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Pollen–Stigma Adhesion in *Brassica* spp Involves SLG and SLR1 Glycoproteins

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The adhesion of pollen grains to the stigma is the first step of pollination in flowering plants. During this step, stigmas discriminate between pollen grains that can and cannot be permitted to effect fertilization. This selection is operated by various constituents of the cell walls of both partners. Several genes structurally related to the self-incompatibility system that prevents self-pollination in *Brassica* spp are known to target their products into the stigma cell wall. We proposed previously that one of these genes, the one encoding the *S* locus glycoprotein (SLG)–like receptor 1 (*SLR1*), which is coexpressed with that encoding SLG, may participate in pollen–stigma adhesion. Here, we exploit a biomechanical assay to measure the pollen adhesion force and show that it is reduced both by transgenic suppression of *SLR1* expression and by pretreatment of wild-type stigmas with anti-SLR1 antibodies, anti-SLG antibodies, or pollen coat-protein extracts. Our results indicate a common adhesive function for the SLR1 and SLG proteins in the pollination process.

INTRODUCTION

Depending on their level of evolution, the various families of flowering plants have pollen and stigmas designed for more or less sophisticated recognition specificity. In families including the Solanaceae, Liliaceae, and Rosaceae, the stigmas are described as "wet" because they are covered with abundant viscous secretions including sugars and glycoproteins (Heslop-Harrison and Shivannah, 1977), permitting the adhesion of pollen grains efficiently but indiscriminately. The dry stigmas of more evolutionarily advanced families, such as the Asteraceae, Brassicaceae, Gramineae, and Papaveraceae, are "dry." The pollen grains adhere specifically onto these dry stigmas owing to the physicochemical complementarity of their cell wall surfaces. In the case of the Brassicaceae, the stigma is covered by a proteinaceous pellicle with waxy secretions. The external layer of the pollen cell wall, the exine, is ornamented by cavities containing a tapetally derived pollen coat lipoproteic complex including many different proteins (Doughty et al., 1993; Ross and Murphy, 1996) and lipids (Preuss et al., 1993) with high affinity for the waxes of the stigma pellicle. A particular class of these pollen coat proteins, the PCPs, is gametophytically expressed and probably excreted from the pollen protoplast (Doughty et al., 1998) to form part of the lipoprotein complex of the pollen exine layer that participates in the early contact with the stigma surface.

In addition, most plant families are able to prevent self-pollination and inbreeding, thereby promoting genetic diversity among the species. In *Brassica* spp, the single *S* locus, which controls self-incompatibility (SI), is a haplotype comprising several different genes. Among these, *SRK* (for *S* receptor kinase) and *SLG* (for *S* locus glycoprotein) are highly polymorphic. *SRK* encodes a receptor-like protein kinase targeted to the plasma membrane of the papillar cells that covers the stigma surface (Stein et al., 1991; Delorme et al., 1995); *SLG* encodes a glycoprotein secreted into the papillar cell wall (Kandasamy et al., 1989; Umbach et al., 1990) that is homologous to the predicted extracellular domain of SRK. This relationship implies that *SLG* and *SRK* have coevolved during the generation of the many *S* haplotypes (Stein et al., 1991). Due to the structure and subcellular location of SRK, Stein et al. (1991) proposed SRK to function in

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the SI signal transduction pathway, which operates after pollen reception and results in self-pollen rejection. Various *B. oleracea* and *B. campestris* mutants or *B. napus* lines that are self-compatible and are presumably deficient in the recognition of self-pollen have severely reduced SRK activity (Goring et al., 1993; Nasrallah et al., 1994).

In contrast, the role of SLGs remains undetermined. On several bases, including the structural homology observed between SLG and the extracellular domain of SRK, the subcellular location of these proteins, and by analogy with the dimerization process demonstrated for many cell surface receptors of protein kinases involved in animal signal transduction (Heldin, 1995), Stein et al. (1991) proposed that SLG and SRK in combination might bind a pollen ligand or dimerize through homophylic binding; there is, however, no experimental evidence for such associations in the case of *Brassica* spp.

Many different genes structurally related to *SLG* and *SRK* form a complex multigene family, expressed in the sexual and/or vegetative tissues of the Brassicaceae and more distantly related botanical families. This suggests that they take part in ubiquitous recognition mechanisms (Elleman and Dickinson, 1994). Among them, the *SLR1* (for *S* locus–related) gene (Lalonde et al., 1989; Trick and Flavell, 1989) is expressed in the stigma and secretes the corresponding protein into the cell walls of mature stigmatic papillae just like *SLG*. It must take some part in the pollination process, although no experimental evidence exists to suggest its role. Because it is unlinked to the *S* locus, *SLR1* cannot direct any *S* haplotype specificity in such SI.

We previously obtained results suggesting that SLR1 proteins participate in pollen–stigma adhesion, because levels of SLR1 accumulation and pollen adhesion were related in intraspecific and interspecific pollinations (Luu et al., 1997a). Candidates for pollen-borne interactors with SLR1, and hence a component of this adhesion, might be found among PCPs (Doughty et al., 1993). The PCP1 class of small (7 kD) PCPs physically interacts with both SLG and SLR1 proteins in vitro (Doughty et al., 1993; Hiscock et al., 1995). Although these particular interactions are not *S*-haplotype specific in vitro and the *PCP1* gene is not linked to the *S* locus (Stanchev et al., 1996), PCPs are potential candidates for pollen-borne ligands in the SI response because an in vivo bioassay showed that the pollen coating contains the male determinant of *Brassica* spp SI (Stephenson et al., 1997).

In this study, we present further evidence for the role of SLR1, using an improved biomechanical assay of pollen–stigma adhesion forces. We demonstrate that antisense *SLR1* plants, with reduced levels of the SLR1 glycoprotein, display significantly altered kinetics of pollen adhesion. Although early pollen adhesion remains unaffected because it is mediated essentially by the outer cell surfaces, unmodified by the transgene, later adhesion involving constituents of the deeper stigma cell wall is significantly reduced. We further show that pretreatment of the stigma surface of wild-type plants with antibodies raised against SLR1 or SLG reduces pollen adhesion, similar to pretreatment with pollen coat protein extracts. We conclude that these effects are due to the masking of the SLR or SLG proteins in the pollen–stigma interface. Our results suggest a common adhesive function for the SLR1 and SLG proteins during pollination in *Brassica* spp.

RESULTS

Transgenic Suppression of *SLR1* Reduces Pollen–Stigma Adhesion in *B. napus*

We compared pollen adhesion in wild-type *B. napus* cv Westar plants and transgenic plants with antisense-suppressed SLR1 glycoprotein levels. We selected plants homozygous for the transgene by DNA gel blot analysis from segregating T_2 progenies of the original transformants 311-10 and 311-17 described by Franklin et al. (1996). Figure 1 demonstrates the presence of the transgene *SLR1* antisense fragment (Figure 1A) and its specific effects in reducing the accumulation of *SLR1* mRNA and SLR1 glycoprotein. The accumulation of *SLG* mRNA and SLG protein is shown to be unaffected by the presence of the *SLR1* antisense transgene (Figures 1B and 1C). Position effects on the expression of the transgene (Figure 1A) probably explain the higher level of suppression of *SLR1* mRNA and SLR1 protein in line 311-17 than in line 311-10.

Theoretical models of pollen–stigma adhesion (Woittiez and Willemse, 1979) postulate that adhesion forces result from surface tension between the fluid interface formed through coalescence of the pollen coating and stigmatic secretions. Adhesion force is thus proportional to the dimensions of the meniscus and to the surface tension of the fluids. We measured the strength of the adhesion in the transgenic plants described above and in untransformed controls by centrifuging pollinated pistils immobilized in a 50% (w/v) sucrose solution, thus inducing progressive detachment of pollen (Figures 2A to 2J) by simple increase of the hydrostatic flotation effect according to Archimedes' principle (Luu et al., 1997b). We found pollen to be captured by the wild-type stigmatic surface within seconds of initial contact through an interaction of the pollen and stigma surfaces. The flow of the lipoprotein pollen coat onto the waxy stigmatic cuticle rapidly produces a meniscus at the site of pollen–stigma contact, as previously described (Dickinson and Elleman, 1985; Elleman and Dickinson, 1990, 1994, 1996; Dickinson, 1995). In the case of wild-type *B. napus*, we established the adhesion force to be $\sim 2 \times 10^{-8}$ Newtons; due to species-specific differences, this value is higher than that reported in the case of *B. oleracea* (Luu et al., 1997b). Pollen grains that were deeply embedded between the papillae and fixed by several points of contact (but not visible at the surface of papillar cell tips) resisted $\sim$10 times

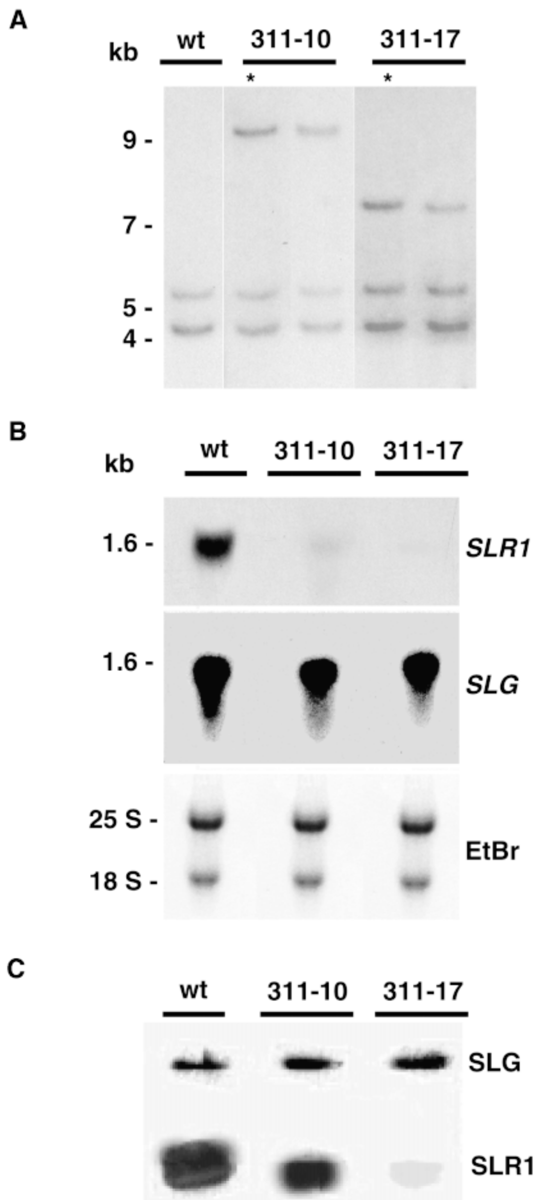

Figure 1. Suppression of *SLR1* in *SLR1* Antisense Transformants of *B. napus.*

(A) Presence of the *SLR1* antisense construct in the progeny of lines 311-10 (band at 9 kb) and 311-17 (band at 7 kb) demonstrated by analysis of HindIII-restricted DNA (10 μg of total DNA per lane). The endogenous *SLR1* genes resulted in bands at 4 and 5 kb. The homozygous plants marked by asterisks were used for subsequent pollen adhesion analyses.

(B) Accumulation of *SLR1* and *SLG* mRNAs in the wild type and lines 311-10 and 311-17 assayed by RNA gel blotting with *SLR1-29* and *SLG-29* probes. The gel at bottom shows rRNAs visualized by ethidium bromide (EtBr) staining, demonstrating equal loading (5 μg) of the lanes; numbers at left identify rRNAs by their sedimentation rate coefficients.

(C) Reduction of SLR1 protein in transformed plants. Isoelectric focusing protein blots of total protein from three stigmas were immunodetected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate staining for the monoclonal anti-SLG antibody 85-36-71 and with Fast Red (Pierce) staining for polyclonal anti-SLR1 antibody (diluted to 1:500 in TBST). The secondary antibodies used alone did not induce any antigen–antibody complex formation on the protein blots.

wt, wild type.

this force (Figure 2E, right) and were not considered further in our analysis. After initial contact, adhesion increased two to threefold over 30 min (Figure 3). However, the size of the meniscus formed at the pollen–stigma interface visualized by scanning electron microscopy (data not shown) did not significantly increase during this period in our experiments. This is in agreement with previous video-microscopic observations of pollen–stigma interactions (Dickinson, 1995). We infer that the kinetic increase of adhesion is due to changes in composition and thus to the intrinsic surface tension of the interface. These changes arose from a reorganization of pollen coat and stigma cell wall elements that has been extensively analyzed by electron microscopic observation (Dickinson and Elleman, 1985; Elleman and Dickinson, 1990, 1994) and must permit hydration of the pollen grain and subsequent pollen germination and penetration of the style.

Figure 3 shows that the strength of the initial capture of pollen grains is quite similar in *B. napus* plants transformed with the antisense *SLR1* gene construct and in untransformed Westar control plants. These results coincide with the absence of morphological differences between the stigma papillar cells of transformed and control plants: on enlargements of scanning electron microscopic images, such as those of Figure 2E, left, we measured the mean diameter of wild-type papillae (20.14 μm; SD = 1.24 μm) versus that of the papillae of transformed plants (20.32 μm; SD = 0.86 μm) and deduced the corresponding mean radius of curvature of the papillar terminal hemispheres (10.07 vs. 10.16 μm). The analysis of the mean did not show significant differences potentially resulting from transformation ($n = 30$; $t = 0.71$; $P < 0.001$), indicating that the surfaces of the stigmas coming into immediate contact with the wild-type pollen grains at the time of pollen capture are quite similar in control and transgenic *B. napus.*

The increase in pollen adhesion that would normally occur 5 min and later after pollination, however, showed a substantial reduction (Figure 3), correlating with the residual levels of *SLR1* mRNA and protein in the two transformed plants 311-10 and 311-17 (Figures 1B and 1C). SLR1 might thus participate in those events associated with the reorganization of the pollen–stigma interface during the late adhesion stages.

Masking SLR1 and SLG Proteins Reduces Pollen–Stigma Adhesion

We confirmed the postulated involvement of SLR1 in pollen–stigma adhesion by complementary experiments in which, during pollination, the binding capacity of wild-type levels of the SLR1 glycoprotein was challenged with PCP extracts and with anti-SLR1 antibodies that acted as competing ligands. Because Hiscock et al. (1995) showed that PCPs interact with both SLR1 and SLG, we used, in addition to an anti-SLR1 antiserum, an anti-SLG monoclonal antibody (Gaude et al., 1993, 1995).

In these experiments, we used *B. oleracea* lines homozygous for the haplotypes S_2, S_3, S_5, S_{29}, and *SC* (a self-compatible line expressing *SLG*, but of undetermined *S* haplotype because it is not self-incompatible; Gaude et al., 1993). We took advantage of the specific genetic and physical properties of the SLG and SLR1 proteins of *Brassica* spp. Nasrallah et al. (1991) defined two classes of haplotypes whose SLGs, although related, have significantly different structures: class I includes the pollen dominant haplotypes displaying strong and complete rejection of self-pollen grains; class II includes the pollen recessive haplotypes (*SC*, S_2, S_5, and S_{15}) with weak and incomplete self-pollen rejection. The antibodies raised against class II SLG proteins (*SC*, S_2, and S_5 in this study) recognize specifically and exclusively class II SLGs but not class I SLGs and vice versa for antibodies raised against class I SLGs (S_3 and S_{29} in this study). On the other hand, the monomorphism and systematic expression of SLR1 protein in all *Brassica* spp lines allow its detection with a single antibody preparation.

First, we verified in our system the in vitro interactions previously demonstrated between PCPs and SLR1 and SLGs (Doughty et al., 1993; Hiscock et al., 1995). PCP extracts from both S_{29} and *SC* lines were prepared according to Doughty et al. (1993); they contain highly basic proteins with pIs ranging from 9 to 9.5 (Figure 4A, Ponceau red stain-

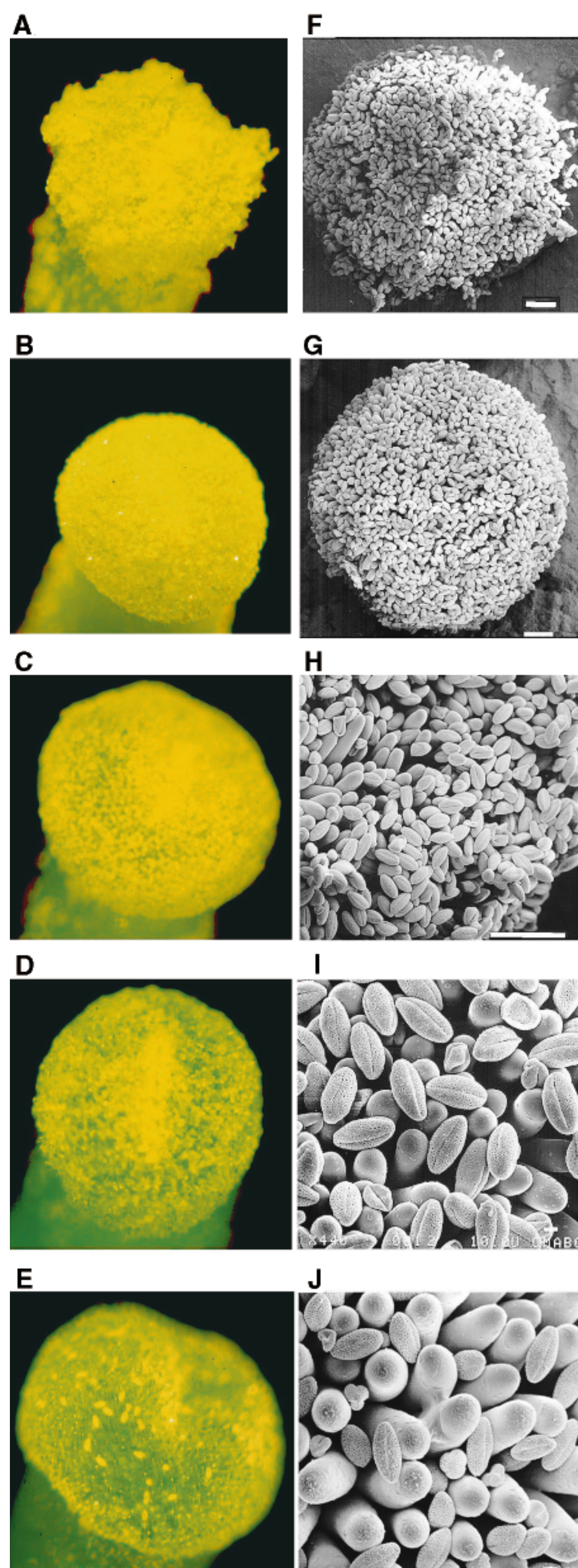

Figure 2. Detachment of Pollen Grains by Flotation from Self-Pollinated Pistils of *B. napus*.

Binocular microscopic (**[A]** to **[E]**) and scanning electron microscopic (**[F]** to **[J]**) views of stigmas progressively depleted of pollen grains.

(A) and **(F)** Pistils pollinated at saturation.

(B) and **(G)** Same pistils as shown in **(A)** and **(F)** and then dipped 30 min after pollination in 50% sucrose.

(C) and **(H)** Same pistils as shown in **(B)** and **(G)** and then centrifuged at 5000*g*, yielding flotation forces of 1.92×10^{-8} Newtons.

(D) and **(I)** Same pistils as shown in **(B)** and **(G)** and then centrifuged at 10,000*g* (3.85×10^{-8} Newtons).

(E) and **(J)** Same pistils as shown in **(B)** and **(G)** and then centrifuged at 15,000*g* (5.78×10^{-8} Newtons).

At 15,500*g* (5.97×10^{-8} Newtons), no more pollen grains were visible at the tips of the papillar cells. The mean diameter of the papillar cells of wild-type and transformed plants was estimated based on enlargements of photographs similar to the one shown in **(J)**. Bars in **(F)**, **(G)**, and **(H)** = 50 μm; bars in **(I)** and **(J)** = 10 μm.

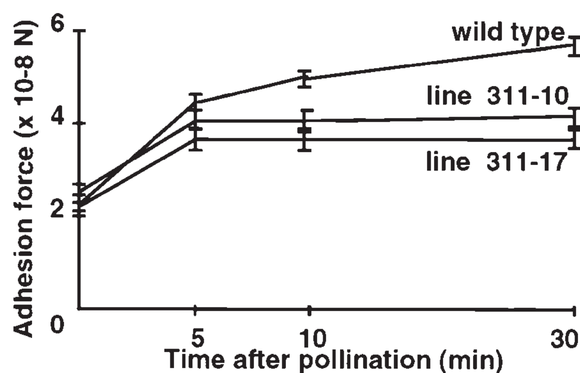

Figure 3. Kinetics of Pollen–Stigma Adhesion in *B. napus* Plants Transformed with *SLR1* Antisense Construct.

The first points (time 0 is initial capture) were measured a few seconds after the pollination of pistils with untransformed pollen. Measurements were repeated with 30 pistils to determine pistil variability. Error bars indicate ±SD. N, Newtons.

ing). We mixed phosphate buffer solutions of PCPs with stigmatic proteins from the same two lines. The protein gel blot of Figure 4B shows the modification of the electrophoretic migration on isoelectric focusing gels of SLR1 and SLG after interaction between PCPs and the stigmatic glycoproteins. All *S* haplotypes express a highly conserved SLR1 protein, and so the anti-SLR1 antibody detected bands in both the S_{29} and *SC* lines. In contrast, the anti-SLG antibody that we used detected multiple isoforms in the stigmatic extract of *SC* (bands α, β, β′, and β″) but not S_{29} (Figure 4B, stigmatic extracts). The antibodies did not react with PCPs alone. When we mixed the stigmatic extracts with PCP extracts, a fraction of the SLR1 proteins in both S_{29} and *SC* lines was complexed into two bands of interaction products with high pIs that were designated IP–SLR1. Two IP–SLG interaction products from an SLG and PCP complex, a minor one with acidic pI and a major one with basic pI, appeared only in the *SC* material (Figure 4B). SDS treatment of IP–SLR1 and IP–SLG (data not shown) resulted in complete dissociation of the complexes, yielding the original, undegraded stigmatic SLR1 and SLG proteins as well as small polypeptides (∼7 kD), with physical characteristics corresponding to those of the PCPs previously described (Doughty et al., 1993; Hiscock et al., 1995).

To evaluate the effects of PCPs on pollen–stigma adhesion, we immersed pistils in PCP solutions derived from S_{29} and *SC* pollen and compared the adhesion forces with those measured after immersion of the pistils in phosphate buffer. Under these unusual pollination conditions, we observed that prewetted pistils captured pollen grains with an increased adhesion strength and allowed very rapid swelling and hydration of the pollen. We observed the same quantitative effects with water, phosphate buffer, and preimmune serum (as a control for treatments with SLG and SLR1 antisera). After this initial acceleration of events immediately after pollination, pollen–stigma complexes did not evolve for ∼2 hr, as previously reported by Zuberi and Dickinson (1985). The pollen adhesion forces remained constant during this period, in contrast with the increase observed in normal pollinations.

We found that PCP pretreatment 10 min before pollination, by immersion of pistils in PCP solutions from S_{29} and *SC* pollen, reduced the force of pollen–stigma adhesion compared with control pistils wetted with water or phosphate buffer (Figure 5). We obtained similar effects in self-pollinations and cross-pollinations between the two lines, in agreement with the lack of *S* haplotype specificity for interactions between the mass of PCPs and stigmatic glycoproteins (Doughty et al., 1993; Hiscock et al., 1995). The subfraction of the PCP family potentially containing *S*-specific male determinants represents only a minor part of total PCPs (Stephenson et al., 1997), undetected in our analysis. We interpret this reduction of adhesion as a result of the masking of SLR1 and/or SLGs by the PCP proteins, making them unavailable or inaccessible to pollen ligands involved in adhesion. Formalin, which is usually added to the solution to block potential evolution of adhesion during centrifugation, had no effect on the final measurements in control experiments.

In additional experiments, we used antibodies to mask SLR1 and SLGs. Protein gel blots (Figure 6) confirmed the class specificity of the antibodies for stigmatic glycoproteins (Gaude et al., 1993, 1995). The SLR1 antiserum bound to protein bands in all four haplotypes S_{29}, S_3, *SC*, and S_5. In contrast, the antibody raised against class II SLG bound exclusively to SLG bands in the recessive class II haplotypes *SC* and S_5 but not to any SLG bands in the dominant class I haplotypes S_3 and S_{29}. Pretreatment of stigmas with SLR1 antiserum significantly reduced pollen adhesion, as compared with water or preimmune treatments, in all *S* haplotypes, including the S_2 line (Figure 7). In contrast, the antibody raised against class II SLG produced a significant decrease in adhesion only in the class II recessive haplotypes S_5 and *SC*. The S_2 line is somewhat different than the other lines studied: we had demonstrated that this haplotype accumulates unusually low levels of SLG_2 glycoproteins and mRNAs but normal amounts of SLR1 (Gaude et al., 1995). We observed a consequent low inhibition (∼10%) of pollen adhesion on S_2 stigmas by the class II SLG antiserum contrasting with a higher inhibition (∼30%) by the SLR1 antiserum, or by the class II SLG antiserum on S_5 and *SC* (Figure 7).

We tested whether the SLR1 and SLG antisera reduced pollen adhesion in an additive manner: we compared pretreatment with an equal mixture of antisera to pretreatments in which each type was diluted by half with preimmune serum. In the recessive class II haplotypes *SC* and S_5, we observed an additive effect (Figure 7); however, in the dominant class I haplotypes S_3 and S_{29}, in which the SLG antibody was not cross-reactive, the mixture was no more

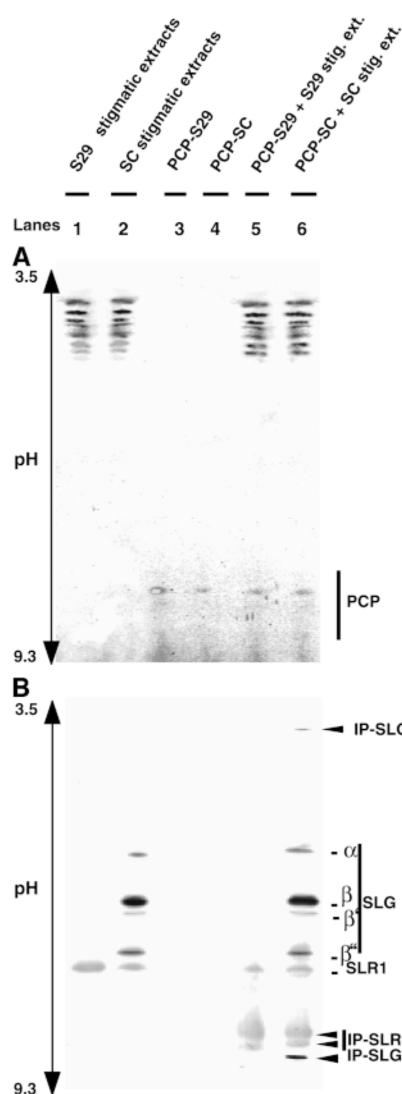

Figure 4. Interaction Products of *B. oleracea* PCPs and Stigmatic Proteins.

(A) Ponceau red staining.

(B) Detection of interaction products.

Ponceau red staining of the filter demonstrates equal loading of the lanes and illustrates the basic pI of PCPs. Lanes 1 and 2 contain control stigmatic proteins from *B. oleracea* (13 μg); class II SLGs were detected only in *SC*, but SLR1 was found in both lines **(B)**. Lanes 3 and 4 contain control PCP proteins from *B. oleracea* (9 μg); the PCPs did not react with SLR1 or SLG antibodies **(B)**. Lanes 5 and 6 contain mixtures of interacting stigmatic proteins (13 μg; stig. ext.) and PCPs (9 μg). Arrowheads in **(B)** indicate the position of the interaction products between SLR1/SLG and PCPs. Fast Red (Pierce)–stained SLR1 and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate–stained class II SLG polypeptides were immunodetected, as described in Figure 1, with 1:500 dilutions of the sera in TBST. Bands α, β, β′, and β″ are isomorphic forms of SLG proteins in *SC* (Gaude et al., 1993).

active in reducing pollen adhesion than was the diluted anti-SLR1 antiserum. These data demonstrate separate contributions to adhesion by each type of stigmatic glycoprotein.

To ascertain that the antibodies used in these studies do not reduce pollen adhesion by blocking the accessibility of the stigma to pollen ligands through simple steric effects, we verified that incubation using an SLG/SLR1 nonimmune rabbit antiserum raised against chicken ovalbumin (Sigma C6534) gives adhesion forces similar to water. In addition, we visualized the penetration of antibodies into the stigma cell wall after serum incubation plus pollination: after fixation, the localization of anti-SLG IgGs inside the cell wall was assessed using a secondary anti–mouse antibody labeled with immunogold (Figures 8A and 8B). The gold particles can be seen only at the places where pollen grains contact the stigma surface, demonstrating that IgGs have not penetrated into the cell wall by simple diffusion but have been translocated into it by the reorganization of the pollen–stigma interface. Control stigmas (not incubated in an anti-SLG antiserum and not pollinated) did not show any non-

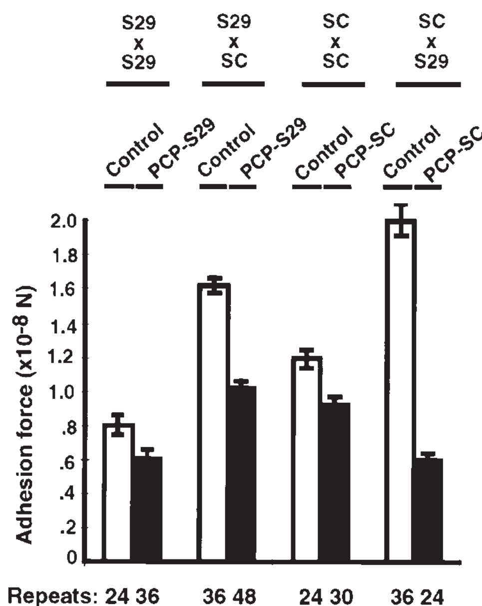

Figure 5. Inhibition of Pollen–Stigma Adhesion after PCP Treatment of *B. oleracea* Pistils.

Six stigmas were immersed in drops of solutions (1.3 μg μL^{-1} in 0.05 M phosphate buffer, pH 7.0) of PCPs extracted from S_{29} pollen (PCP-S29) or *SC* pollen (PCP-SC), air dried for 10 min, and pollinated. Six control stigmas were treated with phosphate buffer. Pollen adhesion forces were determined 30 min after pollination for the four possible self-pollinations ($S_{29} \times S_{29}$ and $SC \times SC$) or cross-pollinations ($S_{29} \times SC$ and $SC \times S_{29}$). Measurements were repeated on as many pistils as indicated by Repeats. Error bars indicate the standard deviation of adhesion forces. N, Newtons.

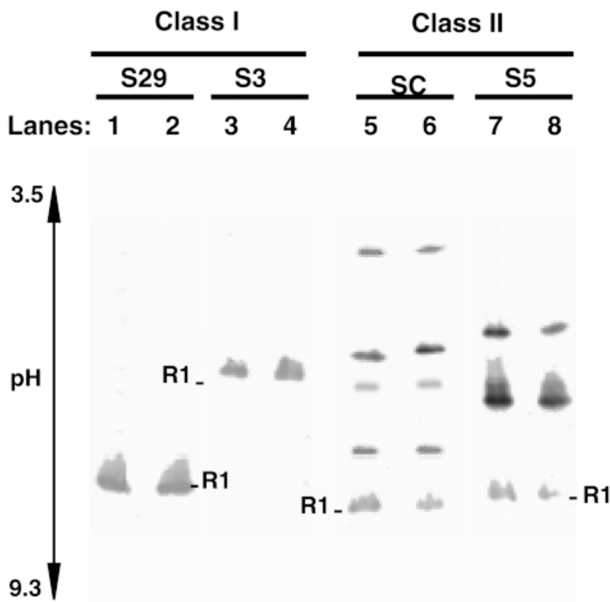

Figure 6. Specificity of Anti-SLR1 and Anti-SLG Antibodies.

Protein gel blot of total stigmatic proteins (10 μg) from lines S_{29} (lanes 1 and 2), S_3 (lanes 3 and 4), *SC* (lanes 5 and 6), and S_5 (lanes 7 and 8), immunodetected as described in Figure 1, with 1:500 dilutions of the sera in TBST. Fast Red–stained SLR1 proteins are present in all four haplotypes and denoted by R1. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate–stained recessive SLG proteins are detected exclusively in the recessive S_5 and *SC* haplotypes (Gaude et al., 1993). The secondary antibodies used alone did not induce any antigen–antibody complex formation on the protein gel blots.

specific interactions of the cell wall with this secondary antibody (Figure 8C).

Finally, we ensured the specificity of antibody inhibition by depleting aliquots of sera against preparative protein blot bands of SLR1 or SLG extracts. The level of antibodies was reduced by ~80 to 90%: pollen adhesion was no longer significantly inhibited in the presence of such antibody-depleted solutions (Figure 7, plants *SC* and S_3).

DISCUSSION

We compared the force of pollen adhesion in wild-type plants under a number of experimental treatments: transgenic suppression of *SLR1* in *B. napus*, pretreatment of *B. oleracea* pistils with solutions of PCPs, and pretreatment with anti-SLR1 or anti-SLG antibodies. Each treatment produced a significant reduction in "late" pollen–stigma adhesion. We obtained somewhat greater effects on *B. oleracea* with the PCPs and antibodies due to the high concentrations of the solutions used here (undiluted rabbit serum or pure mouse ascite, and as highly concentrated a PCP solution as was experimentally feasible) than were obtained on *B. napus* suppressed in SLR1 production. The high titers of masking molecules produced a highly effective competition for the available binding sites of wild-type levels of SLR1 or SLG, whereas transgenic ablation of *SLR1* expression was incomplete, even in the 311-17 line, in which SLR1 protein was only faintly detectable.

The quantitative aspects of pollen–stigma adhesion and of its perturbation analyzed here are, however, not simple: none of the interactions followed the typical and simple laws of mass action, chemical equilibrium, and kinetics. This lack of simplicity has several causes. All biological reactions pertaining to pollen–stigma adhesion occur in a semisolid state, in which the concentrations of the reactants do not necessarily rapidly reach the same values in all cases; although PCPs and antibodies were used in liquid solution, their targets are located in the pollen–stigma interface or in the stigma cell wall. These wall components probably behave as imperfect solvents in biological reactions. Several facts demonstrate that pollen–stigma adhesion is under polygenic control (Preuss et al., 1993), involving many of the various biological components forming both the pollen and the stigma surfaces. We performed experiments and accumulated data on several genotypes or species of Brassica. *B. napus* has the advantage of a higher propensity to transformation; *B. olerac*ea offers well-characterized *S* genotypes, and our laboratory has produced and characterized specific antisera against SLR and SLG proteins (Gaude et al., 1993, 1995; Delorme et al., 1995). These lines, however, are not near-isogenic: in addition to differences concerning their *S* loci, they also differ in their genetic backgrounds and in many components of the pollen and stigmatic surfaces. This partially explains why we observed quantitative differences in the efficiency of the same treatments on different genotypes.

Water itself modifies pollen–stigma interaction in *Brassica* spp (Zuberi and Dickinson, 1985). After an initial acceleration of pollen capture and hydration by wetted stigmas, no morphological changes or increase in adhesion occurred during the first 2 hr, altering the normal kinetics of adhesion, hydration, and germination. However, later stages of pollination and fertilization proceeded normally, and in-the-plant treatment of pistils with the anti-SLR1 antiserum does not significantly affect seed set (Fobis, 1992). Despite this caveat concerning the effects of the experimental treatment, we demonstrate a strict haplotypic specificity of the anti-SLG monoclonal antibody in that pollen adhesion was reduced only in the recessive class II *S* haplotypes (*SC* and S_5). In S_2 plants, which regularly have unusually low amounts of SLG_2 glycoproteins (Gaude et al., 1995), this inhibition of adhesion was itself reduced, just as it was after deliberate depletion of the anti-SLR1 and anti-SLG antisera. This result further demonstrated the specificity of the antisera on pollen adhesion and validates the use of aqueous

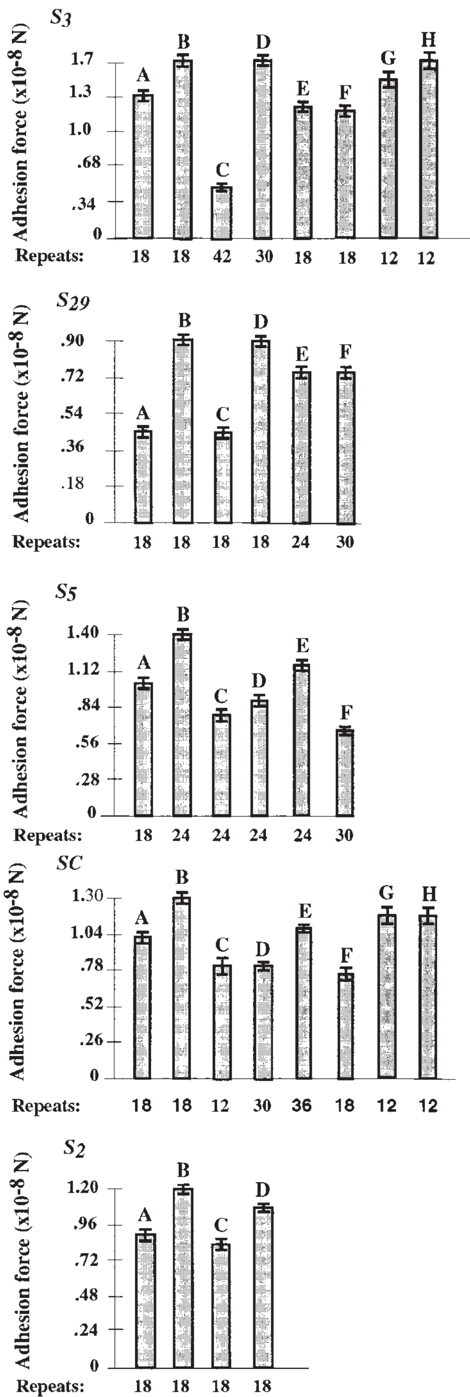

Figure 7. Decrease in Pollen–Stigma Adhesion after Anti-SLR1 and Anti-SLG Antibody Pretreatment of *B. oleracea* Pistils.

Pistils from the dominant class I haplotypes S_{29} and S_3 and from the recessive class II haplotypes S_5, *SC*, and S_2 were pollinated under normal conditions (columns A, normal dry pollination) or dipped for 10 seconds in water or in various sera and air dried for 10 min before pollination. The sera used are as follows: columns B, water or pure undiluted rabbit anti–chicken ovalbumin antiserum (C6534; Sigma); columns C, pure undiluted rabbit anti-SLR1 antiserum or columns D, pure undiluted mouse ascites SLG class II antiserum; columns E, SLR1 antiserum diluted with Sigma C6534 antiserum; columns F, mixture in equal amounts of rabbit anti-SLR1 and mouse ascites anti-SLG antisera; and columns G, rabbit anti-SLR1 antiserum, or columns H, mouse ascites SLG antiserum depleted in specific antibodies. Pollen adhesion forces were determined 10 min after self-pollination. The number of pistils analyzed is indicated by the number of Repeats and was used to estimate the experimental error bars; we consider that adhesion forces are different when the differences are significantly higher than experimental errors. N, Newtons.

solutions of antibodies and PCPs to mask SLG and SLR1 proteins.

We described the kinetics of pollen adhesion in transgenic *B. napus* plants in normal dry pollination conditions: these experiments entirely obviated the compounding effects of water and allowed us to define the stage of pollination at which SLR1 proteins participate in adhesion. The morphologically unmodified stigmas of transgenic plants captured pollen grains with the same efficiency as untransformed Westar plants. But *SLR1* suppression did affect a later stage of adhesion, between 5 to 10 and 30 min after the first pollen contact, resulting in a substantial decrease in pollen adhesion forces relative to untransformed plants. This observation is consistent with the localization of SLR1 and SLG: Kandasamy et al. (1989, 1991) and Umbach et al. (1990) demonstrated by electron microscopy immunocytology that these molecules are located inside the cell wall rather than in the pellicle. SLR1 and SLG probably do not participate in initial pollen capture processes mediated only by external pollen and stigma surfaces. They probably interact later with pollen ligands after the pollen peptides have flowed onto the stigma surface and have been translocated to deeper parts of the cell wall, due to the complete reorganization of the initial pollen–stigma interface (Dickinson and Elleman, 1985; Elleman and Dickinson, 1990, 1994, 1996; Dickinson, 1995).

Our immunocytological analysis demonstrates that the reorganization of the stigma cell wall after pollination also permits the penetration of IgGs (150 kD) deposited on the stigma pellicle: the IgGs do not diffuse freely toward the stigmatic glycoproteins, but they are translocated into the stigma cell wall, where they interact with SLR1 and SLGs. These data contribute to document the potential role of plant cell walls in cell communication.

Our experiments show that two types of ligand molecules, PCP and antibodies, have similar effects on pollen adhesion. Among the pollen components identified thus far, PCPs seem the best candidates as pollen ligands taking part in pollen–stigma adhesion. Like pollen adhesion itself, the greater part of them is not specific to the *S* haplotypes (Doughty et al., 1993), although a subfraction of the PCP family might contain *S*-specific male determinants, as recently shown using an in vivo bioassay (Stephenson et al.,

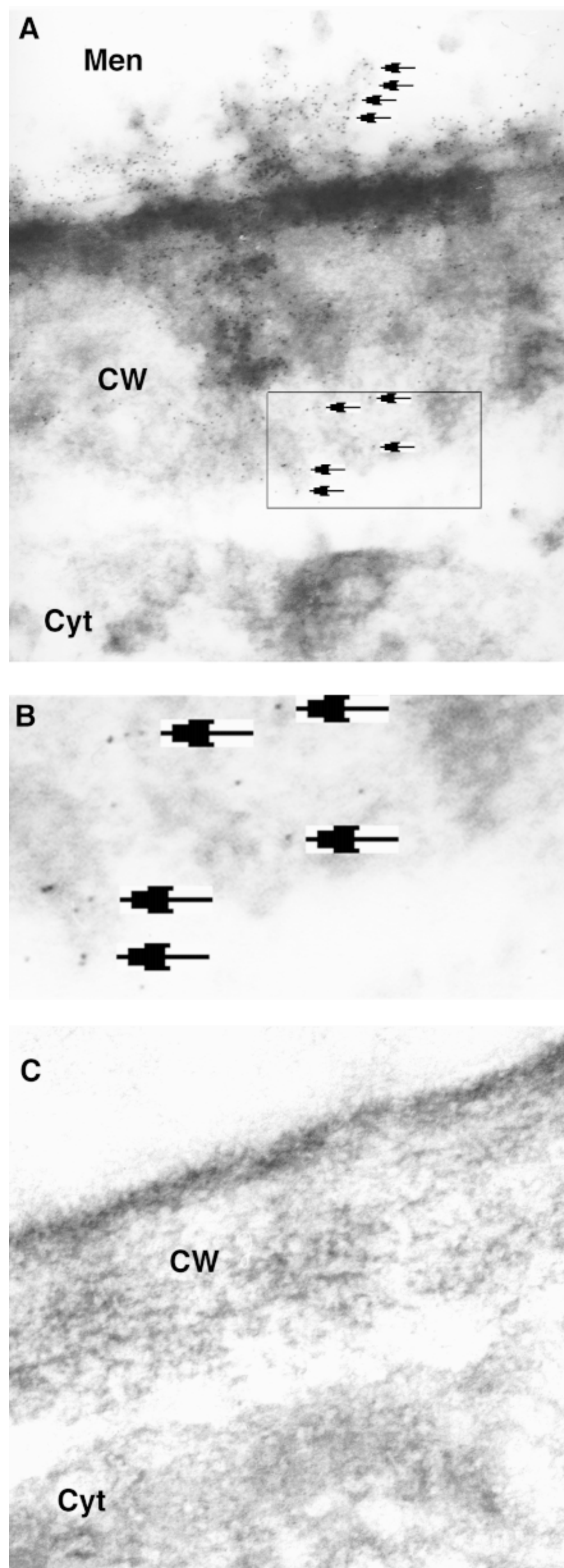

Figure 8. Immunocytological Demonstration of IgG Penetration Inside the *B. oleracea* Stigma Cell Wall.

The results of transmission electron microscopic analysis of cryosections of stigma pollinated after pretreatment with the mouse monoclonal anti-SLG antibody 85-36-71 are shown.

(A) The cell wall, as well as the interface between the stigma papilla and the pollen grain, is labeled with gold particles (arrows).

(B) The boxed area of **(A)** is enlarged for better visualization of the 10-nm gold particles.

(C) The absence of nonspecific interactions between the cell wall and the secondary antibody is demonstrated.

CW, cell wall; Cyt, cytoplasm; Men, meniscus. Magnification for **(A)** and **(C)** is ×52,000; for **(B)**, ×135,000.

1997). They are abundant in the pollen cell wall and contact with abundant SLR1 and SLG proteins in the stigma cell wall. Furthermore, they interact in vitro with SLR1 and SLG, possibly through intermolecular associations between the many cysteine residues present in each type of protein (Stanchev et al., 1996).

Pollen adhesion forces, although apparently mediated by both SLR1 and SLG, do not depend on the specificity of SI in *B. oleracea* (Luu et al., 1997b). This observation would accord with the fact that *SLR1* is unlinked to the *S* locus and so cannot coevolve with the incompatibility haplotype. However, the suggestion that *SLG* is involved in a process unrelated to SI is novel and merits discussion. Nasrallah et al. (1970) first proposed that *SLG* might participate directly in the control of SI because of its linkage to the *S* locus and a correlation between the developmental regulation of its expression and the acquisition of competence for SI. However, this correlation has proved far from perfect. Self-compatible *B. napus* lines express SLGs at high levels (Robert et al., 1994), and several self-incompatible lines of *B. oleracea* (S_2, for instance) produce only very low quantities of SLG (Gaude et al., 1995). The *scf1* (stigma compatibility factor 1) mutation described in *B. campestris* (Nasrallah et al., 1992) breaks down the stigmatic SI response with plants having coordinately reduced levels of *SLG*, *SLR1*, and *SLR2* mRNAs but a wild-type level of *SRK* mRNA. The authors proposed that downregulation of just one of the *S* locus genes (i.e., *SLG*) might be sufficient to disrupt the SI system.

Transformation experiments designed to identify the role of SLG used *SLG* (Toriyama et al., 1991) or *SLG*/*SRK* antisense constructs (Conner et al., 1997); they showed complex, multiple gene-silencing effects that did perturb SI responses but made interpretation difficult.

If the role of SLG were primarily adhesive, then recognition might be exclusively mediated through SRK. SLG diversity, hitherto interpreted as evidence of a role in incompatibility, would simply be the by-product of *SLG*/*SRK* coevolution (Stein et al., 1991). Alternatively, our findings could hint at a dual role for SLG in which ancestral nonspecific pollen adhesion properties (shared with SLR1) have been enhanced with new recognition functions acquired during

the evolution of SI. Recognition would have been supplied through the recruitment of SRK and its protein kinase activity. This evolution would be reminiscent of the relationship between cell adhesion and immunological recognition of self and non-self in vertebrates, where the structural homologies between the cell adhesion molecules and IgGs suggest that the evolution and origin of the immune system were based on precursors of CAMs (or cell adhesion molecules), adhesion molecules of intercellular signaling, and morphogenesis (Edelman, 1987).

Based on our experimental data, SLR1 and SLGs seem to be involved primarily in pollen–stigma adhesion, perhaps through interaction with PCPs. This finding compels us to reconsider the role proposed until now for SLG and, in turn, the interactions, if any, between SLG and SRK during self-pollination and the mechanism of action of SRK itself. We should also take into account the identified function of SLG and SLR1 proteins to better understand the evolution of the multigenic *S* family and the involvement of its members in various systems of cell signaling.

METHODS

Plant Material

The self-incompatible lines S_2, S_{29}, and S_5 of *Brassica oleracea* var *alboglabra* were obtained from D.J. Ockendon (Horticulture Research International, Wellsbourne, UK). The S_3 (self-incompatible) and *SC* (self-compatible) lines already characterized by Gaude et al. (1993) were produced at INRA (Rennes, France). S_{29} and S_3 are pollen dominant, class I haplotypes, whereas *SC*, S_2, and S_5 are pollen-recessive class II haplotypes, according to the classification of *S* haplotypes proposed by Nasrallah et al. (1991). *B. napus* cv Westar plants homozygous for the antisense SLR1 transgene were selected from the T_2 families of the 311-10 and 311-17 primary transformants (Franklin et al., 1996). Untransformed Westar plants were used as controls.

Pollen Coating Extraction

Pollen coating proteins were extracted according to Doughty et al. (1993) from ~300 mg of *B. oleracea* (S_{29} or *P57Sc* lines) pollen suspended in 3 mL of cyclohexane. The supernatant was collected after centrifugation (14,000*g* for 20 sec) and air evaporated. The pollen coat extracts were resuspended in 50 mM phosphate buffer, pH 7.0, with an ultrasonic cell disrupter (Sonics Inc., Danbury, CT). The pollen coating proteins were collected in the supernatant after centrifugation (18,000*g* for 30 min) and adjusted to a concentration of 1.3 mg mL^{-1}.

DNA and RNA Gel Blotting

DNA gel blot analyses were performed with DNA extracted from leaves, digested with HindIII, fractionated by agarose electrophoresis (10 μg per lane), and alkaline transferred onto Hybond N+ nylon membranes (Amersham), according to the manufacturer's instructions. Total stigmatic RNA (5 μg per lane) extracted from stigmas treated with phenol–chloroform (Luu et al., 1997a), digested with RNase-free DNase (Boehringer Mannheim), and fractionated by formaldehyde agarose gel electrophoresis (Sambrook et al., 1989) were alkaline transferred onto Hybond N+ nylon membranes. Hybridization probes were *SLR1-29* and *SLG-29* (Trick and Flavell, 1989) inserts excised from their plasmid vectors. In vitro radiolabeling of the probes was performed by random priming (Boehringer Mannheim labeling kit) and hybridizations (60°C in 4 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate]), and washes (60°C in 0.1 × SSC) were under stringent conditions .

Antibodies and Immunochemistry

Isoelectric focusing gels and protein blots were made according to Gaude et al. (1993). Immunodetection was performed using 1:500 dilutions of a polyclonal anti-SLR1 antibody produced by immunization of a rabbit with the synthetic SLR1 N-terminal peptide TNTLSPNEALTISSN, and of a monoclonal anti-SLG antibody (monoclonal antibody 85-36-71; Gaude et al., 1993, 1995) produced by immunization of mice with the synthetic SLG N-terminal peptide IYVNTLSSSEC. Both peptides were cross-linked to ovalbumin by *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester. The rabbit polyclonal antibody produced during the late stages of immunization was predominantly of the IgG class, as classically described (Harlow and Lane, 1988). The mouse monoclonal antibody was of the IgG subclass 1 with K light chains, as determined using the IsoStrip (Boehringer Mannheim) mouse monoclonal antibody isotyping kit. Antigen–antibody complexes were detected with secondary antibodies directed against rabbit (S372B; Promega) or mouse (S373B; Promega) IgGs and visualized by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad) (SLG) and Fast Red (Pierce Chemical Co., Rockford, IL) (SLR1) staining (Gaude et al., 1993). The secondary antibodies used alone did not induce any antigen–antibody complex formation on the protein blots.

Anti-SLR1 and anti-SLG antisera were depleted of specific IgGs by incubating 10 μL of each serum diluted in 500 μL of TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% [w/v] Tween 20) on nitrocellulose spots containing SLR1 and SLG cut out from a preparative protein blot of *SC* stigmatic proteins (~5 cm^2 containing 60 μg of each protein extracted from 400 stigmas). The incubated sera and the washings of the filters were reconcentrated in TBS to a final volume of 10 μL by centrifugation on Amicon (Beverly, MA) microconcentrators. Immunochemical staining of SLG and SLR1 protein blots with these depleted sera was equivalent to that obtained with 1:2500 to 1:5000 dilutions of normal sera, indicating that 80 to 90% of the specific IgGs had been removed by the treatment.

Scanning Electron Microscopy

Preparation of samples and observations were as described by Luu et al. (1997a, 1997b).

Immunoelectron Microscopy

For electron microscopy, the procedure described by Tokuyasu (1980) was used. The pistils were immersed in freshly prepared 2% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, 0.1 M sodium phos-

phate buffer, pH 7.4, for 6 hr at room temperature. Cryosections were performed essentially as described previously (da Silva Conceição et al., 1997). Grids were floated on drops in successive solutions at room temperature. After blocking in 10% (w/v) BSA in saline phosphate buffer, sections were incubated in 1:50 goat anti–mouse IgG conjugated to 10-nm colloidal gold particles (British Biocell International, Cardiff, UK) diluted in PBS containing 0.1% (w/v) BSA (1 hr at room temperature). The grids were washed in distilled water and stained according to Griffiths et al. (1983). The sections were observed with an electron microscope (model H600; Hitachi, Tokyo, Japan) operating at 75 kV.

Measurement of Pollen Adhesion

Pistils were self-pollinated, except for the case of transgenic plants pollinated by untransformed Westar pollen, or as otherwise indicated in Figure 6. Pollen–stigma adhesion forces were measured by flotation of pollinated pistils in 50% (w/v) sucrose solution with a density ($\rho = 1.19$) greater than that of pollen grains ($\rho = 1.15$). For each measurement, six pollinated pistils were placed in 1.5-mL Eppendorf tubes filled with 2% (v/v) formalin, 50% (w/v) sucrose solution through 0.5-mL tubes punctured at their bottom (Luu et al., 1997b). They were centrifuged at constant acceleration for 10 min at 20°C to remove pollen grains by simple hydrostatic forces. After centrifugation, the pistils were either rinsed in water and observed with a binocular microscope for routine measurements or processed for scanning electron microscopy for fine description of the process. The acceleration necessary to release all the pollen grains from the surface of the papillar tips of the six pistils was recorded and used to estimate the pollen adhesion force (Luu et al., 1997b). In the case of pretreatments with PCPs or antibodies, the stigmas were dipped for 10 sec in a drop of PCP solution or undiluted serum, air dried for 10 min, and then pollinated. To obtain maximal effects on pollen–stigma adhesion, PCP and antibodies were used at the highest concentration experimentally possible, that is, undiluted for the sera and at a concentration of 1.3 mg mL^{-1} for PCPs. The pollen adhesion measurements were made 30 min after pollination. Control pollinations were made on stigmas prewetted in water, 50 mM phosphate buffer, or rabbit anti–chicken ovalbumin serum (C6534; Sigma). Two percent formalin had been added to the sucrose flotation solution to block the potential evolution of adhesion during the flotation process (Luu et al., 1997b). We verified that addition of formalin did not modify the measured adhesion forces, whatever the pollination protocol used (i.e., with or without PCP or antibody solutions, on dry or on prewetted stigmas).

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