Interaction between a coating-borne peptide of the Brassica pollen grain and stigmatic S (self-incompatibility)-locus-specific glycoproteins

(cell recognition/self-incompatibility)

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ABSTRACT Methods are described for the removal of the sporophytic pollen grain coating of Brassica oleracea and for the isolation of coat polypeptides. The coat contains a small number of proteins ranging from 6 to 45 kDa. Many of the larger proteins are glycosylated, while all carry high positive charges resulting in pI values from 8.5 to 11. Polypeptides with pI values of 9.5, 9.0, and 8.5 possess strong esterase activity. No major differences could be detected in either pI values or molecular masses of pollen-coating polypeptides from grains carrying different sporophytically expressed S (self-incompatibility) alleles. Mixing pollen coat proteins with stigmatic extracts results in a conspicuous binding interaction involving female S-locus-specific and perhaps S-locus-related glycoproteins. This interaction, which is reversed by heating in the presence of SDS, results in an apparent charge shift of the female glycoprotein(s) of up to 2 pI units. The male participant in this interaction has been isolated by using a combination of fast protein liquid chromatography and reverse-phase HPLC and was shown to be a 7-kDa nonglycosylated peptide. Experiments with whole pollen cultured in vitro show challenge with stigmatic extracts to stimulate the release of gametophytic and sporophytic polypeptides and to result in the formation of a conspicuous interaction product, demonstrating the 7-kDa peptide to be freely available within the coating of pollen in vivo.

The self-incompatibility (SI) systems of higher plants are emerging as a family of unique signaling systems evolved from mechanisms already operative in the pollen/pistil interaction (1). Thus, in Nicotiana and other members of the Solanaceae, self-pollen is rejected by a mechanism involving a stylar RNase (2, 3), an enzyme common to the styles of many plants and hypothesized to play a role in defense against pathogens. In most species, SI is regulated by a simple genetic system based on few loci and large numbers of alleles (4, 5); generally, if pollen and stigma carry alleles in common, the pollen/stigma interaction is disrupted. The genetics of SI in Brassica and other members of the Cruciferae and Compositae is not so straightforward; the pollen S phenotype is determined by the S-allelic constitution of the parent plant, rather than that of the haploid grain (6).

Through a series of elegant molecular studies, Nasrallah et al. (7) have demonstrated two types of sequence to be linked to the S locus of Brassica. One (S locus glycoprotein; SLG) encodes a 55-kDa glycoprotein (S-locus-specific glycoprotein; SLSG) expressed in the stigmatic papillae, and the other encodes a transmembrane kinase (S receptor kinase; SRK) expressed in the organs of both sexes. Sequence comparisons suggest that the SRK features a domain, which may be extracellular, with maintained homology to the SLG. Further, other gene families with considerable homology to the SLG are also present in the genome (8–10) but unlinked to the S locus (S locus related; SLR). Interestingly, reporter constructs driven by the SLG promoter are expressed in the pistil and microspores of transgenic Nicotiana (11), but in the stigma and anther tapetum of transgenic Arabidopsis and Brassica (12, 13), suggesting that expression is regulated according to whether SI is sporophytically or gametophytically determined. Some expression does however occur in the microspores of transgenic Brassica, pointing to a low level of gametophytic activity. SLG transcripts do not occur in the anther (9), so the promoter must drive the expression of either the SRK and/or an unidentified male determinant.

Complexity at a molecular level is reflected in the cell biology of the SI response in Brassica because, on capture, the pollen draws water and a spectrum of stigmatic molecules through the dry stigmatic cuticle via the pollen coat (14). Following self-pollinations, development is rapidly arrested by a biostatic mechanism requiring protein synthesis and glycosylation (15). The pollen coat is derived from the sporophytic tapetum (16) and the fact that the SLG promoter drives expression in this layer suggests that it may be the location of determinants involved in the male SI response (1, 17). We report here the removal and partial characterization of the pollen grain coating and describe an interaction between a 7-kDa coating peptide and female S-linked and related glycoprotein.

MATERIALS AND METHODS

Plant Material. Greenhouse-grown plants of inbred Brassica oleracea lines homozygous for the S35, S29, and S63 incompatibility alleles (obtained from D. J. Ockendon, Horticulture Research International, Wellesbourne, Warwickshire, U.K.) were used in all the work reported. Stigmas and pollen were collected from newly opened flowers and either extracted immediately or stored at –70°C.

Isolation of Pollen Coat Proteins. Coating was removed from pollen grains by adding 800 μl of cyclohexane to 75 mg of pollen and agitating until suspended (5 sec). After separation by centrifugation (14,000 × g, 20 sec), the cyclohexane fraction was applied to microscope slides upon which it rapidly vaporized. Pollen coat residue was collected and maintained at or near 0°C. To recover coating polypeptides from isolated pollen coat, amassed isolates from a total of 300 mg of pollen were resuspended in 350 μl of a 50 mM phosphate buffer (pH 7.0) with the aid of an ultrasonic cell

Abbreviations: SI, self-incompatibility; SLG, S locus glycoprotein (gene); SLSG, S-locus-specific glycoprotein (protein); SRK, S receptor kinase; IP, interaction product; IEF, isoelectric focusing; SLR, S locus related; pcp, 7-kDa pollen coat protein.

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disruptor (Microson MS-50). Lipids were removed by repeated centrifugations for 20 min at 21,000 \( \times g \).

**Viability Tests for Coatless Pollen.** For structural investigations, pollen grains were vapor-fixed as described by Ellman and Dickinson (14). The integrity of pollen plasma membranes was assayed using the method of Heslop-Harrison and Heslop-Harrison (18). Pollen, with and without coatings, was germinated in vitro [medium: 10 mM Tris/20% (wt/vol) polyethylene glycol 1000/\( \text{CaCl}_2\cdot2\text{H}_2\text{O} \) (243 mg/liter)/\( \text{KNO}_3 \) (100 mg/liter)/\( \text{H}_3\text{BO}_3 \) (10 mg/liter), pH 8.35].

**Isolation of \( S_{25} \) and \( S_{45} \) Stigmatic Glycoproteins.** \( S_{25} \) glycoprotein was eluted from a Mono Q anion-exchange column [fast protein liquid chromatography (FPLC) system, Pharmacia LKB] in 20 mM Tris-HCl at pH 8.0. The \( S_{45} \) glycoprotein was also eluted from the same column, but using a 20 mM ethanolamine buffer at pH 9.5. Fractions containing \( S_{45} \) glycoproteins were further purified by gel filtration using a Superdex 75 HR 10/30 gel-filtration column (Pharmacia FPLC system). Stigmas were harvested immediately prior to fractionation, homogenized in the appropriate elution buffer, and centrifuged at 14,000 \( \times g \), 4°C, to remove cell debris.

**Fractionation of Pollen Coat Polypeptides by Gel Filtration.** Coated proteins were isolated from pollen by using the procedure described above and precipitated by the addition of solid (NH₄)₂SO₄ to saturation. After a 30-min incubation on ice with occasional gentle agitation, proteins were pelleted by centrifugation at 40 min at 21,000 \( \times g \), 4°C, and resuspended in 50 mM phosphate buffer at pH 7.0. Proteins were separated in sample buffer using a Superdex 75 HR 10/30 gel-filtration column.

**Purification of Coat Proteins by HPLC.** Fraction 29 eluted from the Pharmacia Superdex 75 column was run on a Vydac C₄ reverse-phase column (150 \( \times 2 \) mm; HiChrom, Reading, U.K.) equilibrated in 0.1% trifluoroacetic acid in 2% acetonitrile. A gradient of 2–50% acetonitrile was applied over 50 min at a flow rate of 0.2 ml/min. The major peak of activity (peak 16) was identified by interaction with the stigmatic SLG and rechromatographed on an Aquapore RP-300 column (30 \( \times 2 \) mm) (Applied Biosystems) in the same solvents as before but using a much shallower gradient of 10–45% acetonitrile over 80 min.

**Protein Separation and Analysis.** Isoelectric focusing (IEF). Five percent polyacrylamide gels with a pH range of 3.5–10 were run in a Multiphor II unit (Pharmacia LKB). Gel composition and electrophoretic conditions were as described in the manufacturer's instructions.

**SDS/PAGE.** SDS/PAGE was carried out using the Bio-Rad Mini-Protein II dual slab system employing a discontinuous buffer system (19). Separation of low molecular weight fractions obtained following gel filtration was achieved using a Tris/Tricine buffer system (20).

**Nondenaturing native PAGE.** Acetic nondenaturing gels (12% polyacrylamide resolving gel with a 4% stacking gel) were cast and run using the same apparatus as for SDS/PAGE gels. The buffer systems used were based on those described by Chrambach and Overath (21). Gels were run at 200 V constant voltage for 1 hr; polarity was reversed because separation was toward the cathode.

**Electroblotting.** Electroblotting was carried out in the Bio-Rad Mini Trans-Blot electrophoresis transfer cell using the method of Towbin et al. (22).

**Staining of SDS/PAGE, native PAGE, and IEF gels.** For Coomassie staining, gels were fixed and stained in 30% (vol/vol) methanol, 10% (vol/vol) acetic acid, and 0.1% (wt/vol) Coomassie R-250 for 1–3 hr, depending on gel thickness. Destaining was carried out with repeated washes of 25% methanol/10% acetic acid. Silver staining was carried out using the Protein Silver stain system (National Diagnostics, Manville, NJ). However, prior to staining, IEF gels were first treated as follows: gels were fixed in 30% (vol/vol) isopropyl alcohol/10% (wt/vol) trichloroacetic acid/3.5% (wt/vol) 5-sulfosalicylic acid for 1 hr and transferred to 30% isopropyl alcohol/12% trichloroacetic acid; the solution was changed at regular intervals over a 2-hr period.

**Glycoprotein detection (Con A affinity blotting).** Prior to Con A binding, proteins were identified by incubating the blots in 1% acetic acid containing 0.5% (wt/vol) Ponceau S. Blots were destained in 1% acetic acid for 20 min followed by several washes in TBS-Tween 20 [10 mM Tris-HCl, pH 7.4/0.14 M NaCl/0.1% (vol/vol) Tween]. Glycoproteins binding Con A were identified following the methods of Faye and Chriseps (23).

**Radioiodination of Pollen Coat Proteins.** Reverse-phase HPLC-purified 7-kDa pollen coat protein (pcp1) was labeled with \( ^{125}\text{I} \) (0.85 \( \mu \)Ci/\( \mu \)g; obtained from Amersham) by using the iodogen method (24).

**Interaction Between Stigmatic Extracts and Pollen Coat Proteins.** Stigmatic and pollen coat proteins were isolated as described in the previous sections, and interactions were carried out in 18-\( \mu \)l volumes between 20 and 60 min at room temperature. Interaction mixes were then analyzed using PAGE and IEF. Interactions involving radioiodinated peptide were also analyzed using IEF. However, dissociation between coating peptide and stigmatic molecules occurs at the pI of the hybrid molecule, and for this reason these gels were not run to equilibrium but stopped after half the normal running time. Autoradiographs were prepared from these gels using Kodak XAR-5 film and an exposure time of 20 min at \(-70^\circ\text{C}\).

**Protein Emission from Pollen in Aqueous Solutions and "in Vivo" Interaction Between Whole Pollen and Stigmatic Polypeptides.** Ten milligrams of pollen was suspended in 100 \( \mu \)l of 0.3 M mannitol and gently agitated every 5 min over two incubation times (15 and 30 min). After incubation, the pollen was removed by filtration, and the supernatant was analyzed by IEF at pH 3.5–10. For interaction experiments between stigmatic extracts and secreted proteins, 65 \( S_{25} \) stigmas were first homogenized in 100 \( \mu \)l of the mannitol solution; cell debris was removed by centrifugation (total stigmatic protein concentration, 15 mg/ml). Ten milligrams of pollen was then added, and the mixture was treated as above.

**RESULTS**

**Pollen Coating Removal Using Solvent Washes.** Cyclohexane washes resulted in complete removal of the coating with no detectable degradation of the constituent polypeptides and little deleterious effect on the pollen (see below). Approximately 1500 \( \mu \)g of coating polypeptide could be extracted from 300 mg of fresh pollen—far more than is extracted using aqueous media. Extractions in germination or other osmotically balanced media produce a wide spectrum of proteins in which those of gametophytic origin predominate.

After treatment, pollen was examined by scanning and transmission electron microscopy. The exine surface was revealed to be free of coating, whereas cytoplasm of the grains, fixed by dry methods, which prevent pollen hydration (14), appeared to be unaffected by solvent treatment. Coating-free grains both germinated and produced tubes in vitro, but at low levels; 15–20% of the grains generated tube initials and 10% formed elongated tubes. However, the fluorescein diacetate test for viability (18) indicated >90% of the grain protoplasts to be intact and to contain functional esterases.

**The Recovery of Pollen-Coating Polypeptides from Solvent Washes.** The removal of cyclohexane by evaporation, followed by sonication of the residue in aqueous solution, gave good yields of coat polypeptides. However, the procedure could have had a deleterious effect on extracted polypeptides, so stigmatic proteins were exposed to identical treatments. Neither alterations to \( M_r \) nor to \( p_l \) values were noted.
The Polypeptides Present in the Coating of *B. oleracea.* While the majority of the polypeptides run as a single band on acidic native PAGE, a family of smaller subsidiary proteins is also present. A more complex pattern emerges with SDS/PAGE (see Fig. 1, lane a); a number of major polypeptides are present having molecular masses of 30–45 kDa, with other major bands running at 20 kDa and 17 kDa, and a second group of proteins runs close to the front. This latter group of bands with molecular masses of 6–14 kDa was further characterized with PAGE, FPLC, and HPLC (see below). The most abundant of these solvent-extracted coat-polyopeptides can be detected in diffusate from intact pollen grains incubated in osmotically balanced aqueous medium (25), but at very low levels. Interestingly, a major coat polypeptide of 37 kDa was revealed as being composed of a glycosylated and nonglycosylated fraction while other polypeptides of 44, 35, and 32 kDa were also shown to be glycosylated (see Fig. 1, lane b). A 15-kDa polypeptide is also glycosylated; however, this could only be resolved on blots following electrophoresis using the system described by Schägger and von Jagow (20). The low molecular mass peptides (6–10 kDa) are not significantly glycosylated.

Pollen coating extract was also analyzed using IEF (see Fig. 1, lane c). In all the genotypes studied, coat polypeptides run in two main groups, one between pI 8.5 and 9 and another, far more highly charged between pI 9.5 and 11. FPLC on Superdex 75 gave good separation of coat polypeptides on the basis of molecular mass. However, analysis using IEF revealed most fractions to contain several proteins, often of different pl values.

Polypeptides were run on SDS/PAGE, native gels, and IEF from a number of lines of *B. oleracea* homozygous for different *S* alleles. While minor differences could be observed both in molecular mass and pl of polypeptides, no major *S*-specific bands were identified.

The Enzymes of the Pollen Coat. Blotting of pollen and pollen coat proteins from IEF followed by staining for esterase revealed strong activity associated with at least four polypeptides localized in the pollen coat (data not shown). Three of these have pl values between 8.65 and 9.0, while the other focuses at 9.5. No significant differences in enzymic activity could be detected between the *S* genotypes used in this study. It is clear from comparison of lanes containing coat polypeptides with total pollen homogenate that the majority of pollen esterase activity is localized in the coat.

Acidic native PAGE of pollen coat proteins stained for esterase (data not shown) indicates activity to be associated with polypeptides of 30–40 kDa. Acidic native PAGE of pollen coat proteins stained for acid phosphatase detected activity in two polypeptides between 60 and 80 kDa. Again no differences in activity could be detected between *S* genotypes.

The Behavior of Male and Female Polypeptides in Mixtures. Mixture of coat polypeptides with crude stigmatic extracts induced a striking interaction. After IEF, the glycoprotein band regarded as representing the SLSG in our gels decreased in intensity with the generation of a new band at a higher pl (see Fig. 2). Despite careful inspection of the stigmatic protein spectrum following an interaction, no evidence could be found of any other female polypeptides being affected. While SLSGs are identifiable by their segregation with *S* alleles, SLRs are not, and it is possible that if SLSGs and SLRs run close to each other in IEF and PAGE gels the interactions also involve this latter group of glycoproteins.

A less marked interaction takes place with isolated SLSG; under these circumstances, the interaction product (IP) often becomes distributed over a wider pl range. When FPLC-fractionated pollen coat polypeptides were added to female molecules, the most active fractions contained peptides of 6–9 kDa (see Fig. 3). Further separation of the “active” FPLC fractions on reverse-phase HPLC and testing of the fractions resulting has revealed that a peptide of 7000 Da (pcp'2) is responsible for inducing the charge shift in the female glycoprotein (see Fig. 4). Interactions involving radiiodinated pcp'2 first gave confusing results, indicating that the peptide is not associated with the IP, although it must clearly have been responsible for the interaction. Subsequent experiments revealed that the IP dissociates once it reaches
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Hydration of the grain protoplast (32) must involve the passage of stigmatic water to the pollen plasma membrane via a hydrophilic pathway established through the stigmatic cuticle and the cuticle cutates. While there is circumstantial evidence that the cuticle is permeable to water (H.D. un-

published results), the route taken by water through the coating is unknown. However, the abundance of the 30- to 40-kDa peptides suggests that they are distributed throughout the coat, and since many are glycosylated, these proteins could form a domain with high affinity water extending across the cuticle, creating an effective hydration pathway. The stimulation of pollen protein emission by female molecules is potentially significant, especially since it also occurs when coat-less pollen is challenged. This response must involve the release of protoplast or intine held poly

peptides.

Findings reported here strongly indicate that when mole-

cules of the SLSG/SLR family interact with coating poly-

peptides, the SLSGs, and perhaps SLR, bind to the pcp', resulting in a marked alteration in net charge. For this reason, any hypothesis involving the SLSGs/SLRs in physiological and biochemical must now be considered in terms of the IP. Female S-allele specificit

y is known to be carried with the SLSG (33), and it is important to determine whether the peptide interaction is affected by the S allele carried. Certainly the sporophytic origin of pcp' makes it a good candidate for the male SI determinant, but unequivocal evidence for S specificity has proved hard to find. Further, a role for the interaction in SI is called into question by the formation of a clear IP in self-compatible Brassica napus (S. Hiscock, personal communication). However, B. napus is an allotetraploid formed from B. oleracea and Brassica campestris, so the presence of SI-related molecules might be anticipated.

The discovery that the S locus also contains a transmembrane kinase (34) is significant and, although the locations of the SRK gene product in pollen and stigmas are unreported, they must be central to any hypothesis to explain SI. Thus, on the basis of evidence presently available, pollination seemingly involves a complex dialogue between pollen and stigma, complemented with superficial interactions, which establish hydraulic continuity and render the male and female plasma membranes accessible to signals. The precise nature of these signals and their consequences remain largely un-

known, but there is accumulating evidence (7) that SLSG, presumably in the form of the IP, is capable of regulating pollen development according to the S alleles present. SLSG may thus act as a developmental inhibitor, operating through an SRK-encoded kinase at either the pollen or stigmatic plasma membrane. Until pcp's have been sequenced for a number of alleles, we must remain ignorant as to whether they carry S-locus specificity or simply act as a nonspecific cofactor.

Most interestingly, the discovery of sequence homology between the SLG and genes putatively encoding a class of transmembrane kinases was not made in Brassica, but in Zea by Walker and Zhang (35). These authors proposed that the kinase in Zea, which is expressed throughout the plant, is a component of a hitherto undescribed system of intercellular communication. The ubiquitous nature of this class of kinase and its situation at the cell surface suggests that, in Zea at least, it could play a role in defense against pathogens. Thus, as has been the case with the styal RNases of the Solanaceae (2), strong selective pressure for the evolution of outbreeding systems in species with dry stigmas may also have resulted in the adaptation of an already extant system, perhaps involved in pathogen defense, to identify and reject self-
pollen.

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