The Plant Cell, Vol. 16, S84–S97, Supplement 2004, www.plantcell.org

Pollen and Stigma Structure and Function: The Role of Diversity in Pollination

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INTRODUCTION

The ornate surfaces of male and female reproductive cells in flowering plants have long attracted attention for their variety and evolutionary significance. These structures, and the molecules involved in sexual interactions, remain among the most rapidly evolving and diverse characteristics known. As varied as they may be, each element takes part in performing the same functions, protecting pollen and stigma from the environment, delivering and capturing pollen, promoting pollen hydration and germination, allowing the entry of appropriate pollen tubes into the stigma, and guiding the tubes to the ovary (Figure 1A). In this review, we (1) survey recent discoveries of pollen and stigma functions both before and after they make contact, and (2) address the great diversity in pollen and stigma structures across taxa, focusing on how they accomplish key tasks in pollination. This system presents an exciting opportunity for the fruitful unification of cell, genetic, and genomic studies of model organisms with comparative studies of relationship and evolution.

Angiosperm reproduction is highly selective. Female tissues are able to discriminate between pollen grains, recognizing pollen from the appropriate species while rejecting pollen from unrelated species (or from the same plant in self-incompatible species). This selectivity is accompanied by tremendous diversity in the cell surfaces of male and female reproductive structures. The uniquely rich fossil record of pollen wall structures has been of great benefit. Literally hundreds of years of scientific effort have focused on integrating the diversity of pollen form with angiosperm taxonomy. Linking these morphological differences to functional roles is more challenging, requiring molecular and genetic assays that reveal purpose not only within a species but across diverse taxa.

Here, we first survey advances in the cellular and molecular understanding of angiosperm pollen and stigma biology, including pollen–stigma adhesion, pollen hydration and germination, and pollen tube emergence and invasion. By capitalizing on microscopy, molecular, genetic, and genomic resources, it is possible to magnify and dissect cellular functions at the pollen and stigma surfaces both before and after they make contact (Table 1). Recent progress has revealed key molecules and mechanisms and has poised the field for comparative studies across taxa. In the second part of the review, we discuss diversity in pollen and stigma structures, highlighting the coadaptive evolutionary change that supports efficient pollination within a species while restricting pollination between species. Given its combination of facile, genetic model systems, morphological analyses, and well-characterized phylogenies, the study of angiosperm pollination provides an excellent opportunity to unify cell and comparative biology and yields insight beyond pollination to the very mechanisms of evolution and speciation.

POLLEN AND STIGMA CELLULAR FUNCTIONS

Overview of Structures

Mature angiosperm pollen grains are unusual vegetative cells that contain within themselves sperm cells, complete with cell walls and plasma membranes. This arrangement is accomplished soon after meiosis, when an asymmetric mitotic division produces a large cell that engulfs its diminutive sister, the generative cell (Twell et al., 1998; Yang and Sundaresan, 2000). Subsequently, the generative cell undergoes a second mitosis to form the second sperm cell required for double fertilization; ''tricellular'' pollen completes this division before it is released from the anther, whereas ''bicellular'' pollen undergoes this division only later, within the elongating pollen tube. These categories do not apply to gymnosperm pollen grains, which can contain a score of cells and differ from angiosperm pollen in several other respects (Pacini et al., 1999). Pollen cells are contained within a unique pollen wall, whose construction begins when the meiocyte is newly formed, with the surface layers elaborated over time. At maturity, the pollen surface can be divided into three principal strata, with the relative amount of each varying between species: (1) an outer exine wall, itself multilayered, composed of the chemically resistant polymer sporopollenin and interrupted by openings called apertures; (2) an inner intine, also sometimes multilayered, made primarily of cellulose; and (3) a pollen coat, composed of lipids, proteins, pigments, and aromatic compounds, that fills the sculptured cavities of the pollen exine (Figure 1B).

Stigmas, the receptive portions of the female tissues, bind pollen and mediate tube migration into the style. Stigmas generally are classified into two groups: wet stigmas, which are

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.015800.

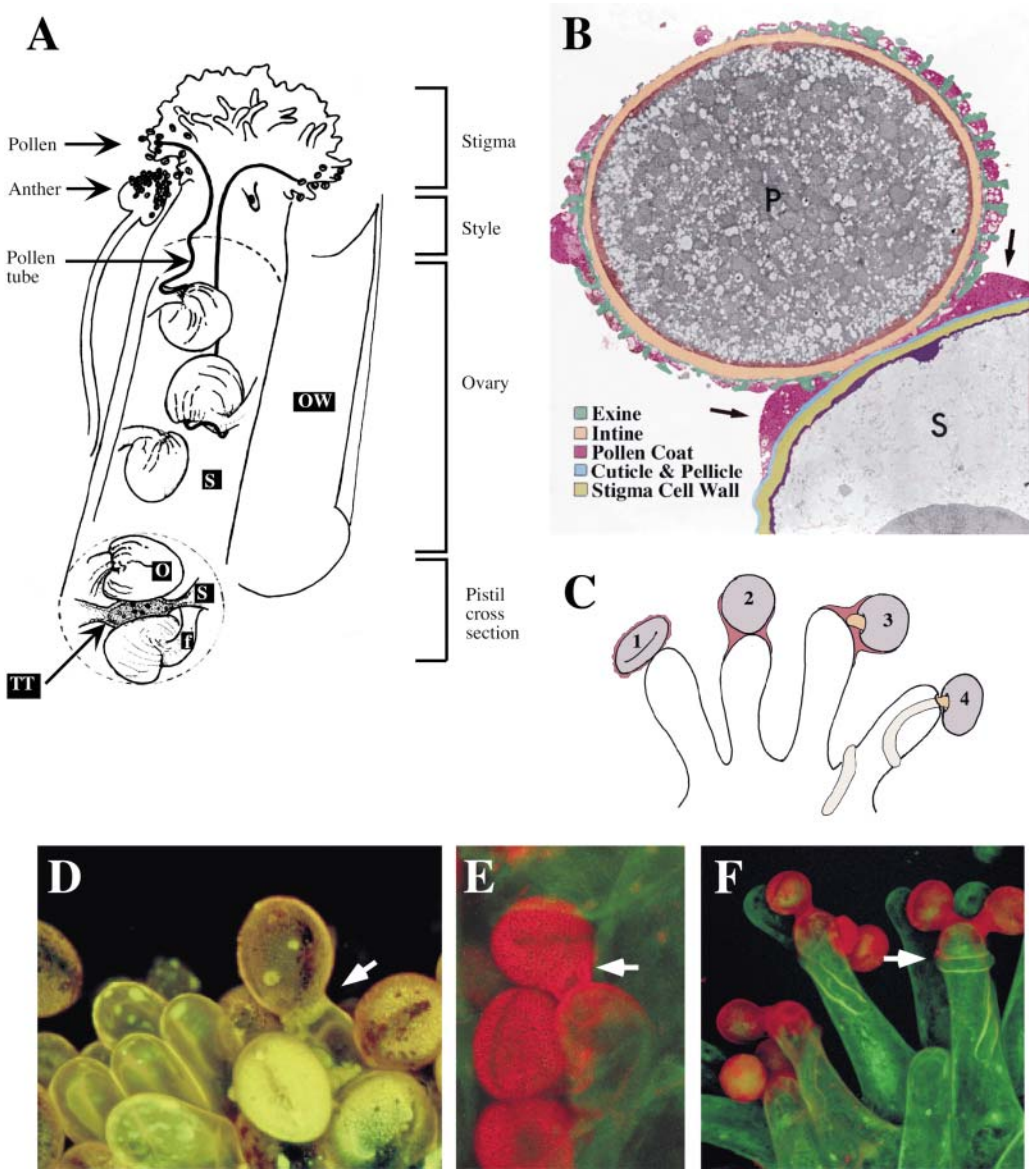

Figure 1. Pollination in Arabidopsis.

(A) Diagram of one of the two ovary chambers after removal of the ovary wall (OW). Pollen lands on the stigma, where it hydrates, germinates, and extends a tube that enters the transmitting tract (TT). The tube emerges onto the septum (S), grows up the funiculus (f), and enters the micropyle opening of the ovule (O), where it fertilizes the egg and central cell. The position of the transmitting tract inside the septum is shown in the cross-section at the bottom of the pistil.

(B) Transmission electron micrograph showing the point of contact between a pollen grain (P) and a stigma papillus (S), colorized to highlight the pollen coat (pink), intine (peach), exine (green), stigma cell wall (yellow), and stigma cuticle (blue). A foot of lipid-rich material (arrows) collects between the two surfaces. The thin stigma pellicle that covers the cuticle is not distinct here.

(C) Diagram of early events on the stigma, including adhesion (1), pollen coat ''foot'' formation and pollen hydration (2), pollen tube emergence from the grain (3), and pollen tube invasion of the papillae cell wall and extension toward the style (4).

(D) to **(F)** Sequence of early events during Arabidopsis pollination.

(D) The pollen coat has mobilized to the site of contact between the pollen and the stigma, forming a foot between the two surfaces (arrow), as visualized with the lipid dye FM1-43.

(E) The pollen tube (arrow) has emerged from the grain's exine wall and projects itself into the stigma papillus, as visualized with the cell wall dye Congo red.

(F) Pollen tubes (arrow) wind their way toward the style, moving between the cell walls of the papillae, where they are protected from the Congo red dye.

Table 1. Pollen and Stigma Structural Features and Their Roles

Structural Feature	Proposed Functional Significance
Pollen grain size	Biotic and abiotic pollinator preference and fluid dynamics
Number of pollen grains per pollination unit	May increase delivery efficiency
Pollen coat	Protects pollen cells from excess desiccation after dehiscence; protects from UV radiation and pathogen attack; stickiness, color, and aroma may affect interaction with pollination vectors; protein components involved in adhesion, signaling, and compatibility; lipids and proteins are necessary for hydration
Exine pattern	Interacts with biotic and abiotic pollination vectors; affects the surface area of the stigma interface; mediates stigma adhesion; retains pollen coat; affects wall strength and elasticity
Exine porosity	Microchannels are sites of water egress and ingress during desiccation and hydration; progressive desiccation limits pollen viability and life expectancy
Aperture size, number, and complexity	Affect environmental vulnerability to desiccation, fungal invasion, and mechanical stress; accelerated desiccation limits pollen viability and life expectancy; sites of focused water ingress during hydration; allow extreme volume changes accompanying desiccation and hydration; serve as portals for pollen tube exit during germination
Intine	Thickness and complexity are inversely coordinated with exine and pollen coat characteristics; specialized layers and inclusions at apertures are involved in pollen tube emergence and invasion of the stigma cell wall
Stigma coat	Defines stigmas as uniquely water-permeant sites on the plant; proteins and lipids are involved in adhesion, hydration, and germination; dry stigmas are pollen compatibility sites, with selective support of pollen hydration and germination, whereas wet stigmas often are covered in exudates from apoptotic cells and block inappropriate pollination only in later steps; at the point of contact between pollen and stigma, the two coatings may mix, a process that mediates coat conversion, hydration, germination, and stigma invasion

covered with surface cells that often lyse to release a viscous surface secretion containing proteins, lipids, polysaccharides, and pigments; and dry stigmas, which have intact surface cells that typically protrude as papillae and are covered by a primary cell wall, a waxy cuticle, and a proteinaceous pellicle (Figure 1B). Stigmas have been studied extensively in plants that exhibit self-incompatibility, a process that restricts inbreeding. Self-incompatible stigmas reject self-pollen by inhibiting pollen hydration, germination, and tube invasion; these processes have been reviewed recently (Nasrallah, 2000; Silva and Goring, 2001; Wheeler et al., 2001; Hiscock and McInnis, 2003; Kao and Tsukamoto, 2004).

Pollen Adhesion to the Stigma: First Contact

To capture pollen grains, stigmas engage biotic and abiotic pollinators (such as insects and wind) and use rapid and strong adhesive interactions to retain pollen grains. The pollen–stigma interface can differ from species to species as a result of the wide variability in the morphology and content of stigma exudates, exine layers, and pollen coats. Several different methods have been devised to investigate and measure pollen–stigma adhesion (Stead et al., 1979; Luu et al., 1997a, 1997b; Zinkl et al., 1999), including chemical and detergent washes and centrifugation-based adhesion assays. Here, we focus on the characterization of pollen adhesion on the dry stigmas of the Brassicaceae, particularly *Arabidopsis thaliana* and *Brassica oleracea*. In both cases, stigmas adhere poorly to pollen grains from other botanical families, demonstrating a specificity that restricts inappropriate pollen access (Luu et al., 1998; Zinkl et al., 1999). Adhesion during downstream stages (such as between pollen tubes and the style) has been reviewed recently (Lord and Russell, 2002; Lord, 2003).

In Arabidopsis, the nature of the pollen–stigma interface has been shown to change as pollination progresses, becoming considerably stronger over time, with different types of adhesive contacts supplementing and supplanting each other (Luu et al., 1997a, 1997b; Zinkl et al., 1999). Zinkl et al. (1999) measured a very rapid ''initial'' adhesion step in Arabidopsis that relies on the pollen exine but not on the pollen coat. This initial adhesion is not likely to be based on protein–protein interactions, because purified exine fragments retained their binding capacity even when washed with a range of compounds, including organic solvents, salts, and reducing and oxidizing agents. Thus, pollen capture in Arabidopsis most likely depends on biophysical and/or chemical interactions between the stigma surface and the polymers of the pollen exine; the nature of these contacts remains unknown. Identification of these selective adhesives requires further analysis of exine structure and characterization of stigma pellicle components. Given the challenge of purifying large quantities of these chemically complex materials, genetic dissection of the pathways required for their synthesis may prove most worthwhile.

After exine-mediated adhesion, mobilization of the pollen coat occurs, leading to mixing of lipids and proteins to form a ''foot'' of contact on the stigma surface (Figures 1C and 1D). There is now extensive evidence that the proteins and lipids in the pollen coat, and proteins on the stigma surface,

also contribute to adhesion, albeit most likely at a later stage than at the time of initial contact. This second stage was measured and shown to require protein–protein interactions, including interactions between pollen coat proteins and the Brassica S-locus–related protein (SLR1) (Luu et al., 1999; Takayama et al., 2000). SLR1 is a stigma-expressed gene related to the S-locus glycoprotein, a protein involved in self-incompatibility (Lalonde et al., 1989). SLR1-mediated adhesion requires some time to establish itself, possibly to allow the mixing of stigma and pollen matrices; consequently, the strength of initial pollen binding in SLR1-deficient plants is the same as in wild-type plants (Luu et al., 1999). SLR1 binds to pollen coat proteins. This binding, along with the enhanced cell-to-cell adhesion, can be blocked by antibody inhibition of the SLR1 proteins (Luu et al., 1999; Takayama et al., 2000). As pollen tubes germinate from the grains, a final stage of adhesion begins in which the pollen tube passes through the foot into the stigma surface, tethering the emptying pollen grain to the stigma via the tube (Zinkl et al., 1999; Dickinson et al., 2000; Heizmann et al., 2000). When inappropriate pollen grains reach this stage, further access is blocked by inhibiting tube growth. This mode is common in plants with wet stigmas, in which pollen grains typically are bound and hydrated without applying species selectivity (Wheeler et al., 2001).

Pollen Hydration: Activating Metabolism

Most pollen grains are metabolically quiescent and highly desiccated, ranging from 15 to 35% water content, when released from the anthers (Heslop-Harrison, 1979a; Buitink et al., 2000). Water immediately surrounds grains that land on a wet stigma, but those that land on dry stigmas mobilize their lipid-rich pollen coat to form an interface between the two cell surfaces. This interface converts to a histochemically distinguishable form thought to promote water flow (Elleman and Dickinson, 1986; Elleman et al., 1992). Water, nutrients, and other small molecules are transported rapidly into the grain from the stigma exudate (wet stigmas) or stigma papillae (dry stigmas) by mechanisms that remain unclear. The discovery of aquaporin expression in the stigma has prompted the exciting model that water channels are involved in the rapid and regulated water release from the stigma to the pollen (Dixit et al., 2001). Also, the stigma cuticle may be uniquely permeable to water traffic (Lolle et al., 1997, 1998; Pruitt et al., 2000). Regardless of the mechanism of transfer, pollen hydration often is regulated, both temporally and spatially. Inappropriate hydration can have disastrous consequences, leading to premature germination within the anther (Johnson and McCormick, 2001) or germination on the wrong surface (Lolle and Cheung, 1993; Lolle et al., 1998). In plants with dry stigmas, regulated pollen hydration provides an effective early barrier to incompatible pollination. This mode is active in self-incompatible crosses (Sarker et al., 1988) and in crosses between species (Lewis and Crowe, 1958; Hülskamp et al., 1995). These processes are remarkably localized: the stigma can hydrate a compatible grain while restricting the hydration of foreign or incompatible pollen on the same papillus (Dickinson, 1995). An understanding of the precise surface changes that provide this exquisite control will require both molecular dissection of stigma and pollen surface components and genetic analyses of their roles.

The genetic and molecular dissection of the lipid-rich matrices found in the pollen coat has progressed considerably in recent years. In Arabidopsis, the pollen coat contains long- and short-chain lipids along with a small set of proteins, including six lipases and eight Gly-rich oleosin proteins that contain a lipid binding domain (Mayfield et al., 2001; Fiebig et al., 2004). The pollen coating of Brassica is structured similarly and also has been shown to contain peptides involved in self-incompatibility (Doughty et al., 1993). Disrupting pollen coat lipids or pollen coat proteins in Brassicaceae species can delay or block pollen hydration. In particular, mutations that impair long-chain lipid synthesis, and consequently the proper assembly of the pollen coating, severely reduce the hydration of Arabidopsis pollen and result in male sterility (Preuss et al., 1993; Hülskamp et al., 1995). Hydraulic contact can be restored to these mutant grains by the addition of purified triacylglycerides (Wolters-Arts et al., 1998). Mutations that affect pollen coat proteins are less extreme, perhaps because of partial functional redundancy. The most abundant protein in the Arabidopsis pollen coat is the Gly-rich oleosin GRP17; seven related proteins have similar oleosin domains but variable hydrophilic tails. Mutations in GRP17 delay the onset of pollen hydration and decrease the ability of the mutant pollen to effectively compete with wild-type pollen (Mayfield and Preuss, 2000). The sequences of the GRP proteins are widely divergent across the Brassicaceae, supporting a model in which pollen coat diversification may contribute to speciation (Mayfield et al., 2001; Fiebig et al., 2004). Unlike the GRP proteins, the pollen coat lipids of most Brassicaceae species are quite similar, making these molecules poor candidates for dictating species specificity (Piffanelli et al., 1998). Consequently, experiments that promote pollen hydration by the application of exogenous lipids (Wolters-Arts et al., 1998, 2002) may bypass crucial regulatory steps that normally occur in vivo, such as those restricting the hydration of incompatible or foreign pollen.

The lipid-rich stigma exudate of plants with wet stigmas is thought to be functionally analogous, in part, to the pollen coat (Dickinson, 1995). Mutations that eliminate this secreted matrix cause female sterility; this defect can be bypassed by adding exogenous lipids (Goldman et al., 1994; Wolters-Arts et al., 1998). The addition of *cis*-unsaturated triacylglycerides is sufficient for hydration and germination, even on stigmaless tobacco plants (Wolters-Arts et al., 1998). These observations suggest that lipids could play an important role in the permeability of the stigma cuticle to water (Lolle et al., 1998; Pruitt et al., 2000). In *fiddlehead*, which is defective in a β-ketoacyl-CoA synthase required for long-chain lipid synthesis, leaf cuticle permeability increases and pollen hydration is stimulated on inappropriate cell surfaces. Based on these findings, it is possible to propose a model in which the presence of lipids, whether provided by the male or the female surface, modulates water transfer to desiccated pollen, while highly diverse proteins and peptides mediate self and foreign pollen recognition.

Pollen Polarization and Germination: Preparing for Pollen Tube Growth

Hydration transforms a pollen grain from a nonpolar cell to a highly polarized cell. Whether tubes emerge on a dry stigma surface, from a grain submerged in stigma exudates, or from pollen germinated in vitro, the grain organizes its cytoplasm and cytoskeleton to support the extension of a single tube. These changes occur within minutes after hydration and include the formation of filamentous cytoskeletal structures that wrap around the nuclei, actin cytoskeleton polarization toward the site of tube emergence (Tiwari and Polito, 1988; Heslop-Harrison and Heslop-Harrison, 1992), reorientation of the large vegetative nucleus so that it enters the extending tube before the generative cells (Heslop-Harrison et al., 1986a; Heslop-Harrison and Heslop-Harrison, 1989; Lalanne and Twell, 2002), assembly of mitochondria and polysaccharide particles at the site of the elongating tube tip (Cresti et al., 1977, 1985; Mazina et al., 2002), and selection of the pollen plasma membrane for secretory vesicle targeting and deposition of callose (β-1→3 glycan) at the site of tube emergence (Johnson and McCormick, 2001).

It is not yet clear how the polarization signal is perceived and subsequently transduced to select a single point for tube emergence. Several candidate signals have been suggested, including water, lipids, and ions (Feijo et al., 1995; Lush et al., 1998; Wolters-Arts et al., 1998). Evidence for water as a polarity signal has come from in vitro experiments with Nicotiana pollen: immersing these grains in purified lipids or in stigma exudates, and providing a nearby aqueous interface, results in polarized growth toward the aqueous medium, mimicking behavior in vivo. The pollen tubes emerge from the aperture closest to the hydrophobic–aqueous interface, suggesting that the source of water provides directional cues that establish polarity (Lush et al., 1998; Wolters-Arts et al., 1998). Not only does this example implicate water, it also suggests a role for lipids in establishing polarity. Additional support for this idea comes from mutants that lack the lipid-rich pollen coating (Preuss et al., 1993; Hülskamp et al., 1995). As described above, such grains are unable to hydrate or germinate on the stigma. Coat-deficient grains do hydrate at increased ambient humidities, but because they lack key axial information, their pollen tubes often emerge at random orientations relative to the stigma (Dickinson, 1995).

Polarization signals ultimately trigger the recruitment of RHO OF PLANTS1 (ROP1), a GTP binding protein involved in F-actin dynamics and the establishment of calcium gradients at tube tips (Gu et al., 2003). ROP1, with its binding partner ROP INTERACTING CRIB-CONTAINING1 protein, localizes to the tip of the growing pollen tube, where they act to focus secretory vesicle delivery (Kost et al., 1999; Li et al., 1999; Wu et al., 2001). Other proteins that preferentially localize to the growing pollen tube tip may play a role in the initial establishment of cell polarity. One candidate, found at the tips of elongating fern rhizoids, is annexin, a protein believed to be involved in tip-oriented exocytosis events; annexins exhibit calcium-dependent binding to phospholipids, affect cytoskeletal structure, and potentially modulate voltage-dependent Ca^{2+} channels (Clark et al., 1995). Pollen activation promises to be a particularly rich subject for studies integrating the external cues and internal signal molecules necessary for global rearrangements and polarized tip growth.

Once the cell has established its internal polarity relative to an external signal, the pollen tube must breach the exine wall to emerge from the grain. Depending on the species examined, pollen tubes either grow out of the apertures or break directly through the exine wall. In rye and eucalyptus, tubes emerge strictly at the apertures, by dissolving apertural intine layers, rupturing the thin sporopollenin wall, and displacing the opercula that guard these sites (Heslop-Harrison, 1979b; Heslop-Harrison and Heslop-Harrison, 1985; Heslop-Harrison et al., 1986b). In Arabidopsis, whose pollen has three distinct apertures, pollen tubes often break directly through interaperture exine walls precisely at the site of contact with the stigma surface (Figure 1E). Regardless of exit site, pollen tube escape requires either (1) exine weakening by enzymatic digestion from the inside or outside of the wall, or (2) exine tearing by local gel-swelling forces or focused turgor pressure. Evidence for the former possibility comes from reports of significant exine remodeling after contact with the stigma (Gherardini and Healey, 1969; Dickinson and Lewis, 1974). Gel-swelling forces or turgor pressure also could play important roles, but because such forces radiate in all directions, they must be focused at the site of emergence. A combination of mechanisms is most likely, with the increased turgor pressure of the pollen grain contributing to the rupture of a patch of partially degraded exine at the pollen–stigma interface. There is evidence that pollen grains are preset to harness biomechanical forces for pollen tube emergence; dead pollen grains are able to hydrate to the same extent as living grains, swell, alter apertural coverings, and even germinate short tubes before the tubes ultimately rupture (Heslop-Harrison, 1979b).

The challenge of purifying factors that regulate cell polarity and tube emergence calls for the use of complementary genetic strategies. For example, mutations that upset the physical association between the two Arabidopsis sperm cells and the vegetative pollen nucleus have been identified. These mutations disrupt the polarized transport of the male germ unit to the pollen tube tip by (1) separating the vegetative nucleus from the two sperm cells (*germ unit malformed1* [*gum1*] and *gum2*) or (2) mislocalizing an intact male germ unit to the pollen wall (*mgu displaced1* [*mud1*] and *mud2*) (Lalanne and Twell, 2002). Other visual screens have identified mutations that allow tubes to grow without emerging from the grain or disrupt the accumulation and deposition of callose before pollen tube emergence (Johnson and McCormick, 2001). In addition, screens for pollen gametophytic mutations have yielded mutants altered in germination, polarity, and tube emergence (Ryan et al., 1998; Grini et al., 1999; Johnson and McCormick, 2001; Procissi et al., 2001; Johnson and Preuss, 2002; Lalanne and Twell, 2002). Similar approaches have not yet yielded stigma-specific mutations, despite numerous screens to isolate sporophytic sterile mutants. This deficiency suggests functional redundancy in the pollen-recognition machinery involved. On the other hand, the fertility of Arabidopsis flowers with genetically ablated stigma cells (Kandasamy et al., 1993; Thorsness et al., 1993) calls into question the absolute requirement for the tissue.

Pollen Tube Invasion: Growing into the Stigma

After crossing the exine wall, pollen tubes can only enter the style after transiting the stigma barrier. The details of this process vary considerably from species to species. In plants with open styles, the stigma is covered with an epidermis that is continuous with the style, but in species with closed stigmas, pollen tubes grow through the outer cuticle and cell wall of the stigma papillae to enter the style. For >100 years, enzymes secreted by pollen have been proposed to play an important role in pollen tube invasion of the stigma surface (Green, 1894). Acid phosphatase, ribonuclease, esterase, amylase, and protease activity have been localized to pollen intines and tubes (Knox and Heslop-Harrison, 1970). Esterases, particularly those known as cutinases, are important for breaching the stigma cuticle; they have been identified in the pollen of Brassica, Tropaeolum, and many other taxa (Knox and Heslop-Harrison, 1970; Shaykh et al., 1977; Hiscock et al., 1994) as well as in the proteinaceous pellicles of dry stigma surfaces (Heslop-Harrison and Shivanna, 1977; Hiscock et al., 2002b). Removing or disrupting the pellicle prevents compatible pollen tubes from entering the stigma, despite normal germination (Heslop-Harrison and Heslop-Harrison, 1975; Heslop-Harrison, 1977; Heslop-Harrison and Shivanna, 1977). In addition, cutinase inhibitors significantly reduce the ability of pollen tubes to penetrate Brassica stigmas (Hiscock et al., 2002a). Hydrolysis of pectin in the stigma cell wall also is necessary, for which pollen expresses genes encoding pectin esterase and pectate lyase (Kim et al., 1996; Wu et al., 1996). A pollen-specific Brassica polygalacturonase also has been detected at the tip of pollen tubes as they enter the stigma papillar cell walls (Dearnaley and Daggard, 2001).

Enzymatic penetration of the stigma surface is precisely controlled to not expose the pistil to pathogenic or inappropriate invasion. This control likely requires constant communication between the pollen tube and the stigma. Recently, receptor kinases have been identified as candidate mediators of communication between the pollen tube and the stigma. Members of the large Leu-rich repeat receptor kinase family (*Lycopersicon esculentum* protein receptor kinase1 [LePRK1] and LePRK2) localize to the growing pollen tube (Muschietti et al., 1998). Yeast two-hybrid experiments using the extracellular domains of these kinases yielded many candidate ligands, including cell wall–remodeling proteins and Cys-rich proteins implicated in extracellular signaling. One pollen-specific, Cys-rich protein, LATE ANTHER TOMATO52 (LAT52), interacts in vivo with LePRK2; LAT52 is essential for pollen hydration and germination in vitro and for normal tube growth in vivo. This finding suggests that LAT52 and LePRK2 are members of an autocrine signaling cascade that may regulate and maintain pollen tube growth (Tang et al., 2002).

POLLEN AND STIGMA STRUCTURAL DIVERSITY

Although the processes discussed above are conserved across angiosperms, they are accomplished by an astounding variety of pollen and stigma structures. Male and female reproductive tissues evolve rapidly; yet, to maintain their interactions, they also evolve coordinately. This evolution is evident in angiosperm pollen grains, which are remarkably diverse, varying in size (from 20 to 250 μm in diameter), in their pigments (Lunau, 2000), in their aromas (Dobson and Bergstrom, 2000), in their nutritional content for pollinators (Roulston and Cane, 2000), and in the patterns of their ornate exine structures (Figures 2A to 2D). Many of these characteristics are important for pollen delivery and for modulating the adhesion, hydration, and emergence of pollen tubes. Stigma cells are similarly diverse (Figures 2E and 2F); they vary in form, are either unicellular or multicellular, and differ in wall structure and cuticle pattern while remaining compatible with their pollen counterparts. The taxonomic relationships of a wealth of angiosperms are understood (Qiu et al., 1999). With the availability of model systems that reveal gene functions, the power of comparative genomics can now be harnessed to investigate the role of genetic diversity in plant reproduction. This system represents an opportunity with enormous potential: it is now possible to merge an understanding of cell biology with the immense evolutionary diversity seen in nature.

Pollen Walls: Generating Structural Diversity

Pollen wall patterns are so diverse and often so characteristic of each species that they have long been used for taxonomic classification and even for forensic identifications (Szibor et al., 1998). Complete understanding of the evolution of diversity in pollen wall characteristics calls for the study of the genes and mechanisms behind their development. Species-specific pollen patterns are established early in meiosis (Sheldon and Dickinson, 1983). Microsporogenesis can be divided into two broad classes: (1) simultaneous, in which the first meiotic division is followed immediately by the second, with a wall of callose that is deposited subsequently around all four tetrahedrally arranged microspores; and (2) successive, in which a callose wall is deposited around the dyad formed by the first meiotic division, before the second division occurs. Simultaneous microsporogenesis usually is associated with eudicots, whereas successive microsporogenesis is associated with monocots, although to a lesser extent, because of several evolutionary reversals (Furness et al., 2002). Microspore alignment within the callose wall is important on several levels, including internal vegetative and generative cell polarity (Twell et al., 1998), the release of pollen as a monad, tetrad, or cluster, and exine sculpting and aperture position. A primexine matrix, the precursor of exine, typically is deposited between the callose wall and the plasma membrane of the developing microspores. The callose wall later is degraded, enabling the pollen grains to be released as monads (Paxson-Sowders et al., 1997). In some species, including a number of ancient flowering plants, pollen grains instead remain in fused clusters, an arrangement thought to increase pollination efficiency. Mutants defective in the early stages of pollen development may provide insight into genes that are modulated during evolution, resulting in the striking variation of pollen form. For instance, mutations that mimic natural tetrad clusters have been found in Arabidopsis. In *quartet* mutants (Figure 3), callose deposition is disrupted during microspore development, causing the exine layers of pollen tetrads to merge (Preuss, 1994; Rhee and Somerville, 1998; Rhee et al., 2003). *quartet* mutations have been useful for genetically dissecting

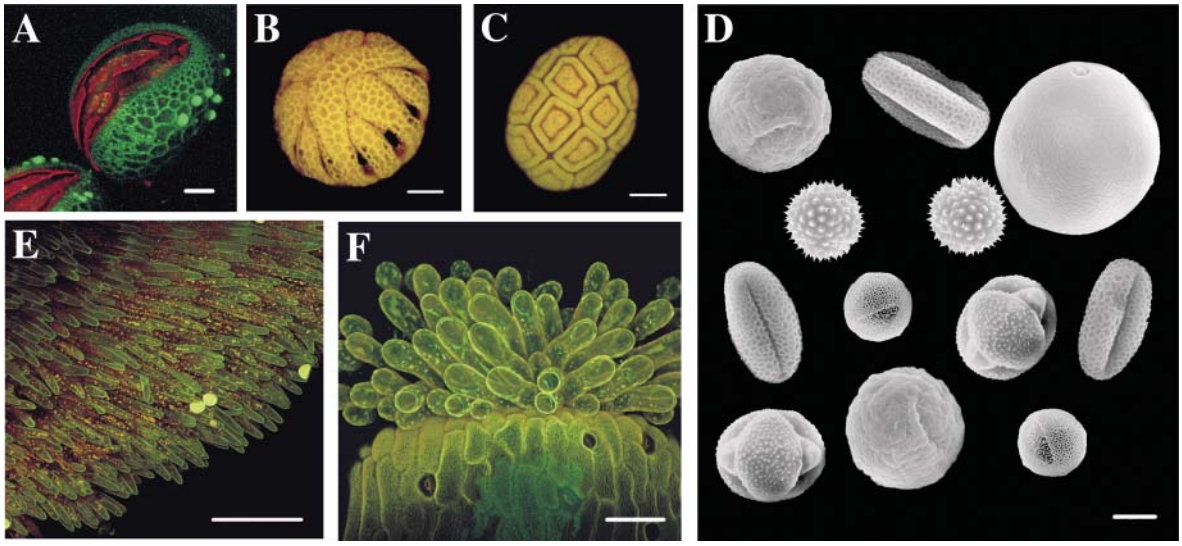

Figure 2. Examples of Angiosperm Diversity in Pollen and Stigma Structures.

(A) Hydrated *Lilium longiflorum* pollen grain stained with Congo red. The intine (red staining) contains several fissures at the single large aperture; the pollen coating (green) forms droplets on the surfaces of the ornamented exine walls.
(B) Multiapertured pollen of *Passiflora fanguinolenta*, hydrated and stained with exine-specific Auramine O dye.
(C) Polyad pollen of *Acacia retinoides*, stained with Auramine O. Thirteen small, interconnected pollen grains are visible.
(D) Composite scanning electron micrographs of several pollen types, including Arabidopsis, *Ambrosia* (ragweed), maize, *Plumbago*, and *Artemisia douglasiana* (mugwort), which vary in size, exine ornamentation, and number and arrangement of apertures.
(E) A portion of the stigma of *Torenia fournieri*, visualized with Auramine O.
(F) Stigma papillae from Arabidopsis visualized by FM1-43.
Bars = 10 μm in **(A)** to **(D)** and 200 μm in **(E)** and **(F)**.

chromosome segregation during meiosis (Copenhaver et al., 2000; Copenhaver, 2003). Other Arabidopsis mutations have a more severe fusion defect. Pollen from *tetraspore/stud* (*tes/stud*) mutants fail to undergo cytokinesis, resulting in the release of a giant pollen grain that contains four vegetative nuclei and up to eight sperm (Hulskamp et al., 1997; Spielman et al., 1997; Yang et al., 2003). Although these mutations have led to interesting insights, there are likely additional genes to be found; screens for *tes* or *quartet* phenotypes were not performed to saturation. Such genes would serve as appealing candidates for comparative pollen development studies across taxa.

Patterning events that affect exine sculpting and aperture position also occur during or soon after meiosis. Haploid pollen typically produces a primexine, establishing an early pattern. After callose wall degradation, diploid tapetal cells elaborate this pattern with their deposition of sporopollenin material (Paxson-Sowders et al., 1997). Temporally regulated construction and degradation of the callose wall that surrounds pollen occurs in angiosperms (Heslop-Harrison, 1971; Munoz et al., 1995) and resembles the patterns of spore wall development in ferns and bryophytes (Pettitt and Jermy, 1974). The initial species-specific pattern of the primexine requires coordination between the pollen plasma membrane and its underlying cytoskeleton, vesicles, and endoplasmic reticulum (Dickinson and Sheldon, 1986; Takahashi and Skvarla, 1991; Fitzgerald and Knox, 1995; Perez-Munoz et al., 1995; Paxson-Sowders et al., 2001). For example, the Arabidopsis *DEFECTIVE IN EXINE1* gene product, a predicted membrane-associated protein with limited similarity to animal integrins, appears to interact with the plasma membrane to nucleate sporopollenin deposition (Paxson-Sowders et al., 2001). An exine pattern mutant also has been described in *Haplopappus gracilis*, whose normal surface pattern of densely packed spines is reduced to a random scatter of sporopollenin deposits with few interspersed spines (Jackson et al., 2000). Similarly, mutation in the Arabidopsis *less adherent pollen1* gene yields mutant pollen grains with discontinuous exine walls composed only of globular sporopollenin deposits (Zinkl and Preuss, 2000). With the identification of additional mutants involved in exine development, it will become possible to distinguish those genes required for sporopollenin synthesis from those required to establish patterns. Patterning genes, in particular, may be highly variable across species, either in their coding sequence or in their site and timing of expression. Because these genes are likely altered through evolution, exine patterns discovered by mutation and considered aberrant in one species may be normal in another species.

Exine also varies in the number, distribution, and architecture of the apertures that interrupt it (Figure 2). Apertures are diverse across taxa, within families, within species, and even within a single plant (Mignot et al., 1994). Pollen from monocots characteristically has a single aperture, a trait considered to be ancestral. Most dicot pollen grains have three apertures, although in both instances, aperture numbers have increased or decreased repeatedly and independently during the course of

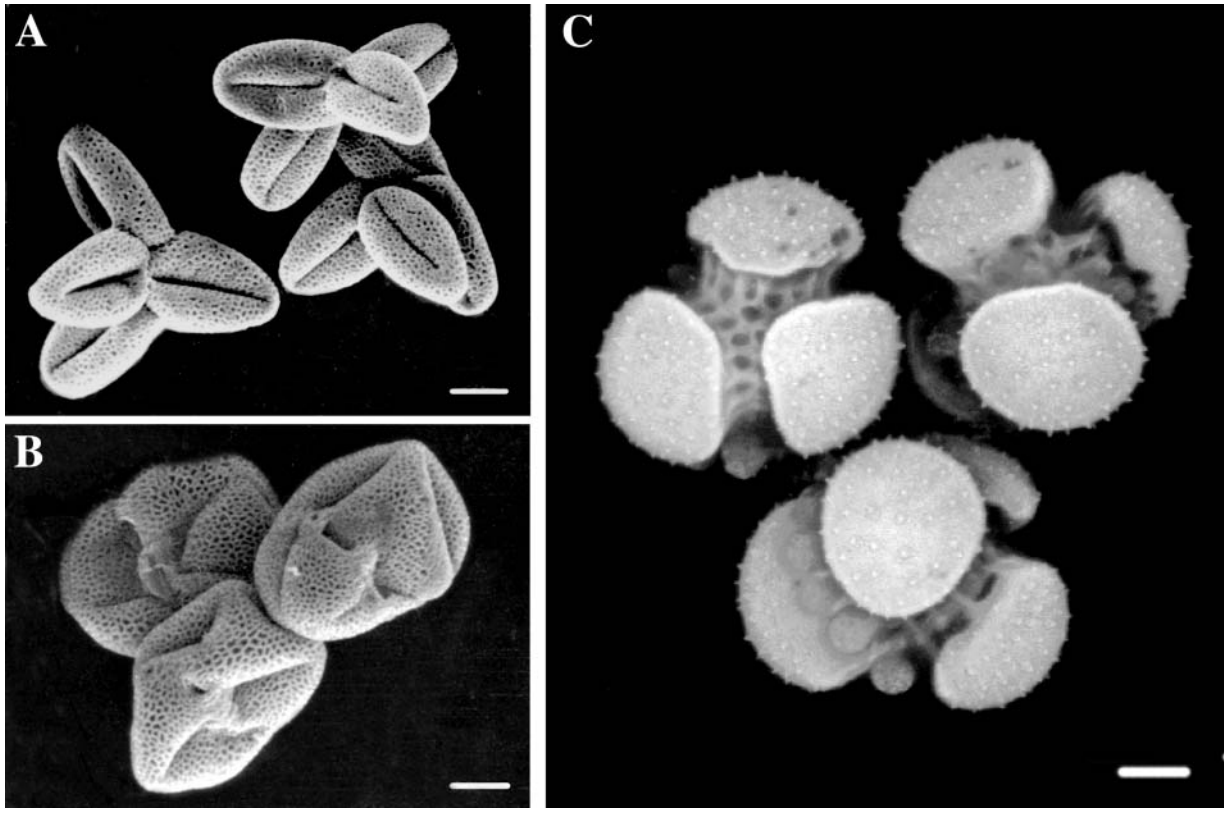

Figure 3. Pollen Tetrads and Clusters.

(A) Pollen tetrads from the Arabidopsis mutant *quartet*. Four complete pollen grains are connected by exine bridges.
(B) Fused pollen grains from the Arabidopsis mutant *tes/stud*. Cytokinesis does not occur after meiosis, resulting in a multinucleate cytoplasm.
(C) Natural pollen tetrads from *Drocera binata* (sundew).
Bars = 10 μm.

evolution. At the extremes, pollen exines from some species lack apertures entirely, whereas others are ''omniaperturate,'' lacking exine or coated everywhere with a very thin exine layer (Pacini, 1991; Rowley et al., 1997). The positions of the apertures are set during the tetrad stage, when gaps in the exine form at sites of close apposition between the plasma membrane and the adjacent callose wall (Heslop-Harrison, 1968). The mechanism of aperture placement is still unclear, but it has been linked to microsporocyte cytokinesis. Indeed, in the *tes/stud* mutant, a cytokinesis defect results in aberrant aperture positions (Hulskamp et al., 1997; Spielman et al., 1997; Yang et al., 2003).

Several aperture functions seem likely, although it has been difficult to link them experimentally to aperture position and number. Three logical functions for apertures are (1) sites for egress and ingress of water during pollen desiccation and hydration, (2) structural components that distribute stresses caused by grain shrinkage and swelling (termed harmomegathy), and (3) portals for the exit of the pollen tube. First, although the exines of some pollen grains are perforated with microchannels (Rowley et al., 1959), others are impermeant to water and rely on apertures to regulate grain desiccation and hydration. Consequently, some types of apertures are associated with certain plant types, especially with wet or dry stigmas. For instance, Heslop-Harrison (1979) discussed the association between multiple circular aperture (forate) exines and dry stigmas, hypothesizing that their adaptive value lies in their ability to focus water entry into the grain. Second, rigid walls are liabilities for pollen grains that undergo enormous volume decreases in the anther and subsequent increases on the stigma surface. In this context, apertures are analogous to architectural spacers in concrete, absorbing and permitting necessary shifts without breakage. The elasticity of the sporopollenin wall itself also is valuable for these deformations (Rowley et al., 2000). Third, it has long been recognized that pollen tubes exit through apertures in many pollens. Curiously, this behavior is by no means universal; some pollens lack apertures altogether, whereas others, such as Arabidopsis pollen, have apertures but do not regularly use them for tube emergence (Figure 1E). A comparative study of tube emergence across taxa could yield insight into the evolution of the mechanisms necessary for different forms of tube emergence. Although apertures provide an important means of communication between pollen and the outside world, they also are sites of vulnerability to excess desiccation and invasion by fungal hyphae (Bih et al., 1999); consequently, some plants cover

the apertures with specialized intines, lipid-rich pollen coatings, or opercula. Mutations that disrupt the regular pattern of apertures have been identified, but it is not currently feasible to use genetics to remove or add these features. Such resources would clarify the requirement for apertures in water transport, communication, and adaptation to specific types of stigmas.

Unlike the exine, and similar to most other cell walls, the cellulosic intine is constructed by the pollen cells themselves. Intine structure often is coadapted with the overlying exine structure. For example, pollen grains that have smooth or reduced exines and are unable to bind significant quantities of pollen coat have thickened or complex intines that frequently contain inclusions of hydrolytic enzymes (Heslop-Harrison, 1975b, 1977). Moreover, at the apertures, intine thickening complements exine thinning. Intine elaboration at the apertures can include extra layers between the innermost intine proper and the thin layer of aperture exine. Some of these intine layers contain enzymes and potent allergens, whereas others appear to play a role in cell wall swelling and tube emergence (Howlett et al., 1973; Heslop-Harrison, 1979b). Cellulose fibers within the intine wall radiate toward the aperture sites, generating hoops oriented around the grain's circumference that are thought to strengthen the polarized structure of the elongating pollen tube (Christen and Horner, 1974; Heslop-Harrison, 1979b; Heslop-Harrison and Heslop-Harrison, 1982). As the pollen tube emerges, the intine layer becomes continuous with the cell wall of the tube, with new wall material deposited at the growing tip. The enzymatic activity localized within the intine then is incorporated into the lateral tube walls, potentially playing a role in tissue invasion and self-incompatibility reactions. To date, few mutants have been identified that alter intine function in pollination, likely because of multiple, redundant pathways. Genomic and biochemical analyses promise to provide important clues to the identities of key intine activities.

Pollen Structure and Delivery

Pollen grains are transported to the stigma by a variety of biotic and abiotic mechanisms, for which their structures are believed to be uniquely adapted (Ackerman, 2000; Dobson and Bergstrom, 2000; Lunau, 2000). In some flowers, the selection for attracting animal pollinators is so strong that pollen grains are produced that are infertile and that function only for pollinator attraction and reward. Such grains are seen in *Lagerstroemia*, whose dimorphic anthers produce blue fertile pollen and yellow feeding pollen; although the feeding pollen can germinate, its tubes never reach the style (Pacini and Bellani, 1986). Similarly, cashew trees produce four types of pollen that are comparable in dimensions, structure, and staining characteristics but different in their abilities to germinate on and penetrate the stigma (Wunnachit et al., 1992). Beyond these extreme examples, many studies have argued for pollinator preference in the evolutionary selection for a given pollen characteristic, reporting correlations between such characteristics as size or surface features and pollination by beetles, flies, bees, moths, or birds (Skvarla et al., 1978; Hesse, 2000). The evolutionary selection of other pollen characteristics appears linked to their delivery by abiotic vectors; ~20% of all angiosperm families contain wind- or water-pollinated flowers (Ackerman, 2000). Most likely, biomechanical principles contribute to the small size of many pollens delivered by wind and to the filamentous structures of many aquatic pollens (Cox and Knox, 1989; Ackerman, 2000).

Diversity in Pollen Coat Composition

The structure of the pollen coat also is adapted to different delivery mechanisms. Insect pollination is correlated with an abundant pollen coat (Pacini and Franchi, 1996). For example, the Brassica pollen coat can constitute 10 to 15% of the mass of the pollen grain (Dickinson et al., 2000). By contrast, grains carried by the wind often have a more limited coating, such as that seen among the grasses (Heslop-Harrison, 1979b). Because lipids appear to be necessary at the interface between pollen and stigma, those pollens with limited pollen coats may depend on lipid-rich stigma exudates to assume the functionally equivalent role in germination (Wolters-Arts et al., 1998). Pollens also differ dramatically in the character and composition of their pollen coats, with grass pollen coats (pollenkitts) often lacking inclusions, whereas more complex Brassica coatings (tryphines) contain protein and lipid bodies (Pacini, 1997; Dickinson et al., 2000). Several resources provide exciting opportunities to experimentally investigate the role of some pollen coat in pollen delivery, including (1) the identification of mutants with globally depleted pollen coats, such as the *eceriferum* defects in Arabidopsis (Preuss et al., 1993; Hülskamp et al., 1995); (2) the ability to engineer mutants lacking specific proteins with reverse genetics (Mayfield and Preuss, 2000) or RNA interference techniques; and (3) the opportunity to explore pollen coat evolution through comparative genomics (Hall et al., 2002; Fiebig et al., 2004).

The pollen coat is generated by the tapetal cells that surround developing pollen grains. These cells secrete oil droplets and proteins and subsequently degenerate, filling the cavities of the exine with their cytoplasmic contents. Compared with sporopollenin-rich exines, pollen coats are better characterized; they can be extracted and their contents can be purified in sufficient quantity for identification (Doughty et al., 1993; Ross and Murphy, 1996; Ruiter et al., 1997; Murphy and Ross, 1998; Mayfield et al., 2001). The first biochemical isolations were in Liliacea, in which pollen coats were found to be largely lipidic, with carotenoids as major pigments (Heslop-Harrison, 1968). Further studies have identified medium-chain, long-chain, and very-long-chain fatty acids, proteins, glycoconjugates, carotenoids, and flavonoids (Piffanelli et al., 1998). In maize, the major protein component in the pollen coat is xylanase, which, upon its release at stigma binding, facilitates pollen tube invasion by hydrolyzing the xylan on the stigma surface (Bih et al., 1999). In *B. oleracea*, >100 pollen coat proteins, ranging in size from 5 to 50 kD, have been separated on denaturing gels. These include proteins involved in pollen–stigma adhesion or self-incompatibility responses (Preuss et al., 1993; Ross and Murphy, 1996; Ruiter et al., 1997; Murphy and Ross, 1998). Analysis of higher molecular mass proteins from Arabidopsis yielded a simpler mixture, including lipases, Gly-rich oleosins, calcium binding proteins, and proteins with similarity to the extracellular domains of receptor kinases, although lacking the kinase domain

(Mayfield and Preuss, 2000; Mayfield et al., 2001). Pollen coats also contain volatile lipids for pollinator attraction, such as polyunsaturated C18 free fatty acids, which are known to be potent honeybee attractants (Hopkins et al., 1969). Chemical analyses of 15 plant species from 10 families revealed that the pollen of each species emits its own characteristic mixture of volatiles, with the three major classes of volatiles seen in floral scents, isoprenoids, fatty acids, and benzoids, present in pollen volatiles to different degrees depending on the species (Dobson and Bergstrom, 2000). Although the set of known pollen coat proteins in Arabidopsis and Brassica is substantial, it represents only a small subset of the proteins from the tapetal cytoplasm. Extensive degradation of the released tapetum materials likely occurs before dehiscence, with the surviving coat components assembling into structures that are resistant. Support for this idea is provided by Arabidopsis mutants lacking long-chain pollen coat lipids; in these cases, proteins are deposited on the pollen surface but disappear from the coating before the pollen is released (Preuss et al., 1993).

Stigma Diversity and Development

A survey of 1000 species has shown that stigma structure generally correlates with taxonomic subdivision and argues for the coevolution of pollen and stigma structures. For example, angiosperm species that produce trinucleate pollen typically have dry stigmas, whereas binucleate pollens often interact with wet stigmas (Heslop-Harrison, 1977). As diverse as they may be, all stigmas perform the same functions. Stigmas capture pollen, support hydration and germination, and offer entry points and guidance to pollen tubes en route to the ovaries. Stigmas do not only discriminate between different pollen grains; they also promote outcrossing or self-fertilization by the coordinated timing of their maturation with pollen release. This phenomenon, termed dichogamy, has been investigated for >200 years. Two forms of dichogamy have been recognized: protandry and protogyny, in which the male or female structures, respectively, are the first to mature (Bertin and Newman, 1993). Standard tests for stigma maturation and receptivity are routinely conducted based on (1) the presence of simple enzymatic activity, (2) the ability to support pollen germination, and (3) the ability to support fertilization (this third assay being the most stringent) (Dafni and Maues, 1998). Stigma development is linked to the development of other floral organs. In Arabidopsis, immature stigmas appear to be able to promote pollen tube growth, although the immature pistil then is unable to support navigation to the ovules (Nasrallah et al., 1994). As the pistil matures, the papillae extend to form elongated cells receptive for pollination. Mutations that alter pistil structure also disrupt stigma structure; *crabs claw*, *spatula*, and *ettin* mutants have unfused carpels, open styles, and two clusters of stigma papillae, each at a carpel apex. *spatula* mutations have additional effects on stigma papillar maturation (Alvarez and Smyth, 1998, 1999), as do mutations in dynamin (ALD1), a GTP binding protein involved in membrane trafficking. ALD1 localizes to the developing cell plate during cytokinesis and maintains the plasma membrane during cell expansion (Kang et al., 2001, 2003). Transmission electron microscopy analyses of *ald1* mutants revealed defects in cell wall and plasma membrane integrity that result in the formation of short papillae cells and infertility (Kang et al., 2003). Because stigma and carpel structures vary across taxa, mutants that superficially mimic naturally occurring structures may highlight the very genes that have been modified through evolution.

At maturity, the receptive surface of a stigma presents secretions and enzymes required for pollination. Some plants that separate male and female flowers (monoecious angiosperms) have been found to retain their stigmas in the functionally male flowers but to delay the maturation of these stigmas. Likewise, some sterile cultivars lack the stigma secretions visible in their fertile counterparts (Heslop-Harrison and Shivanna, 1977). Other structural changes accompany late stigma development, including modifications of the papillar cells and their cuticles. Also, in plants with wet stigmas, apoptotic events release stigma fluids from internal reservoirs (Heslop-Harrison, 1977, 1981). A great challenge in investigating stigma function is the dearth of informative mutants, a lack likely attributable to pathway redundancy. Exploring stigma function with reverse genetics and comparative genomics is likely to yield valuable insights and to provide a necessary link between evolutionary investigations of morphology and genetic analyses of functional cell biology.

CONCLUSIONS

Here, we surveyed recent discoveries of pollen and stigma functions, including (1) pollen adhesion, a multiphase process that is initially independent of protein–protein interactions but later involves pollen coat proteins; (2) pollen hydration, a step for which the presence of lipids, whether provided by the male or the female surface, likely modulates water transport while diverse proteins mediate pollen incompatibility; (3) pollen activation and germination, processes dependent on polarizing cues, cytoplasmic rearrangements, and the harnessing of biomechanical forces for the focused breaching of the exine wall; and (4) stigma invasion, a stage that involves the localized activity of digestive enzymes. We also addressed the great diversity in pollen and stigma structures used across taxa to accomplish pollination, including diversity in pollen wall development, pollen delivery, pollen coat composition, and stigma development.

Studies of model organisms have revealed key molecules and mechanisms, setting the stage for comparative studies across taxa. For example, in pollen wall development, the patterning genes identified through Arabidopsis screens are likely to be highly variable between species, either in their coding sequences or in the sites and timing of their expression. Work in comparative genomics is called for not only within the angiosperms but between pollen, fern spores, and even diatoms (Schmid et al., 1996), in which similar cytoskeletal and plasma membrane shifts are likely associated with wall construction, sculpting, and aperture placement. Because genes that play roles in determining wall morphologies are the very genes altered through evolution, patterns considered aberrant or mutant to one species may be considered normal in another. Suppositions such as that sculpted exine is required to hold an abundant pollen coat could

be addressed using Arabidopsis mutations, such as *lap1* (Zinkl and Preuss, 2000), that affect exine patterning. The roles of pollen coat or pollen dispersal unit size in pollinator preference and pollen delivery could be addressed using knowledge of Arabidopsis mutants, such as those in *ECERIFERUM*, *TES/STUD*, or *QUARTET* genes (the former affects pollen coat quantity and the latter two affect the release of monad pollen, respectively). Comparative genomics and RNA interference technology then could be exploited to knock out these genes in insect-pollinated flowers, such as those of Brassica. Integrating cell, genetic, and genomic studies of model organisms with comparative studies of relationship and evolution allows pollen and stigma sophistication to be viewed in the context of coadaptive or mutual evolutionary adjustments. Because pollination mechanisms also are key to reproductive isolation and speciation, attention focused across a variety of species also will help to complete any model of the success and diversity of angiosperms.

ACKNOWLEDGMENTS

We are grateful to members of the Preuss laboratory, especially Kiera Von Besser, Mark Johnson, and Ravishankar Palanivelu, for their helpful discussion and review of this article. We also thank horticulturalist Steven Meyer at the Lincoln Park Conservatory, Chicago Park District, for flowers with tetrad and polyad pollens. This work was supported in part by grants from the Howard Hughes Medical Institute (A.F.E.), the U.S. Department of Agriculture (2002-35318-12560) (R.S.), the National Science Foundation (MCB-0077854), and the University of Chicago Materials Research Science and Engineering Center (National Science Foundation DMR-0213745).

Received July 28, 2003; accepted January 15, 2004.

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