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Pollen-Wall Proteins: Exine-Held Fractions Associated with the Incompatibility Response in Cruciferae

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Summary. During maturation of the pollen in species of *Iberis* (Cruciferae) materials from the anther tapetum are transferred to cavities of the exine. These sporophytically-derived materials are responsible for the rejection response induced in the stigmatic papillae within 4–6 h. following incompatible pollination, a response which leads to the occlusion of the stigma papillae by the polysaccharide, callose. The rejection reaction can be induced with agar or agarose gels into which pollen-wall materials have been allowed to diffuse, and also with isolated fragments of the tapetum itself taken from anthers of incompatible plants before transfer of its contents to the pollen. Partial fractionation of the exine-held materials using thin-layer gel filtration suggests that the incompatibility reactions are mediated by one or more "recognition" proteins or glycoproteins in a group with molecular weights in the range 10,000–25,000 daltons.

The rejection response has been found to be a rapid and sensitive assay for incompatibility in several Cruciferae.

Introduction

Flowering-plant pollen grains carry proteins in two extracellular sites, in the cavities of the sculptured layer of the exine, the sexine, and embedded between polysaccharide layers of the intine, particularly in the vicinity of germination apertures (Heslop-Harrison, Heslop-Harrison, Knox and Howlett, 1973). The exine proteins are synthesized in the tapetum, and are transferred with lipoidal and other material during the final phase of maturation of the pollen; they are accordingly sporophytic (diplophase) in origin. The intine proteins are incorporated during wall growth, and are the product of the spore, or, after pollen mitosis, of the vegetative cell of the pollen grain; they are therefore gametophytic (haplophase) products. We have suggested that the wall proteins in these two sites are likely to be concerned in the recognition responses involved in inter- and intraspecific incompatibility reactions (Heslop-Harrison, 1967; Knox and Heslop-Harrison, 1971a; Knox, Willing and Ashford, 1972; Knox, 1973). In the sporophytic type of intraspecific incompatibility system, the genetical control of pollen behaviour is imposed by the diploid parent, and the inhibition in an incompatible pollination is characteristically at the stigma surface (Linskens and Kroh, 1967). These facts would be neatly explained were the sporophytically-synthesised exine proteins responsible for the recognition function (Heslop-Harrison, 1967, 1968). We show in this report that an exine-borne fraction is indeed involved in the Cruciferae, a family with a sporophytic incompatibility system.

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Materials and Methods

The principal observations were made on clonal material of two species of *Iberis*, *I. semperflorens* L. and *I. sempervirens* L. The genetics of incompatibility in the genus *Iberis* are known from the work of Bateman (1954) and Sampson (1962).

The fluorescent protein "probe" 1-anilinonaphthylsulphonic acid (1-ANS), (Stryer, 1968) was used to follow the synthesis and transfer of the exine-borne proteins and the subsequent release on the stigma. Freeze-sectioned anthers (Knox and Heslop-Harrison, 1970), whole pollen grains and whole stigmas were transferred without any fixation to 1-ANS at 0.001% in 0.01 M phosphate buffer at pH 6.8 containing 15% methanol. This stain-fixing medium was withdrawn after 3–5 min, and replaced by water or 15% methanol. Observations were made with the Vickers Photoplan system with incident illumination using exciter filter no. 1 and barrier filter no. 3. Of many methods tested none apart from immunofluorescence proved as effective as this in stabilising and revealing the highly mobile wall proteins (see Howlett, Knox and Heslop-Harrison, 1973).

The formation of callose in stigma hairs provided a rapid assay for the incompatibility response (Knox, 1973; Dickinson and Lewis, 1973). In a compatible pollination in the Cruciferae, pollen tubes emerged and penetrated neighbouring stigmatic papillae after erosion of the cuticle and then grew down between the lamellae of the cellulosic stigma cell wall (Kroh, 1964). The papillae form no internal callose, although plugs are produced at intervals in the growing pollen tubes in the usual manner. In incompatible pollinations on the other hand, callose lenticules or plugs are formed in a few hours within the stigma papillae in contact with the pollen grains. If the tubes emerge, these also become occluded with callose after making a certain amount of distorted growth. This "rejection reaction" is readily revealed using the decolorised aniline blue method (Arens, 1949) for callose localisation. In the present experiments, tests were made for the activity of extracts and separated protein fractions using unpollinated stigmas from intact flowers, while excised stigmas were used to assay the activity on chromatograms. In most cases the stigmas

were examined for callose formation 4–6 h after treatment, since it is known from the work of Dickinson and Lewis (1973) that secondary changes may occur later in the occluded cells, the callose reaction ultimately disappearing. The selection and handling of stigmas for use in the assay was done with care. Stigmas from early buds did not produce the reaction, and spurious responses could be obtained from stigmas taken from unprotected open flowers, the consequence of accidental selfing or minor surface damage.

For bulk extraction, the exine proteins were stripped from mature pollen in isotonic medium for 5 min so as to avoid disruption of the grains (see Knox, Willing and Ashford, 1972) and the extracts were then concentrated using an Amicon pressure ultrafiltration cell no. 10 PA and UM 10 membranes which allow passage of molecules less than 10,000 daltons.

The pollen-print technique (Heslop-Harrison *et al.*, 1973; Howlett *et al.*, 1973) provided a very delicate and speedy way of separating small amounts of the wall proteins for testing effects on the stigma directly. The pollen to be used was collected on a strip of single-sided sellotape, and this was then pressed into contact with an agar or agarose film (1% in water and c. 0.5 mm thickness) for an appropriate period of time. Under these conditions, the grains hydrated from the film, and the wall materials were printed off into the gel. The sellotape was then pulled away, carrying with it the adhering pollen grains and leaving the prints. Cubes cut from 10 min pollen-print gels were applied to stigmas to test the capacity of the transferred materials to induce the rejection reaction.

The proteins of wall leachates were partly fractionated and characterised by thin-layer gel filtration (Pharmacia, Uppsala). The most satisfactory separation was obtained using Sephadex Superfine Grades G75, G100 and G150, the chromatograms being developed with 0.05 M tris buffer pH 7.5 at an angle of 10° for periods up to 5.5 h. As molecular weight markers ribonuclease A, cytochrome C, ovalbumin and soybean trypsin inhibitor were used at concentrations of 5 mg/ml. For comparison, some runs were made with Antigen E, an allergenic fraction from the pollen of *Ambrosia* spp. (ragweeds), now known to be held in wall sites (Knox and Heslop-Harrison, 1971b; Howlett *et al.*, 1973). Replicas from the gels were made on Whatman 3MM paper and stained with 0.25% Coomassie Blue RL (Searle Scientific Services, High Wycombe) and scanned with a Joyce-Loebl Chromoscan, operated in the reflectance mode.

Results

Synthesis and Transfer of the Exine Materials

As in species of the Malvaceae for which a detailed description has been given elsewhere (Heslop-Harrison *et al.*, 1973), the materials ultimately lodged in the cavities of the exine of the cruciferous pollen grain are synthesised in the tapetum of the anther in the interval following immediately upon meiosis. At the time of pollen mitosis, lipid droplets and protein-containing cisternae of the endoplasmic reticulum are conspicuous in the tapetal cells. At this time the exine sculpturing is fully developed, but no 1-ANS staining material is present in the cavities (Fig. 1). The transfer starts in *Iberis* some 60–70 h before anthesis, when the dissolution of the tapetum begins. Thereafter, although normal fixation and staining procedures do not effectively reveal the tapetum-derived proteins in the reticulations of the exine, they are dramatically exposed by the 1-ANS procedure (Fig. 2). A developmental account of the tapetum and the transfer process will be given in another paper.

Emission Patterns in Pollen Prints and on the Stigma

A pollen print made in agar from a pollen grain of *I. sempervirens* is illustrated in Fig. 3. The time of exposure was 3 min, and the emitted proteins were stained with a Coomassie Blue stain-fixing medium. The effectiveness of the transfer to the gel and the remarkable resolution obtainable with the method is evident. It may be noted that with this time of exposure there has been no discharge of intine proteins from the colpus in contact with the gel. Lipoidal *Pollenkitt* is also present in the print (Heslop-Harrison, 1968; Heslop-Harrison *et al.*, 1973) but this is not revealed by the Coomassie Blue staining.

Fig. 4 shows an unpollinated stigma and Fig. 5a stigma surface 30 min after pollination, both stained under identical conditions with the 1-ANS stain-fixing medium for 10 min. On the unpollinated stigma surface 1-ANS-coupling material is seen in the crevices between the papillae. A thin proteinaceous surface pellicle is also present, but is not visible in this preparation. The release of the exine-borne proteins on to the surface of the pollinated stigma is shown in Fig. 5, and also residues in the exine cavities not in contact with the stigma papillae.

Initially the pollen-wall proteins are not held at all tenaciously on the stigma, and may be removed by brief rinsing. Hower, binding soon begins where the grains have been in close contact, as may be seen in Fig. 6. The binding sites are at or very near the surface of the stigma papillae. The events associated with the binding and with erosion of the cuticle of the papillae preparatory to the penetration of the pollen tubes will be described in a later paper.

Induction of the Rejection Reaction by Separated Exine Proteins

A typical rejection reaction elicited by incompatible pollen in *I. sempervirens* is illustrated in Fig. 7. This may be compared with the micrograph of Fig. 8, made under identical conditions following a compatible pollination, where callose is absent from the hair in contact with the grain.

The callose response is induced when gel cubes cut from pollen prints made from incompatible pollen are placed upon the stigma, but not when cubes from prints of compatible pollen are used (Fig. 9). Similarly, the rejection reaction is developed when the stigma is flooded with crude leachate of wall materials prepared under conditions which would preserve the integrity of the vegetative cells of the pollen grains.

As may be seen in Fig. 7, the callose induced by incompatible pollen tends to be formed in lenticules near the point of contact with the stigmatic papillae

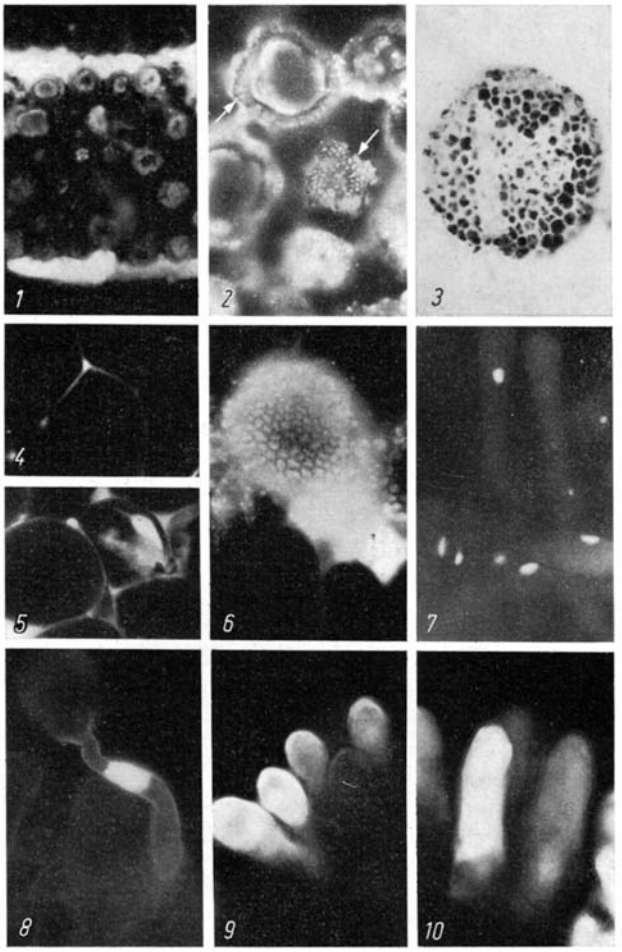

Fig. 1. Localization of proteins in developing anther of *Iberis sempervirens*, 1-ANS procedure, freeze-sectioned without prior fixation. Proteins present in the tapetal cells show intense fluorescence with 1-ANS, while only cytoplasmic activity is detectable in the pollen grains (Xc. 250)

Fig. 2. Localization of proteins in mature pollen just before anther dehiscence, 1-ANS procedure as Fig. 1. The tapetum is absent, and intensely fluorescing proteins fill the exine cavities of the pollen (Xc. 600)

Fig. 3. Pollen-print from pollen of *I. sempervirens* after 3 min exposure to agar gel, Coomassie Blue procedure. The sites of the wall-proteins in the exine cavities are clearly demonstrated (cf Fig. 6). The slit running longitudinally down the print is the site of one of the germinal apertures, but release of the intine proteins has not commenced (Xc. 1200)

Fig. 4. Whole mount of unpollinated stigma of *I. sempervirens* seen in surface view, 1-ANS procedure. Faint specific fluorescence is detectable in the interstices between the stigma hairs, and is presumably associated with the external surface (Xc. 1200)

Fig. 5. Surface view of stigmas, 2 h after self pollination, 1-ANS procedure as in Fig. 4. After this period the stigmas were laved for 1 h in saline to remove proteins not tenaciously bound to the surface of the stigma hairs. Fluorescent proteins remaining can be seen in this view (Xc. 1200)

Fig. 6. Release of exine-held proteins on surface of stigma, 1-ANS procedure as in Fig. 5, without saline washing (Xc. 950)

Fig. 7. Rejection response induced in unpollinated stigma hairs of *I. sempervirens*, 4 h after self pollination, decolorized aniline blue technique. The hairs have been teased out so that several callose lenticules are in the same focal plane, and the pollen grains have been lost into the medium (Xc. 430)

Fig. 8. Acceptance of compatible pollen of *I. sempervirens*, decolorized aniline blue procedure as in Fig. 7, fixed 4 h after pollination. No callose can be seen in the stigma hair through which the pollen tube has penetrated, and a plug of callose has formed only in the pollen tube (Xc. 600)

Fig. 9 and 10. Rejection response induced in unpollinated stigma hairs of *I. semperflorens*, decolorized aniline blue procedure as in Figs. 7 and 8. Fig. 9 shows the result of exposure of the stigma hairs to agarose gels diffused with pollen-wall materials by the pollenprint technique with 10 min exposure to the pollen. Fig. 10 shows stigma hairs exposed directly to hand sections of tapetal tissue from anthers with developing pollen prior to transfer of the exine proteins (Xc. 600)

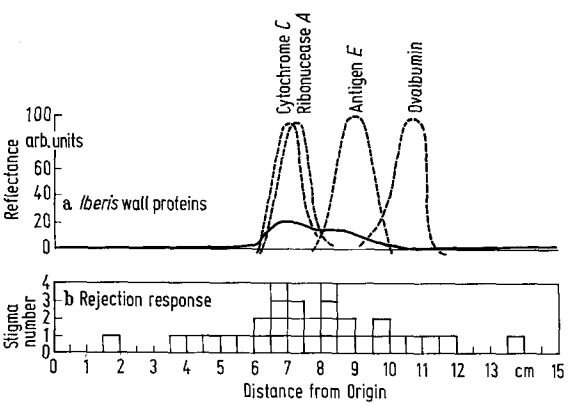

Fig. 11

Fig. 11. Thin-layer gel filtration fractionation of the wall proteins from *I. semperflorens* pollen, together with the response of unpollinated stigmas placed directly on the gel.

Fig. 11a shows a scan of a Coomassie-Blue stained replica run on the same gel as used for Fig. 11b. Solid line shows the pattern obtained for the concentrated pollen extract, broken lines the patterns of standard, marker proteins applied at a concentration of 5 mg/ml to the same gel: cytochrome c (MW 12,400); ribonuclease A (MW 13,700); the ragweed pollen allergen, Antigen E (MW 38,000); and ovalbumin (MW 45,000).

Fig. 11b shows diagrammatically the actual number of stigmas exhibiting the rejection response following exposure to the gel for 4 h, using the decolorized aniline blue procedure. Samples of four excised stigmas were placed in 30 parallel rows 5 mm apart to cover every 5 mm of the gel surface, c. 2×15 cm. The squares on the diagram indicate stigmas that showed a demonstrable response. Sephadex G150 Superfine gel, 0.8 mm thickness, 10° angle, run for 5.5 h

(Dickinson and Lewis, 1973). The gels tend to envelop the whole surface of the papillae in contact, and under these conditions the callose reaction is more generally distributed (Fig. 9).

Induction of the Rejection Reaction with Isolated Tapetum Fragments

Proof that the materials responsible for the re jection reaction are derived from a sporophytic tissue, the tapetum, was obtained by dissecting out the tissue just before the onset of dissolution and applying this to the stigma of a flower from the same plant. Again a vigorous rejection reaction was elicited (Fig. 10).

Preliminary Fractionation of the Exine Proteins

The presumption in the foregoing is that a protein or glycoprotein fraction of the exine-borne materials is concerned in the recognition response of sporophytic incompatibility. The experiments do not, however, exclude the possibility that other constituents are involved, since lipid and carbohydrate moieties are also transferred to the wall from the tapetum. Assays have therefore been carried out using extracts fractionated by thin-layer gel filtration (Radola, 1968). Two major and two minor fractions were detected using Sephadex G150 Superfine gel (Fig. 11a). From comparison with the reference proteins run on the same gel, the molecular weights of the fractions range from 10,000 to about 40,000 daltons. Fractionation on Sephadex G75 Superfine gels confirmed this pattern, but showed that the bulk of the proteins are within the molecular weight range 10,000—25,000. Fig. 11b shows the results of one of three experiments which we have carried out to determine whether the fractionated proteins are active in inducing the rejection response in unpollinated stigmas of *I. semperflorens*. The zones with stigmas showing the rejection reaction can be directly compared with the scan of Coomassie Blue-stained self proteins run from a neighbouring gel strip. There is a clear association between the zone of maximal stigma response and the sites of the fractionated pollen-wall proteins. The technique is as yet too crude to assign the response to a particular fraction, but the greatest response was obtained from the stigmas in contact with the fractions with molecular weights between 10,000 and 25,000 daltons.

Discussion

Dickinson and Lewis (1973) have fully characterised the callose rejection reaction for *Sinapis alba*, and in experiments similar to those reported here but using somewhat different techniques Dickinson (in preparation) has shown that here also the response in incompatible pollinations is determined by exine-borne materials. The earlier postulation that tapetum-derived fractions housed in exine cavities must be concerned in sporophytic incompatibility systems (Heslop-Harrison, 1967, 1968) seems therefore to be justified.

Evidence given in this paper supports the earlier assumption that the fractions concerned are proteins or glycoproteins, a finding in full accordance with earlier views on the nature of the pollen contribution, notably those expressed by Linskens and Kroh (1967) and Sampson (1962). Presumably the initial reaction is between the pollen wall protein and a stigma fraction, such as those studied by Nasrallah, Barber and Wallace (1969).

There are striking parallels between the stigma response to self and not-self pollen proteins revealed in this report, and the reactions found in mammalian immunology between sensitised lymphocytes and antigen (see Burnet, 1971); lymphokines are released that may inhibit or promote cell proliferation. Also, in plant disease resistance, Frank and Paxton (1971) have implicated a glycoprotein (WM 10,000—30,000) released by the soybean stemrot fungus, *Phytophthora megasperma* as the inducer of the phytoalexin involved in host defence reactions in resistant soybean plants.

The stigma rejection response, detectable within 4—6 h after incompatible pollination in crucifers, may well have considerable practical use in plant breeding techniques as an assay for incompatibility in sporophytic self-incompatible plants. Marrow-stemkale, *Brassica oleracea* var. *acephala* has a well-defined sporophytic self-incompatibility system (Thomspon 1957; Thompson and Howard, 1959). Recent work, in collaboration with Dr. K. F. Thompson, has demonstrated a similar rejection response in the stigmas to that reported here in *Iberis*, following selfings in S genotypes high in the dominance series. Matings between these genotypes are compatible, and the stigmas showed no rejection response. The response does not seem to be confined to the Cruciferae since the callose occlusion of both stigma hairs and pollen tubes has been observed following selfings in the composite, *Cosmos bipinnatus* (Knox, 1973) and even in interspecific incompatibility in *Populus* spp. (Knox, Willing and Pryor, 1972).

Work is now being directed at a fuller fractionation and characterization of the exine-held proteins, and also towards the precise identification of the receptor sites on or in the stigmatic papillae.

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