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

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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...
and coordinately with SRK in stigmatic papillar cells (Stein et al., 1996), and accumulates in the papillary 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 ΔS-1668 that carries a deletion encompassing the SLG gene. This mutant was identified in a screen of F1 plants generated by using γ-irradiated pollen from a self-incompatible S13\S13 plant to pollinate stigmas from plants homozygous for the Sf1 haplotype and selecting for mutant self-compatible plants in the otherwise self-incompatible F1 generation (Nasrallah et al., 2000). The Sf1 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 ΔS-1668 strain had shown that it carries a mutant S13 haplotype in which all but 500 bp at the 5′ end of the SLG13 gene was deleted but which retained an intact SRK13 gene (Boyes et al., 1997). Thus, this strain is expected to express transcripts and proteins derived from SRK13 but to lack the 1.6-kb transcripts derived from SLG13. ΔS-1668 also produces SLG13 protein but not SRK13 protein due to the presence of the Sf1 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 ΔS-1668 is a highly self-fertile strain, routinely producing >300 pollen tubes/stigma upon self-pollination. Furthermore, ΔS-1668 stigmas were fully compatible with pollen derived from plants bearing the S13 haplotype (>300 pollen tubes produced/stigma), in contrast to wild-type S13\S1 and S13\S13 stigmas, which inhibited the development of S13-derived pollen. However, ΔS-1668 pollen failed to germinate on stigmas carrying the S13 haplotype, an incompatible reaction identical to that exhibited by pollen from wild-type S13\S1 and S13\S1 plants. Thus, DNA encompassed by the deletion in ΔS-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, ΔS-1668 stigmas, which are null for
SLG₁₃ (and only produced a low level of SLG₁₃ transcripts) accumulated SRK₁₃ transcripts to levels indistinguishable from control S₁₃S₁₃ 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 S₁₃S₁₃ and SCF₁ control stigmas, was undetectable in stigmas of the ΔS-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 ΔS-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 ΔS-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 suggests of an interaction between the two proteins. To test the possibility that...
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 (Holleck, 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,
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 ΔS-55. ΔS-55 is a self-compatible strain identified in the same screen as ΔS-1668. The ΔS-55 strain is deleted for both SLG₁₃ and SRK₁₃ (Nasrallah et al., 2000) and possesses the Sf₁ haplotype with its non-functional SRK₁₃ gene and its functional SLG₁₃ gene (see Table I). This plant hence produces only SLG₁₃ 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 stigmas as well as stigmas obtained from a deletion mutant designated ΔS-55. ΔS-55 is a self-compatible strain identified in the same screen as ΔS-1668. The ΔS-55 strain is deleted for both SLG₁₃ and SRK₁₃ (Nasrallah et al., 2000) and possesses the Sf₁ haplotype with its non-functional SRK₁₃ gene and its functional SLG₁₃ gene (see Table I). This plant hence produces only SLG₁₃ protein and allows unambiguous characterization of SLG properties in native tissue.

Maturation of the Brassica S-Locus Receptor Kinase
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 ΔS-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 reextracting 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-
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).

Figure 5. Oligomerization of membrane-associated SLG. Whole cell extract (CE), soluble, and microsome (Micro) fractions obtained from ΔS-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.

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
expression studies are now recognized as an essential and convenient tool for the biochemical analysis of plant proteins (for review, see Frommer and Ninne
mann, 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; Mont
tamat et al., 1999), Xenopus (Cao et al., 1992; Maurel et al., 1993), and mammalian COS cells (Kammerlo
her et al., 1994). Most pertinent to this study, expression of storage proteins of the maize kernel in trans
genic tobacco plants was used to demonstrate that β-zein expression has a stabilizing effect on δ-zein
(Bagga et al., 1997).

Therefore we retransformed SRK6-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, and generated 12 inde
pendent 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 SRK6-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 ex
pressed 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 pro
duced in the [SRK] transformants. They also pro
duced 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 condi
tions is specifically modified when SRK is co-expressed with SLG. When [SRK] and [SRK+SLR1] extracts were analyzed under non-reducing condi
tions, 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

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Figure 6. Chemical treatment of stigma microsome fractions. Equal amounts of microsome fraction obtained from Brassica stigmas were treated with 1 M H2NO, 50 mM DTT, 8 mM urea, 0.1 M Na2CO3, 0.2% (w/v) SDS, 1% (w/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.
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.

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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 ΔS-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 papillary 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 ε-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_11 haplotype, SLG_11 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 SLG8 and SLG13, 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 wild-type plants (Jeong et al., 1999) may contribute to the stabilization of the full-length receptor. It is also possible that in some strains, other classes of S proteins might contribute to the stabilization of SRK in the strains we analyzed. For example, the stigmas of *B. rapa* sclf1 mutant strain have been described previously (Nasrallah et al., 1988, 1992, 1994b). Pollination phenotypes of reciprocal crosses involving ΔS-1668, S13S13, and S15S13 plants were determined by monitoring pollen tube behavior by UV-fluorescence microscopy (Kho and Baer, 1968).

### MATERIALS AND METHODS

#### Plant Material and Pollination Assays

*Brassica oleracea* plants bearing the *S*, *S*13, and *S*1 haplotypes and the *Brassica campestris* (syn. *B. rapa*) sclf1 mutant strain have been described previously (Nasrallah et al., 1988, 1992, 1994b). Pollination phenotypes of reciprocal crosses involving ΔS-1668, S13S13, and S15S13 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 SLG13 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 SRKs 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 metabisul- fite, 1 mM phenylmethylsulfonyl fluoride, 10 μM 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 from *S*13, *S*15 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 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-Mₚ 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.
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 S6S6 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 (H3NO, 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 Na2CO3, 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 non-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.

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 electrophorased 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., 1989) 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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