Isolation and characterization of pollen coat proteins of *Brassica campestris* that interact with *S* locus-related glycoprotein 1 involved in pollen–stigma adhesion

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Adhesion of pollen grains to the stigmatic surface is a critical step during sexual reproduction in plants. In *Brassica*, *S* locus-related glycoprotein 1 (SLR1), a stigma-specific protein belonging to the *S* gene family of proteins, has been shown to be involved in this step. However, the identity of the interacting counterpart in pollen and the molecular mechanism of this interaction have not been determined. Using an optical biosensor immobilized with *S* gene family proteins, we detected strong SLR1-binding activity in pollen coat extracts of *Brassica campestris*. Two SLR1-binding proteins, named SLR1-BP1 and SLR1-BP2, were identified and purified by the combination of SLR1 affinity column chromatography and reverse-phase HPLC. Sequence analyses revealed that these two proteins (*i*) differ only in that a proline residue near the N terminus is hydroxylated in SLR1-BP1 but not in SLR1-BP2, and (*ii*) are members of the class A pollen coat protein (PCP) family, which includes PCP-A1, an SLG (*S* locus glycoprotein)-binding protein isolated from *Brassica oleracea*. Kinetic analysis showed that SLR1-BP1 and SLR1-BP2 specifically bound SLR1 with high affinity (K_d = 5.6 and 4.4 nM, respectively). The *SLR1-BP* gene was specifically expressed in pollen at late stages of development, and its sequence is highly conserved in *Brassica* species with the A genome.

Sexual reproduction in plants depends on highly specific interactions between pollen and the pistil. These interactions are the basis of interspecific and intraspecific recognition systems, which allow the pistil to distinguish among genetically diverse ranges of pollen grains arriving at the stigma. Germination and growth of "appropriate" pollen grains are selectively promoted, whereas "inappropriate" pollen grains are selectively inhibited. One notable example of an intraspecific recognition system is self-incompatibility (SI) (1).

In *Brassica*, SI is controlled by a single polymorphic locus, termed the *S* locus. Two stigmatically expressed polymorphic genes have been identified at the *S* locus. One is the *S* locus glycoprotein (*SLG*) gene, which encodes a secreted glycoprotein (2, 3), and the other is the *S* locus receptor kinase (*SRK*) gene, which encodes a receptor-like serine/threonine protein kinase (4). SRK is the sole determinant of the *S* haplotype specificity of the stigma (5); its extracellular domain, which is highly similar to SLG, is thought to interact with the pollen *S* determinant of the same *S* haplotype. Recently, the gene encoding the pollen *S* determinant has been identified (6–8). This gene, designated *SP11* (*S* locus protein 11) or *SCR* (*S* locus cysteine-rich), encodes a novel class within a family of proteins named the pollen coat protein (PCP) family (9, 10). It is hypothesized that interactions between SP11/SCR and SRK elicit a signaling cascade within the surface cells of the stigma, the papillae, leading to the rejection of self-pollen. The role of SLG in SI is enhancing the recognition process between the stigma and self-pollen (5).

The *Brassica* genome contains a number of *SLG*-like genes that have been grouped in the *S* gene family (or *S* multigene family) (11, 12). Two such members, *SLR1* (13–16) and *SLR2* (17), have been shown to be expressed specifically in the stigmatic papillar cell. They are not linked to the *S* locus (15, 17), but they also encode secreted glycoproteins that share very similar primary structural features with SLGs. Because the sequences of SLR1 and SLR2 are highly conserved among *Brassica* species, they are thought to play essential roles in pollination (18). To test this hypothesis, transgenic plants of *Brassica napus* carrying an antisense *SLR1* gene were analyzed (19, 20). The stigmas of those that produced reduced levels of SLR1 were found to have significantly reduced ability in pollen adhesion, suggesting that SLR1 is involved in the pollen–stigma adhesion process.

Although how SLR1 is involved in pollen–stigma adhesion has not been elucidated, it is predicted that SLR1 interacts with some components on the pollen surface during adhesion. Doughty *et al.* (9, 10) analyzed pollen coat extracts of *Brassica oleracea* by using a band-shift assay in IEF (isoelectric focusing) gels and identified a basic 7-kDa protein, termed PCP-A1 (protein 1 of class A PCP), which interacted with SLGs. They also suggested that the pollen coat of *B. napus* contains proteins that bind SLG and SLR1; however, the identity of these proteins and their binding affinities for SLG and SLR1 were not determined (10).

In this study, we used an optical biosensor along with chromatographic methods to identify and purify two pollen coat proteins of *Brassica campestris*, named SLR1-binding protein 1 (SLR1-BP1) and SLR1-binding protein 2 (SLR1-BP2), both of which specifically bound SLR1 with high affinities. Sequence analyses revealed that these two proteins were essentially identical in their sequences, except that a proline residue near the N terminus was hydroxylated in SLR1-BP1, but not in SLR1-BP2. Database searches showed that these two proteins are new members of the class A PCP family of proteins. These results suggest that the interactions of the *S* gene family of proteins and the PCP family of proteins, in addition to their presumed role in mediating the signaling pathway during incompatible pollina-

Abbreviations: SI, self-incompatibility; SLG, *S* locus glycoprotein; SRK, *S* locus receptor kinase; SP11, S. locus protein 11; SCR, *S* locus cysteine-rich; PCP, pollen coat protein; SLR1, *S* locus-related glycoprotein 1; SLR1-BP1 (or -BP2), SLR1-binding protein 1 (or 2); MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB035560–AB035564).

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tion, also might be involved early in the process of compatible pollination.

Materials and Methods

Plant Materials. *B. campestris* (syn. *rapa*) S_8 and S_9 homozygotes have been described previously (21). *B. oleracea*, *B. napus*, *Brassica nigra*, *Brassica juncea*, and *Raphanus sativus* used in this study were from the genetic stock maintained in Takii Seed (Kyoto).

Preparation of SLGs and SLR1. SLG_8 and SLG_9 were purified from stigmas of *B. campestris* S_8 and S_9 homozygotes by using a previously described procedure (21). SLR1 (previously named *NS*-glycoprotein) (22) was purified from stigmas of *B. campestris* as described previously (13).

Biosensor Measurements. All measurements were performed on BIAcore 2000 equipped with a CM5 sensor chip (Amersham Pharmacia). SLG_8, SLG_9, and SLR1 were immobilized separately to the parallel flow cells of a sensor chip. The amount of each immobilized protein was adjusted to approximately 2,000 response units. One parallel flow cell was derivatized under identical conditions in the absence of protein and used as a sham-derivatized control cell. All binding curves were corrected for background by subtracting those measured from the control cell. Binding experiments were performed with a constant flow rate at 10 μl/min in HBS-EP buffer (10 mM Hepes/150 mM NaCl/3 mM EDTA/0.005% surfactant P20, pH 7.4; Amersham Pharmacia). The sensor chip was regenerated after each run with 10 μl of HBS-EP containing 2 M NaCl. Kinetic constants were calculated by using BIAEVALUATION 3.0 software.

Extraction of Pollen Coat Proteins. Pollen coat proteins were extracted from *B. campestris* S_8 and S_9 homozygotes by using cyclohexane according to the procedure described by Doughty *et al.* (9). After cyclohexane was vaporized, the residue was resuspended in HBS-EP. Lipids and insoluble materials were removed by centrifugation and subsequent filtration with a 0.22-μm filter unit (Milex GV; Millipore). Protein concentrations were determined by Bradford assay (Bio-Rad).

Purification of SLR1-BPs. SLR1 affinity column chromatography was performed on a SMART system (Amersham Pharmacia). Purified SLR1 (1 mg) was coupled to *N*-hydroxysuccinimide-activated Superose PC 3.2/2 column according to the manufacturer's instructions. The pollen coat extract (0.5 ml, containing about 200 μg of protein) from S_9 homozygotes was passed through the column at a flow rate of 0.1 ml/min. The column was washed with 1.3 ml of HBS-EP and eluted with HBS-EP containing 2 M NaCl. The column eluate then was separated by reverse-phase HPLC by using an octadecyl silica (ODS) column (VP-318, 4.6 × 250 mm; Senshu Scientific, Tokyo). The column was eluted with a 40-min linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

Purification of BcPCP-A1. BcPCP-A1 was purified from pollen coat extracts of *B. campestris* S_9 homozygotes. Crude extracts first were fractionated by the ODS column by using the same conditions as described for reverse-phase HPLC above. SLG-binding activity was detected in a broad range (20–35 min). The fraction (24–25 min) with the strongest binding activity was applied to a Mini S PC 3.2/2 column (Amersham Pharmacia) and eluted by a linear gradient (0–0.5 M) of NaCl in 20 mM sodium acetate buffer, pH 6.0. The main active fraction (≈0.16 M NaCl) was purified further by an ODS column (Develosil 300 ODS-HG-5, 2.1 × 250 mm; Nomura Chemical, Seto, Japan) with a 40-min linear gradient of 0–40% acetonitrile in 0.05% heptafluoro-*n*-butyric acid at a flow rate of 0.2 ml/min.

Amino Acid Sequence Analysis. Amino acid sequencing was performed on a 492A Procise protein sequencer (Perkin–Elmer). Pyroglutamate aminopeptidase (Takara Shuzo, Kyoto) digests were conducted in 50 mM sodium phosphate, pH 7.0/1 mM EDTA/10 mM DTT (1:10 enzyme/substrate ratio; 42°C for 2 hr). Lysyl endopeptidase (Wako) digests were conducted in 50 mM Tris·HCl, pH 8.5/0.6 M urea (1:10 enzyme/substrate ratio; 37°C for 3 hr). Before proteolytic digestion, the cysteine residues were reduced with DTT and alkylated with iodoacetamide. The peptide fragments obtained were fractionated by the ODS column (Develosil 300 ODS-HG-5, 2.1 × 250 mm) with a 40-min linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min.

Molecular Mass Analysis. Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) analysis was done on a Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, MA). Sinapic acid was used as a matrix for ionization.

PCR Cloning of *SLR1-BP* and *BcPCP-A1*. Total RNA was extracted from anthers (collected from buds at 0–9 days before anthesis) of *B. campestris* S_9 homozygotes by using Isogen (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized by using an oligo(dT) primer. A partial sequence of *SLR1-BP* was obtained by PCR by using degenerate primers 5′-GT(A/T/G/C)GA(A/G)CA(A/G)TA(C/T)CC(A/T/G/C)GA(C/T)CC(A/T/G/C)AA-3′ and 5′-TC(A/G)CA(A/T/G/C)C(G/T)(A/G)CA(C/T)TGCAT(A/G)TG-3′, which were designed based on two internal amino acid sequences, QCVEQYPDPNGK and GHMQCRCDYHC, respectively, of SLR1-BP. Based on the partial sequence, 3′ or 5′ extension of the cDNA was performed by using the rapid amplification of cDNA ends technique. The genomic DNA sequences were obtained by using *SLR1-BP* specific primers, 5′-CACTATTAGTTCATTATTTA-3′ and 5′-TTATTTGAGTTTCAACGTAG-3′. Molecular cloning of *BcPCP-A1* (GenBank accession no. AB035564) has been described previously (8).

RNA Gel Blot Analysis. Total RNA was isolated by using Isogen. The probe was ^{32}P-labeled by random priming (Roche Diagnostics). The RNA (20 μg) was size-fractionated on 1.2% agarose/formaldehyde gels, transferred to GeneScreen*Plus* (Du Pont) membranes, hybridized overnight (in 5× SSC/5× Denhardt's solution/1% SDS at 60°C), and washed at high stringency [in 0.1× SSPE (0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/2% SDS at 60°C].

***In Situ* Hybridization.** *In situ* RNA hybridization was performed by using the method described previously (10). A full-length coding region of *SLR1-BP* cDNA was subcloned into pBluescript SK(+) (Stratagene). Both antisense and sense probes were synthesized by using an SP6/T7 digoxigenin RNA-labeling kit (Roche Diagnostics).

Results

SLR1- and SLG-Binding Activities in Pollen Coat Extracts of *B. campestris*. Three channels of the BIAcore sensor chip were immobilized separately with SLR1, SLG_8, and SLG_9 proteins of *B. campestris*. Using this sensor chip, we found strong SLR1-binding activity and weak SLG-binding activity in pollen coat extracts of *B. campestris* (Fig. 1*A*). To evaluate binding activity, the relative response unit was used, which is defined as the response 220 sec after sample injection relative to the response before the injection. The relative response unit values showed a clear dependence on protein concentrations (Fig. 1*B*).

S haplotype specificity was not observed in the interaction between SLGs and the pollen coat extracts. The S_9 pollen coat

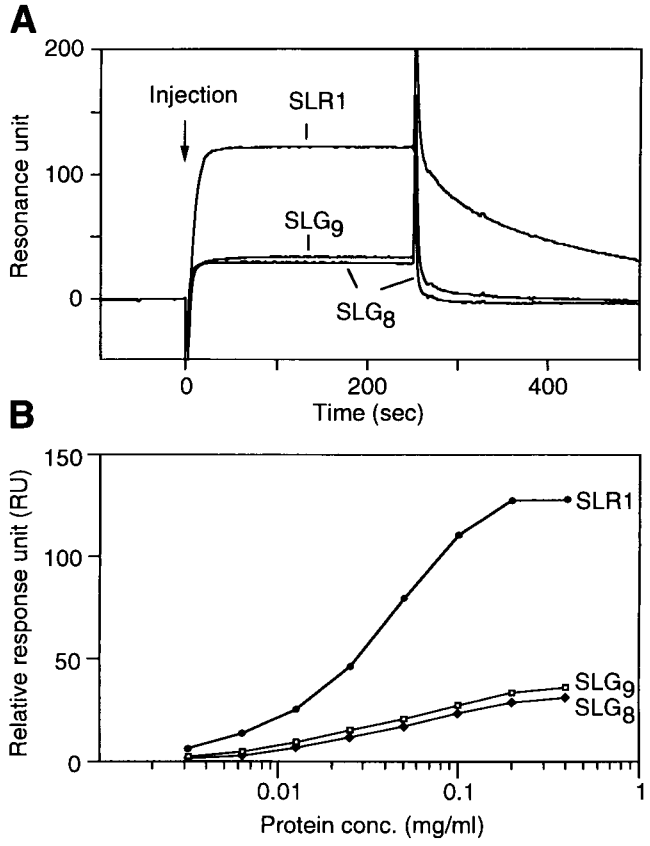

Fig. 1. Surface plasmon resonance measurement of the interaction between pollen coat extract from *B. campestris* S_9 homozygotes and immobilized SLG_8, SLG_9, and SLR1. (*A*) An overlay of sensorgrams. The pollen coat extract (0.4 mg of protein per ml) was injected between 0 and 240 sec. (*B*) Concentration dependence of relative response units.

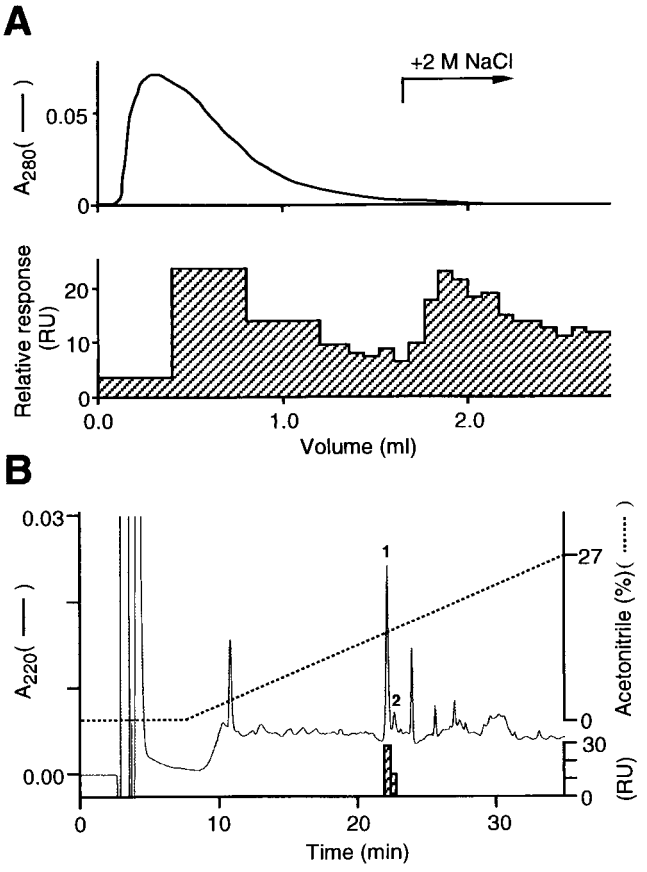

Fig. 2. (*A*) SLR1 affinity column chromatography. Chromatogram monitored at 280 nm (*Upper*). SLR1-binding activity measured by BIAcore (*Lower*). The arrow indicates the position at which the elution started. (*B*) Reverse-phase HPLC (on VP-318 column) purification of the SLR1-binding fractions. The dotted line shows the concentration of acetonitrile. Hatched bars indicate SLR1-binding activity. SLR1-BP1 (**1**) and SLR1-BP2 (**2**) were eluted at 22.2 min and 22.7 min, respectively.

extract showed a slightly higher affinity for SLG_9 than for SLG_8 (Fig. 1); however, the S_8 pollen coat extract also showed the same trend (data not shown).

Purification of SLR1-Binding Proteins. Because the pollen coat extracts showed high binding activity for SLR1, we employed affinity column chromatography as the first step for the purification of SLR1-binding proteins (Fig. 2*A*). The S_9 pollen coat extract was applied directly to the affinity column with immobilized SLR1, and almost all the molecules absorbing at 280 nm were recovered in the flow-through fractions. The bound molecules were then eluted with a high-salt buffer (2 M NaCl). The SLR1-binding activity appeared to be highly enriched in the high-salt eluted fractions. Some SLR1-binding activity was detected in the flow-through fractions, so these fractions were chromatographed repeatedly until all the binding activity had been recovered in the high-salt eluted fractions (data not shown). The affinity-purified fractions were pooled and subjected to reverse-phase HPLC (Fig. 2*B*). Strong SLR1-binding activity was detected in two peaks at retention times of 22.2 min and 22.7 min. Proteins in these two peaks were named SLR1-BP1 and SLR1-BP2, respectively.

Protein Analysis of SLR1-BP1 and SLR1-BP2. N-terminal sequences of SLR1-BP1 and SLR1-BP2 were determined after treatment with pyroglutamate aminopeptidase. They differed only at the fourth Edman degradation cycle, where the phenylthiohydantoin (PTH) derivatives of hydroxyproline and proline were detected in SLR1-BP1 and SLR1-BP2, respectively (Table 1). MALDI-TOF MS analysis of SLR1-BP1 and SLR1-BP2 gave protonated molecular mass $(M+H)^+$ peaks at m/z 6317.6 and 6301.9, respectively. This difference in the molecular mass (16 mass units) was the same as the difference between the hydroxylated proline in SLR1-BP1 and the proline in SLR1-BP2. To confirm that this posttranslational modification of a proline residue was the only difference between SLR1-BP1 and SLR1-BP2, proteolytic digests of these two proteins were conducted and the internal peptide fragments obtained were sequenced. Reverse-phase HPLC of the lysyl endopeptidase digests of SLR1-BP1 and SLR1-BP2 gave the same chromatogram pattern with four separate peaks (Table 1). The amino acid sequences of the corresponding peptide fragments of SLR1-BP1 and SLR1-BP2 were found to be identical. All these results taken together strongly suggest that SLR1-BP1 and SLR1-BP2 have identical amino acid sequences and that the only difference between them is that the proline residue mentioned above is hydroxylated in SLR1-BP1 but not in SLR1-BP2. Thus, SLR-BP1 and SLR1-BP2 are encoded by the same gene, *SLR1-BP*. Hereafter, "SLR1-BP" will be used to denote both proteins in cases in which distinction between them is not necessary.

Cloning and Sequence Analyses of *SLR1-BP* Gene. A partial *SLR1-BP* cDNA fragment was amplified from the anther cDNA pool by using primers designed on the basis of two internal amino acid

Table 1. Summary of MALDI-TOF MS and amino acid sequence data for SLR1-BP1 and SLR1-BP2

	SLR1-BP1	SLR-BP2
Protein mass* $(M + H)^+$	6317.6 (6317.1)	6301.9 (6301.1)
N-terminal sequence	(Q†)KGGP‡VRKQCVEQYPDPNGKCVIDQCKAQCA	(Q†)KGGPVRKQCVEQYPDPNGKCVIDQCKAQCA
Internal fragments§	Peak 1 (28.8 min) GHMQCRCDYHC	Peak 1 (28.8 min) GHMQCRCDYHC
	Peak 2 (29.4 min) CVIDQCK	Peak 2 (29.4 min) CVIDQCK
	Peak 3 (30.3 min) QCVEQYPDPNGK	Peak 3 (30.3 min) QCVEQYPDPNGK
	Peak 4 (32.4 min) GGLARCIDTGK	Peak 4 (32.4 min) GGLARCIDTGK

*Experimental masses obtained by MALDI-TOF MS. Values in parentheses are the relative masses calculated from the deduced amino acid sequences shown in Fig. 3 and based on all eight cysteines forming intramolecular disulfide bridges.
†N-terminal sequences were obtained after pyroglutamate aminopeptidase treatment.
‡The phenylthiohydantoin derivative of hydroxyproline was detected.
§Lysyl endopeptidase fragments were separated by reverse-phase HPLC and sequenced (see *Materials and Methods*). Values in parentheses indicate the retention time of each fragment.

sequences (fragments 1 and 3). The full-length *SLR1-BP* cDNA sequence was determined by 5′ and 3′ rapid amplification of cDNA ends, and it contained an ORF of 249 bp encoding a small, 83-aa protein (Fig. 3). The deduced amino acid sequence included all the determined peptide sequences shown in Table 1, suggesting that the *SLR1-BP* cDNA indeed encodes SLR1-BP. The sequence also contained a putative 26-aa hydrophobic signal peptide, with the most likely cleavage site in agreement with the determined N-terminal amino acid sequence of the mature SLR1-BP. Database searches revealed that the amino acid sequence of SLR1-BP is the same as that of a pollen extracellular protein PEC-3 (23) except for 1 amino acid difference in the signal peptide (Fig. 3*B*). PEC-3 was identified as a potential pollen allergen from *B. rapa*, but its biological function is unknown.

Further sequence analysis revealed that SLR1-BP shares the following features with PCP-A1, an SLG-binding protein of *B. oleracea* (10): N-terminal hydrophobic putative signal sequences, hydrophilic mature protein with a small molecular mass (relative mass: 6,300 Da for SLR1-BP2 and 6,401 Da for PCP-A1), basic in nature (predicted pI: 9.03 for SLR1-BP and 9.26 for PCP-A1), and eight conserved cysteine residues (Fig. 3*B*). However, despite these structural similarities, the overall amino acid sequence similarity between SLR1-BP and PCP-A1 is very low, with 41% sequence identity in the mature protein.

The genomic structure of *SLR1-BP* also was determined by PCR by using primers designed based on the extreme 5′ and 3′ ends of the coding region. A 595-bp fragment was obtained that had a sequence identical with that of SLR1-BP cDNA, except for the presence of a single, 268-bp intron located close to the 3′ end of the signal peptide-coding region (Fig. 3*A*). The intron region is AT-rich (77%), and the consensus intron splice donor and acceptor sequences, GT and AT, are present at both ends (24). A single intron with a similar size and sequence is also present at the equivalent positions of the *PCP-A1* and *PCP-1* genes, suggesting that this genomic structure is common among the members of the class A PCP family (10, 25).

Characterization of SLG-Binding Protein in *B. campestris*. We also attempted to purify the SLG-BPs from the pollen coat of *B. campestris*; however, the affinity purification of SLG-BPs was not successful, perhaps because of their low affinities or low levels in the pollen coat. We therefore fractionated the pollen coat extract with conventional chromatographic methods and assayed the SLG-binding activity of each fraction by using a BIAcore biosensor. At least 10 proteins in the pollen coat were found to interact with SLGs; all of them were small, basic 4- to 8-kDa proteins containing eight cysteine residues, but their positions were not always conserved (S.T., unpublished results). A major component of these SLG-binding proteins was found to be 89% identical in the amino acid sequence (95% identical in the nucleotide sequence) to PCP-A1 (Fig. 3*B*). This protein is likely to be an ortholog of PCP-A1 and is designated BcPCP-A1 (*B. campestris*-derived PCP-A1).

Kinetic Analysis of SLR1-BP and BcPCP-A1. To compare quantitatively the affinity of SLR1-BP and BcPCP-A1 for SLR1/SLG, their binding kinetics were analyzed by BIAcore. Various concentrations of SLR1-BP1, SLR1-BP2, and BcPCP-A1 were

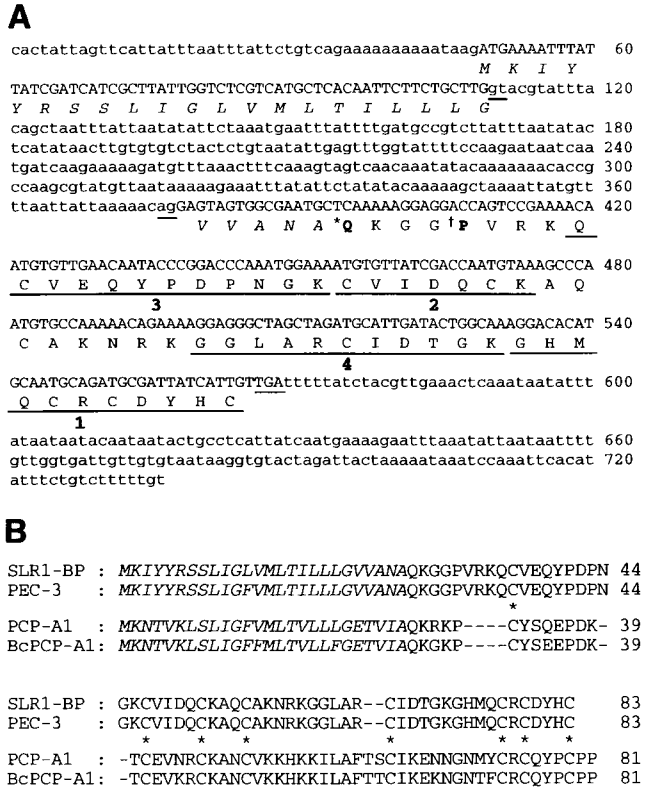

Fig. 3. (*A*) Nucleotide sequence of *SLR1-BP* and its deduced amino acid sequence. The noncoding regions are shown in lowercase letters, with the intron splice donor/acceptor sequences (*gt* and *ag*) demarcating the intron. The coding regions are shown in uppercase letters, with the underlined TGA sequence indicating the stop codon. The putative signal peptide is shown in italic letters; the four underlined peptide sequences (numbered 1–4) match those obtained from internal amino acid sequence analysis (see Table 1). *Q denotes the N-terminal glutamine that is modified to pyroglutamic acid in the mature protein; †P denotes the proline residue that is modified to hydroxyproline in SLR1-BP1. (*B*) Alignment of the amino acid sequences of SLR1-BP, PEC-3 (23), PCP-A1 (10), and BcPCP-A1. Eight conserved cysteine residues are indicated by asterisks.

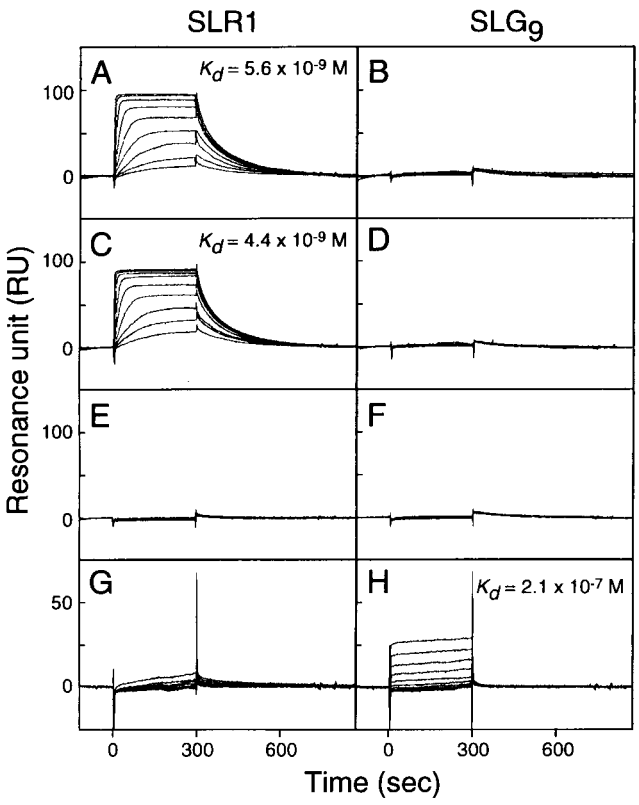

Fig. 4. Kinetic analyses of SLR1-BP1 (*A* and *B*), SLR1-BP2 (*C* and *D*), reduced and alkylated SLR1-BP1 (*E* and *F*), and BcPCP-A1 (*G* and *H*). SLR1-BP1, SLR1-BP2, and reduced and alkylated SLR1-BP1 were serially diluted (1, 2, 4, 8, 16, 32, 64, 128, and 256 nM) and applied to a sensor chip immobilized with SLR1 (*A*, *C*, and *E*) and SLG_9 (*B*, *D*, and *F*). BcPCP-A1 also was diluted (4, 8, 16, 32, 64, 128, 256, 512, and 1,024 nM) and applied to a sensor chip immobilized with SLR1 (*G*) and SLG_9 (*H*).

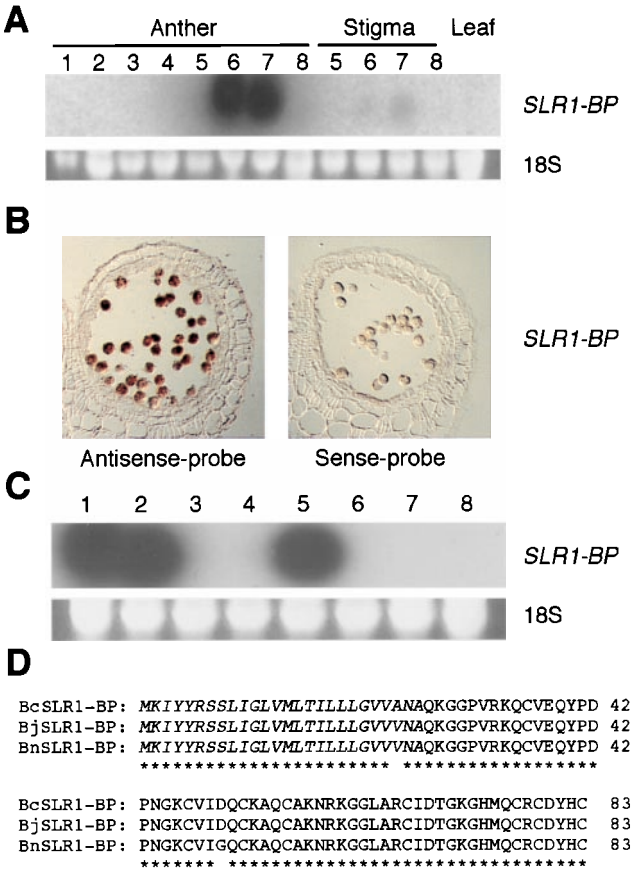

Fig. 5. (*A*) Tissue-specific expression of *SLR1-BP*. An RNA gel blot containing total RNA from anther, stigma, and leaf tissues was hybridized with the *SLR1-BP* coding region probe (*Upper*). Numbers 1–8 represent different bud sizes, with 1 = 0–1 mm, 2 = 1–2 mm, 3 = 2–3 mm, 4 = 3–4 mm, 5 = 4–5 mm, 6 = 5–7 mm, 7 = 7–10 mm, and 8 = open flower. The RNA gel was stained with ethidium bromide before blotting (*Lower*). (*B*) *In situ* hybridization of *SLR1-BP*. Anther sections derived from 5- to 7-mm-long flower buds were hybridized with an *SLR1-BP* antisense riboprobe and an *SLR1-BP* sense riboprobe (negative control). (*C*) Distribution analysis of *SLR1-BP*. An RNA gel blot containing total anther RNA from *B. campestris* (AA, 2*n* = 20; lane 1), *B. napus* (AACC, 2*n* = 38; lane 2), *B. oleracea* (CC, 2*n* = 18; lanes 3 and 4), *B. juncea* (AABB, 2*n* = 36; lane 5), *B. nigra* (BB, 2*n* = 16; lane 6), *R. sativus* (lane 7), and *Arabidopsis thaliana* (lane 8) was hybridized with the *SLR1-BP* coding region probe (*Upper*). The RNA gel was stained with ethidium bromide before blotting (*Lower*). (*D*) Alignment of the deduced amino acid sequences of SLR1-BP from *B. campestris* (BcSLR1-BP), *B. juncea* (BjSLR1-BP), and *B. napus* (BnSLR1-BP). Conserved amino acid residues are indicated by asterisks.

sequentially injected over the sensor surface immobilized with SLR1 and SLG_9, and their kinetic rate constants were determined (Fig. 4). SLR1-BP1 and SLR1-BP2 specifically bound SLR1 with similarly high affinities; their dissociation constants (K_d) were 5.6×10^{-9} M and 4.4×10^{-9} M, respectively. Thus, hydroxylation of the proline residue in SLR1-BP did not seem to have any significant effect on the SLR1-binding activity. However, when SLR1-BP1 was reduced with DTT and then alkylated with iodoacetamide, the obtained form (reduced and alkylated SLR1-BP1) completely lost binding activity for SLR1 (Fig. 4*E*). BcPCP-A1 specifically bound SLG, but its binding affinity was much lower ($K_d = 2.1 \times 10^{-7}$ M).

Temporal and Spatial Expression and Distribution of *SLR1-BP* Gene. RNA gel blot analysis of *SLR1-BP* was performed to determine its temporal and spatial expression pattern. Total RNA from anthers and stigmas at different developmental stages as well as from leaves was hybridized with an *SLR1-BP* cDNA probe (Fig. 5*A*). High levels of *SLR1-BP* mRNA were detected only in anthers at late developmental stages, and the size of the *SLR1-BP* transcript was approximately 480 bp, consistent with the size deduced from the *SLR1-BP* cDNA sequence. *In situ* hybridization showed that the antisense probe hybridized only to the cytosol of the microspores and not to any sporophytic tissue of the anther (Fig. 5*B*). Thus, like *PCP-A1* (10), *SLR1-BP* showed strictly gametophytic expression.

To determine the distribution of the *SLR1-BP* gene in Brassicaceae, we analyzed *SLR1-BP* expression in the anthers of various species of Brassicaceae by using RNA gel blot analysis (Fig. 5*C*). Strong hybridization was observed in *B. campestris*, *B. napus*, and *B. juncea*, but no hybridization was detected in two other species of *Brassica* (*B. oleracea* and *B. nigra*) or in two other genera (*Raphanus* and *Arabidopsis*) of Brassicaceae. The same results were obtained from DNA gel blot analysis (data not shown). To confirm this distribution pattern of *SLR1-BP*, we also performed PCR-based analysis. *SLR1-BP* gene fragments were amplified from anther cDNAs of *B. campestris*, *B. juncea*, and *B. napus*, but not from those of *B. oleracea*, *B. nigra*, *Raphanus*, or *Arabidopsis*. The deduced amino acid sequences of SLR1-BPs from *B. juncea* (BjSLR1-BP) and *B. napus* (BnSLR1-BP) showed 98.8% and 97.6% identity, respectively, with that from *B. campestris* (BcSLR1-BP) (Fig. 5*D*). Because both *B. napus* (AACC, 2*n* = 38) and *B. juncea* (AABB, 2*n* = 36) contain *B. campestris* (AA, 2*n* = 20)-type chromosomes (A genome) (26), *SLR1-BP* is likely to be specific to the A genome and be highly conserved among these *Brassica* species.

Discussion

In this paper, we report the isolation and characterization of SLR1- and SLG-binding proteins from the pollen coat of *B. campestris*. We first used an optical biosensor to monitor the interactions between the pollen coat proteins and stigmatic *S* gene family proteins, and this methodology proved to be effective in detecting such interactions. Using this methodology along with chromatographic methods, we showed that the pollen coat contains many molecules that interact with SLR1 and SLGs. Among them, SLR1-BP1 and SLR1-BP2 were purified by affinity chromatography because of their strong SLR1-binding activity. Sequence analyses revealed that these two proteins are members of the class A PCP family, which includes PCP-A1. A key feature of the PCP family of proteins is the presence of eight cysteine residues (10). The protonated molecular masses of SLR1-BP1 and SLR1-BP2 determined by MALDI-TOF MS (6317.6 and 6301.9 Da, respectively) are very close to the molecular masses calculated for these two proteins based on all the cysteine residues forming intramolecular disulfide bridges (6317.1 and 6301.1 Da, respectively; see Table 1). The class A PCP family of proteins is expected to share the same three-dimensional structure that is stabilized by these disulfide bonds. We found that reduction of the disulfide bonds of SLR1-BP1 followed by the alkylation of sulfhydryl groups abolished its SLR1-binding activity, suggesting that the three-dimensional structure is essential for this activity. Despite their expected structural similarity, the amino acid sequence identity between SLR1-BP and PCP-A1 is very low. The binding specificity of each protein must be determined by the amino acid residues located at its surface.

Biochemical analysis revealed that the major component of SLR1-BPs, SLR1-BP1, is hydroxylated at a proline residue, a posttranslational modification with interesting biological significance. The kinetic analysis indicated that this modification does not affect the binding activity of SLR1-BP. Hydroxyproline residues in some plant proteins are known to be glycosylated. Although we did not detect the glycosylated form of SLR1-BP1 in this study, we cannot rule out the possibility that it exists in the pollen coat.

We also found a number of pollen coat proteins that interact with SLGs, one of them being BcPCP-A1, a putative *B. campestris* ortholog of PCP-A1 of *B. oleracea*. Although BcPCP-A1 specifically interacts with SLG, the K_d of the BcPCP-A1/SLG_9 interaction is about 40 times higher than that of the SLR1-BP1/SLR1 interaction. All of the other SLG-binding proteins obtained thus far also show weak SLG-binding activity (S.T., unpublished data). Because PCP-A1 and these other SLG-binding proteins bind SLG with low affinity, it remains to be determined whether the binding observed *in vitro* has any biological relevance. In this study, we also compared the pollen coat extracts of S_8 and S_9 homozygotes to identify any protein(s) that bound SLGs in an *S* haplotype-specific manner. However, no such proteins were found. Moreover, the predicted mature form of the pollen *S* determinant, SP11/SCR (6–8), was not identified in this study, suggesting that either the amount of SP11/SCR in the pollen coat is very low or SP11/SCR does not show *S* haplotype-specific binding activity for SLGs. It would be of interest to use the same approaches to examine whether SRK, the expected receptor of SP11/SCR, interacts with SP11/SCR and, if so, whether this interaction is *S* haplotype-specific.

Luu *et al.* (19, 20) have shown previously that SLR1 is involved in pollen–stigma adhesion, and it would seem reasonable to hypothesize that the interaction between SLR1 and SLR1-BP observed in this study contributes to the pollen–stigma adhesion process. However, although the *SLR1* gene is widely distributed among the *Brassica* species, our distribution analysis revealed that the *SLR1-BP* gene is present only in the *Brassica* species containing the A genome. For example, the *SLR1-BP* gene was not found in *B. oleracea*, although alleles of *SLR1* of *B. oleracea* are more than 90% identical to those of *B. campestris* (16, 18). It is possible that some other proteins in the pollen coat of *B. oleracea* may have a function similar to that of SLR1-BP in *B. campestris.* Indeed, we have found SLR1-binding activity in the pollen coat extract of *B. oleracea* (S.T., unpublished observation). It would be of interest to examine whether the ability of SLR1-BP (or its functional homolog) of one *Brassica* species to strongly interact with SLR1 of another *Brassica* species might determine whether the pollen of the former can adhere to the stigma of the latter. The precise biological role of SLR1-BP will have to be established in future studies through analysis of transgenic plants by using gain-of-function or loss-of-function approaches.

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