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Cell recognition in plants: Determinants of the stigma surface and their pollen interactions

(pollen–stigma interactions/concanavalin A/cell surface receptors/binding sites/incompatibility)

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ABSTRACT **The interaction between compatible pollen grains and the female stigma of *Gladiolus gandavensis* has been used as a model system for investigation of cell recognition in plants. The molecular architecture of the receptive stigma surface has been investigated, and determinants binding to both concanavalin A and β-glucosyl artificial carbohydrate antigen, as well as esterase activity, have been characterized, and conditions for their isolation have been established. The stigma surface, before and after modification, was found to bind ^{125}I-labeled proteins nonspecifically. Pollen tube penetration of the papillar cuticle is prevented when the receptor sites for concanavalin A are occupied. The concanavalin-A-binding determinants of the stigma surface have been fractionated to reveal several glycoproteins in the molecular weight range 43,000–93,000 and a group of glycolipids of molecular weight approximately 22,000. These results are interpreted in terms of two major recognition events regulating pollination.**

Cells of virtually all eukaryotic organisms possess some capacity to distinguish self from not-self (1). Processes reflecting this recognition capacity have been extensively explored in animal systems (2), but plants have been largely neglected. However, recognition between compatible pollen and the female stigma in plants provides a model system for cell recognition which is genetically specified (3), and in which transfer of information has been demonstrated both for pollen, which carries the male gametes (4), and the stigma surface (5).

In this communication we describe the recognition events in the self-compatible system of *Gladiolus*, whose pollen walls are known to contain a range of proteins in both exine and intine sites (6). We have investigated the molecular architecture of the external stigma surface with a view to demonstrating surface receptors.

MATERIALS AND METHODS

Cut flowers of *Gladiolus gandavensis* were maintained in 0.01% 8-hydroxyquinoline citrate and 1% sucrose solution. Stigmas were removed from flowers open for 24 hr and placed upright in a 1% agar plate. Pollen was collected from dried anthers.

Preparation of Pollen Extracts. Pollen was extracted for 10 min at room temperature with 10 ml of 0.05 M Tris·HCl, 0.15 M NaCl, 0.001 M $CaCl_2$, pH 7.4, subsequently referred to as Tris–saline buffer. The filtrate was dialyzed and freeze dried. (Yield: 15 mg/g of pollen.)

Preparation of Stigma Extracts. Groups of 30 mature stigmas were washed in 6 ml of Tris–saline buffer at 4° by repeated dipping over a period of 15 min and the filtrate was dialyzed and freeze dried. (Yield: 2 mg per 100 stigmas.) Extraction was repeated for 15 min with 0.5% sodium deoxycholate (Merck) in the same buffer at 4°, and this extract was dialyzed and freeze dried. (Yield: 10 mg per 100 stigmas.) Analysis by the phenol-sulfuric acid method (7) showed that the dialyzed and dried extract contained 25% sodium deoxycholate and 17% carbohydrate (as glucose).

Binding of Concanavalin A Conjugated to Fluorescein Isothiocyanate (FITC-Con A) to *Gladiolus* Stigma Surfaces. Stigmas were fixed by treatment with 0.2% (vol/vol) glutaraldehyde in Tris–saline for 5 min, drained, and washed in Tris–saline containing 50 mM lysine (8) and twice in distilled water. The stigmas were drained onto Whatman no. 1 filter paper between washes. The stigmas were then dipped into FITC-Con A (13.5 mg/ml, in Tris–saline) for 30 min at room temperature, washed with the same buffer, and examined by reflected light incident fluorescence microscopy.

Esterase Activity of Stigma Surfaces. Esterase activity was detected as described (5, 6).

Binding of Yariv Artificial Carbohydrate Antigens. Yariv artificial carbohydrate antigens (9), supplied by Dr. M. A. Jermyn, were used as described previously (10).

Pollination Experiments. The patency of the stigma surface after various treatments was assessed in terms of pollen tube growth and penetration of the stigma surface. Stigmas were pollinated with pollen from fresh anthers. Pollen tube growth was allowed to take place for 18 hr at 25° in a moist chamber, then stigmas were treated with decolorized aniline-blue:glycerol 9:1. Under these conditions, pollen tube growth was observed by the fluorescent staining of callose in the pollen tube wall (11). For treatment, the stigmas were dipped in solutions of the agents for 5 min at 20°, drained, and washed three times before pollination.

Binding of Labeled Proteins to Stigma Surfaces. Groups of six to ten stigmas were lightly fixed by treatment with glutaraldehyde as described above. They were then incubated with ^{125}I-labeled protein solution, labeled by the lactoperoxidase technique (12) for 5 min, washed three times, and counted in a deep well gamma counter. Each protein solution was diluted to 6×10^6 cpm/ml before use.

Preliminary experiments indicated that binding of labeled proteins to the *Gladiolus* stigma surface was complete within 5 min. No change in the amount of radioactive material bound could be detected after incubation of the stigma and protein for 1 hr, or after washing for periods up to 1 hr at room temperature. Binding at 4° was about half as efficient as binding at 20°. Each binding experiment was repeated on at least two occasions.

Isolation of Concanavalin A (Con A)-Binding Components of Stigma Surfaces. Con-A-binding material from buffer extracts of stigmas was isolated by affinity chromatography using

Abbreviations: Con A, concanavalin A; FITC-Con A, Con A conjugated with fluorescein isothiocyanate; M_r, molecular weight.

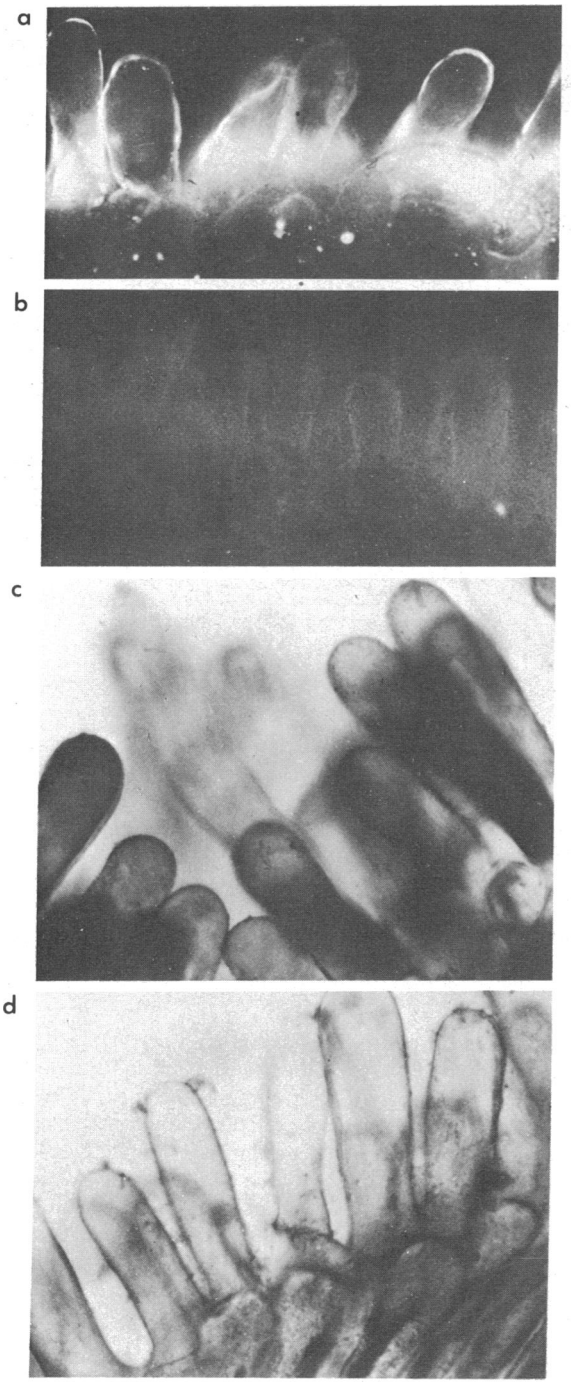

FIG. 1. Cytochemical localization of stigma surface markers. (a) Binding of FITC-Con A to glutaraldehyde-fixed stigmas. Fluorescence is associated with the surface of the papillae. Stigma surfaces treated with 0.25 M galactose showed similar intensity of fluorescent binding. ×170. (b) Binding of FITC-Con A to glutaraldehyde-fixed stigmas in the presence of 0.25 M α-methyl-D-glucoside. Only autofluorescence of the stigma surface was detected. ×170. (c) Esterase activity on fresh stigma surface. ×210. (d) Esterase activity of the stigma surface that had been pretreated with 0.5% sodium deoxycholate in buffered saline for 15 min at 4°. Only wisps of reaction product are apparent at the stigma surface. ×210.

Con-A-Sepharose. Columns (10 × 1 cm) of Con-A-Sepharose were packed in Tris–saline containing 0.1% Nonidet P40 (NP40); the ^{125}I-labeled sample (10 μg) was applied; and the column was washed successively with buffer, α-methyl-D-mannoside (4%), and sodium thiocyanate (3.5 M). The three

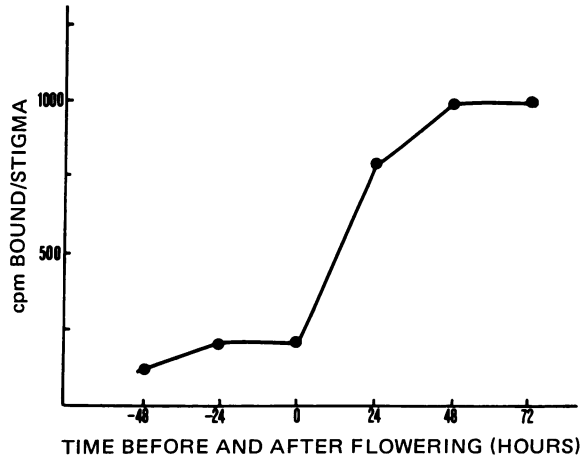

FIG. 2. Binding of ^{125}I-labeled *Gladiolus* pollen extract to stigmas at times before and after flower opening. Measurements were made on groups of six stigmas (SEM = 93 cpm).

fractions were separately collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (13). The gels were cut into 1 mm sections which were separately counted.

RESULTS

Cytochemical localization of stigma surface receptors

FITC-Con A is bound specifically to mature stigma papillae (Fig. 1a), indicating the presence of surface acceptors for this lectin. The specificity of binding was demonstrated by the lack of binding to FITC-Con A preincubated with 0.25 M α-methyl-D-glucoside (Fig. 1b) and the persistence of binding to FITC-Con A preincubated with 0.25 M galactose. The binding was restricted to the surface layer of the papillae that is supported by the cuticle.

Esterase activity was shown to be present both at the stigma surface and in the cell walls (Fig. 1c). Fresh stigma surfaces were stained intensely by the β-glucosyl antigen. The corresponding α-galactosyl antigen did not stain the surfaces under the same conditions.

The effects of the removal of esterase activity and surface determinants binding to Con A and artificial carbohydrate antigens was followed. Washing of the stigma surface with cold Tris–saline did not perceptibly alter the binding of FITC-Con A, although some Con-A binding material was released, which precipitated with Con A. The final concentration of Con A was 1 mg/ml and stigma extract contained 60 μg/ml of carbohydrate (as glucose). Turbidity measured as OD_{420} = 0.07, while glycogen at the same glucose concentration gave OD_{420} = 0.50.

These surface determinants were all progressively removed by treatment with buffered 0.5% sodium deoxycholate. Removal was apparent after 2 min treatment and complete after 15 min treatment at 4° (Fig. 1d). The nonionic detergent NP40, at concentrations up to 1.0% in the same buffer, did not remove any of these determinants.

Binding characteristics of stigma surface

During maturation of the stigma after opening of the flower, a layer is secreted onto the papillar surface. The appearance of this layer coincides with development of the capacity to bind labeled protein and to receive compatible pollen. The period of maximum receptivity was 24–48 hr after flower opening as shown by the ability of the stigma to bind ^{125}I-labeled *Gladiolus* pollen extract (Fig. 2). In all subsequent experiments stigmas

Table 1. Binding of Con A to stigma surfaces

	Treatment	cpm per stigma
1.	Fresh stigmas + ^{125}I-labeled Con A	868 ± 102
2.	Fixed stigmas + ^{125}I-labeled Con A	2062 ± 562
3.	Con A + ^{125}I-labeled Con A	1650 ± 176
4.	^{125}I-labeled Con A + α-methyl-D-glucoside	966 ± 142
5.	^{125}I-labeled Con A + galactose	1392 ± 153
6.	^{125}I-labeled Con A + maltose	593 ± 77
7.	^{125}I-labeled Con A + cellobiose	1504 ± 266

Stigmas were treated with ^{125}I-labeled Con A solution for 5 min at room temperature. The effect of various sugars was investigated by adding the sugar at a final concentration of 0.25 M to the ^{125}I-labeled Con A solution. Treatments 2 to 7 were carried out using glutaraldehyde-prefixed stigmas.

at this stage of maturity were used. Specificity of pollen protein binding to the stigma surface was tested by measuring the binding of various labeled protein preparations. Fresh stigmas bound labeled *Gladiolus* pollen extracts to the extent of 4488 ± 230 cpm per stigma, while fixed stigmas bound to a similar extent (4100 ± 540 cpm per stigma). Pollen extracts from sunflower (*Helianthus annuus*) and ryegrass (*Lolium perenne*) showed no significant difference in binding capacity (5316 ± 600 and 4728 ± 360 cpm, respectively). Protein preparations of animal origin were also found to bind to the stigma surface, although to a more variable extent: bovine serum albumin, 2412 ± 60; cytochrome *c*, 4218 ± 120; and *Limulus* hemocyanin, 6108 ± 132 cpm per stigma.

Binding of Con A to stigma surfaces

Evidence for the specificity of binding of Con A to the stigma surface was obtained using ^{125}I-labeled Con A (Table 1). Fixed stigmas showed a 2-fold increase in Con A binding and a considerable degree of specificity of binding was demonstrated by the inhibition of binding in the presence of α-methyl-D-glucoside and maltose, both of which bind to Con A. Under the same conditions, sugars which do not bind Con A, e.g., galactose and cellobiose, were ineffective as inhibitors of Con A binding.

Binding of pollen proteins to modified stigma surfaces

The receptive stigma surface was modified by buffer washing, by detergent washing, and by binding of Con A to the surface. The ability of the stigma surface to bind ^{125}I-labeled *Gladiolus* pollen extracts after these modifications was measured. Buffer washing of the stigma surface did not alter its capacity to bind

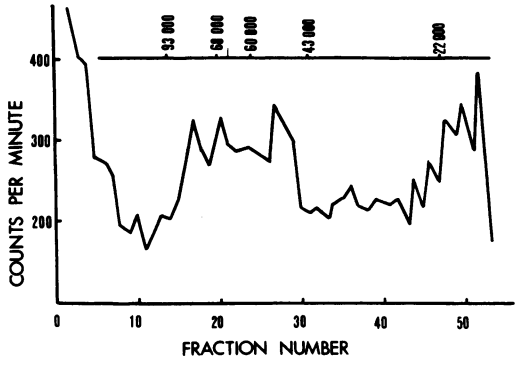

FIG. 3. Polyacrylamide gel electrophoresis of the fraction of ^{125}I-labeled stigma extract eluted from Con-A-Sepharose with 0.25 M α-methyl-D-mannoside. The top scale gives the migrations of marker proteins with the indicated molecular weights.

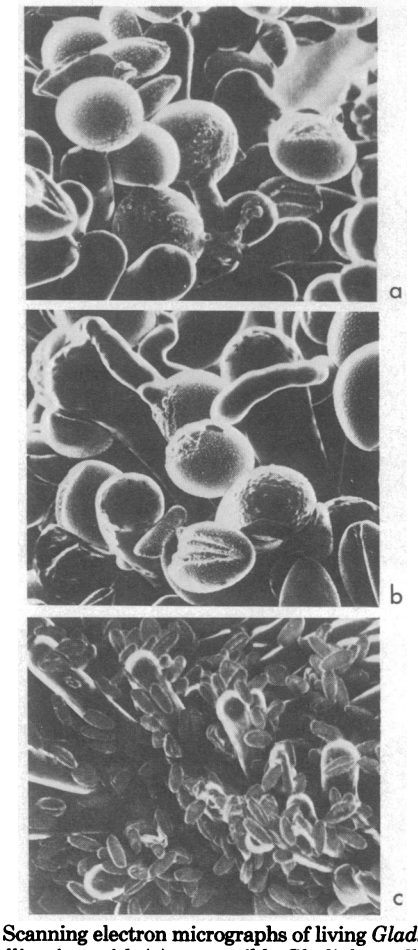

FIG. 4. Scanning electron micrographs of living *Gladiolus* stigma, 2 hr after pollination, with (a) compatible *Gladiolus* pollen ×115, (b) *Crocosmia aurea* pollen ×160, (c) *Gloriosa rotschildiana* pollen ×75.

labeled pollen protein (5244 ± 906 cpm before wash, 4674 ± 864 cpm after wash). Treatment with 0.5% sodium deoxycholate effectively doubled the protein binding capacity (11,460 ± 2370). Binding of labeled pollen protein to stigma surfaces pretreated with unlabeled Con A at 5 mg/ml (6288 ± 480 cpm) was not significantly different from that measured without Con A pretreatment. Pretreatment of stigmas with Con A in the presence of inhibitory or noninhibitory sugars did not significantly alter the extent of binding. Pretreatment of stigmas with unlabeled *Gladiolus* pollen protein at 5 mg/ml did not affect subsequent binding (4218 ± 690 cpm).

Isolation of Con-A-binding components of stigma surfaces

Polyacrylamide gel electrophoresis of the components of labeled stigma extract that were eluted with α-methyl-D-mannoside from Con-A-Sepharose showed that there are at least four well-separated components with molecular weights (M_r) between 43,000 and 93,000 as well as a group of lower M_r components about M_r 22,000 (Fig. 3). These were distinct from the components that were not bound to the column or were eluted with 3 M sodium thiocyanate.

Response of self and foreign pollen to *Gladiolus* stigmas

Gladiolus gandavensis, being self-compatible, accepts its own pollen and that of other genotypes of the same species (Fig. 4a). Within 20 min of landing on the stigma, the pollen hydrates, swells, and produces a short pollen tube which penetrates the

papilla cuticle after 45–60 min. Pollens from related members of the family Iridaceae, such as *Crocosmia aurea*, also hydrate and swell, and a pollen tube grows and coils around the papilla without penetration (Fig. 4b). Pollen from other families, e.g., Liliaceae, is not accepted at all and fails to hydrate (Fig. 4c).

Qualitative observations of the behavior of *Gladiolus* pollen on modified *Gladiolus* stigma surfaces suggest that the stigma surface plays a vital role in these recognition processes. Following deoxycholate treatment and extensive washing of the stigma surface, pollen germination is completely inhibited, whereas after only buffer and water washing of the surface, normal pollen tube growth was observed. Binding of Con A to the stigma surface, followed by extensive washing, left a surface that allowed pollen germination and tube growth in a random fashion across the stigma surface, without penetration of the cuticle. This growth resembled that observed for other members of the Iridaceae on untreated stigmas of *Gladiolus*.

DISCUSSION

Cell surface acceptors for Con A have been demonstrated for many animal cells, and we now report the presence of similar acceptors bearing glycosyl moieties on a plant cell surface. Previously, binding of Con A to plant cells has only been observed with isolated protoplasts (14). In most situations the plant cell membrane is protected by the cell wall, and is not accessible to direct chemical examination. The outer layer of the stigma papillae provides a model system for the investigation of plant cell surfaces: as well as being directly accessible, it performs a defined function—the recognition of compatible pollen (3).

The processes involved in pollination of *Gladiolus* are outlined in Fig. 5, which is based on recent proposals (3, 15–17). When pollen alights on the stigma surface, components of the outer pollen wall layer (exine) make contact with the receptors of the stigma surface (system 0). If the pollen is positively recognized, fluid is taken up from the stigma surface. If, however, foreign pollen, e.g., *Gloriosa*, is received by the stigma, recognition system 0 is inoperable since no hydration of the pollen grain or other metabolic processes have been observed. One explanation is that compatibility results from complete fit or mutual recognition by pollen–stigma receptors (1), whereas the passive behavior of foreign pollen is explained in terms of receptor misfit.

Following acceptance by system 0, proteins from the inner layer of the pollen wall are released, and germination and pollen tube growth commences. At this point, a second recognition event occurs for compatible pollen, system I. This allows activation of a cutinase system which digests the cuticle (18) and enables the pollen tube to penetrate the stigma surface. *Gladiolus* pollen fulfils the requirements of system I, while pollen of other genera within the Iridaceae, e.g., *Crocosmia*, previously accepted by system 0, does not fulfil the requirements of system I. Again these events may be ascribed to fit or misfit of receptors (1).

The nonacceptance options of Fig. 5 are cut-off points which do not appear to lead to further metabolic events. In contrast, active rejection responses can be demonstrated in certain incompatibility situations, for example, the callose rejection response in intergeneric and self matings of various Cruciferae and Compositae (3, 15, 19). In these cases, recognition can lead to either acceptance or active rejection.

Both recognition systems 0 and I involve reactions at the stigma surface. Transmission electron micrographs of the stigma surface layer of *Raphanus* and *Silene* (6, 15, 17, 19) show that this is not a classic bilayered membrane; it is apparently secreted on the surface of the cuticle and makes contact with the stigma

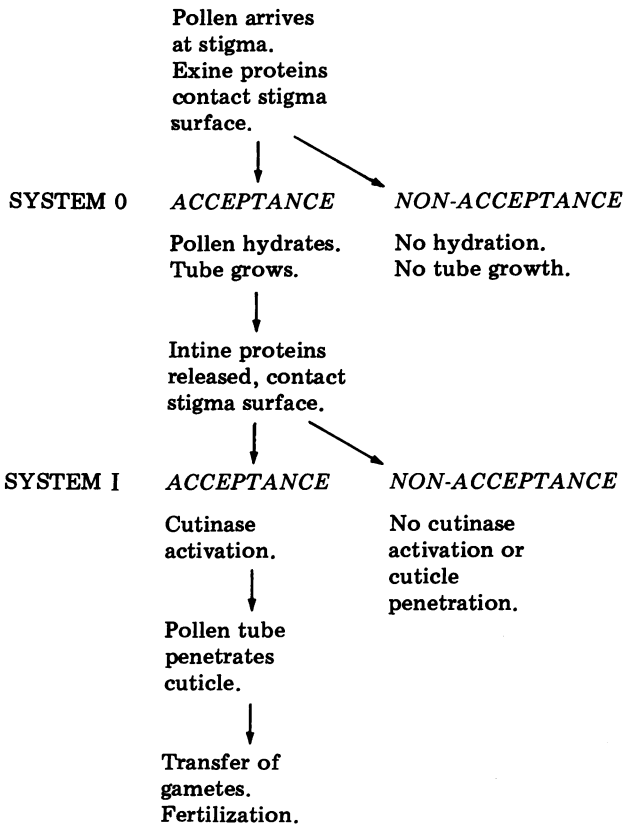

FIG. 5. Sequence of recognition events leading to successful compatible pollination in *Gladiolus*, showing the two points where discrimination of foreign pollen from other families (0) and from other genera (I) occurs.

cell membrane via channels in the cuticle and cell wall. In a study of the mechanism of pollination in *Gladiolus*, we have shown that the cuticle is the only physical barrier to fertilization (20). After penetrating the cuticle, pollen tubes grow through the mucilage-filled guide direct to the ovary. Pollen recognition occurs at the stigma surface.

The components of the papillar surface include a β-glycosyl binding polymer, the "all-β lectin" described in the seeds of many plants (21). This polymer is apparently identical with a component of the guide mucilage[‡] and is therefore unlikely to be involved in the recognition events, but is more likely to function as a nutrient source and physical support for the developing pollen tube, as well as acting as an adhesive for capturing pollen grains at the stigma surface.

The presence of nonspecific esterases in both the pollen wall emissions and on the stigma surface suggests that these enzymes may be involved in the active "cutinase" complex, which is essential for breakdown of the cuticle to allow pollen tube entry (5, 18). Surface determinants, including those exhibiting esterase activity, were removed by treatment of the stigmas with sodium deoxycholate, a detergent used extensively for solubilization of animal membranes. After such treatment, no germination of compatible pollen was detected, indicating removal of receptors for both systems 0 and I. These receptors are presumably removed along with other components of the papillar surface layer, exposing the cuticle surface, which has very different

[‡] A. E. Clarke, S. Harrison, J. A. Considine, and R. B. Knox, "Cell recognition in plants: Lectins and pollen-stigma interactions," manuscript submitted.

binding characteristics, so that it bound labeled proteins twice as effectively as the intact surface.

Binding Characteristics of Stigma Surface. Experiments with ^{125}I-labeled proteins indicate that the stigma surface is generally "sticky" and able to bind nonspecifically to a wide range of proteins and glycoproteins. *Gladiolus* pollen extract did not bind to any greater extent than other pollen extracts tested, so that any specific binding of informational molecules in the pollen extracts is masked by nonspecific binding of other components. The lectin Con A binds to acceptor glycoproteins on the stigma surface and this has been demonstrated both with fluorescent labeling techniques and with binding of ^{125}I-labeled Con A to stigma surfaces. Both techniques revealed the specificity of this binding. Moreover, the presence of this lectin on the stigma surface effectively blocked system I, that is, although compatible pollen germinated and produced long tubes, these failed to penetrate the cuticle.

Isolation of Stigma Surface Receptors. The multicomponent nature of the stigma surface receptors is revealed by the polyacrylamide gel electrophoresis of the ^{125}I-labeled stigma surface extract. This is analogous to the complexity of mammalian cell membrane components. Separation of the Con-A-binding components provides a simplification of the pattern and presumably one of these components (Fig. 3) is crucial to successful completion of system I. The high M_r components are glycoprotein in nature, while the low M_r components are probably glycolipids. Because of the difficulties in obtaining sufficient quantities of the stigma surface, it has not yet been possible to define which of the components are involved in the binding to pollen.

These experiments provide an insight into the biochemical mechanisms involved in the discrimination of self from not-self in plants, and may have application to analysis of receptors involved in sporophytic incompatibility (3, 15, 16), where inhibition of pollen tube growth occurs at the stigma surface.

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