Post-Transcriptional Maturation of the S Receptor Kinase of *Brassica* Correlates with Co-Expression of the *S*-Locus Glycoprotein in the Stigmas of Two *Brassica* Strains and in Transgenic Tobacco Plants[1]

Ram Dixit, Mikhail E. Nasrallah, and June B. Nasrallah*

Department of Plant Biology, Cornell University, Ithaca, New York 14853

The *S*-locus-encoded S receptor kinase (SRK) is an intrinsic plasma membrane protein that is viewed as the primary stigma determinant of specificity in the self-incompatibility response of *Brassica* spp. We analyzed two self-compatible mutant strains that express low levels of the *S*-locus glycoprotein (SLG), a cell wall-localized protein also encoded at the *S* locus that is coordinately expressed with SRK. We found that mutant stigmas synthesized wild-type levels of *SRK* transcripts but failed to produce SRK protein at any of the developmental stages analyzed. Furthermore, SRK was shown to form aberrant high-molecular mass aggregates when expressed alone in transgenic tobacco (*Nicotiana tabacum*) plants. This aggregation was prevented in tobacco plants that co-expressed SRK and SLG, but not in tobacco plants that co-expressed SRK and SLR1, an SLG-related secreted protein not encoded at the *S* locus. In analyses of protein extracts under reducing and non-reducing conditions, evidence of intermolecular association was obtained only for SLG, a fraction of which formed disulfide-linked oligomers and was membrane associated. The data indicate that, at least in plants carrying the *S* haplotypes we analyzed, SRK is an inherently unstable protein and that SLG facilitates its accumulation to physiologically relevant levels in *Brassica* stigmas.

Plants possess a large number of genes encoding transmembrane receptor-like protein kinases (Stone and Walker, 1995; Becraft, 1998; Hardie, 1999). These genes can be classified into distinct families on the basis of the sequence of their predicted extracellular domain (Becraft, 1998; Hardie, 1999) and are thought to play important roles in a variety of biological processes in view of the diverse expression patterns exhibited by their transcripts. However, a biological function is actually known for only a few of these genes, and attempts to identify the receptor protein and investigate its biochemical properties have been made for an even smaller subset of the genes. Among these are the *Brassica S*-locus receptor kinase (SRK) (Stein et al., 1991), which functions in the self-incompatibility response, and the Arabidopsis CLAVATA1 (CLV1) protein, which is required for normal development of the shoot meristem (Clark et al., 1997). SRK, a member of the *S* gene family, which is characterized by an "S" domain containing a conserved array of Cys residues, has been shown to be an integral membrane protein in *Brassica* stigmas (Delorme et al., 1995; Stein et al., 1996), to be targeted to the plasma membrane when expressed in transgenic tobacco (*Nicotiana tabacum*) plants (Stein et al., 1996), and as predicted from its sequence, to be oriented in the plasma membrane with its "S" domain to the outside of the cell (Letham et al., 1999). CLV1, the predicted extracellular domain of which contains Leu-rich repeats, has been shown to occur in complexes with other proteins in vivo (Trotochaud et al., 1999) and to require CLV2 for its stability (Jeong et al., 1999). CLV2 is predicted to be a transmembrane protein with an extracellular domain containing Leu-rich repeats and a very short cytoplasmic domain lacking a kinase domain (Jeong et al., 1999).

The *Brassica* self-incompatibility response prevents the development of genetically related pollen on the epidermal (papillar) cells of the stigma (for review, see Nasrallah and Nasrallah, 1993; Nasrallah et al., 1994a). This response is controlled genetically by haplotypes of the *S* locus, and a self-incompatibility (SI) response is instigated if the pollen and pistil are derived from plants sharing an identical *S* haplotype. Recent work has demonstrated that specificity in the SI response is determined by two highly polymorphic proteins encoded by the *S* locus: the *S* receptor kinase discussed above determines SI specificity in the stigma (Takasaki et al., 2000), and the SCR (*S*-locus Cys-rich) protein, a small highly polymorphic Cys-rich protein expressed specifically in anthers and proposed to be a ligand for SRK, is necessary and sufficient for SI specificity in pollen (Schopfer et al., 1999).

In addition to SRK and SCR, the *S* locus encodes a third protein, the *S*-locus glycoprotein (SLG). SLG shares a high degree of sequence similarity with the SRK ectodomain (Nasrallah et al., 1987; Stein et al., 1991; Kusaba et al., 1997), is expressed specifically

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* Corresponding author; e-mail jbn2@cornell.edu; fax 607–255–5407.

and coordinately with SRK in stigmatic papillar cells (Stein et al., 1996), and accumulates in the papillar cell walls to high levels (Kandasamy et al., 1989), often reaching a 100-fold excess over SRK. However, the role of SLG is not understood. Its requirement for SI has been questioned on the basis that self-incompatible plants homozygous for some *S* haplotypes express low levels of SLG (Tantikanjana et al., 1993, 1996; Gaude et al., 1995), that an *S* haplotype seems to lack an *SLG* gene (Okazaki et al., 1999), and that sequence analysis of some *SLG/SRK* gene pairs reveals a more robust correlation between sequence divergence and SI specificity for *SRK* than for *SLG* (Kusaba and Nishio, 1999; Kusaba et al., 2000). Nevertheless, it remains possible that *SLG* performs another function in SI. Such a role is suggested from the fact that the majority of *Brassica S* haplotypes analyzed contains a highly expressed *SLG* gene and that this gene also occurs in self-incompatible strains of *Raphanus* (Sakamoto et al., 1998) and thus has persisted through events of speciation. Furthermore, transgenic plants that express both SLG and SRK exhibit an enhanced SI response relative to transgenic plants that express SRK alone (Takasaki et al., 2000).

We have been analyzing the expression of *SRK/SLG* transcripts and proteins in *Brassica* mutant strains that exhibit a stigma-specific breakdown of SI to elucidate properties of the SRK receptor and define parameters required for its proper maturation and function. In this paper, we report on our analysis of two mutant strains bearing defects in the structure or expression of the *SLG* gene. We show that SRK does not accumulate in stigma cells when *SLG* expression is dramatically reduced, providing a biochemical basis for the requirement of SLG in SI. Together with results of expression studies in transgenic tobacco plants, our data reveal that the SRK isoforms we analyzed require accessory molecules for their accumulation and proper maturation. Thus, these isoforms may be inherently unstable, as has been demonstrated for CLV1 (Jeong et al., 1999) as well as for many of the receptors and other intrinsic membrane proteins analyzed in animal systems (Yoshimura et al., 1990; Ward and Kopito, 1994; Centrella et al., 1996). The requirement of molecules related to the receptor extracellular domain may represent a common mechanism for the proper maturation and accumulation of plant receptor protein kinases.

RESULTS

Analysis of Self-Compatible Mutant *Brassica* Strains

Two self-compatible (SC) mutant *Brassica* strains that exhibit defects in the structure or expression of *SLG* were used in this study: (a) *Brassica campestris* (syn. *B. rapa*) strain homozygous for *scf1*, a recessive mutation at a trans-acting locus unlinked to the *S* locus that leads to a dramatic reduction in the levels of *SLG* transcripts and transcripts encoded by two other stigma-specific members of the *S* gene family, but does not affect the levels of *SRK* transcripts (Nasrallah et al., 1992) and (b) a *B. oleracea* mutant designated Δ*S*-1668 that carries a deletion encompassing the *SLG* gene. This mutant was identified in a screen of F_1 plants generated by using γ-irradiated pollen from a self-incompatible $S_{13}S_{13}$ plant to pollinate stigmas from plants homozygous for the S_{f1} haplotype and selecting for mutant self-compatible plants in the otherwise self-incompatible F_1 generation (Nasrallah et al., 2000). The S_{f1} haplotype is a naturally occurring non-functional (self-fertile) haplotype that carries a null *SRK* allele and as such does not encode SRK protein, but it does contain a functional *SLG* gene and thus encodes SLG protein (Nasrallah et al., 1994b). Previous DNA gel-blot analysis of the Δ*S*-1668 strain had shown that it carries a mutant S_{13} haplotype in which all but 500 bp at the 5′ end of the SLG_{13} gene was deleted but which retained an intact SRK_{13} gene (Boyes et al., 1997). Thus, this strain is expected to express transcripts and proteins derived from SRK_{13} but to lack the 1.6-kb transcripts derived from SLG_{13}. Δ*S*-1668 also produces SLG_{f1} protein but not SRK_{f1} protein due to the presence of the S_{f1} haplotype.

We had previously reported that the *scf1* mutation causes the stigma to be receptive to self-pollen but does not affect the pollination phenotype of the male gametophyte (Nasrallah et al., 1992). Pollination analysis revealed that Δ*S*-1668 is a highly self-fertile strain, routinely producing >300 pollen tubes/stigma upon self-pollination. Furthermore, Δ*S*-1668 stigmas were fully compatible with pollen derived from plants bearing the S_{13} haplotype (>300 pollen tubes produced/stigma), in contrast to wild-type $S_{13}S_{f1}$ and $S_{13}S_{13}$ stigmas, which inhibited the development of S_{13}-derived pollen. However, Δ*S*-1668 pollen failed to germinate on stigmas carrying the S_{13} haplotype, an incompatible reaction identical to that exhibited by pollen from wild-type $S_{13}S_{13}$ and $S_{13}S_{f1}$ plants. Thus, DNA encompassed by the deletion in Δ*S*-1668 is required for SI in the stigma but not in pollen.

Analysis of *SRK* Transcripts and Proteins in *SLG*-Deficient Mutants

We performed RNA gel-blot analysis of wild-type and mutant stigmas at different stages of development to determine if the levels and developmental regulation of *SRK* transcripts were similar in wild-type and mutant plants. As illustrated in Figure 1A, *scf1* stigmas from open flowers and from buds at 1 d prior to anthesis (−1 stage) exhibited a depletion of the 1.6-kb *SLG* transcripts relative to wild-type *SCF1* controls (Fig. 1A, left panel). In contrast, the levels of the 3.0-kb *SRK* transcripts were comparable between the mutant and wild-type stigmas (Fig. 1A, center panel). Similarly, Δ*S*-1668 stigmas, which are null for

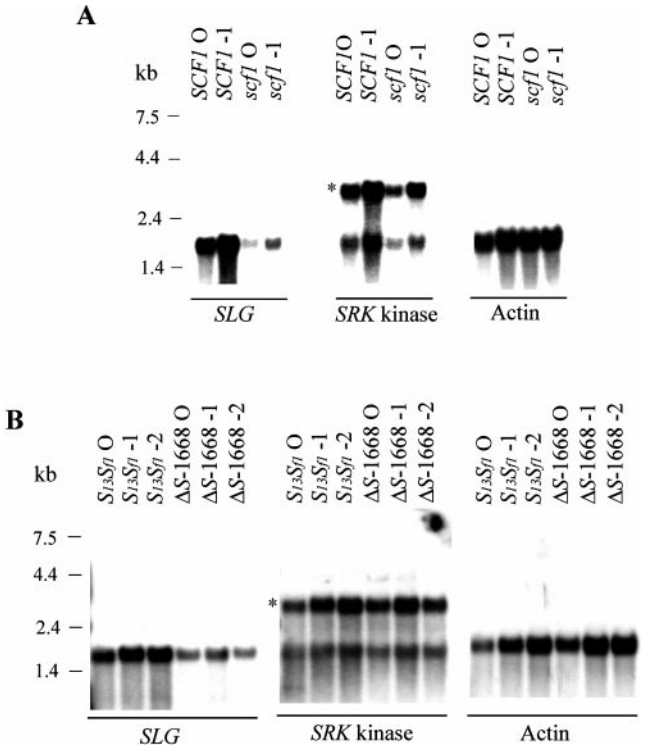

Figure 1. RNA gel-blot analysis of *Brassica* strains. Poly(A$^+$) RNA was prepared from stigmas isolated from wild-type *SCF1* and mutant *scf1* homozygotes (A) and wild-type $S_{13}S_{f1}$ and mutant Δ*S*-1668 plants (B) (approximately 3 μg of RNA per lane for both experiments). The blots were hybridized sequentially with a DNA probe specific for *SLG* (derived from the 3′-untranslated region of *SLG*) and a probe corresponding to the kinase domain of *SRK*, followed by a *Brassica* actin probe to confirm equal loading of RNA. The developmental stage of isolated stigmas is indicated above the lanes as 0 (open flowers at anthesis) or as negative numbers corresponding to days before anthesis. The asterisk in the center panel indicates the position of the 3.0-kb *SRK* transcripts. The 1.5-kb band detected with the *SRK* probe probably corresponds to a related kinase gene. Molecular length markers in kb are indicated to the left.

SLG_{13} (and only produced a low level of SLG_{f1} transcripts) accumulated SRK_{13} transcripts to levels indistinguishable from control $S_{13}S_{f1}$ stigmas (Fig. 1B, center panel). The genotype and *S* transcript species produced by both wild-type and mutant strains used in this study are shown in Table I.

To determine if attenuation of *SLG* transcripts (and thus SLG protein) in the mutant strains affected the levels of SRK protein, we performed protein immunoblot analysis of open flower stigmas from wild-type and mutant plants using monoclonal antibody MAb/H8. We have previously demonstrated that MAb/H8 detects SRK as a discrete band of approximately 108 kD and SLG as a cluster of glycoforms in the size range of approximately 40 to 65 kD (Stein et al., 1996). Figure 2A shows that the approximately 108-kD SRK protein, which is clearly visible in whole cell extracts of $S_{13}S_{f1}$ and *SCF1* control stigmas, was undetectable in stigmas of the Δ*S*-1668 and *scf1* mutants, either in whole cell extracts or in microsome fractions. Furthermore, whereas SRK is enriched in plasma membrane fractions obtained from wild-type self-incompatible stigmas (Fig. 2B), it remains undetectable in plasma membrane fractions purified from mutant stigmas (as shown for Δ*S*-1668 in Fig. 2C). The low level of SRK visible in the endosome fraction in Figure 2B is probably reflective of its presence in the secretory pathway in transit to the plasma membrane.

Instability of membrane proteins has been shown to be developmentally regulated (Kearse et al., 1994). To test if SRK is initially expressed in mutant stigmas at early stages of flower bud development and subsequently degraded as the stigmas mature, we performed immunoblot analysis of stigmas at various maturation phases. SRK was undetectable in *scf1* stigmas (Fig. 3A) and Δ*S*-1668 stigmas (Fig. 3B) at all developmental stages tested, which is in contrast to wild-type stigmas in which SRK was detected throughout development. The consistent correlation we observed between the diminished levels of SLG protein and the absence of detectable SRK protein despite the presence of wild-type levels of *SRK* transcripts indicates that post-transcriptional processes regulate SRK accumulation in *Brassica* stigmas.

Formation of Disulfide-Linked Oligomers of *S*-Locus Glycoprotein in the Stigma

The observation that SRK does not accumulate in the absence of SLG is suggestive of an interaction between the two proteins. To test the possibility that

Table I. *Wild-type and mutant Brassica strains analyzed in this paper*

Strain (Phenotype)	*S* Haplotype	Transcript Species
SCF1SCF1 (SI)	S_8S_8	Wild-type levels of SRK_8, SLG_8, *SLR1*, and *SLR2* transcripts
scf1scf1 (SC)	S_8S_8	Wild-type levels of SRK_8 transcripts; low levels of SLG_8, *SLR1*, and *SLR2* transcripts
$S_{13}S_{f1}$ (SI)	$S_{13}S_{f1}$	Wild-type levels of SRK_{13}, SLG_{13}, SLG_{f1}, *SLR1*, and *SLR2* transcripts; no SRK_{f1} transcripts
Δ*S*-1668 (SC)	$S_{13\Delta}S_{f1}$	Wild-type levels of SRK_{13}, SLG_{f1}, *SLR1*, and *SLR2* transcripts; no SRK_{f1} and SLG_{13} transcripts
Δ*S*-55 (SC)	$S_{13\Delta}S_{f1}$	Wild-type levels of SLG_{f1}, *SLR1*, and *SLR2* transcripts; no SRK_{f1}, SRK_{13}, and SLG_{13} transcripts

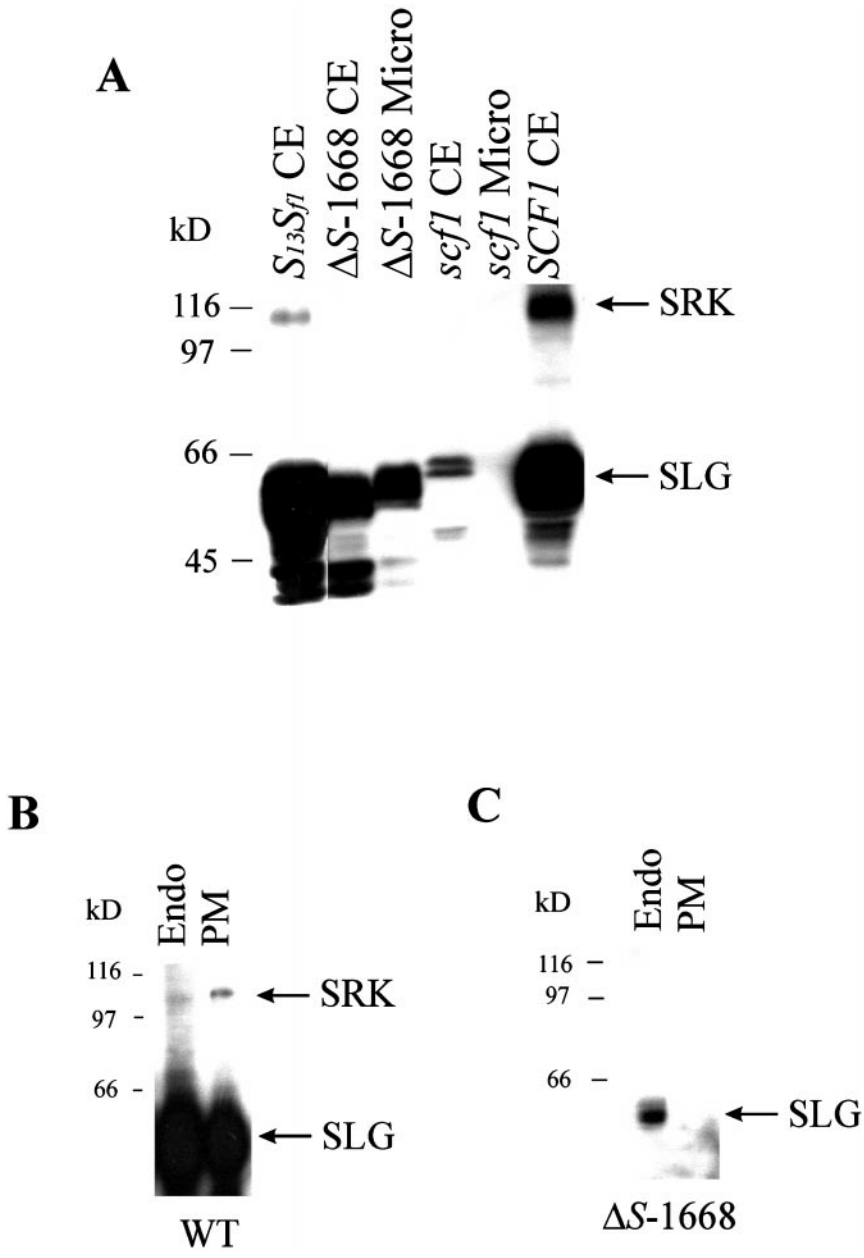

Figure 2. Immunoblot analysis of SLG and SRK in *Brassica* stigmas. A, Whole cell extracts (CE) or microsome fractions (Micro) prepared from $S_{13}S_{f1}$, Δ*S*-1668, *scf1scf1*, and *SCF1SCF1* stigmas were subjected to immunoblot analysis using MAb/H8. Each lane contains 50 μg of protein. B and C, Microsome fractions isolated from wild-type (WT) stigmas (B) and from Δ*S*-1668 stigmas (C) were partitioned into endomembrane-enriched (Endo) and plasma membrane-enriched (PM) fractions and the blot probed with MAb/H8. Each lane contains 10 μg of protein. SRK is detected in wild-type but not in mutant stigma extracts. SLG observed in Δ*S*-1668 stigmas is the product of the SLG_{f1} gene. The lower level of SRK in $S_{13}S_{f1}$ plants is due to the presence of only one functional copy of the *SRK* gene. Molecular mass markers in kD are indicated to the left of each panel.

such an interaction might occur through the formation of inter-molecular disulfide bonds, stigma cell extracts were subjected to SDS-PAGE analysis under reducing (+dithiothreitol [DTT]) and non-reducing (−DTT) conditions, followed by immunoblot analysis. As shown in Figure 4A, the apparent molecular mass of both SLG and SRK observed under non-reducing conditions was decreased by approximately 5 to 10 kD relative to that observed under reducing conditions. In addition, we observed a significant difference in electrophoretic mobility between reduced and alkylated SLG relative to unreduced and alkylated SLG using acid-urea gel electrophoresis (data not shown). Both electrophoretic properties are indicative of intra-molecular disulfide bonding (Hollecker, 1997). This occurrence of intra-molecular disulfide bonds appears to be a general feature of proteins within the *S*-gene family. Similar electrophoretic shifts were also noted for SLG and SRK from the S_8, S_{13}, and S_{22} haplotypes (data not shown) as well as for the *S*-locus related SLR1 glycoprotein (see below), a molecule that is expressed specifically in papillar cells and accumulates to high levels in the cell wall like SLG but is encoded by a gene unlinked to the *S* locus (Umbach et al., 1990).

It is interesting that there was a significant enhancement of the SRK-containing 108-kD band in wild-type stigma extracts run in the absence of DTT (Fig. 4A). This enhancement could result from the formation of SLG oligomers, possibly homodimers,

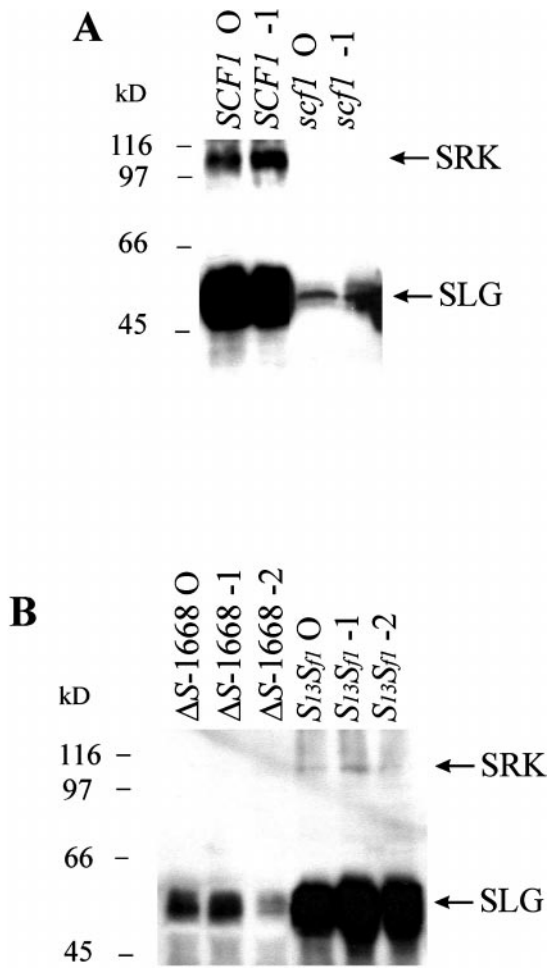

Figure 3. Developmental analysis of SLG and SRK in *Brassica* stigmas. Microsome fractions (40 μg of protein in each lane) isolated from stigmas of wild-type *SCF1* and mutant *scf1* homozygotes (A) and wild-type $S_{13}S_{f1}$ and mutant Δ*S*-1668 plants (B) at various developmental stages were subjected to immunoblot analysis using MAb/H8. Developmental stage designation is as in Figure 1.

which would be expected to migrate at approximately the same position as SRK. Indeed, SLG fractions isolated by preparative isoelectric focusing and shown to be free of contaminating proteins by silver staining (see "Materials and Methods") were also found to contain an approximately 108-kD species upon electrophoresis under non-reducing conditions (Fig. 4A). These results strongly suggest that the apparent enhancement in SRK signal under non-reducing conditions is due to the presence of SLG homodimers. However, neither this study nor another study that also suggested the occurrence of SLG dimers (Doughty et al., 1998) can categorically rule out the possibility that the approximately 108-kD SLG fraction represents heterodimers (or oligomers) between SLG and one or more unidentified stigma protein(s) with the same pI point and molecular mass as SLG.

In addition to the approximately 108-kD band, stigma whole cell extracts running under non-reducing conditions also contained minor bands that migrated with an apparent molecular mass of approximately 120 kD and approximately 140 kD (Fig. 4A). Because bands with the same mass were also observed in purified SLG fractions under non-reducing conditions (Fig. 4A), these bands likely represent higher-order SLG oligomers. It should be noted that we did not detect immunoreactive bands >200 kD in size that might represent SRK homodimers or SRK complexed with other as-yet-unidentified molecules, contrary to a recent study that reported the detection of >200-kD SRK species upon cross-linking of un-pollinated stigma extracts (Giranton et al., 2000). If such SRK complexes prove to be of general occurrence in *Brassica* strains, our inability to detect these complexes would suggest that their formation is not mediated by disulfide bridges.

To ascertain that disulfide bonds did not artificially arise during preparation of stigma extracts, *Brassica* stigmas were pretreated with a high concentration of iodoacetate (IAc) in the presence or absence of DTT. IAc quenches free sulfhydryl side chains on proteins (Hollecker, 1997) and will hence prevent their participation in disulfide bond formation during preparation of stigma extract. The analysis was carried out using wild-type stigmas as well as stigmas obtained from a deletion mutant designated Δ*S*-55. Δ*S*-55 is a self-compatible strain identified in the same screen as Δ*S*-1668. The Δ*S*-55 strain is deleted for both SLG_{13} and SRK_{13} (Nasrallah et al., 2000) and possesses the S_{f1} haplotype with its non-functional SRK_{f1} gene and its functional SLG_{f1} gene (see Table I). This plant hence produces only SLG_{f1} protein and allows unambiguous characterization of SLG properties in native tissue.

Stigma proteins were either reduced and alkylated by immersion in buffer containing both DTT and IAc or alkylated in the absence of DTT by immersion in buffer containing IAc alone. Control stigmas were immersed in buffer containing no DTT or IAc for an identical time period. The results of this experiment are shown in Figure 4B. Stigma extracts in which proteins were reduced and alkylated by treatment of stigmas with DTT and IAc prior to extraction exhibited the expected electrophoretic patterns under reducing conditions: i.e. wild-type stigma extracts exhibited the approximately 108-kD SRK band and the cluster of SLG forms (Fig. 4B, +DTT lanes), and Δ*S*-55 stigma extracts exhibited SLG monomers (Fig. 4B, +DTT lanes). Pretreatment of wild-type or Δ*S*-55 mutant stigmas with high concentration of IAc did not prevent the approximately 5- to 10-kD shift in mobility of SLG and SRK observed under reducing versus non-reducing conditions (Fig. 4B, compare +DTT and −DTT lanes) and also did not prevent the formation of SLG oligomeric forms under non-reducing conditions (Fig. 4B, −DTT lanes). Hence, the observed disulfide-bonded forms of SLG do not

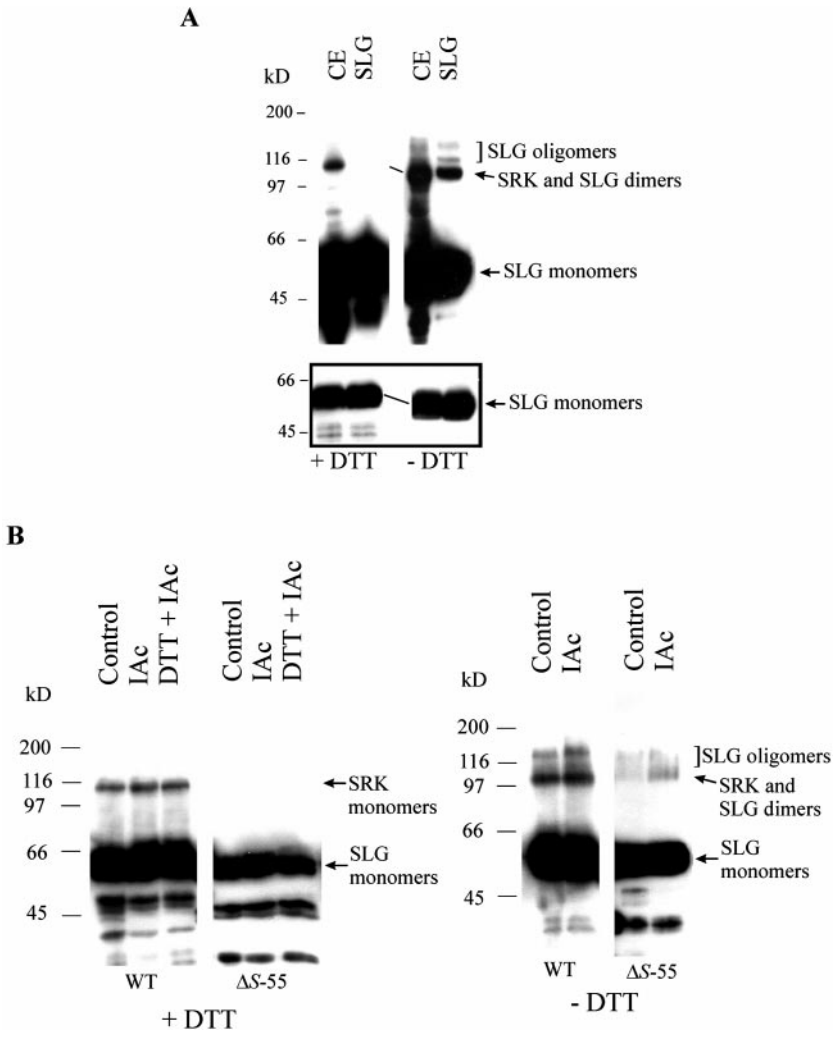

Figure 4. Analysis of stigma proteins under reducing and non-reducing conditions. Stigma proteins were separated by SDS-PAGE under reducing (+DTT) or non-reducing (−DTT) conditions and subjected to immunoblot analysis using MAb/H8. A, Wild-type stigma whole cell extract (25 μg, CE) and isoelectric focusing-purified SLG (2 μg, SLG). The box shows the SLG signal after a short exposure of the immunoblot to x-ray film. The oblique lines indicate the observed differences in mobility of SRK and SLG under reducing and non-reducing conditions. B, Whole cell extracts obtained from wild-type (WT; 20 μg of protein) or Δ*S*-55 (50 μg of protein) stigmas treated with buffer alone (Control), buffer with 100 mM IAc, or buffer with 50 mM DTT and 100 mM IAc (DTT + IAc).

result from oxidative processes induced during cell extraction.

Identification of a Membrane-Associated Fraction of SLG

Figure 4, A and B demonstrate that only a fraction of the SLG population is represented as oligomers. Such a result might arise if the disulfide bond-induced oligomerization is dynamic in nature or it may be indicative of a heterogeneous SLG population, only a subset of which is competent for oligomerization. Experiments testing the electrophoretic behavior of SLG in soluble or microsome fractions obtained from Δ*S*-55 stigmas under non-reducing conditions revealed that it is only the microsome fraction-associated SLG that is capable of forming disulfide bond-mediated oligomers (Fig. 5A). The result is all the more striking since the bulk of SLG is retained in the soluble fraction. Hence, SLG occurs as a heterogeneous population in *Brassica* stigmas: Only a subset of SLG glycoforms can associate with the microsome fraction and it is this subset that forms disulfide-linked oligomers. This property of SLG membrane retention was also observed for SLR1 (Fig. 5B) and as such may be a general feature of the "soluble" S-family proteins. However, the capacity to form inter-molecular disulfide bonds is perhaps distinctive of SLG since we have failed to detect oligomeric forms of SLR1 under non-reducing conditions (Fig. 5B).

To determine the nature of the forces resulting in SLG-membrane association, we attempted to disrupt this association by treating stigma microsome fractions with various chemicals. The microsome fractions for this experiment were prepared using a relatively hypotonic buffer (lacking glycerol) to prevent the formation of intact membrane vesicles, which might trap proteins inside. As shown in Figure 6, all of the treatments resulted in some release of the membrane-associated SLG, but in most cases only to an extent equivalent to that achieved simply by re-extracting microsomes with the homogenization buffer (HB) (Fig. 6). However, significantly greater dissociation of SLG from the microsomes was achieved by treatment with detergents: SDS treat-

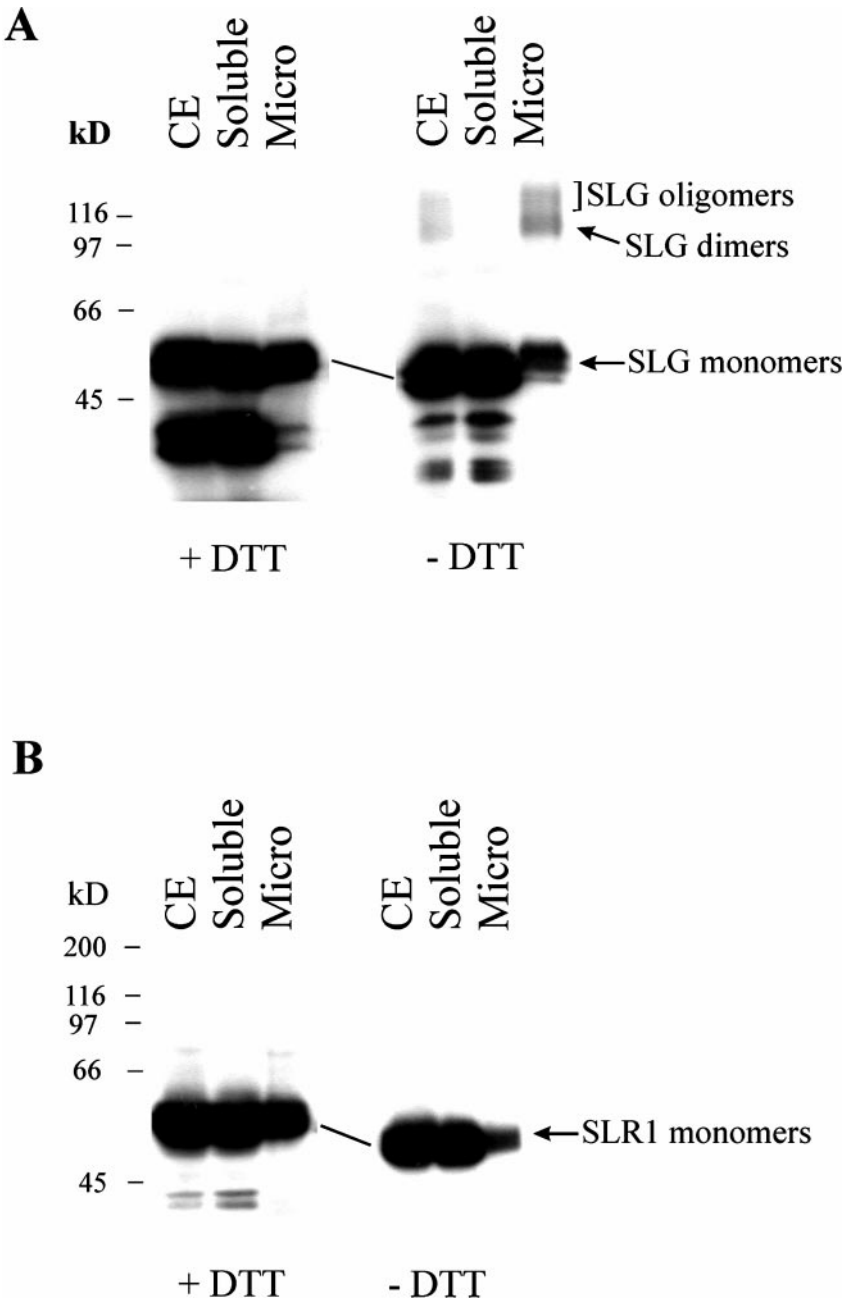

Figure 5. Oligomerization of membrane-associated SLG. Whole cell extract (CE), soluble, and microsome (Micro) fractions obtained from Δ*S*-55 stigmas were subjected to electrophoresis under reducing (+DTT) or non-reducing (−DTT) conditions followed by immunodetection using MAb/H8 (A) or anti-SLR1 serum (B). Each lane contains 100 μg of protein. The oblique lines indicate the observed differences in mobility of SLG and SLR1 under reducing and non-reducing conditions.

ment resulted in near complete release of SLG from the membranes (Fig. 6). It should be noted that SLG membrane association is also insensitive to the inclusion of 50 mM DTT in the extraction buffer prior to stigma homogenization (data not shown) and hence the association of SLG with the membrane fraction is non-covalent in nature and is probably mediated by hydrophobic forces. Similar membrane associative properties have been described for animal Cys string proteins, which are predicted to be soluble proteins but nonetheless associate with cellular membranes via the Cys string domain (Mastrogiacomo et al., 1998).

Co-Expression of SRK with SLG or SLR1 in Transgenic Tobacco Plants

To investigate further the effect of SLG on SRK accumulation, we used a heterologous tobacco expression system. We had previously shown that transformation of tobacco with chimeric genes consisting of the cauliflower mosaic virus (CaMV) 35S promoter fused to either *SRK* cDNA (Stein et al., 1996) or *SLG* cDNA (Perl-Treves et al., 1993) resulted in the production of SLG and SRK proteins that were indistinguishable from stigma-expressed proteins on reducing SDS-PAGE gels. Furthermore, heterologous

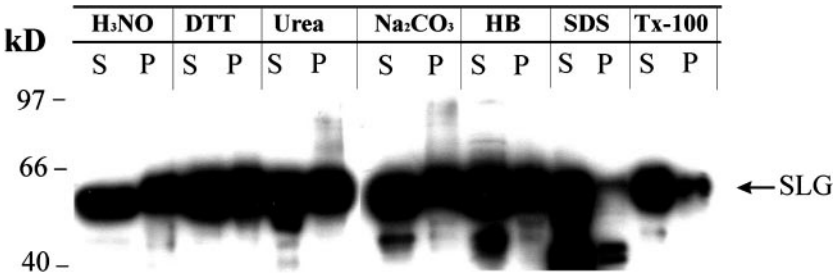

Figure 6. Chemical treatment of stigma microsome fractions. Equal amounts of microsome fraction obtained from *Brassica* stigmas were treated with 1 M H_3NO, 50 mM DTT, 8 M urea, 0.1 M Na_2CO_3, 0.2% (w/v) SDS, 1% (v/v) Triton X-100 (Tx-100), or HB (see "Materials and Methods") and centrifuged at 100,000*g* for 1 h to obtain supernatant (S) and pellet (P) fractions. The fractions were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and the immunoblot was probed with MAb/H8.

expression studies are now recognized as an essential and convenient tool for the biochemical analysis of plant proteins (for review, see Frommer and Ninnemann, 1995) and have been performed using tobacco plants (Kaye et al., 1998; Veena Reddy and Sopory, 1999), as well as yeast (Chen and Halkier, 1999; Montamat et al., 1999), Xenopus (Cao et al., 1992; Maurel et al., 1993), and mammalian COS cells (Kammerloher et al., 1994). Most pertinent to this study, expression of storage proteins of the maize kernel in transgenic tobacco plants was used to demonstrate that β-zein expression has a stabilizing effect on δ-zein (Bagga et al., 1997).

Therefore we retransformed SRK_6-expressing transgenic tobacco plants previously generated in our laboratory (Stein et al., 1996) with a chimeric gene consisting of the double CaMV 35S promoter fused to the coding region of SLG_6, and generated 12 independent transformants (designated [SRK+SLG]) that expressed both *SLG* and *SRK*. Two classes of control transgenic plants were also generated: One class consisted of nine independent transgenic plants (designated [SRK]) expressing only SRK that were produced by retransforming the *SRK*-expressing plants with vector lacking the *SLG* transgene, and a second class comprised of nine independent transformants (designated [SLG]) expressing only *SLG* that were obtained by introducing the *SLG* transgene into tobacco plants containing vector lacking SRK_6. In addition, the SRK_6-expressing tobacco plants were retransformed with another chimeric gene construct consisting of the *SLR1* cDNA inserted downstream of a double CaMV 35S promoter as a control for the specificity of any effect SLG might have on SRK properties. Four independent transformants that expressed SRK and SLR1 (designated [SRK+SLR1]) were obtained and used for the analyses.

Microsome fractions obtained from all independent [SRK+SLG], [SRK], and [SRK+SLR1] transformants were subjected to immunoblot analysis with MAb/H8. As shown in Figure 7A (lanes 1–7), the [SRK+SLG] lines produced SRK at levels similar to those produced in the [SRK] transformants. They also produced high amounts of SLG, a significant fraction of which was associated with the microsome fraction as observed in *Brassica* stigmas (Fig. 5A). Similarly, the [SRK+SLR1] plants expressed SRK (Fig. 7A, lanes 8 and 9) as well as high amounts of SLR1, a fraction of which was also membrane associated (Fig. 7A, boxed panel).

To determine if the SLG and SRK proteins produced in transgenic tobacco displayed electrophoretic properties similar to those observed in *Brassica* stigmas, microsome fractions isolated from the [SRK+SLG], [SRK], [SRK+SLR1], and [SLG] tobacco plants were tested by immunoblot analysis following SDS-PAGE under reducing and non-reducing conditions. As shown in Figure 7B (left panel), tobacco-expressed SLG and SRK migrated to their expected positions under reducing conditions. In addition, under non-reducing conditions, the SLG protein expressed in either the [SLG] or [SLG+SRK] plants exhibited the same approximately 5- to 10-kD difference in electrophoretic mobility relative to reduced SLG as observed in *Brassica* stigmas. Furthermore, a fraction of unreduced SLG migrated as bands of approximately 120 kD, which likely represent SLG oligomers similar to those observed in *Brassica* stigmas, because bands of similar size appear in extracts from tobacco plants expressing SLG alone (Fig. 7B, compare the lanes "SLG" and "SRK+SLG" in the right panel).

It is interesting that the electrophoretic behavior of tobacco-expressed SRK under non-reducing conditions is specifically modified when SRK is co-expressed with SLG. When [SRK] and [SRK+SLR1] extracts were analyzed under non-reducing conditions, SRK protein did not migrate to the expected position. Instead, under optimal protein-blotting conditions, SRK was detected as a very high molecular mass band at the top of the separating gel (Fig. 7B, the "SRK" and "SRK+SLR1" lanes in the right panel). This SRK band exceeds in mass that expected for SRK dimers and likely consists of multimeric aggregates of SRK. It is significant that no such SRK aggregates were detected in extracts of the [SRK+SLG] plants (Fig. 7B, the "SRK+SLG" lanes in

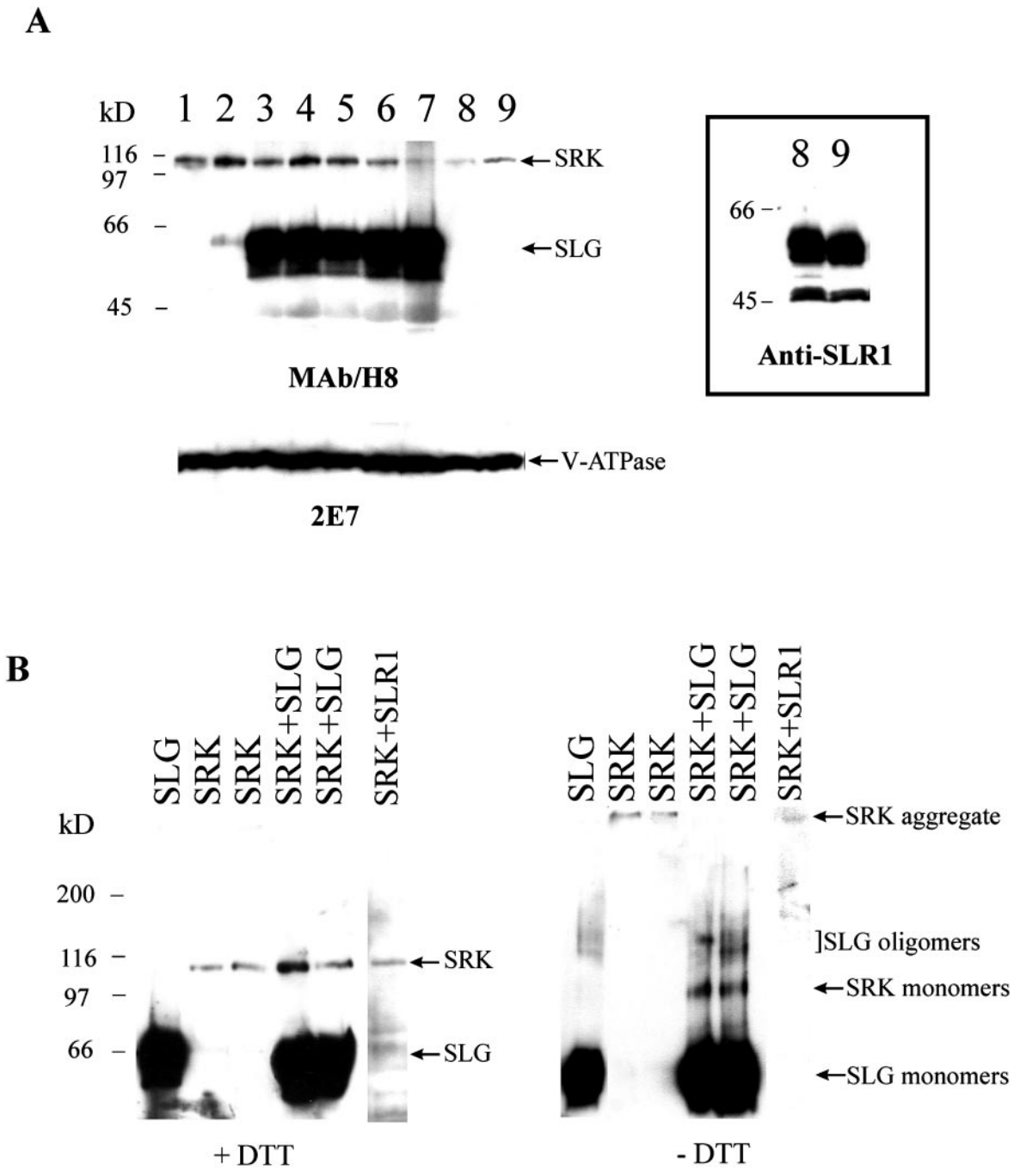

Figure 7. Immunoblot analysis of transgenic tobacco plants. A, Microsome fractions (150 μg of protein in each lane) were isolated from transgenic tobacco seedlings that express either SRK alone (lanes 1 and 2), both SRK and SLG (lanes 3–7), or both SRK and SLR1 (lanes 8 and 9). Each lane represents an independent transformant. The blot was sequentially probed with MAb/H8 to identify SLG and SRK (top panel) and with the anti-vacuolar H^+-ATPase 2E7 antibody as a loading control (bottom panel). The boxed panel to the right shows samples from plants represented in lanes 8 and 9 probed with anti-SLR1 serum to demonstrate the accumulation of SLR1 protein. B, Microsome fractions (150 μg of protein in each lane) isolated from transgenic tobacco seedlings expressing SLG alone, SRK alone, SRK and SLG, or SRK and SLR1 were subjected to electrophoresis under reducing (+DTT) and non-reducing (−DTT) conditions followed by immunodetection with MAb/H8. The results shown are representative of each class of plants and each lane represents an independent transformant.

the right panel); rather, in these extracts, the mobility of SRK was restored to that exhibited by stigma SRK run under non-reducing conditions. It should be noted that we have never detected SRK aggregates in non-reduced extracts of wild-type *Brassica* stigmas that express substantial levels of SLG.

DISCUSSION

The analysis of *Brassica* self-compatible mutants described in this paper suggests that the SRK receptor protein kinase isoforms we analyzed are regulated post-transcriptionally and may be inherently unstable molecules. In two independent mutant strains, there was a correlation between the depletion of SLG protein and the failure of stigmas to accumulate detectable levels of SRK protein despite the synthesis of normal amounts of *SRK* transcripts. The breakdown of SI in the *scf1* and Δ*S*-1668 mutant strains may thus be a direct consequence of the absence of the SRK receptor in mutant stigmas. These molecular defects are associated with a breakdown of SI in the stigmas but not the pollen of the mutant strains. Therefore, our results also show that the *SLG* and *SRK* genes function in the stigma but not in pollen, in support of biochemical (Stein et al., 1996), genetic (Nasrallah et al., 1992, 1994b; Goring et al., 1993), and transgenic (Toriyama et al., 1991; Conner et al., 1997; Stahl et al., 1998; Cui et al., 2000; Takasaki et al., 2000) studies.

Taken together with the observation that co-expression of SLG (but not SLR1) with SRK in tobacco cells prevents the aggregation of SRK, our results suggest that in the strains we used and with the *SRK/SLG* alleles we analyzed, SLG plays a major role in the stabilization of SRK molecules, possibly by facilitating their proper maturation. Such a role would provide a molecular basis for the breakdown of SI in plants that express little or no SLG, and for the observed but as-yet-unexplained enhancement in the intensity of the acquired SI response in transgenic *B. campestris* plants that express both SRK and SLG relative to transgenic plants that express SRK alone (Takasaki et al., 2000).

Post-transcriptional regulation of proteins is a well-known phenomenon. In particular, proteins that are part of heterodimers or higher-order complexes are often degraded when another protein in the complex is absent (Halban and Irminger, 1994; Wickner et al., 1999). Thus, our results provide circumstantial evidence that, in the strains we analyzed and in the tobacco expression system, SRK interacts with SLG either directly or indirectly. Such an interaction would presumably occur through the extracellular domain (ectodomain) of SRK, which occupies the same topological space as SLG (Letham et al., 1999). It would not, however, be mediated by disulfide bridges between the conserved Cys residues contained in both the SRK ectodomain and SLG, because we found no evidence for the occurrence of SLG-SRK disulfide-linked dimers in stigma extracts and in transgenic tobacco plants expressing SRK and SLG. Further, because SRK and SLG are coordinately regulated in papillar cells, the interaction might occur between the immature proteins either co-translationally or as they migrate through the secretory pathway, or between the mature proteins at the papillar cell surface to which both are targeted.

Several transmembrane proteins have been shown to be inherently unstable, with a substantial fraction of the newly synthesized protein targeted for degradation (Yoshimura et al., 1990; Ward and Kopito, 1994; Centrella et al., 1996). By analogy to processes described in the maturation of receptors in animal systems, SLG may assist in SRK folding by transient binding as described for receptor-associated protein in the folding and trafficking of the low density lipoprotein and very low density lipoprotein receptors (Savonen et al., 1999). An oligomerization-assisted folding mechanism alternatively may operate as described for the T-cell receptor (TCR) complex (Bonifacino and Klausner, 1994), procollagen (Bulleid et al., 1997), and the secreted immunoglobulin, IgM (Reddy and Corley, 1998). It is interesting that both the receptor-associated protein and ϵ- and ζ-subunits of TCR are very stable molecules compared with the corresponding unstable low density lipoprotein/very low density lipoprotein receptors or α-, β-, and δ-TCR subunits (Bonifacino and Klausner, 1994; Savonen et al., 1999). With this perspective, it is interesting to note that SLG is a highly stable protein and its accumulation is insensitive to the absence of SRK both in *Brassica* stigmas (Nasrallah et al., 1994b) and transgenic tobacco plants (Perl-Treves et al., 1993; this study). The accumulation of the SRK isoforms we analyzed may hence be viewed as correlated with the co-expression of highly stable SLG protein.

Based on our results, it is possible to infer some features required for the stabilization of SRK in *Brassica* stigmas expressing the *S* haplotypes investigated in this study. First, the amount of SLG protein appears to be critical, because SRK does not accumulate in *scf1* stigmas that do produce low levels of SLG. Second, qualitative properties of SLG may be important since, in ΔS-1668 stigmas, molecules contributed by the S_{f1} haplotype, SLG_{f1} in particular, failed to complement the mutation in SLG_{13} and to allow the accumulation of SRK_{13}. Thus, only some allelic forms of SLG might contribute to the stabilization of a particular SRK protein. It is also possible that only a subset of SLG functions in SRK stabilization, namely the SLG fraction that is membrane associated and capable of dimerizing. Membrane association of SLG would limit its diffusion to the two-dimensional space of the membrane and hence is likely to influence the frequency and character of SLG interaction with the transmembrane SRK protein. In this regard it is of interest to note that membrane-bound and soluble forms of various growth factors have been shown to display different potencies in activating the corresponding transmembrane receptors and can hence have distinct functional roles (Miyoshi et al., 1997; Takemura et al., 1997; Mueller et al., 1999).

It is interesting that SLG-related molecules cannot effect stabilization of SRK in the strains we analyzed. Several secreted SLG-related proteins are expressed in *Brassica* papillar cells. These include soluble glycoproteins encoded by the *SLR1* (Umbach et al., 1990) and *SLR2* (Boyes et al., 1991; Tantikanjana et al., 1996) genes that share approximately 70% sequence identity with SLG_8 and SLG_{13}, and also possibly SLG-like soluble forms of SRK, designated sSRK, which were predicted based on the occurrence of truncated *SRK* transcripts (Stein et al., 1991) and were indeed detected in at least one *Brassica* strain (Giranton et al., 1995). However, in the mutant stigmas we analyzed, such SLG-related molecules could not substitute for SLG in allowing normal accumulation of SRK. In addition, the formation of aberrant SRK aggregates in transgenic tobacco was prevented specifically by co-expression of SLG, but not by co-expression of SLR1, which is consistent with a specific role for SLG in the stabilization of SRK.

Whether the various allelic forms of SRK will all prove to require accessory molecules for their accumulation to physiologically relevant levels remains to be determined. *S* haplotypes are extremely diverse, and *SRK* and *SLG* genes exhibit extraordinarily high levels of sequence polymorphisms (Nasrallah et al., 1987; Chen and Nasrallah, 1990; Stein et al., 1991; Kusaba et al., 1997). Remarkably, they can also vary in their organization and in the classes of transcripts and proteins they produce (Tantikanjana et al., 1993, 1996; Gaude et al., 1995; Cabrillac et al., 1999). It is thus possible that some allelic forms of SRK are inherently more stable than the SRK_8 and SRK_{13} analyzed in our study, or that some *SRK* genes produce relatively high levels of sSRK that might contribute to stabilization of the full-length receptor. It is also possible that in some strains, other classes of S proteins that share a high degree of sequence identity with SLG may contribute to the stabilization of SRK. For example, the stigmas of *B. oleracea* S_2S_2 homozygotes produce low levels of secreted SLG (Tantikanjana et al., 1993, 1996; Gaude et al., 1995). However, these stigmas express SLR2, a protein that shares >90% sequence identity with SLG_2 (Boyes et al., 1991) and hence could potentially substitute for SLG_2. S_2S_2 stigmas also express a membrane-anchored form of SLG_2 consisting of the SLG_2 S domain fused to a transmembrane domain and a short cytoplasmic tail (Tantikanjana et al., 1993). This protein, which is structurally similar to the CLV2 protein (Jeong et al., 1999), would have the same diffusional constraints as SRK and therefore might be effective in stabilizing SRK even when present at relatively low levels.

It is significant that the correlation we observed between SRK and SLG is similar to that observed between the Arabidopsis CLV1 receptor kinase and the CLV2 protein (Jeong et al., 1999), even though the SRK and CLV1 receptors belong to very different families of receptor protein kinases. Analysis of *clv2* mutant strains revealed a dramatic (> 90%) decrease in the levels of CLV1 protein, although *CLV1* transcript levels were unaffected (Jeong et al., 1999). It is intriguing that the residual CLV1 protein was detected as a novel high-M_r complex that was absent in wild-type plants (Jeong et al., 1999). The strong parallels between these results and the ones described in this paper indicate that plant transmembrane receptor kinases are characterized by the same inherent instability described for receptors in animal systems. Further, the requirement of molecules related to the receptor extracellular domain, either in the form of a soluble protein (as in the case of SRK) or of a membrane-anchored protein (as in the case of CLV1), may represent a common mechanism for the sustained accumulation of plant receptor protein kinases.

MATERIALS AND METHODS

Plant Material and Pollination Assays

Brassica oleracea plants bearing the S_6, S_{13}, and S_{f1} haplotypes and the *Brassica campestris* (syn. *B. rapa*) *scf1* mutant strain have been described previously (Nasrallah et al., 1988, 1992, 1994b). Pollination phenotypes of reciprocal crosses involving Δ*S*-1668, $S_{13}S_{f1}$, and $S_{13}S_{13}$ plants were determined by monitoring pollen tube behavior by UV-fluorescence microscopy (Kho and Baer, 1968).

Isolation of RNA and Protein from *Brassica* Stigmas

Isolation of poly(A^+) RNA from *Brassica* stigmas and subsequent gel-blot analysis were performed as described (Stein et al., 1991). The gel blots were hybridized with a probe derived from *SLG* (probes derived from several *SLG* genes produce equivalent hybridization signals; an SLG_{13} 3′-untranslated region probe was used in this study), and a probe corresponding to the kinase domain of *SRK* (again kinase probes derived from several *SRK* alleles are equivalent; an SRK_6 kinase domain probe was used in this study). An actin probe was used as a loading control.

Stigma protein extracts were prepared by homogenizing stigmas in buffer containing 30 mM Tris (tris[hydroxymethyl]aminomethane)-HCl, pH 7.5, 75 mM NaCl, 10 mM EDTA, and 10% (v/v) glycerol. The buffer was supplemented with 5 mM ascorbate, 2.5 mM potassium metabisulfite, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 μg/mL pepstatin A just before use. Whole cell extracts and microsome samples were isolated using conditions described previously (Stein et al., 1996). Plasma membrane-enriched fractions were prepared by two-phase partitioning of stigma microsome pellets using a scaled-down version of the protocol previously described for tobacco (*Nicotiana tabacum*) tissue (Stein et al., 1996).

Purification of SLG_6 from S_6S_6 stigmas by isoelectric focusing was performed on a pH 3.5 to 9.5 gradient in flat beds of Sephadex G50 (Pharmacia Biotech, Piscataway, NJ) as described by Nasrallah et al. (1985). The purity of the SLG fraction was determined by silver staining of various

amounts of purified SLG following electrophoresis under reducing conditions.

Alkylation of Stigma Proteins Using IAc

Brassica stigmas obtained from open flowers were incubated with 100 mM IAc in the presence or absence of 50 mM DTT in the above-mentioned extraction buffer (10 stigmas in 30 μL of extraction buffer) containing 0.05% (v/v) Tween 20 as a surfactant. Alkylation of the stigma proteins was carried out for 20 min at room temperature in the dark. Stigmas immersed in extraction buffer lacking DTT and indole-3-acetic acid were used as a control. After the incubation period, the stigmas were homogenized in the respective buffers to obtain whole cell extracts as described above that were subjected to electrophoresis under reducing or non-reducing conditions.

Chemical Treatment of Stigma Microsome Fractions

Microsome fractions were isolated from *B. oleracea* S_6S_6 stigmas as described above using an HB consisting of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM EDTA along with the anti-oxidative and protease inhibitor supplements. Equal quantities of microsome pellets were resuspended in the following solutions: (a) 1 M hydroxylamine (H_3NO, prepared in HB and pH adjusted to 7.1 using NaOH), a deacylating agent, to test for the presence of acyl moieties on SLG that may result in its membrane attachment; (b) 50 mM DTT in HB to test for the covalent attachment of SLG to integral membrane proteins via disulfide bonds; (c) 8 M urea in HB, to test for hydrogen bond-mediated SLG-membrane association; (d) 0.1 M Na_2CO_3, pH 11.5, to test for ion-sensitive peripheral attachment of SLG to membranes; (e) 0.2% (w/v) SDS in HB to test the sensitivity of SLG-membrane attachment to treatment with ionic detergent; (f) 1% (v/v) Triton X-100 in HB to test the sensitivity of SLG-membrane attachment to treatment with non-ionic detergent; and (g) HB with no additives as a control for the extent of SLG released during resuspension of the microsome pellet. Equal volumes of all solutions were used to resuspend the microsome pellets. All treatments were incubated for 16 to 18 h at 4°C except for the SDS treatment, which was carried out at room temperature. The samples were subsequently centrifuged at 100,000*g* for 1 h and the supernatant and pellet fractions were analyzed by SDS-PAGE to determine the extent of SLG solubilization.

Co-Expression of SLG or SLR1 with SRK in Transgenic Tobacco

SLG_6 or *SLR1* cDNA was inserted between the duplicated CaMV 35S promoter (Kay et al., 1987) and *nos* terminator of a pBIN19 (Bevan, 1984) derived plant transformation vector bearing a hygromycin-resistance cassette. Cells of *Agrobacterium tumefaciens* strain GV3101 (Katavic et al., 1994) containing these constructs were used as described (Horsch et al., 1988) to transform tobacco (*N. tabacum* cv Petit Havana) plants expressing SRK_6 (Stein et al., 1996) or tobacco plants previously transformed with vector lacking the SRK_6 transgene. As an additional control, we used the vector backbone to transform the SRK_6-expressing tobacco plants.

Kanamycin- and hygromycin-resistant transformants were analyzed by DNA gel-blot and immunoblot analysis to confirm the presence and expression of SLG_6 or *SLR1* transgene. Seeds obtained from the primary transformants were surface sterilized and germinated on Murashige and Skoog medium (Murashige and Skoog, 1962), containing 50 mg/L kanamycin and 10 mg/L hygromycin. Tobacco shoot tissue from seedlings grown for 30 to 45 d on selection medium was used to obtain microsome fractions as described previously (Stein et al., 1996).

Protein-Gel Electrophoresis and Immunoblot Analysis

Samples containing equal amounts of protein were resolved by SDS-PAGE on 7.5% (w/v) or 10% (w/v) gels and electroblotted onto PVDF membranes using a semi-dry transfer technique. Protein quantification was carried out according to the Bradford technique (Bradford, 1976) using the Bio-Rad (Hercules, CA) dye reagent. Bovine serum albumin was used as a standard for protein quantification. Electrophoresis was performed either under reducing conditions by inclusion of DTT (100 mM) or under non-reducing conditions by omission of DTT from the protein-loading buffer. All experiments entailing the comparison of protein mobility under reducing versus non-reducing conditions were performed on the same gel with empty lanes separating the reduced versus non-reduced samples (to limit diffusion of DTT). The monoclonal antibody MAb/H8, which recognizes SLG and SRK (Kandasamy et al., 1989; Stein et al., 1996), was used at a concentration of 1:50 and the polyclonal anti-SLR1 serum (Umbach et al., 1990) was used at a concentration of 1:1,000. The 2E7 serum, which recognizes vacuolar H^+-ATPase (Ward et al., 1992), served to verify equal loading between lanes for the transgenic tobacco microsome samples and was used at a concentration of 1:500. Immunoblots were developed using the Boehringer Mannheim (Indianapolis) chemiluminescence western-blotting kit according to the manufacturer's instructions.

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