

Pollen-stigma adhesion in *Arabidopsis*: a species-specific interaction mediated by lipophilic molecules in the pollen exine

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SUMMARY

To investigate the nature and role of cell adhesion in plants, we analyzed the initial step of pollination in *Arabidopsis*: the binding of pollen grains to female stigma cells. Here we show this interaction occurs within seconds of pollination. Because it takes place prior to pollen hydration, it also requires adhesion molecules that can act in a virtually dry environment. We developed assays that monitored adhesion of populations of pollen grains and individual cells. Adhesion between pollen and stigma cells is highly selective – *Arabidopsis* pollen binds with high affinity to *Arabidopsis* stigmas, while pollen from other species fails to adhere. Initial binding is independent of the extracellular pollen

coat (tryphine), indicating that adhesion molecules reside elsewhere on the pollen surface, most likely within the exine walls. Immediately after pollination, the stigma surface becomes altered at the interface, acquiring a pattern that interlocks with the exine; this pattern is evident only with pollen from *Arabidopsis* and its close relatives. Purified exine fragments bind to stigma cells, and biochemical analyses indicate that this specific, rapid and anhydrous adhesion event is mediated by lipophilic interactions.

Key words: Adhesion, *Arabidopsis thaliana*, Pollen, Stigma, Reproduction, Exine, *CER* mutants

INTRODUCTION

Cell recognition plays a key role in biology: cells of the immune system can identify foreign invaders, targeted cell migrations are essential for animal development, and gametes bind in a species-specific manner. Here we characterized the nature of cell recognition at the earliest stage of *Arabidopsis* reproduction, namely the binding of pollen grains to female stigma cells. This interaction occurs in a relatively dry environment yet it is highly specific, enabling flowers to selectively bind pollen from appropriate species.

Male and female plant cells form de novo contacts requiring cell recognition at many stages of plant reproduction. Pollen hydration occurs following signal exchange with the stigma, pollen tubes germinate and are guided through female tissues to the ovules, and sperm fuse with the egg and central cell, yielding a zygote and endosperm (Wilhelmi and Preuss, 1999). Because inappropriate pollen tubes would deplete female tissue resources, many plants, including *Arabidopsis*, regulate pollen development at the stigma surface. Plants employing this early discrimination typically have a dry stigma, allowing precise control of pollen adhesion and hydration (Heslop-Harrison, 1981). In contrast, wet stigmas are covered with carbohydrate- and lipid-rich secretions that hydrate pollen indiscriminately; consequently, such plants inhibit incompatible pollen at later stages.

Early stages of pollination for plants with dry stigmas, including pollen binding to the stigma surface, diffusion of the

extracellular pollen coat along the stigma cells, and pollen hydration, require pollen-stigma signaling. Molecules that mediate these interactions likely reside on extracellular surfaces or within the underlying cell walls. Pollen grains have two cell walls: an inner layer, intine, and an outer lattice network, exine (Dobson, 1989). Intine is composed primarily of cellulose, while exine is rich in sporopollenin, a highly stable, mixed polymer containing long-chain fatty acids and phenolics (Bedinger et al., 1994; Thom et al., 1998; and references therein). In addition, exine is typically covered with a lipid and protein-rich matrix known as tryphine or pollenkitt (Dobson, 1989). Papillae cells from dry stigmas are coated with an external proteinaceous pellicle (Elleman et al., 1992). Cell-cell interaction molecules that reside on these cell surfaces must function in a relatively dry environment and be appropriately displayed within the complex extracellular matrices.

Stigma molecules that play a role in pollen-stigma interactions have been uncovered through studies of the natural variation in plants displaying self-incompatibility (SI) in *Papaver* (Franklin et al., 1995) and *Brassica* (Stein et al., 1996; Stephenson et al., 1997). The *Brassica* SI locus encodes two stigma surface proteins, a transmembrane receptor kinase (SRK) and an extracellular glycoprotein (SLG); a related cell wall protein, SLR1, is encoded by an unlinked gene (McCormick, 1998). An SLG-related protein with unassigned function, AtS1, has been identified in *Arabidopsis* flowers (Dwyer et al., 1994).

Pollen molecules critical for communication with the stigma

reside within the lipid-rich pollen coat. *Arabidopsis* mutants lacking a normal coat are defective in pollen hydration and are male-sterile. These coat defects arise from alterations in *CER* genes which function in the synthesis of long-chain lipids (Hülkamp et al., 1995; Preuss et al., 1993). Inhibition of *cer* pollen hydration can be bypassed by increasing ambient humidity or by adding large quantities of triacylglycerols (Wolters-Arts, et al., 1998); the absence of triacylglycerols in the wild-type coat (Hernández-Pinzón, et al., 1999) suggests their effect is not specific. The pollen coat of some *Brassica* species carries cysteine-rich peptides (PCPs) that play a direct role in SI: adding PCP-containing fractions to pollen can alter its SI phenotype (Stephenson et al., 1997). Interestingly, PCP proteins bind SLG and SLR1 in vitro (Doughty et al., 1993; Hiscock et al., 1995).

Although the signaling machinery described above is critical for pollen recognition, few studies have focused on the contribution of cell adhesion molecules to pollination. Such components are likely required to stabilize pollen-stigma contacts. Recently, Luu et al. (1999) implicated SLR1 and SLG in pollen-stigma adhesion. Applying antibodies raised against SLR1 and SLG to stigmas diminished pollen adhesion by four fold, and stigmas from an SLR1-deficient plant displayed a two-fold decrease. Although adhesion defects were undetected immediately after pollen-stigma contact, they were significant at later stages.

In this study, we identified an earlier adhesion event that allows plants to capture appropriate pollen. This binding interaction occurs within seconds of contact between pollen and the stigma; subsequently, adhesive contacts are remodeled upon pollen hydration and pollen tube growth. Two simple adhesion assays were devised: one is suitable for surveying populations of pollen grains, and the other measures the adhesive force between individual pollen and stigma cells in their native dry environment. Pollen-stigma adhesion was found to be highly species-specific; unexpectedly, it is mediated by lipophilic molecules found within the exine wall. The adhesion interaction that occurs in *Arabidopsis* pollinations may be representative of all plants with dry stigma surfaces.

MATERIALS AND METHODS

All reagents were from Sigma (St. Louis, MO), except for octylglucoside and octylthioglucoside (Pierce, Rockford, IL).

Plant materials

Arabidopsis thaliana strains were Landsberg *erecta* (Ler-0). Wild-type (CS20) and male sterile *ms1* (CS75) strains were from the *Arabidopsis* Stock Center (Columbus, OH). *cer6-2* was previously described (CS6242; Preuss et al., 1993). *Arabidopsis lyrata* was from Dr Rodney Mauricio (University of Georgia). Pollens were from Greer Laboratories (Lenoir, NC), Biopol Laboratories (Spokane, WA), or greenhouse collections (*Petunia* and *Arabidopsis*). *Arabidopsis* growth conditions were previously described (Preuss et al., 1993).

Liquid assay for monitoring adhesion of populations of pollen grains

Unpollinated pistils were harvested from *ms1* or wild-type flowers. Pistils of uniform age (0–8 hours after flowering, stages 14–15; Bowman et al., 1989) were saturated with approximately 5000 pollen grains; pollinations required ≤ 1 minute. Pollinated pistils were washed in 200 μ l of phosphate buffer (50 mM potassium phosphate, pH 7.4) containing 0.01% NP-40 (low stringency) or 1% Tween 20 (high stringency). Samples were mixed for 5 seconds and centrifuged

for 1 minute at 7200 g. Washed pistils were fixed (90% ethanol, 10% acetic acid) and stained with 5 mg/ml 3, 3'-diethylthiocarbocyanine iodide (DiOC₂) dissolved in phosphate buffer containing 90% glycerol (Regan and Moffatt, 1990). Adhering pollen grains were counted with bright-field illumination using a Zeiss Axioskop.

Dry assay for monitoring adhesion of individual pollen grains

Adhesive force was measured with a custom-built device (Fig. 2B) designed on the same principles as an atomic force microscope (Sarid, 1994). One pistil was mounted on the free end of a flame-drawn glass fiber using cyanoacrylate glue (Pronto CA40 Instant Adhesive, 3M, St. Paul, MN), and a second was mounted with double-stick tape onto a glass slide positioned on a three-axis translation stage. The pistil on the stage was pollinated, and a pollen grain bound to an isolated papillae cell was selected for analysis. Seconds after pollination, the stage was maneuvered to bring the grain into contact with a stigma papillae cell from the pistil on the glass fiber. Once contact was made, the position of the micrometer drive controlling the stage was noted (± 5 μ m). Subsequently, the stage was retracted at a constant rate (6.7 ± 2.1 μ m/seconds), bending the glass-fiber cantilever. When the adhesive contact between the pollen grain and one of the stigma cells was broken, the cantilever snapped back to its equilibrium position, and the stage was arrested immediately (within 2 μ m). The total displacement is proportional to the bending force on the cantilever and provides a direct measurement of the adhesion force.

The force exerted by the bent cantilever at the adhesive contact can be described by Hooke's law, $F(z) = -kz$, where z represents the displacement and k is the glass fiber's spring constant. The maximum displacement z_{\max} therefore provides a measure of the adhesive force. Estimating the adhesion force requires an accurate assessment of the spring constant, k . For a cylindrical glass fiber, the value of k can be described by $(3\pi d^4 E)/64l^3$ (Sarid, 1994), where d is the diameter of the fiber, l is its length, and E is the Young's modulus of glass. The diameters of the fibers ranged from 60 to 150 (± 5) μ m, measured with a digital micrometer gauge, and lengths from 40 to 75 (± 0.3) mm. Glass has a typical Young's modulus of $E = 6 \times 10^{10}$ N/m². This suggests a typical spring constant of roughly $k \approx 50$ nN/ μ m. Because the Young's modulus can change as glass is drawn into a fiber, we determined the spring constant for each cantilever by measuring its resonance frequency, f . This natural vibration frequency is related to the spring constant by $k = 0.24\pi^3 d^2 l \rho f^2$ (Sarid, 1994), where $\rho = (2.20 \pm 0.04) \times 10^3$ kg/m³, the measured mass density for our glass fibers. To determine a fiber's resonance frequency, we monitored its motion by reflecting a laser beam off its tip onto a position-sensitive photodetector. Vibration of the cantilever created a sinusoidal photodiode signal as the reflected laser beam swept back and forth. A signal analyzer (HP 35670A, Hewlett Packard, Palo Alto, CA) isolated this sinusoidal component from the photodiode signal and thus measured the fiber resonance frequency. Resonance frequency can be determined from thermally-driven vibrations, but a gentle stream of filtered air was blown across the fiber to drive larger amplitude oscillations. Resonance frequencies averaged 25 (± 1) Hz.

Combining the measurements of diameter, length, density, and resonance frequency provided us with an estimate of the cantilever's spring constant, accurate to within 10%. When combined with the roughly 0.5 mm error in estimating the pollen's position on the cantilever and the 5 μ m error in z_{\max} , we estimated relative errors in the measured adhesion force to be $\leq 20\%$.

Pollen coat extraction and exine purification

Pollen was harvested by clipping *Arabidopsis* inflorescences, washing them in phosphate buffer, filtering through cheesecloth, and centrifuging to obtain a pollen pellet. The pollen coat was removed by washing three times in 10 volumes of cyclohexane (Doughty et al., 1993). Before performing adhesion assays, delipidated pollen was washed in phosphate buffer and water and dried on a microscope slide.

For exine purification, pollen pellets were suspended in Tris buffer (100 mM Tris-Cl (pH 8.0) containing protease inhibitors (0.2 mg/ml iodoacetamide, 10 mg/ml aprotinin, 17.5 mg/ml benzamidine, 1 mg/ml antipain, 1 mg/ml pepstatin and 200 mM phenylmethylsulfonyl fluoride). The suspension was circulated in a 5 ml nebulizer (Baxter Healthcare, Corp., Valencia, CA) using nitrogen gas, lysing most grains within 5 minutes. Wall fragments were collected by centrifugation at 7,500 g, and the pellet was washed three times in Tris buffer. To remove intact pollen, the pellet was loaded onto a sucrose step gradient (30, 40, 50, 60, 70%) and centrifuged (20,000 rpm in a SW27 rotor (Beckman Instruments, Palo Alto, CA) for 30 minutes at 4°C; Chay et al., 1992). *Arabidopsis* exine fragments migrated to the interface of the 50% and 60% steps; other pollen species partitioned differently. Exine fractions were washed three times in Tris buffer, incubated at 4°C for 2 hours in 0.05% sodium deoxycholate to remove contaminating cytoplasm, washed three times in Tris buffer, and incubated for 12 hours at 4°C in Tris buffer containing 2 M NaCl. After washing three times with distilled water, fragments were dried onto glass under vacuum and used for morphological analysis or in adhesion assays.

Electron microscopy

For anhydrous fixation, pistils were pollinated and immediately placed upright under a glass dish with subliming OsO₄ crystals (Polysciences, Warrington, PA) for 2 hours at 22°C, washed three times with cacodylate buffer (100 mM sodium cacodylate, pH 7.4) and post-fixed in 2.5% glutaraldehyde (Polysciences) in cacodylate buffer (Elleman and Dickinson, 1986). Routine fixation used 2.5% glutaraldehyde for at least 2 hours at 22°C and post-fixation for 2 hours in 2% OsO₄ in cacodylate buffer. For scanning electron microscopy, fixed samples were washed three times in cacodylate buffer and incubated in an ethanol series (50%, 70%, 85%, 95% and 100%) at -20°C; each incubation was for 10 minutes and was repeated once. Samples were washed three times in 100% ethanol at room temperature for 10 minutes each, incubated in 100% ethanol for 20-30 minutes, and washed twice with amyl acetate (10 minutes each) and dried to critical point in a DCP-1 vacuum apparatus (Denton Vacuum, Inc., Moorestown, NJ). Stigmas were excised, coated with 8 nm gold, and observed on a Jeol JSM-8404 microscope.

For transmission electron microscopy, anhydrous fixation was as described except after post-fixation, samples were washed three times in cacodylate buffer, then in maleate buffer (50 mM sodium maleate, pH 6.0). Subsequently, they were incubated in 1% uranyl acetate in maleate buffer for 30-60 minutes, washed three times with maleate buffer, dehydrated in the ethanol series (-20°C), and infiltrated with Polybed 812 resin (Polysciences). After polymerization overnight at 60°C, 50-65 nm sections were cut on a Reichert-Jung Ultracut E ultramicrotome and counterstained with 2% uranyl acetate in 100% methanol and 1% dimethyl sulfoxide, followed by Sato's triple lead stain (Sato, 1968). Sections were examined with a Jeol JEM-100CX II.

RESULTS

Pollen-stigma adhesion is rapid and highly selective

To monitor pollen adhesion to *Arabidopsis* stigmas, we devised a convenient and quantitative assay, using stigmas from either emasculated wild-type flowers or from the male-sterile mutant, *ms1*, both of which yielded similar results. *ms1* affects early stages of pollen development and thus provides a ready supply of unpollinated stigmas (van der Veen and Wirtz, 1967). Mature pistils were collected and their stigma

surfaces completely covered with dry pollen grains. The entire pollinated pistil was washed vigorously in buffer containing 0.01% NP40, and adhering grains were counted (see Materials and Methods). Because this assay applied strong shear forces, only pollen grains with high-affinity binding were recovered.

An average of 208 (±70) *Arabidopsis* pollen grains remained bound to each stigma (n=38) after the detergent wash (Fig. 1A,C). Importantly, pollen from an unrelated plant, *Petunia hybrida*, did not bind strongly to *Arabidopsis* stigmas (Fig. 1B,C); on average, only 7 pollen grains (±3) were detected per stigma (n=33). These results indicate that *Arabidopsis* stigmas preferentially bind *Arabidopsis* pollen. Similar results were obtained when pollen was incubated on the stigma for 5 minutes or for as little as 30 seconds

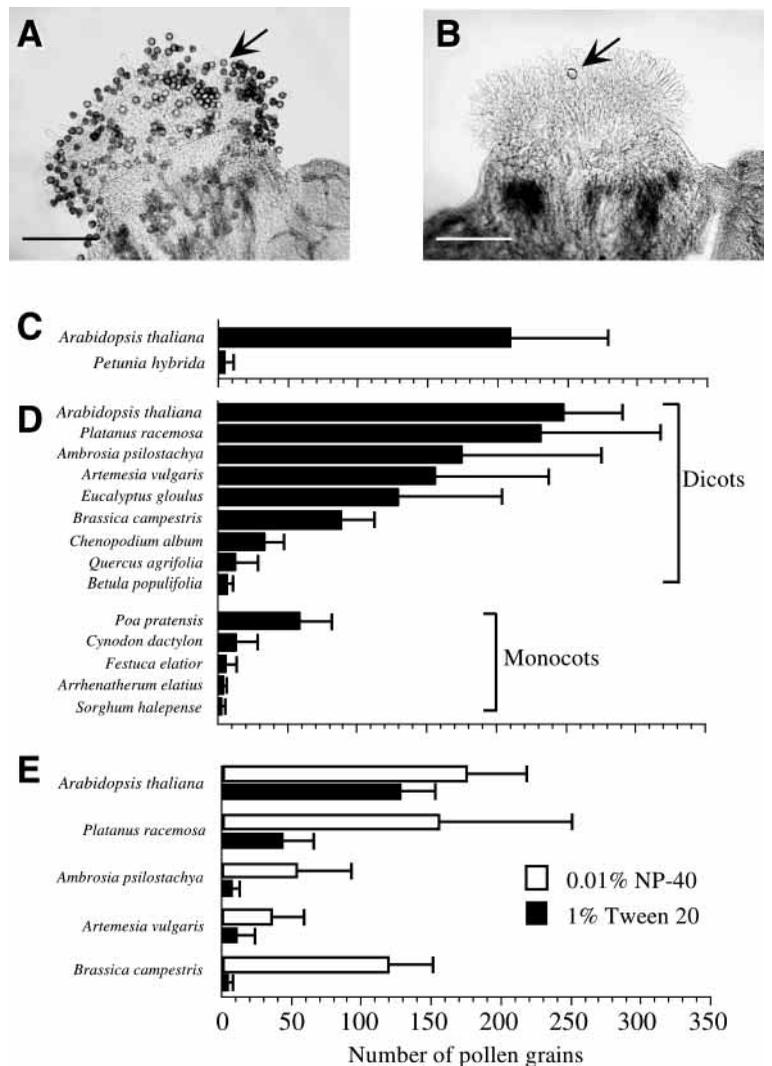


Fig. 1. Assessment of pollen adhesion using a liquid assay. (A,B) *Arabidopsis* (A), but few *Petunia* (B) pollen grains (arrows) adhered strongly to *Arabidopsis* stigmas. Bar, 0.5 mm. (C-E) Mean number of pollen grains bound per stigma (error bars, standard deviation). (C) As in A and B; n=38 (*Arabidopsis*) or n=33 (*Petunia*). (D) Indicated pollen was applied to *Arabidopsis* stigmas; n≥10 assays. Pollen from monocots generally displayed weaker adhesion than pollen from dicots. (E) Comparison of low stringency adhesion assay (0.01% NP40) with a high stringency assay (1% Tween 20). Foreign pollen that showed binding in (D) was efficiently removed under high stringency conditions while *Arabidopsis* grains remained.

(160 ± 65 ; $n=28$), indicating that the adhesion reaction is rapid.

To monitor the selectivity of *Arabidopsis* stigmas, we used our assay to measure the adhesion of a variety of pollen species. Many of these pollen are common allergens and can be obtained commercially. Pollen from monocotyledonous plants displayed little or no detectable binding (Fig. 1D), while pollen from some dicots showed significant binding. Interestingly, pollen from a close relative, *Brassica campestris*, did not bind as tightly as *Arabidopsis* pollen. Of the foreign pollen grains exhibiting some adhesion, no simple correlation between binding affinity and phylogeny could be derived.

We tested the adhesion affinity of those foreign pollen displaying significant binding by increasing the stringency of the assay by washing for an hour in 0.01% NP40 or 1% Tween 20 (Fig. 1E). These conditions did not significantly affect the number of *Arabidopsis* pollen grains remaining attached to the stigma, but dramatically reduced binding of foreign pollen by approximately 70–95%. In addition, washing in 1% Tween 20 easily removed *Brassica campestris* pollen from *Arabidopsis* stigmas, whether the incubations were for 1 hour (Fig. 1E) or as little as 2 minutes. Thus, the adhesion step occurring in the first few seconds of *Arabidopsis* pollination selectively discriminates among diverse pollen grains. Although some foreign pollen is able to attach to the stigma, none exhibits the high affinity binding characteristic of *Arabidopsis*.

Monitoring the force of pollen-stigma adhesion

The assay described above is convenient for measuring the adhesion of many pollen types and for monitoring the affinity of cell populations. As an alternative, we developed an instrument to measure the magnitude of the binding force between an individual pollen grain and the stigma. Unlike optical tweezers or atomic force microscopes previously used to monitor cell adhesion (Dammer et al., 1995; Schmidt et al., 1993), this device enabled us to analyze pollen binding in its native environment – the dry stigma surface.

Although the results presented in Fig. 1 demonstrate that foreign pollen binds *Arabidopsis* stigmas only weakly, we could not predict whether similar results would be obtained in a dry environment. Consequently, we first demonstrated that a high-pressure air stream could easily dislodge *Petunia* pollen grains from *Arabidopsis* stigmas, while *Arabidopsis* pollen remained bound. Selectivity in plant pollination is often attributed to interactions with specific pollinators and to regulation of flowering time. Our results indicate that shear forces generated by wind, coupled with a selective cell adhesion system, could play a critical role in capturing appropriate pollen and removing foreign grains.

To monitor the binding force between individual cells, we used a sensitive spring gauge consisting of a fine glass fiber (Fig. 2). Because the physical properties of the fiber are known, its spring constant can be calculated (Fig. 2A, see Materials and Methods). One cell is mounted onto this glass fiber, and a second, mounted on a motorized stage, is brought into contact (Fig. 2B). Subsequently, the stage supporting the second cell is slowly retracted, and the point at which the cells break contact is noted; this displacement is proportional to the binding force.

For this experiment, it is critical that any compound used to attach the cells to the apparatus does not, itself, promote adhesion. For example, liquid adhesives could spread to the

pollen-stigma interface, confounding the force measurements. Consequently, we mounted only the lower portion of each pistil (the ovary), providing a natural support for the stigma and pollen cells to be tested. One pistil was glued to the glass fiber,

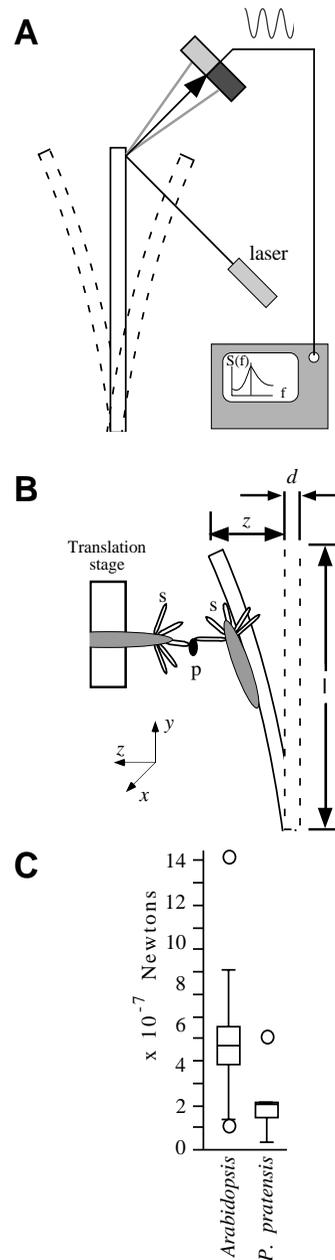


Fig. 2. Assessment of pollen adhesion using a dry assay. (A) The resonance frequency of glass fiber cantilevers was measured by reflecting a laser beam off their tips and detecting the oscillations with a position-sensitive photodetector. (B) Instrument used to monitor pollen-stigma adhesion forces. Stigmas with bound pollen grains were maneuvered with a translation stage. The cantilever bends as the stage is retracted; the maximal displacement (z) is proportional to the binding force. d , fiber diameter; l , fiber length. (C) Measurements of pollen-stigma binding force showing the median 50% of data points (box), the remaining quartiles (lines), and outlying points not included in the calculations (open circles). Measurements with *Arabidopsis* pollen employed either wild-type or the hydration-deficient *cerb-2* mutant, giving comparable results.

and a second pistil to a glass slide mounted on the stage (Fig. 2B). Pollen was applied to a stigma cell on one of these pistils and then a stigma cell from the other pistil was contacted. This design relies on two cell-cell interactions (stigma-pollen-stigma), and the force measured reflects the weaker interface.

The binding force between *Arabidopsis* pollen and stigma cells averaged $5.0 (\pm 2.6) \times 10^{-7}$ N ($n=20$; Fig. 2C). Because the instrument has an error $\leq 20\%$ (see Materials and Methods), the observed variation in binding force likely arose from other factors. In particular, the magnitude of adhesion is expected to vary with the surface area of the pollen-stigma interface which ranged from 8-40 μm^2 . The observed binding force is consistent with the viscous Stokes' drag exerted upon a pollen grain by a strong breeze. By analogy, if this adhesive were spread onto an area of 0.1-0.5 m^2 , it would be sufficiently strong to suspend a 100 kg object.

We tested whether this single cell assay, like the liquid adhesion assay, detects species-specific differences in pollen adhesion. As shown in Fig. 2C, the adhesion force of *Poa pratensis* pollen to *Arabidopsis* stigmas was $1.5 (\pm 0.8) \times 10^{-7}$ N ($n=5$), a decrease of approximately three fold relative to *Arabidopsis*. Similar tests with *Petunia*, *Sorghum* and *Cynodon* revealed less striking differences. These results indicate that this assay, which mimics physiological conditions, uncovers species-specific differences in pollen adhesion. In contrast, the liquid adhesion assay is more disruptive, but allows differences among a larger variety of pollen grains to be detected.

Initial pollen-stigma adhesion is independent of the pollen coat

The interactions between the pollen coat and the stigma have led to the suggestion that pollen coat molecules mediate pollen-stigma adhesion (Luu et al., 1997; Stead et al., 1979). We used the binding assays to monitor initial adhesion in grains lacking a coat. The *Arabidopsis* pollen coat, consisting of clear lipid droplets and proteins embedded on the exine surface (Fig. 3A), can be removed by two different means, either through genetic alterations or chemical extraction. Pollen from *cer6-2* mutants has an extreme coat defect that eliminates all detectable lipid droplets and most proteins (Fig. 3B). Alternatively, cyclohexane efficiently extracts the coat (Doughty et al., 1993; Fig. 3C).

Surprisingly, pollen coat removal did not significantly affect adhesion (Fig. 3D). An average of 208 ± 25 *cer6-2* pollen grains ($n=29$) and 209 ± 25 cyclohexane-extracted pollen grains ($n=19$) remained bound to each stigma, as compared to wild-type (225 ± 25 grains, $n=35$). Even high stringency washes in 1% Tween 20 did not dislodge *cer6-2* pollen (not shown). Similarly, wild-type and *cer6-2* pollen displayed a comparable affinity for the stigma in the single-cell assay (Fig. 2).

These results indicate that the initial adhesive interaction does not depend on an intact pollen coat. Consequently, the relevant adhesion molecules are likely localized within other pollen surface layers, potentially within the exine. Although it is possible our methods left behind residual coat components that mediate adhesion, this seems unlikely since genetic and chemical techniques yielded similar results.

The magnitude of pollen adhesion increases during pollination

Although *cer6-2* pollen binds to the stigma, the absence of a pollen coat inhibits subsequent steps in pollination – the stigma

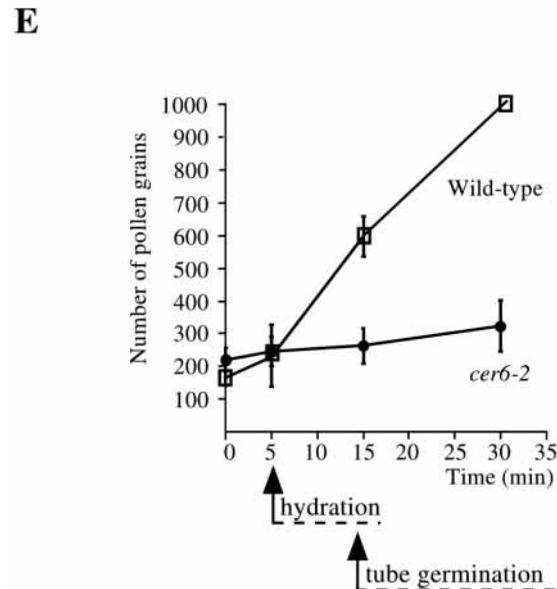
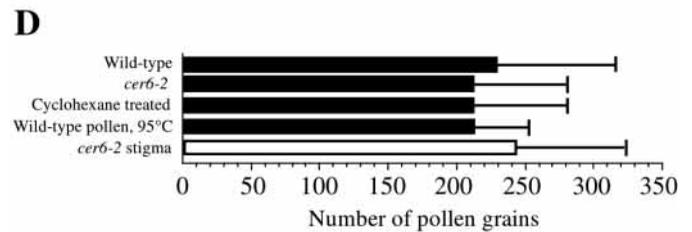
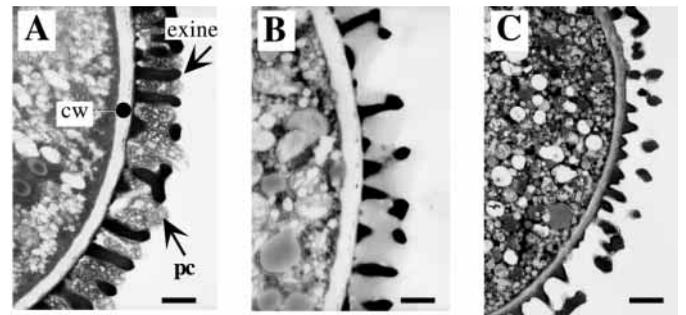


Fig. 3. The pollen coat is unnecessary for adhesion. (A-C) Transmission electron micrographs of wild-type (A), *cer6-2* (B), and cyclohexane-extracted (C) *Arabidopsis* pollen. Bar, 1 μm ; cw, cell wall; pc, pollen coat. (D) Results of adhesion assays as in Fig. 1, $n \geq 10$. Pollen applied to *ms1* *Arabidopsis* stigmas, black bars; wild-type pollen applied to *cer6-2* stigmas, white bar. (E) Liquid adhesion assay monitoring the number of pollen grains bound per stigma ($n=5$ stigmas per data point; error bars, standard deviation). Onset (arrows) and duration (dashed lines) of pollen hydration and pollen tube germination are indicated.

cells do not transfer water to the pollen and pollen tubes do not germinate (Preuss et al., 1993). A comparison of adhesion of wild-type and *cer6-2* grains thus makes it possible to distinguish between early adhesion events and events necessary for pollen hydration and tube invasion.

At different times following pollination, stigmas were washed to remove weakly bound grains (Fig. 3E). As pollen hydration progressed, the number of wild-type grains that bound with high affinity increased four-fold (15 minutes; Fig. 3E). This change

in adhesion may reflect the spreading of the pollen coat onto the stigma, conversion of the coat into a different form, or structural alterations in the stigma wall (Elleman et al., 1992). After pollen grains hydrated, wild-type adhesion continued to increase; 30 minutes after pollination, >1000 grains remained firmly attached. Importantly, adhesion of *cer6-2* pollen did not change significantly throughout the time course. Foreign pollen, which did not hydrate or germinate, also displayed a consistent, albeit low, level of adhesion; the number of adhering *Petunia hybrida* pollen grains never exceeded 9 per stigma. These results imply that measurements of wild-type pollen adhesion, if performed within a few minutes of pollination (Fig. 1), reflect events independent of pollen hydration.

Previously, Luu et al. (1997) used a different assay and reported a four-fold weaker binding affinity for *cer* mutant pollen compared to wild type, even with measurements performed immediately after pollination. They anchored inflorescences to the bottom of microcentrifuge tubes and floated pollen from the tips of stigmatic papillae by centrifugation at 900 g in 50% sucrose, 2% formalin. In contrast, we used *cer6-2*, the most extreme pollen coat mutant known, yet observed an initial binding affinity equivalent to wild type. Although the different results could be explained by differences in centrifugal force, the adhesion assays reported here employed a comparable force and more stringent wash conditions. Instead, it is possible that the measurements obtained by Luu et al. (1997) were confounded by the onset of pollen hydration. Alternatively, the presence of formalin in their buffer might have crosslinked the wild-type pollen coat to the stigma, resulting in an apparent adhesion difference. Nonetheless, in over 50 measurements of *cer6-2* and wild-type pollen adhesion, we detected no difference in initial binding affinity. Importantly, similar conclusions were drawn from the single-cell assay, which monitors adhesion under physiologically relevant conditions.

Structure of the pollen-stigma interface

The adhesion of *cer* mutants and cyclohexane-treated pollen suggests exine, rather than the pollen coat, plays a primary role in initial cell adhesion. To investigate the ultrastructure of contact sites between exine and the stigma, we fixed *Arabidopsis* stigmas immediately after pollination. Because aqueous fixatives could alter these interactions, we used an anhydrous technique (Elleman and Dickinson, 1986).

Some plants decorate their surfaces with micron-sized projections that attach to animal hair. Although it is conceivable that exine projections could similarly mediate stigma attachment during pollination, this is unlikely because ultrastructural analysis revealed no

obvious stigma features that could account for species-specific binding (Fig. 4). Consequently, pollen-stigma adhesion results from molecular, rather than large-scale structural interactions.

The wild-type pollen coat is apparent at the pollen-stigma interface (Fig. 4A, arrow), obscuring visualization of the exine. However, some wild-type grains detach under strong vacuum, revealing impressions on the papillae surface with a pattern

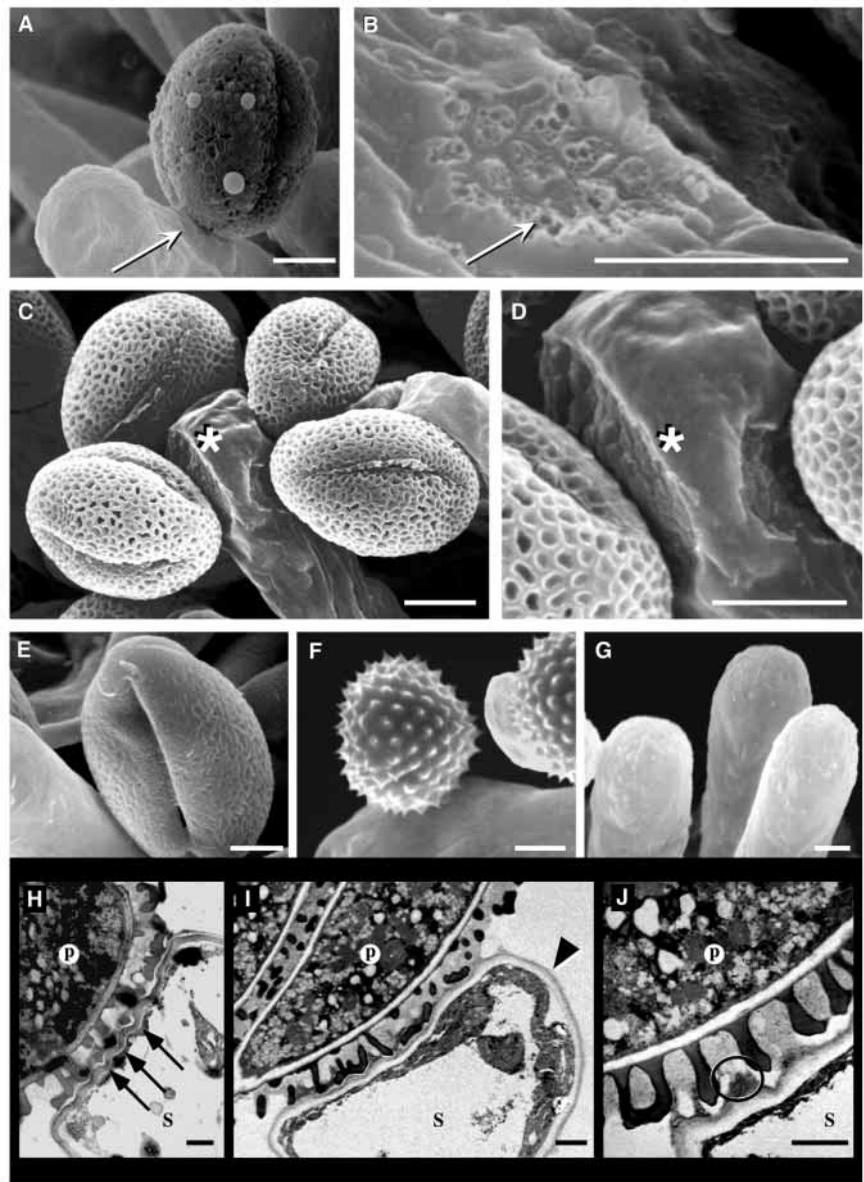


Fig. 4. Analysis of the pollen-stigma interface, using anhydrous fixation. (A-G) Scanning electron micrographs of pollinated *Arabidopsis* stigmas. (A) Wild-type *Arabidopsis* pollen with an intact coat displaying numerous lipid droplets. A portion of the pollen coat (arrow) migrated onto the stigma surface. (B) Surface of a stigma cell that had previously contacted a wild-type pollen grain; notable impressions (arrow) were common, but never observed on unpollinated controls. (C-D) Pollination with *cer6-2* pollen revealed dramatic impressions in the stigma surface (*). (E-F) Control pollinations with *Petunia* (E) and *Ambrosia psilostachya* (F) pollen. (G) Surface of an unpollinated *Arabidopsis* stigma. (H-J) Transmission electron micrographs of the interface between wild-type *Arabidopsis* pollen and the stigma. Arrows in H indicate areas of the stigma cell wall in direct contact with pollen exine which appear thinner (reduced 37-52%) than in unpollinated regions (I, arrowhead). (J) High magnification view showing intermingling of the stigma pellicle material with the pollen coat (circle). Size bars, 5 μ m (A-G); 1.5 μ m (H-J); S, stigma; p, pollen.

reminiscent of the exine structure (Fig. 4B). Similar impressions, though less distinct, were observed with aqueous fixation (not shown). These indentations were more obvious when pollen without a coat was analyzed; patterns corresponding to a negative imprint of the exine lattice were apparent (Fig. 4C-D). Pollen from species within the same genus (*Arabidopsis lyrata*) left similar marks.

We tested whether alterations in the stigma surface could be detected when other pollen species were applied to *Arabidopsis* stigmas. Few *Petunia* pollen grains were retained on the stigma following anhydrous fixation, but remaining grains left no detectable patterns (Fig. 4E). Similarly, pollen from *Ambrosia psilostachya*, *Artemisia douglasiana*, *Arrenathrun elatius*, *Sorghum halepense*, and *Platanus racemosa* left no impressions, indicating that the observations with *Arabidopsis* pollen most likely were a consequence of a highly specific interaction. This was most striking with pollen from *A. psilostachya*, which has a surface covered with pointed bacula (Fig. 4F); if the imprints with *Arabidopsis* pollen were due to artifacts of fixation, *A. psilostachya* pollen would be expected to leave characteristic marks. Finally, impressions were never detected in the surface of unpollinated stigmas (Fig. 4G), confirming that pollen imprints result from interaction with pollen.

The patterns observed in the stigma surface could reflect alterations in the extracellular protein pellicle or the underlying cell wall. To determine which stigma structures were affected by pollen binding, we examined cross sections, using anhydrous fixation and transmission electron microscopy. Dramatic undulations in the stigma pellicle and cell wall were detected with *Arabidopsis* pollen (Figs. 4H-J). Stigma surfaces that did not contact pollen were uniformly smooth (Fig. 4I, arrowhead). When in contact with pollen, the stigma surface protruded into spaces between exine bacula, and these protrusions were associated with changes in cell wall thickness (thickness reduced 37-52%; Fig. 4H, arrows). No changes in pollen wall morphology were observed.

In addition to the pollen imprints in stigma cells, components from the stigma pellicle diffused from the stigma surface and intermingled with wild-type pollen coat material (Fig. 4J, circle). Similar fusions between the stigma pellicle and the superficial layer of the pollen coat have been observed in *Brassica* (Elleman and Dickinson, 1986). Some intermingling of stigma material and residual pollen coat components was also observed with *cer6-2* pollinations (not shown).

The remodeling of the stigma surface occurred rapidly – all observed changes took place prior to fixation (within 5 minutes). Thus, these changes reflect an early step in pollen adhesion: they are immediate, involve the exine and stigma surfaces, and are associated with undulations in the cell wall that increase the contact surface area.

Chemical nature of pollen-stigma adhesion

The exine and stigma-associated molecules that mediate pollen adhesion have extraordinary characteristics. They interact within seconds of pollination and mediate large-scale changes in cell surface structure. More striking, these adhesives function in an environment with limited water; those on pollen survive baking under a vacuum at 95°C for 1 hour (Fig. 3D).

Few functional homologs of animal adhesion molecules are known in plants, and pollen-stigma binding is unlike any

previously characterized adhesion interaction. Consequently, rather than testing the role of candidate molecules, we initiated a chemical approach to characterize pollen adhesion. We tested whether a wide spectrum of compounds known to disrupt electrostatic or hydrophobic interactions could break the adhesive contacts. Importantly, we monitored adhesion with *cer6-2* pollen, trapping pollination at the first step. Stigmas from *cer6-2* mutants are fully functional; they are fertile when pollinated with wild-type pollen (Preuss et al., 1993) and display normal levels of pollen adhesion (Figs 2C, 3D). Thus, to determine the nature of the adhesive interaction, we collected self-pollinated *cer6-2* stigmas and incubated them for ≥1 hour in various reagents to test for pollen removal (Tables 1, 2).

As shown in Table 1, most treatments did not remove pollen from the stigma. High concentrations of salts, including 8 M LiCl or chaotropes such as 6 M urea and 2 M guanidine, failed to fully dislodge pollen, suggesting electrostatic interactions are not solely responsible for pollen binding. Although adhesive interactions mediated by cadherins and integrins can be disrupted by divalent cation chelators (Argraves et al., 1987; Nose and Takeichi, 1986), those compounds had no effect on pollen adhesion. Pollen-stigma adhesion was also resistant to strong reducing or oxidizing agents, suggesting independence, respectively, of disulfide bonds or carbohydrate conformation. In addition, ethanol or many detergents had no effect, even at high concentrations; neither did high and low pH buffers (4.0-10.0), nor temperatures ranging from -20°C to 60°C (not shown). Finally, enzymatic treatments with protease or lipase did not remove bound pollen grains, although these enzymes might have had limited access to the adhesion molecules. Taken together, our results suggest adhesion is unlikely to be mediated solely by a protein-protein interaction; if proteins are involved, they are remarkably stable.

Acid or base was capable of removing pollen (Table 2), but only at concentrations that compromised tissue integrity,

Table 1. Chemical treatments that did not disrupt pollen-stigma adhesion

Reagent	Range of concentrations
LiCl	0.01-8 M
Sodium chloride	0.01-2 M*
Urea	0.01-6 M*
Guanidine hydrochloride	0.01-2 M*
EDTA	0.01-0.5 M
EGTA	0.01-0.5 M
EDTA:EGTA (1:1)	0.01-0.25 M
Sodium- <i>m</i> -periodate	0.01-0.25 M
Sodium borohydride	0.01-0.25%
Dithiothreitol (DTT)	1-200 mM
Ethanol	10-100%
NP-40	0.01-2.5%
Brij 35	0.01-5%
Triton X-114	0.01-10%
Octylglucoside	0.01-5%
Sodium deoxycholate	0.01-5%
CHAPS	0.01-5%
Trypsin	0.1-2.5 mg/ml
Proteinase K	100 µg/ml
Phospholipase A2	0.01-0.1 mg/ml
Phospholipase C (Type I)	0.1-1 mg/ml

*At maximum concentrations, pollen was partially removed.

Table 2. Chemical treatments that disrupted pollen-stigma adhesion

Reagent	Minimum concentration to reduce adhesion*	Minimum concentration for complete pollen removal
Acetic acid	0.5 M	1 M
Hydrochloric acid	0.5 N	1 N
Sodium hydroxide	0.2 N	2 N
Sodium dodecyl sulfate (SDS)	0.1%	0.1%
Brij 58	0.1%	0.2%
Triton X-100	0.25%	0.5%
Octylthioglucoside	0.5%	1%

*Concentration at which 50% of bound pollen was removed.

suggesting their effects were not specific. In contrast, some agents were effective at low concentrations. These included 0.1% SDS, an anionic denaturing detergent, as well as the nonionic detergents, 0.2% Brij 58, 0.5% Triton X-100, and 1% octylthioglucoside. Strikingly, closely related nonionic detergents (Brij 35, Triton X-114 and octylglucoside) were not effective at pollen removal, indicating the length or structure of the hydrophobic moieties was critical for solubilizing the adhesive molecules. These results indicate that pollen-stigma adhesion is likely mediated by lipophilic molecules that assume conformations sensitive to certain detergents. Alternatively, it is possible that molecules mediating adhesion are anchored to the cell surface through a hydrophobic interaction and detergents liberate an entire adhesion complex.

Purified exine binds to stigma cells

Precise identification of the adhesion molecules can be achieved with a purified system. As a first step, we tested whether exine alone adhered to the stigma surface. We purified pollen exines from *Arabidopsis* and for controls, chose pollen from several other species (*A. douglasiana*, *Cynodon dactylon*, *P. pratensis* and *Quercus agrifolia*) which did not display adhesion to *Arabidopsis* stigmas in earlier assays (Fig. 1D). Briefly, pollen grains were fragmented in a nebulizer and fractionated on sucrose gradients (see Materials and Methods). Wall fractions were collected, washed to remove cytoplasmic components, dried, and applied to stigma surfaces. After 5 minutes, pistils were washed under low or high stringency conditions (as in Fig. 1E) and examined in the light microscope (Fig. 5). *Arabidopsis* wall fragments bound with high affinity, even under high stringency conditions (Fig. 1A). While wall fragments from some pollen species remained bound after a low stringency wash, none adhered under stringent wash conditions (Fig. 1B-E). Ultrastructural examination revealed that the exine surface of the *Arabidopsis* wall fragments associated with the stigma pellicle (Fig. 5F). These results indicate that adhesion molecules can be preserved through extensive in vitro manipulation.

DISCUSSION

Cell adhesion in pollination

Cell adhesion plays important roles in all biological systems, whether to anchor organisms, cell differentiation, or cell

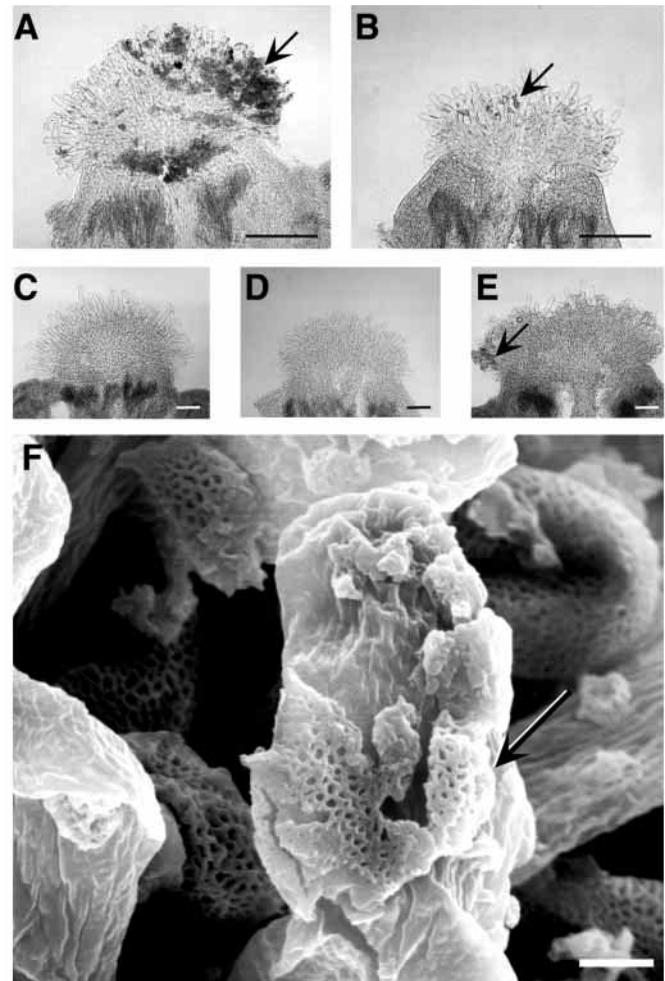


Fig. 5. Adhesion of pollen exine fragments to *Arabidopsis* stigmas. (A-E) Light micrographs illustrating exine fragment binding following a wash in 1% Tween 20 for 1 hour. (A) *Arabidopsis*, (B) *Artemisia douglasiana*, (C) *Cynodon dactylon*, (D) *Poa pratensis* and (E) *Quercus agrifolia* pollen. Size bars, 0.5 mm. (F) Scanning electron micrograph of *Arabidopsis* exine fragments (arrows) bound to stigma cells. Size bar, 5 μ m.

migration. Although most plant cells do not migrate, reproductive cells establish de novo adhesive contacts. Plant gametes are brought into proximity through pollen tube guidance – a process requiring cell-cell adhesion (Lord and Sanders, 1992; Wilhelmi and Preuss, 1996). We have defined another aspect of adhesion in plant reproduction. This adhesion is unusual: it occurs within seconds, generates a strong binding interface, and is species-specific. Moreover, this interaction occurs on a dry surface, employing lipophilic molecules. The outer pollen wall harbors these molecules: pollen grains lacking an extracellular coat bind stigma cells, and purified exine fragments exhibit species-specific binding.

Interactions between the exine and stigma cause imprints on papillar cell surfaces, undulations in the stigma cell wall, and intermixing of stigma and pollen surface material, but distantly related pollens did not alter the *Arabidopsis* stigma surface. In mammals, contacts between cells and the extracellular matrix induce cytoskeleton remodeling and signaling cascades (Gumbiner, 1996; O'Toole, 1997). Likewise, pollen-stigma

adhesion events may trigger cytoplasmic reorganization (Elleman et al., 1992) and initiate signaling that regulates pollen hydration and pollen tube invasion. However, it is not clear whether signaling via the tryphine occurs simultaneously with, or immediately after, adhesion.

Nature of pollen-stigma adhesion molecules

Chemical tests revealed the nature of the adhesion reaction. Pollen binding is insensitive to agents that disrupt electrostatic interactions; however, a subset of hydrophobic reagents dislodged bound pollen, indicating lipophilic molecules either mediate adhesion or anchor adhesives.

The array of reagents that failed to disrupt adhesion illustrate the unique properties of the pollen-stigma interface. Binding is resistant to classic adhesion-disrupters, such as divalent cation chelators, proteases, chaotropic agents, reducing and oxidizing agents, and salts. In addition, pollen adhesion molecules were resistant to temperatures of 95°C, and bound pollen-stigma complexes were stable from -20° to 60°C. These tests suggest the initial adhesion interaction is not mediated by proteins; or, if proteins are involved, they are either inaccessible or highly stable.

The non-denaturing detergents, Brij 58, Triton X-100, and octylthioglucoside removed bound pollen, while the structurally related detergents Brij 35, Triton X-114, or octylglucoside and compounds that disrupt lipid bilayers (ethanol, lipase and phospholipases) were not effective, suggesting that disrupting detergents specifically perturb adhesion molecules rather than the pollen surface. Differences between disruptive and non-disruptive detergents can be subtle. For example, octylthioglucoside substitutes a sulfur atom for oxygen in octylglucoside.

Although cell recognition is often mediated by proteins, hydrophobic molecules can provide a high degree of specificity. Lipo-oligosaccharides of specific chain length and substitution mediate species-specific interactions between *Rhizobium* and their hosts (Fisher and Long, 1992). Free lipid molecules can also serve as species-specific recognition factors; the coral *Montipora digitata*, employs a di-unsaturated C₁₄ alcohol (Coll and Miller, 1991) sperm attractant. Moreover, C₂₄-C₃₂ lipids on the surface of avocado (*Persea americana*) promote the germination and differentiation of fungal spores in a species-specific manner (Podila et al., 1993). Thus, a hydrophobic molecule, such as a lipid or lipopolysaccharide, could conceivably provide the necessary specificity for pollen-stigma adhesion.

Plant cell adhesion molecules

Adhesion-mediating proteins in other plants may serve as models for cell adhesion in *Arabidopsis*. Although not displaying SI, genomic sequencing has revealed many *Arabidopsis* genes related to *Brassica SLG* (Dwyer et al., 1994), a gene implicated in pollen adhesion (Luu et al., 1999). Although their biological role is unknown, sequence analysis of a maize exine protein (*Pex1*) revealed hydroxyproline-rich domains common to animal extracellular matrices (Rubinstein et al., 1995). Some animal extracellular matrix molecules have plant homologues. The *Arabidopsis* Wak1 protein has predicted transmembrane, integrin and kinase domains (He et al., 1996) suggesting integrin-like receptors may function in plants.

While we focused on the initial step in adhesion, it is possible our measurements reflect the sum of many chemical reactions that occur in rapid succession. For example, pollen contact could initiate the release of enzymes from the stigma that cement the adhesion interface. Such a response would resemble the tissue remodeling observed in animal embryo implantation where metalloproteinases reorganize the extracellular matrix (Basbaum and Werb, 1996). Candidate remodeling enzymes have been identified in pollination; a *Brassica napus* cutinase (Hiscock et al., 1994) and a maize metalloproteinase-like enzyme (Radlowski et al., 1996).

Assays that monitor pollen-stigma adhesion

The liquid-based adhesion assay has the merits of speed, reproducibility and simplicity, while the single-cell assay measures the binding force in a physiologically relevant environment. Importantly, the *cer6-2* mutant facilitated measurements by arresting downstream stages of pollination. Previous attempts to measure adhesion monitored wild-type pollen grains and were thus limited to events occurring after hydration initiates (Stead et al., 1979). In addition, measuring pollen-stigma adhesion in a dry environment provides an opportunity to eliminate the confounding effects of water. Previous studies of *Brassica oleracea* pollination showed that stigmas treated with water are less competent to germinate pollen tubes (Zuberi and Dickinson, 1985).

Although *Arabidopsis* is primarily a self-pollinating organism, we detect a substantial pollen adhesion that may mediate pollen capture during cross-pollination. Other species which rely more strongly on outcrossing may have a similar, if not stronger, adhesion response.

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