Biochemistry and Physiology of Recognition in Pollen-Stigma Interactions

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Discriminative interactions between cells can explain recognition phenomena that occur in nearly all life forms. In higher plants, the gametic life-cycle phase is characterized by numerous cell-to-cell (surface-to-surface) contact relationships. In facilitating fertilization, intercellular developments arise from information transfers between the male gamete (the pollen grain and its extending tube) and the cells of the female tissues (the pistil). There is need to understand how recognition events regulate the cell-to-cell interactions that guide pollen tube development from the receptive surface of the female (the stigma) to the specific termination site in the ovary (the egg).

Integrated among these complicated interactions is a genetically regulated self-recognition system that prevents self-fertilization (reviewed in cited references 1 and 24). The phenomenon, which is called self-, generative-, or sexual-incompatibility, exists in flowers of nearly half of all plant species (2). Genetic studies indicate that molecular components of the self-recognition system are coded by multiple alleles of a complex gene called the *S*-gene (18). Within a species, pollen will germinate on the stigma, grow through the style and effect fertilization in ovaries of plants carrying a different S-alleles(s), but, pollen is specifically prevented from effecting fertilization following self-pollination or following crosses between sexually related plants carrying an identical S-allele (17), unless that allele is recessive or inactive (30).

Sexual incompatibility begins with a "recognition" between pollen and pistil when the interacting cells either possess a common S-allele or, as occurs in sporophytically controlled *Brassica*, when the haploid pollen grain develops from a diploid cell with an identical ("self") S-allele. We proposed (7) that this recognition event involves a genotype-specific interaction of a mobile informational molecule on the stigma papillae, with a receptor molecule permanently located in or on the pollen grain. With sexual incompatibility, this recognition reaction is prerequisite to manifestation of the biochemical event(s) or physiological process(es) that prevents continued pollen development.

Evidence is presented that this self-incompatibility system, which is genotype specific, involves recognition of self and is controlled by a stigmatic S-allele-coded glycoprotein. We hypothesize that the glycoprotein blocks continued development of self pollen by specifically interfering with attachment of pollen grains and tubes to the female tissues.

Prior to discussing self-incompatibility, we first describe distinct, early stages of pollen germination and tube development. This includes the reporting of two additional recognition systems, thigmotropism and contact guidance, that control male gamete (pollen tube) orientation and its progress from one developmental stage to the next.

Stages of pollen germination and tube development. By controlling environmental conditions, five developmental stages have been identified for Brassica pollen germination in vitro. The first four stages are autotrophic. They are programmed within the mature grain to occur before pollen tube penetration into female tissues. Except for recognition of self (7), development of these stages does not require specific messages from female tissues. Pollen tube development has been arrested at these different stages, depending on the substratum and relative humidity.

Primary binding stage. The first postpollination stage of pollen development is a primary binding of the grain to the stigma papillae. The first recorded difference between compatible and incompatible pollinations occurs within minutes at this primary binding stage. For cross pollen, binding to the stigma immediately after pollination is significantly greater than binding of self pollen (28). Little is known of how this primary binding stage controls development of compatible pollen or the incompatible response of self pollen. This primary binding is probably a loose, sticky adhesion of the pollen to the papillae that occurs as the proteinaceous components of the stigma pellicle and pollen wall mix and flow onto and around the pollen grains (11,28).

Hydration stage. Pollen of *Brassica* is relatively desiccated at maturation. The second postpollination stage of pollen grain development involves water uptake. As measured by grain swelling (ratio of major vs minimum axis), hydration begins in vitro in atmospheres at 50% relative humidity (RH) or higher (28). Our observations indicate that following hydration, germ tube formation consistently occurs at a higher RH of about 98%. At 88% RH the grain swells (hydrates) but tube formation is erratic, and below 88% RH hydration but not germination occurs. Thus, in vitro, it appears that a requisite level of cell turgor is essential for germ tube formation.

In vivo pollen hydration may be mediated by a stigmatic hydration factor. After Sephadex chromatography of whole stigma extracts (5), we identified a hydrophilic factor in the low-molecular-weight region of chromatograms that (after freeze-drying) rehydrated within minutes (even seconds) after exposure to the atmosphere. For "dry" stigmas (like those of *Brassica*) this factor might be responsible for reducing "hydraulic resistances" in part(s) of the "water pathway" leading from the stigmatic cells to the pollen grain (12). In addition, we suggest that it might facilitate an increase in the relative humidity at the stigma surface by attracting water vapor from the surrounding atmosphere. Thus, the factor would act as a hygroscopic agent and augment water uptake by the grain prior to its making a more intimate cell-to-cell (wall-to-wall and heterotrophic) contact with female tissues. We feel that the heterotrophic processes must be preceded by hydration and penetration of the hydrophobic cuticle, albeit discontinuous, which covers stigma papillae (12). In situ germination of *Dieffenbachia maculata* pollen requires an atmosphere at 100% RH (9).

Probe tube stage. This third identified stage of pollen tube development is readily observed when pollen is kept at 98% RH on an inert substratum, such as a glass slide. Here, a short probe tube emerges from most grains, attaining a maximum length of about one grain diameter. This probe tube emergence is an autotrophic developmental stage; no exogenous signal or nutrients are required besides contact and water in the vapor state at 98% RH.

The probe tubes always originate at and grow toward the surface of the glass slide, whether the pollen grain is on the top or underside. Similarly, tubes grow toward the substratum when germinated at 98% RH on opposite sides of a nylon strand (Fig. 1). That all probe tubes originate at and develop toward the substratum interface indicates that a recognition system must "dictate" the direction of probe tube growth. Thigmotropism is the phenomenon governing orientation when contact of a cell or tissue with a solid body is the directive factor (29).

Generally, as observed with *Crocus* (10) in vivo, each probe tube develops at or near the contact surface. Some contractile mechanism and penetration or pushing force must be involved in this or the next stage of pollen tube development. The emerging tube actually forces the pollen grain to "rock back" on its axis. This force causes the pollen tube to appear to bend toward the

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substratum (Fig. 1). A role for this penetration force in making firm contact of the probe tube tip with the subcuticular, but extracellular, wall of papillae is intimated. An effective penetration force can occur only if the pollen grain binds tightly to a receptive substratum.

Probe tube attachment stage. If the probe tube contacts a receptive plant tissue, germ tube attachment develops. This occurs when probe tubes strike compatible stigmas, or if they contact the middle lamellar region of elongated cells on the epidermis of anther filaments or flower petals (Fig. 2). Attachment does not occur on other regions of the cell surface. When attachment does not occur, tube elongation ceases at the probe stage and callose deposition fills the probe tube (Fig. 3).

Epidermal cells on the petals of Brassica flowers show a gradual progressive differentiation from elongated cigar-shaped cells at the base to rounded nearly isodiametric cells at the distal margin. Pollen attachment and tube elongation occur with decreasing frequency as the cells become progressively more rounded. Occasionally, germ tubes form between rounded cells, where they elongate in a distorted zig-zag manner as the tubes grow along the zig-zag path of the middle lamella between cells (Fig. 4).

Filaments of the spider plant (*Chlorophytum* spp.) are composed of rounded cells. Pollen of *Brassica* placed (*unpublished observation*) on such tissues developed a probe tube, but did not elongate. Possibly, the surface topography of rounded cells prevents firm attachment of tube tips.

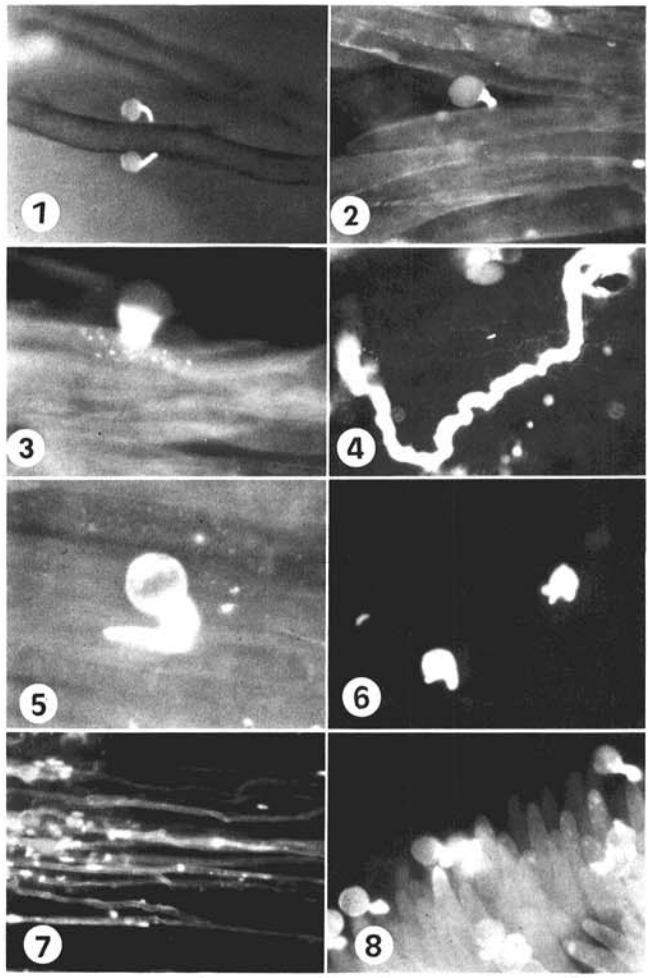
Probe tubes about to attach to the middle lamellar region can be identified by lack of staining and fluorescence for callose at their tips (Fig. 2). Completion of tube attachment is evinced by the uplifting or raising of the pollen grain above the surface of the tissue on which the probe tube attaches (Fig. 5). Lifting of the grain and thickening of the probe tube (compare Figs. 2 and 5) are probably a combined manifestation of the contractile and penetration forces discussed above and involve tube attachment and increasing cell turgor. The lifting seems to result from a weakening of the primary binding of the pollen grain to the papillae.

During tube attachment, an appressoriumlike structure develops (Fig. 5). A similar pollen tube structure has been identified on *Crocus* papillae (10). Beyond this stage, we presume an intimate partner relationship must develop, which coordinates transfer of nutritive precursors from the female tissues, permitting the developing germ tube to continue to grow. Thus, completion of the probe tube attachment stage signals the beginning of the male gamete's heterotrophic association with the female tissues and an end to its autotrophic phase.

Probe tube inhibition stage. We have observed probe tube formation at 98% RH on surfaces of leaves, stamens, petals, sepals, leaf petioles, flower pedicels, incompatible stigmas, and the sides of pistils. Except for specific regions between elongated cells of stamens and petals, pollen tube development proceeds to the probe tube stage but seldom goes beyond it. Growth ceases after about 3 hr, whereupon the primary probe tube becomes occluded with callose. Occasionally a second and (more rarely) a third and fourth probe tube sequentially emerges from the pollen grain (Fig. 6). Thus an endogenous, probe-tube-localized inhibitory mechanism must communicate to the pollen grain that binding and tube attachment cannot occur, which then blocks continued tube development. That, in turn, causes callose production and sometimes the formation of additional tubes from the same grain (Fig. 6). We do not know what triggers the inhibition mechanism, but biochemical evidence (6) suggests that an inhibitor of pollen germination is programmed to arise via protein synthesis in the pollen.

We observed that the incompatibility reaction and tube growth through pistillar tissues (but not hydration, probe tube formation, attachment, and grain lifting) are blocked by pretreating pollen grains with cycloheximide (8). Thus, development of the inhibition stage and pollen tube development of pistillar penetration capabilities must depend on new protein synthesis. Development up to that point must be programmed during microsporogenesis to occur in the developing male gametophyte. If development of the inhibition stage requires pollen protein synthesis, then one would expect that plants defective in that feedback inhibitory mechanism might fail to attach to papillae (as a result of self-recognition), yet be self-compatible because hydration and development beyond the attachment stage could no longer be prevented. A genotype of *Brassica oleracea* has been reported (28) that attaches poorly, yet is self-compatible.

Pollen tube elongation stage. As described above, inhibition of pollen tube development at the probe tube stage occurs when Brassica pollen is germinated in vitro at 98% RH on glass slides, or



Figs. 1–8. 1, Pollen grain probe tube formation on a nylon strand. *Brassica campestris* pollen was germinated in petri plates on a nylon cloth held in an atmosphere maintained at 98% RH by saturated $K_2Cr_2O_7$. Plates were sealed with parafilm during overnight incubation at 20–22 C. The nylon strand in the figure is from the perimeter of the cloth. Pollen callose was selectively stained by applying a mist of aniline blue in K_3PO_4 and viewing with fluorescence microscopy (16). Except where noted otherwise, germination conditions were similar for the remaining figures. Pollen grains are 40 μm in diameter. **2,** Early stage of pollen grain probe tube attachment. Pollen was applied to excised filaments of Brassica stamens. Prior to staining for callose, tissue was softened by incubation for 30–40 min at 60 C in 1.0 N NaOH. During slide preparation, tissue became partially separated along middle lamellar region of cigar-shaped epidermal cells, revealing the site of tube attachment. Note lack of fluorescence at the tube tip. Results were similar for pollen germination and attachment for cells at base of flower petals. **3,** Pollen grain probe tube formation on an "incompatible" region of a filament epidermal cell. The cell contacted by the probe tip has reacted by formation of numerous callose spots. **4,** Pollen tube elongation (zig-zag) over a middle lamella region. The nearly isodiametric cells are at the distal portion of a Brassica flower petal. **5,** Formation of "appressoriumlike" structure by a pollen grain probe tube attached to a middle lamellar region. A sieve-platelike structure is at the pollen tube-filament cell surface. The pollen grain has been lifted, an indication that the attachment stage has been completed. **6,** Multiple probe tubes from pollen germinated on a glass slide. **7,** Unidirectional tube elongation on Brassica filaments. **8,** Stigmatic papillae and incompatible pollen grains arrested at the probe stage.

on the more-or-less round cells of the several tissues mentioned above. In contrast, a pollen tube elongation stage occurs when the pollen is germinated in 98% RH and in contact with the highly elongated cells of anther filaments (no genotype specificity was observed). After probe tube attachment to the filaments, tubes frequently attain lengths of more than 100 grain diameters. Attachment and elongation occur in the region above the middle lamella between cells of filament and petal tissues (Fig. 7). As with probe tube formation, tube elongation on filament tissue also was unidirectional (Fig. 7); elongation occurred along the longitudinal axis of the subjacent "cigar-shaped" cells. Tube elongation occurred in the middle lamella area; ie, in the groove between two adjacent filament cells. Near the ends of the supporting filament segments a few tubes reoriented and grew in opposite directions. Most tubes stopped growing at the filament ends and developed swollen tips and other irregularities. Occasionally, after making contact with a filament cell, the tube split and "twin" tubes grew in opposite directions.

It is unlikely that nutrition, orientation, or attachment is mediated by extracellular low-molecular-weight substances on these filaments; washing the filament tissue for 3–24 hr with water or phosphate buffer (0.01 M, pH 7.0, ±0.15 M NaCl) did not interfere with or alter pollen tube development. Further, anther filaments are not the normal substrates on which pollen tubes develop.

In vitro tube elongation occurred on the filament surface, but no penetration into that tissue occurred. Since penetration of tissues in compatible pistils does occur in vivo, development of subsequent stigmal and then stylar penetration stages must require activation by conditions not present on anther filaments.

Each stage discussed so far occurs extracellularly relative to the female tissues. We have observed (*unpublished*) that on papillae and within the pistil, there appears to be a unique pollen tube shape associated with cells in each tissue the tube is growing in. The following tube shapes occur: papillar (the broadest tubes), stigmatic (typically slightly distorted), and conducting (the thinnest). A callose plug frequently delineates the point of transition between tissue types. In the stigmas of cotton pistils, the shape of the tube also seems to be determined by mechanical restraints placed on it by the surrounding cells (15).

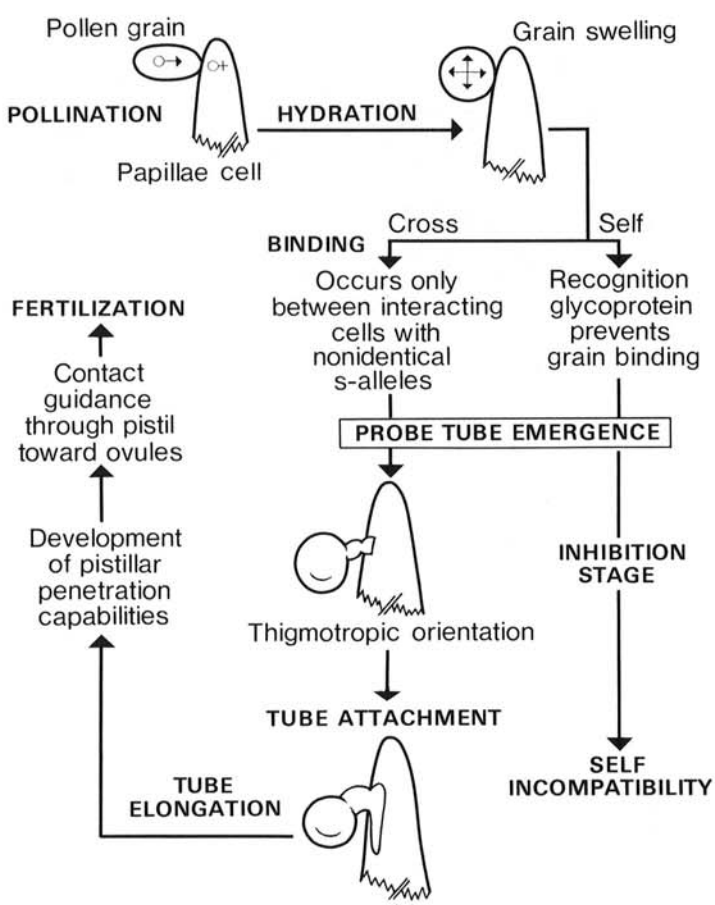


Fig. 9. Summary of developmental stages of pollen germination and tube development.

From these results we propose that pollen tube orientation during elongation involves *contact guidance*, a cell-cell recognition system encompassed by a morphogenetic phenomenon that "... covers all aspects of the way tissues and cells create and respond to mechanical forces in developing spatially patterned structure"(14). Contact guidance by mechanical substrate is the mechanism proposed for controlling orientation of infection haustoria by germinating bean rust spores (31) and of developing optic and nerve fibers (14). Contact guidance is an alternative to chemotropism as a means for regulating pollen tube development, a hypothesis that recently has been challenged (19). For the pollen-stigma interactions, we hypothesize that a morphogenetic phenomenon controls recognition responses that, for the most part, are responsible for determining tube shape, development of penetration capabilities, and for guiding pollen tubes the long distance from stigma surface, through stylar tissues, to the ovule. As suggested by Mascarenhas (19), we do not exclude the possibilities that biochemical or chemospecific gradients might affect some orientations or tube growth, such as when a change is necessitated in growth direction toward the ovules.

Self-recognition and control of the incompatibility response. Based on the similar appearances of pollen grains and probe tubes on all living and nonliving substrata tested, as compared to those on incompatible stigmas (Fig. 8), we conclude that pollen tube development beyond the probe tube stage is prevented by the consequence of self-recognition, and that this results in incompatibility. Specifically, in vivo, we propose that binding of the grain and attachment of its probe tube to stigmatic papillae are both interfered with by a genotype-specific stigma recognition molecule following self-pollination. In the absence of grain binding and tube attachment, whether the substratum is an incompatible stigma, a leaf surface, a glass slide, or nylon strand, etc., continued tube development is blocked by the subsequently occurring but preprogrammed stage of pollen tube inhibition (Fig. 9). As evidence of lack of grain binding and tube attachment, most self-incompatible grains are washed away when stigmas are fixed for cytological observation. Quantitatively, binding of pollen grains to compatible stigmas is much greater than that for incompatible stigmas (28).

Glycoprotein control of self-recognition. Cell-to-cell recognition in plants and animals has been hypothesized to involve binding of an external ligand produced by one cell to a complementary molecule on another (3). Candidates for such extracellular molecules include antigenic determinants, lectins, arabinogalactan proteins, arabinoxylans, and allergens (4). Evidence presented below and elsewhere (5) in detail indicates that S-allele-specific molecules from stigmas of *Brassica* are glycoproteins that are involved with self-recognition and control of pollen incompatibility.

Serologically and electrophoretically detected macromolecules isolated from stigmas have been correlated with S-allele self-incompatibility genotypes (13,21,23,25). The S-allele-specific molecules of the stigma segregate in absolute correlation with the self- and cross-incompatibility phenotypes of each F_2 plant (22). Very low quantities of the molecule are present on stigmas of immature flower buds, where self pollen functions normally (20,25). The quantity increases as buds develop into mature flowers, this being accompanied by increased expression of incompatibility (20,25). A suppressor mutant with low quantities of the stigma molecule was self-compatible (21). Stigmas homozygous for a particular S-allele generally contain twice as much of the stigma molecule as heterozygotes (13,26,27). The molecule is not detected in pollen or other tissues of the same plants (21,25,27). S-allele genotype-specific molecules from stigmas of *Brassica campestris* were precipitated by concanavalin A and stained PAS positive (26). In situ control of pollen incompatibility by such a genotype-specific, S-allele-coded macromolecule isolated from

stigmas of *Brassica* was reported recently (5). Criteria presented (5) were consistent with this stigma recognition molecule being a highly purified lectinlike glycoprotein.

CONCLUSIONS

Cell-to-cell contact interactions and the self-incompatibility system of plants control relationships between a haploid male gametophyte and the diploid female tissue of the sporophyte. Thus developmental stages of the male gametophyte mimic, with incompatibility vs compatibility, respectively, those of fungal spores attempting to infect resistant vs susceptible tissues of plants. An overall justification for basic studies of the self-incompatibility system in *Brassica* is that it can provide information to guide research relative to cell-to-cell recognitions by pathogenic and symbiotic organisms and by other angiosperm systems. One merit of the system in *Brassica* is that the methods of procaryotic molecular biology can be applied to the haploid pollen grain and its germ tube. Bridges are needed to facilitate comparison of knowledge of procaryotic systems with knowledge of eucaryotic systems. The self-incompatibility system in *Brassica* appears to provide such a bridge.

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