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Control of pollen hydration in *Brassica* requires continued protein synthesis, and glycosylation is necessary for intraspecific incompatibility

(cell recognition/plant glycoprotein/self-incompatibility)

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ABSTRACT **Pollen hydration and self-incompatibility (SI) in *Brassica* have been studied by using a combination of *in vivo* video-microscopy and experiments with metabolic inhibitors. Experiments with cycloheximide confirm earlier observations that pollen hydration is regulated through protein synthesis. No protein or glycoprotein has positively been identified with this event; however, it is unlikely that the total pool of any particular glycoprotein is involved, but rather a newly synthesized or otherwise activated fraction. Micromanipulation of pollen on the stigmatic papillae suggests that access to this hydration regulation system is limited to members of the Brassicaceae: pollen grains of other species—even those possessing dry stigmas—fail to hydrate. It is proposed that an interaction between enzymes of the stigma surface and the superficial layer of the pollen grain coating creates continuity between the content of the papillar wall and the grain protoplast. Inhibition of protein synthesis also overcomes SI, and since the advent of regulated hydration and synthesis of the so-called *S*-gene glycoproteins coincide with the acquisition of the SI system, there is strong circumstantial evidence that the same molecular species is involved in both processes. Experiments with tunicamycin, which prevents glycosylation of glycoproteins, indicate that the glycosyl groups of the *S*-gene glycoprotein are required for the operation of the SI system but not for the regulation of hydration. Further experiments suggest that pollen is positively inhibited on incompatible papillae but that this inhibition is biostatic. Recovery from the effects of the SI system appears to involve the metabolism of an inhibitor by the pollen. SI in *Brassica* thus emerges as a sophisticated process under dynamic control in both the female and male partners. The evolutionary advantages of such a system are discussed.**

The regulated passage of water from stigma to pollen is an essential prerequisite for successful pollen tube development (1). Controlled hydration is particularly important for pollen grains of *Brassica*, for, once they have adhered by means of a surface coating (2, 3), they draw water through the "dry" surface of the stigmatic papillae. Members of the Brassicaceae also possess a self-incompatibility (SI) system, which prevents self-pollination by inhibiting development of the pollen grain on the stigma surface, and a range of data now indicates that there is a link between the operation of the SI system and pollen hydration (4, 5). Recent structural studies involving anhydrous fixation (6, 7) have provided dramatic evidence in support of Heslop-Harrison's (1) contention that dry pollen extracts water from the stigma first by means of matric potential and then, on formation of an intact plasma membrane, by a system based on turgor pressure differentials. To achieve regulated flow, the hydraulic "pull" exerted by the pollen grain must be matched by a resistance to flow on the part of the stigma.

The structures involved in this process are quite complex; the pollen is bounded by lipidic coating (2) invested by a membrane-like coating superficial layer (CSL) (7), while the stigma surface consists of an enzyme-rich superficial pellicle (8) investing an irregular cuticular layer. The papilla boundary is well adapted for the regulated passage of water, for the cellulosic wall possesses a number of microchannels visible in freeze etching preparations (9), and osmotic changes in the papillae may open cracks in the cuticle itself (8). Nothing is known of the physiology of hydration and, in particular, of the manner by which hydration of pollen grains on "dry" stigmas (10) is linked with the SI system.

MATERIALS AND METHODS

Plant Material. *Brassica oleracea* (cabbage, kale) plants homozygous for the incompatibility alleles *S25*, *S29*, and *S63* (supplied by D. J. Ockendon, Institute of Horticulture, Wellesbourne, Warwickshire, U.K.) and *Brassica napus* (rape), *Brassica campestris* (turnip), *Raphanus sativus* (radish), and *Sinapis* sp. (mustard) plants were raised from seed in a heated greenhouse.

Study of Pollen Grain Hydration. All pollinations involved stigmas from freshly opened flowers. After the careful removal of petals, calices, and anthers, pistils were affixed to a microscope slide with a thin band of Blu-tack (Bostik, Leicester, U.K.). Hydration of the pistils was maintained by applying a small piece of tissue paper soaked in water to the cut ends. Using carefully drawn (terminal diameter 20 μm) glass needles fitted to a micromanipulator (Narishige Optical Instrument Services, London), pollen grains were moved to the papillar surface to effect pollinations. Operations were viewed by means of an inverted microscope (Leitz Labovert) linked to video recording apparatus (Sony U matic). The accuracy of the system was such that pollen grains from different sources could be placed adjacent to one another on a single papilla; even chains of several pollen grains could be affixed to papillae. Between observations, pistils were removed and maintained under a normal vapor pressure deficit [VPD; normal VPD = 11–14 millibars (11) (1 millibar = 100 Pa]. Pollen grains were recorded as hydrated only when the ratio of their axes approached 1 (12).

The manipulations involved in the pollen transfer experiment were carried out as described above, but in the cases in which pollen was removed from a stigma and then maintained in a normal VPD for a period, grains were transferred to a glass slide for the appropriate interval.

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Abbreviations: SI, self-incompatibility; CH, cycloheximide; TM, tunicamycin; GPs, glycoproteins; SGPs, *S*-gene GPs; VPD, vapor pressure deficit.

Study of Pollen Germination and Tube Growth *in Vivo*. Excised pistils were placed in the wells of polystyrene microtiter plates (Titertek, Flow Laboratories) containing glass-distilled water and stigmas were pollinated by touching their surfaces with newly dehisced anthers. When cyclohexeximide (CH) treatment of the stigmas was required, the drug was added to the water to give a concentration of 10 mM. (Our recently obtained results indicate that all the effects of CH are precisely paralleled when either 0.24 mM blasticidin S or 0.1 mM puromycin is used in place of CH.)

To determine pollen development and tube growth, stigmas were either mounted directly in decolorised aniline blue (BDH) and stained as described by Linskens and Esser (13) or fixed in chloroform/absolute ethanol/acetic acid (6:3:1, vol/vol), softened with 1 M NaOH at 60°C for 1 hr, and then stained. After staining, material was mounted in a drop of 50% (vol/vol) aqueous solution of glycerol prior to examination in a Light Dialux microscope fitted with an incident UV illumination system (Leitz; excitation 450–490 nm, beam split 520 nm, and suppression 575 nm). Pollen germination and tube growth were scored in all circumstances.

***In Vitro* Pollen Germination.** Pollen of *B. oleracea* was germinated in a culture medium previously described (14). Where appropriate, CH was added to the germination medium to a final concentration of 10 mM.

Treatment with Tunicamycin (TM). TM is not easily translocated through the pistil vascular system and, for this reason, stigmas were excised together with 0.5 mm of the style and the cut end was swiftly transferred to the surface of a piece of filter paper soaked in 0.12 mM TM (Sigma) in water. Stigmas were pollinated after 2 hr of treatment. Control stigmas received identical treatment but were supplied only with glass-distilled water.

Pollen Hydration on TM-Treated Stigmas. Narrow glass tubes, 2 cm long with a diameter of 0.75 mm, were placed into the wells of microtiter plates containing 0.12 mM TM. Capillary action drew the liquid up to the top of the tubes. Stigmas, prepared as previously described, were then inserted into the top of the tubes such that continuity was established between the solution and the cut surface. This arrangement ensured that pollen on these stigmas experienced normal VPDs rather than the hydrated atmosphere characteristic of the earlier experiments.

Owing to the delicacy of the system, and the fact that progress of hydration was followed by video microscopy, individual pollinations had to be made by using the micromanipulator under an inverted microscope. Pollinations were always made 120 min after treatment commenced to ensure that the drug was present in the papillae.

RESULTS

Pollen Hydration on the Stigma Surface. The examination of living pollinated stigmas in a dry environment (see Fig. 1) clearly indicates pollen hydration kinetics to be dependent upon the *S* genotype of the pistil. Indeed, *S* genotypes can be arranged in a ranking of the speed with which they promote the hydration of pollen grains (see Table 1, row 3). An average hydration rate for compatible pollen under normal conditions (VPD ≈ 11–14 millibars) is of the order of 90 min. Mature pollen placed on buds of plants containing any *S* genotype hydrates very quickly, generally being completely rounded within 25 min (see fourth row of Table 1). However, even in these circumstances, some slight genotype-specific differences are observed.

Differences in hydration rate between plants with different *S* alleles are clearly obvious when self-pollinations are studied. While penetration of the stigma by self-tubes is rare in *B. oleracea*, the extent of self-pollen development on the stigma very clearly depends upon the *S* alleles present. For example,

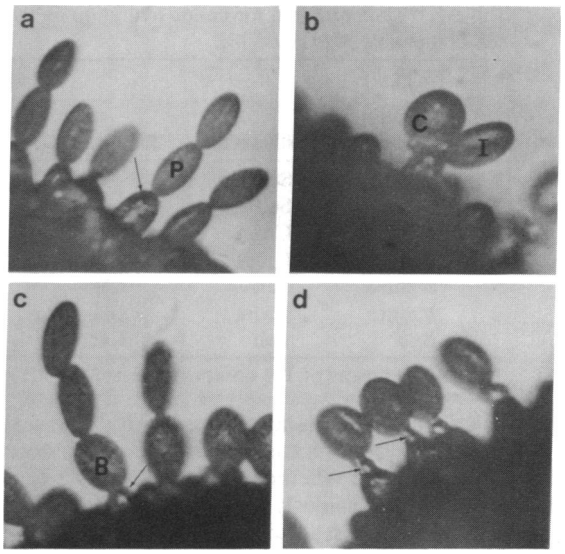

FIG. 1. Micrographs taken directly from the video monitor screen. (×950.) (*a*) Self-pollen grains (P; *S25* genotype) placed individually and in chains on stigmatic papillae (arrow). No hydration has occurred. (*b*) Compatible (C) and incompatible (I) grains on the surface of the same stigmatic papilla 24 hr after pollination. The cross pollen has hydrated and developed a tube, whilst self-pollen remains fusiform. (*c*) Compatible pollen grains (B) placed in chains on stigmatic papillae (arrow). Only the grain adjacent to the papilla has hydrated in the 10 hr since pollination took place. (*d*) Self-pollen on a CH-treated stigma. In the first hour after pollination, all the grains have hydrated and produced short tubes (arrows).

self-pollen grains on stigmas with *S25* alleles show little or no sign of hydration (Fig. 1*a*), while selfing of plants with *S63* alleles results in considerable hydration of pollen grains, emergence of pollen tubes, and the growth of some of these tubes along the stigma surface. Hydration of pollen normally occurs after interspecific pollinations amongst close relatives of *Brassica*, but no subsequent development takes place.

When a compatible pollen grain and incompatible one are placed side by side on the same stigmatic papilla they behave entirely independently; the compatible hydrates within 90 min, while the incompatible swells slightly but retains its fusiform image for up to 24 hr (Fig. 1*b*). When pollen grains are placed on the stigma in chains, but with only one pollen grain touching the stigma surface, only that grain in contact reacts according to its compatibility, while all others remain dehydrated (Fig. 1*c*). "Compound" pollinations made with grains from other closely related species also result in the sole hydration of the grain adjacent to the stigmatic papilla.

When CH is applied at 10 mM, very rapid hydration of pollen grains ensues. Whether pollination is intraspecifically incompatible or completely compatible, the pollen grains hydrate more rapidly than controls (see Table 1, rows 5 and 6), and will often rapidly extend a pollen tube onto the surface of the stigma (Fig. 1*d*). From fluorescence microscopy it is difficult to determine whether pollen germination in the presence of high concentrations of CH produces tubes that penetrate the stigma surface because, under all circumstances, tubes cease growth at a length approximately equivalent to that of the stigmatic papillae; however, transmission electron microscope observations clearly show the presence of pollen tubes in the papillae walls. Although long tubes are produced when *Brassica* pollen is germinated *in vitro* in a specially formulated culture medium (14), only short tubes emerge when 10 mM CH is added to this medium. Compound pollinations have also been carried out with CH-treated stigmas, and, once more, exclusively the pollen grain adjacent to the pollen surface hydrates very rapidly.

Table 1. Time required for pollen hydration on the surface of stigmas of three *S* genotypes and a self-compatible *Brassica*

	Flower	Pollination	Treatment	Hydration time, min *S63*	*S25*	*S29*	Self-compatible
1	Mature	Self	—	Variable	—	—	88 ± 5
2	Bud	Self	—	15.7 ± 0.6	29 ± 1	27 ± 1	32 ± 0.8
3	Mature	Cross	—	70.5 ± 5	102.6 ± 4.5	95 ± 5	93 ± 0.8
4	Bud	Cross	—	15 ± 0.6	24.5 ± 0.6	26 ± 0.8	32.2 ± 1
5	Mature	Self	CH	23 ± 0.8	34 ± 1.4	24.3 ± 1	35 ± 1.2
6	Mature	Cross	CH	25.8 ± 0.1	31.4 ± 1	24.8 ± 0.8	36.2 ± 1
7	Bud	Self	CH	15.2 ± 0.6	25 ± 0.8	26 ± 1	29 ± 1

Data are means of 100 observations and the 95% confidence limits are given.

The only exception to this accelerated hydration caused by CH is in buds, where hydration of mature pollen occurs at exactly the same rate, whether or not the stigma has been treated with CH (see Table 1, rows 2, 4, and 7). CH-treated bud stigmas often possess somewhat flacid papillae with irregular outlines, and experiments using solutions of equivalent molarity to the CH suggest that this effect is likely to result from turgor pressure differentials acting on the thin cell wall rather than from the action of the drug.

Surprisingly, CH treatment of stigmas of *B. oleracea* also promotes the germination of pollen of other species, including *B. napus*, *B. campestris*, *B. alboglabra*, *R. sativus*, and a species of *Sinapis* (Table 2). In all cases the pollen tubes extend to a length normally observed after this type of pollination in *B. oleracea*.

Effect of Inhibitors on SI. When CH is supplied to stigmas already pollinated with self-grains, rapid hydration ensues and tubes emerge about 120 min after treatment, even if the pollen has been present on the stigma for up to 24 hr. After 24 hr on a self-stigma, subsequently treated with CH, 70% germination and 60% tube production for self-pollen were observed. Since hydration of grains applied to the surface of CH-treated stigmas occurs almost immediately, it is reasonable to assume that the delay of 120 min between CH application and the "release" of self-pollen from the SI system relates to the time required for inhibitor transport to the stigma surface.

In an attempt to discover whether self-pollen is actively inhibited on the papillar surface, rather than simply failing to "make contact" with the female cell, stigmas of *S25* were self-pollinated for 3 hr and the pollen was transferred to CH-treated stigmas of the same genotype. In the controls, CH-treated *S25*, stigmas were self-pollinated with grains directly from the anther. The results from this work (see Fig. 2*a*) show that 180-min exposure on a self-stigma causes grains to develop far more slowly when transferred to the CH-treated papillae. Fresh grains, on the other hand, germinate directly and produce tubes. This delay in development seems to result solely from an interaction between the self-grains and the stigma surface, for pollen removed from the anther and held in a dry environment for an equivalent period shows no such delay in hydration or germination when placed on CH-treated stigmas.

These results indicate that the inhibition of self-grains is reversible but provide no indication as to whether pollen itself plays a part in this process. *S25* stigmas were therefore self-pollinated and, after 180 min, pollen was removed and held on a glass slide or micromanipulation needle for a further 180 min. Finally, the grains were transferred to CH-treated *S25* stigmas. When the response of these grains was compared with that of those transferred directly from the self-stigma, the delay induced by the experience on the self-stigma was observed to be lost. The grains behaved exactly as though they had been transferred directly from the anther.

Although early experiments using the glycosylation inhibitor TM provided only equivocal results (15), the apparent importance of the *S*-gene glycoproteins (SGPs) in pollen–stigma interactions is such that further study was merited. Further, our experiments with CH indicated that translocation of molecules within the body of the stigma itself was very slow, and that the likelihood of a larger molecule such as that of TM (M_r of 817 as compared with 281 for CH) being transported from the base of the stigma to the papillae was

Table 2. Percentage germination obtained on *B. oleracea* (*S63*) stigmas with pollen grains from other *Brassica* species and closely related species

Pollen parent	% germination Control	CH-treated
B. oleracea	3	73
B. napus	2.5	69
B. campestris	1	71
B. alboglabra	5.4	68
R. sativus	2	68
Sinapis sp.	4	70

The controls are on untreated stigmas and the CH-treated stigmas received CH for 2 hr before pollination.

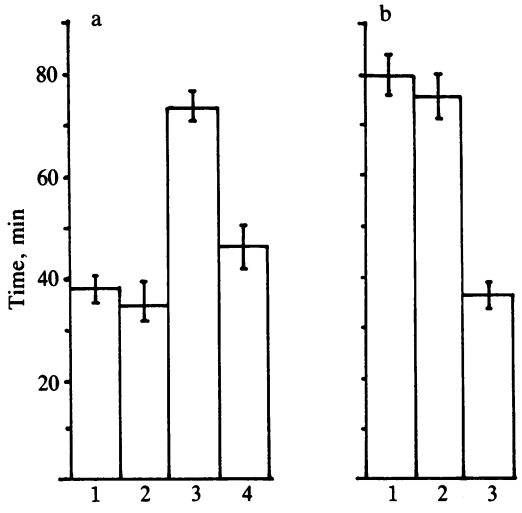

FIG. 2. (*a*) Hydration times of self-pollen after transfer experiments. Bars represent 95% confidence limits. Column 1, self-pollen on CH-treated stigma. Column 2, self-pollen, excised from the anther and maintained in dry environment for 180 min before transfer to CH-treated stigma. Column 3, pollen exposed to self-stigmas for 180 min and then transferred to CH-treated self-stigma. Column 4, pollen exposed to self-stigma for 180 min, removed, maintained in a dry environment for 180 min, and finally transferred to a CH-treated self-stigma. (*b*) Comparison of the effect on hydration of TM and CH in pollinations in *B. oleracea*. Column 1, control cross-pollination (*S25* × *S63*). Column 2, TM-treated self-pollination (*S25*). Column 3, CH-treated self-pollination (*S25*).

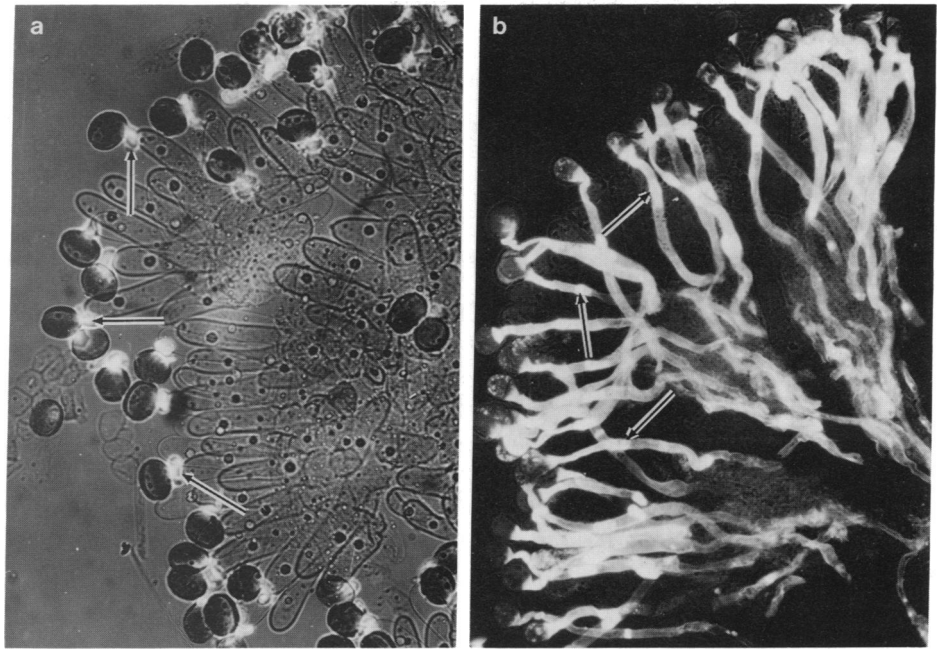

FIG. 3. Effect of TM on self-pollinations in *B. oleracea*. Pollinations were stained with aniline blue and viewed under UV illumination with phase-contrast background. (×680.) (*a*) Control self-pollination. The small callose-invested tubes (arrows) are inhibited at the surface of the stigmatic papillae. (*b*) TM-treated stigma after self-pollination. Large numbers of callose-invested tubes have advanced into the main body of the pistil. Callosic plugs (arrows) are clearly visible within these tubes.

small. From an alternative approach (see *Materials and Methods*), it can be seen (Fig. 2*b*) that TM exerts a very real effect on pollen–stigma interactions in *Brassica*. The SI mechanism appears completely suppressed, and pollen germinates to produce a tube in a manner identical with that in compatible pollinations (see Fig. 3). TM performs even better than CH as an SI suppressor, for glycosylation seems not to be needed for extended tube growth, and the tubes grow well down into the body of the stigma. When the rates of hydration of pollen placed on either CH- or TM-treated stigmatic papillae are charted (see Fig. 2*b*) it emerges that pollen on the CH-treated pistils hydrates quickly, often within 20 min, while that on the TM-treated papillae hydrates at a rate comparable with normal cross-pollination.

DISCUSSION

Mechanism of Pollen Grain Hydration. Studies of pollen hydration in *Brassica* are complicated by the fact that stigmas of different plants hydrate pollen at various rates. These differences may well be independent of the SI gene expression, and recent information suggests that they can result from differences in thickness of the papillar cuticle (16). Nevertheless, in all plants studied, pollen hydrates slower and in a more regular fashion on mature rather than bud stigmas. This difference cannot result from a modification of the wall during maturity of the pistil, for hydration accelerates to "bud rates" when the papillae are treated with CH. Because pollen hydrates at the same speed on bud stigmas, whether CH-treated or not, it is likely that the drug exerts its effect upon the stigma rather than the pollen. These data also provide incontrovertible evidence that the measured hydration on the surface of mature stigmatic papillae requires continuous synthesis of a protein. It could reasonably be argued that, in addition to inhibiting protein synthesis, CH induces other physiological changes in the stigma or pollen. However, our recent experiments (unpublished) with blasticidin S and puromycin, protein synthesis inhibitors with very different structures from CH, indicate that the effects observed result only from the inhibition of protein synthesis.

The stigmatic papillae of *Brassica* contain a number of proteins, the most highly characterized of which are glycoproteins (GPs) thought to be associated with the SI system (SGPs) (17–19). Importantly, GPs similar to these are also present in self-compatible varieties and in other *Brassica* species that do not possess operational incompatibility systems—e.g., *B. napus*. However, since CH-treated pistils can be shown to contain large quantities of these SGPs or their equivalents (R.H.S. and H.G.D., unpublished data), regulation of hydration might be mediated either by newly synthesized GP, which is later returned to an inactive pool in the cytoplasm, or by a small fraction of the GP that is in some way activated through protein synthesis. Circumstantial evidence for the involvement of newly synthesized protein with the pollen–stigma interaction has also come from autoradiographic work (9), which demonstrated that incorporated amino acid is first localized in the wall of the papillae and then translocated to the vacuole. It is clearly premature to hypothesize how this system might operate, but the simplest model would involve the active protein, perhaps accompanied by a considerable hydration shell, being translocated from the stigma to the pollen grain with the stigmatic water. On arrival at the grain, it would be metabolized within either the pollen coating or the protoplast itself. Since it is known that GPs have a high water-trapping capacity, the so-called SGPs might seem well adapted for this role. However, as is discussed later, it appears that hydration can still be regulated even if glycosylation is inhibited in the papillae. Despite the clear indication provided by these results, they do not exclude the possibility that the CH is translocated to the pollen and takes its effect there. Were this to be the case hydration would have to be regulated by an interaction between molecules produced by the mature stigma and a mechanism in the pollen requiring the synthesis of protein.

CH has also proved a useful tool in investigating the physiology of interspecific pollinations. It is well known that the response of pollen in interspecific pollinations is very variable, and much of this variability lies in the hydration rates of these pollen grains. The use of CH circumvents these problems, and hydration after an interspecific cross—if it is

to occur—takes place quickly. Our results suggest that both stigma and pollen surfaces have a unique part to play in the hydration process, for dehydrated pollen placed on a fully hydrated grain remains incapable of drawing water from it. Stigma surfaces do contain an active esterase (8), and this enzyme may act on a surface component of the coating such that the main body of the coat makes direct contact—through the papillar cuticle—with the contents of the cell wall beneath. The evolution of such relatively simple barriers to pollen development must serve not only to promote the identity of that species but also to prevent infection by microorganisms.

Relationship Between Hydration and SI. Developmentally, it is impossible to separate the acquisition of the SI mechanism from the appearance of "regulated" hydration. If the proteins central to these two processes are the SGPs, they might be assumed to carry an *S*-specific message into the grain in addition to regulating the flow of water. This message would be identified by a component in the pollen and, depending upon the nature of the interaction, pollen development would either be inhibited or permitted to proceed. It is of importance to ascertain whether the "SI role" of this molecule is mediated through the proteinaceous or glycosyl groups or a combination of the two. The effect on hydration and SI of TM, which is held to inhibit glycosylation of GPs (20), confirms unambiguously that the glycosyl groups are essential for the successful operation of the SI but not hydration. This experiment fails to confirm absolutely that the recognition sequence is carried in the glycosyl groups, but it does suggest that they play a key part in either recognition or response. Certainly glycosyl groups of glycoproteins have been implicated in a number of other recognition systems and, in particular, in the Ia antigens of the mouse histocompatibility system (21).

If, indeed, it does emerge that the same family of glycoproteins is involved in both the regulation of hydration and SI, a pointer is perhaps provided towards the mechanism by which SI has evolved in more primitive species with gametophytically controlled SI. If the possession of these molecules conferred the ability of plants to regulate the growth of pollen tubes in their styles either through osmotic or metabolic control, they would represent ideal "vectors" for the SI message. This would be not only on account of their being constantly supplied to the developing pollen tubes but also in their possession of glycosyl groups featuring many possibilities of rearrangement into different "recognition sequences." Thus, as sporophytically controlled SI evolved, and plants acquired the ability to regulate pollen development from the earlier stages of germination on a dry stigma, it is not surprising to find the same families of molecules involved, but instead transferred to the grains from a "periplasmic" pool in the papillar wall rather than being secreted into a transmitting tissue. The inference that these GPs may possess a role other than in SI is underlined by their presence in all self-compatible *Brassica* species so far studied.

Further details of recognition and response in *Brassica* SI remain unclear. The simplest explanation of these events would be, after a self-pollination, for a stigmatic SGP to bind specifically with a component of the grain coating. This hybrid molecule would then move to the pollen protoplast, where it would act as an inhibitor. Hypothesizing along these lines is much hampered by the fact that we know nothing of the male molecules involved, except that GPs of molecular weight equivalent to that of female SGPs have been found in the pollen. Other mechanisms are, of course, possible, including one in which the female SGPs are cleaved by a pollen coat enzyme to produce oligosaccharides. These would be drawn to the pollen grain surface, where they would be recognized and, after a self-pollination, act as inhibitors. The involvement of such oligosaccharides in cell communication has been examined in some detail in the recent literature (22).

Whatever the nature of the male protagonists in SI, the combination of evidence (23) pointing to the presence of an inhibitor in self-pollinated stigmas, combined with the data presented here, indicates that pollen is actively inhibited by a biostatic mechanism. Further, our work suggests that whatever inhibitor is generated during the "experience" on the incompatible papilla, the pollen is capable of degrading it over a comparatively short period.

SI in *Brassica* is emerging as a finely balanced physiological system, possibly being under dynamic control both in the stigmatic papillae and in the pollen grain. Clearly, any change to the continuing turnover processes in either pollen and stigma due to environmental conditions, or developmental changes in the plant, could render the system pseudocompatible and thus capable of producing viable seed. Such a sensitivity to external circumstances might well confer a considerable evolutionary advantage on plants possessing these systems.

Recent information on the genetical control of SI in *Brassica* (24) points to the involvement of a second gene, which is oppositional in its operation but appears to exert its effect gametophytically. It is therefore perhaps naive to expect SI in *Brassica* to involve solely the interaction of a stigmatic GP with an element of the pollen grain coating. The participation of gene products within the pollen protoplast may well be required, either through the generation of hybrid molecules from sporophytic and gametophytic components or by some process so far unknown.

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