6.2% (w/w) polyethylene glycol 3350 (omitting phenylmethylsulfonyl fluoride). Three successive rounds of partitioning yielded a colorless upper phase enriched in plasma membranes and a green lower phase containing intracellular membranes. After washing, pelleting, and resuspending in 250 mM sucrose, 5 mM Bis-Tris-propane (BTP)/Mes, pH 7.2, the partitioned membranes were assayed for marker enzyme activity and stored in liquid nitrogen.

Marker Enzyme Assays

Vanadate-sensitive ATPase was assayed by a protocol modified from Hodges and Leonard (1972). Membranes were added to a reaction mixture containing 3 mM ATP-2Na, 3 mM $MgSO_4$, 50 mM KCl, 25 mM Hepes, 0.03% (v/v) Triton X-100 (pH adjusted to 6.5 with BTP), with or without 100 μM sodium orthovanadate. The reaction proceeded at 30°C for 15 min and was stopped by the addition of 0.25% (w/v) ammonium molybdate, 1.3% (w/v) SDS, and 1.4% (v/v) sulfuric acid. Activity was represented by the difference in amount of phosphate released (Fiske and Subbarow, 1952) in the presence and absence of vanadate.

Triton-stimulated UDPase was assayed by a protocol modified from Nagahashi and Kane (1982). Membranes were added to a reaction mixture containing 3 mM UDP-Na, 3 mM $MnSO_4$, 50 mM KCl, 25 mM Hepes (pH adjusted to 7.4 with BTP), with or without 0.03% (v/v) Triton X-100. Reactions proceeded as described above for 10 min. Activity was represented by the difference in activity observed in the presence and absence of Triton X-100.

Spectrophotometric assays for NADH cytochrome *c* reductase and cytochrome *c* oxidase were performed essentially as described by Yoshida (1979).

Preparation of Brassica Stigma Microsomes

All steps were performed at 4°C or less, and all buffers contained the protease inhibitors listed above. For routine preparation of microsomes, 30 stigmas (~6 mg fresh weight) were ground with a Teflon (Kontes, Vineland, NJ) pestle in a homogenization buffer (2.5 μL per stigma) containing 30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% (v/v) glycerol. For most experiments, it was necessary to include DTT (5 mM), potassium metabisulfite (2.5 mM), ascorbate (5 mM), and polyvinylpyrrolidone (3 to 5% [w/w]) to prevent the artifactual formation of high molecular mass SLG protein. Centrifugation was carried out at 4000*g* for 5 min (7000 rpm; Eppendorf centrifuge), and the recovered supernatant was recentrifuged to remove cell debris and large organelles. The supernatant was fractionated into soluble and microsomal membrane fractions by centrifugation at 100,000*g* (49,000 rpm, TLA 100.4 rotor; Beckman) for 1 hr. After washing with homogenization buffer, the pelleted membranes were solubilized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [w/v] Nonidet P-40 (Pierce), 0.5% [w/v] deoxycholate, 0.1% [w/v] SDS) for 30 min and then centrifuged at 100,000*g* for 1 hr. The supernatant, containing solubilized membrane proteins, was stored at −80°C.

Membrane Protein Solubility Studies and Concanavalin A Binding

Tobacco microsomes were divided into three equal pellets. One pellet was extracted directly with SDS-PAGE sample buffer to give a total

microsome fraction. The second pellet was resuspended with 100 mM sodium carbonate, pH 11.5, and incubated on ice for 30 min (Fujiki et al., 1982). The third pellet was resuspended with RIPA buffer and incubated on ice for 30 min. Tubes were centrifuged at 150,000*g* at 4°C (60,000 rpm, TLA 100.4 rotor; Beckman) for 1 hr. Solubilized proteins were recovered in the supernatant, and the insoluble pellets were dissolved in SDS-PAGE sample buffer. Equal volume proportions of each fraction were analyzed by immunoblotting. Solubility studies with Brassica stigma microsomes were carried out as above, except that 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.5% (v/v) Triton X-100 was used instead of RIPA buffer.

RIPA-solubilized tobacco membranes were incubated with concanavalin A (ConA)–Sepharose beads (Sigma) at 4°C for 1.5 hr. Beads were washed four times with 1 mL of RIPA. Bound material was released by boiling with SDS-PAGE sample buffer and analyzed by immunoblotting.

Other Protein Extraction Procedures

For the experiment depicted in Figure 10, stigmas and leaves were ground in 30 mM Tris-HCl, pH 7.5, 75 mM NaCl, 10 mM EDTA, 10% (v/v) glycerol, and protease inhibitors. Pollen was ground in 20 mM Tris-HCl, pH 8.0, 1% (v/v) β-mercaptoethanol, 10 mM EDTA, 2.5 mM potassium metabisulfite, and protease inhibitors. Homogenates were centrifuged at 4000*g* for 5 min (7000 rpm; Eppendorf centrifuge). The supernatants were boiled with SDS-PAGE sample buffer and analyzed by immunoblotting.

Protein Immunoblots and Antibodies

Proteins were quantified with the Bio-Rad (Hercules, CA) protein assay dye reagent (Bradford, 1976) with BSA as a standard; for protein solutions containing detergent, a modified Lowry assay was employed (Bensadoun and Weinstein, 1976). Proteins were resolved by electrophoresis on 7% SDS–polyacrylamide gels (unless otherwise specified) and electroblotted onto PVDF membranes. Membrane blocking for 30 min and antibody binding for 1 hr were carried out in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% [v/v] Tween 20) containing 5% (w/v) nonfat dry milk at room temperature. After primary and secondary antibody treatments, membranes were washed three times for 10 min each with TBST. As specified in the text, blots were either probed with alkaline phosphatase–conjugated second antibodies and developed with chromogenic substrates 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium (Bio-Rad) or probed with horseradish peroxidase–conjugated secondary antibody and developed with the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim, Indianapolis, IN). Blots were stripped and reprobed using the method described by Kain et al. (1994).

MAb/H8, raised against SLG (Kandasamy et al., 1989), was used at a 1:50 dilution. MAb 2E7, raised against the 60-kD subunit of vacuolar H^+-ATPase (Ward et al., 1992), was used at a 1:200 dilution. Rabbit antiserum raised against the plasma membrane H^+-ATPase (Harper et al., 1990) was used at a 1:10,000 dilution. The use of rabbit antiserum R1-254, raised against residues 790 to 803 within the C-terminal region of SRK_6, was described by Stein and Nasrallah (1993). R1-254 was affinity purified over a column containing the synthetic peptide antigen covalently coupled to an agarose matrix. The column was prepared by using SulfoLink Coupling Gel (Pierce) and resulted in 3.3 mg of peptide immobilized on 2 mL of gel. The column was loaded with antiserum and washed with PBS, pH 7.4. Fractions were eluted with 0.2 M glycine, pH 2.8, 0.5 M NaCl, and neutralized with 1.0 M Tris-HCl, pH 9.0. Fractions containing protein were pooled and concentrated with a Centricon 10 (Amicon) device. The affinity-purified antibody was used at a concentration of 5 μg/mL.

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