RESEARCH ARTICLE

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Pollen from *Arabidopsis thaliana* and other Brassicaceae are functionally omniaperturate[1]

Anna F. Edlund[2,7], Qin Zheng[3], Nancy Lowe[4], Skye Kuseryk[2], Krystle L. Ainsworth[4], Robert H. Lyles[5], Steven J. Sibener[3], and Daphne Preuss[6,8]

PREMISE OF THE STUDY: Most pollen walls are interrupted by apertures, thin areas providing access to stigmatic fluids and exit points for pollen tubes. Unexpectedly, pollen tubes of *Arabidopsis thaliana* are not obligated to pass through apertures and can instead take the shortest route into the stigma, passing directly through a nonaperturate wall.

METHODS: We used stains and confocal microscopy to follow early pollen tube formation in *A. thaliana* and 200+ other species. We germinated pollen in vitro and in situ (at control and high humidities) and also used atomic force microscopy to assay material properties of nonaperture and aperture walls.

KEY RESULTS: Pollen tubes of *A. thaliana* breached nonaperture walls despite these being an order of magnitude stiffer than aperture walls. Breakout was associated with localized swelling of the pectin-rich (alcian blue positive) intine. The precision of pollen tube exit at the pollen–stigma interface was lost at high humidity. Pollen from ~4% of the species surveyed exhibited breakout germination behavior; all nine breakout species identified so far are in the Brassicaceae family (~25% of the Brassicaceae sampled) and are scattered across seven tribes.

CONCLUSIONS: The polarity of pollen germination in *A. thaliana* is externally induced, not linked to aperture location. The biomechanical force for breaking nonaperture walls is found in localized swelling of intine pectins. As such, the pollen from *A. thaliana*, and likely many Brassicaceae family members, are functionally omniaperturate. This new mechanism for germination between extant apertures raises questions about exine porosity and the diversity of mechanisms across taxa.

KEY WORDS atomic force microscope; biomechanics; pollination; sporopollenin; stigma

The outer exine of living pollen is durable and strong, but also dynamic, flexible, and elastic (Bolick and Vogel, 1992; Rowley and Skvarla, 2000). Pollen wall materials and architectures permit large volume changes during grain desiccation and hydration, termed harmomegathy (Wodehouse, 1935; Payne, 1972; Heslop-Harrison, 1976, 1979a, c; Blackmore and Barnes, 1986; Scotland et al., 1990), and allow grains to be resilient to shock (Rowley and Skvarla, 2000). The exine walls in most angiosperm species are also interrupted by openings or apertures, which can support harmomegathy and provide paths of least resistance for emerging pollen tubes. Pteridophyte spores, likewise, bear monolete or trilete scars, which often serve as rupture points, and gymnosperm pollen contain either thin regions or true apertures in their walls (Walker, 1974; Kuprianova, 1974; Furness and Rudall, 2004; Rudall and Bateman, 2007). Nonetheless, not all pollen and spore walls have apertures. Inaperturate angiosperm pollen grains, whose walls can only be escaped by wall rupture or disintegration are found in 18 orders containing 54 families (Kress and Stone, 1983; Thanikaimoni, 1986; Zavada and Anderson, 1997; Blackmore and Crane, 1998; Furness and Rudall, 1999; Stone, 1987; Dobritsa and Coerper, 2012). Several examples are known of pollen wall disintegration after pollination; spines may melt, or walls become amorphous and porous (Gherardini and Healey, 1969; Dickinson and Lewis, 1974; Rowley and Rowley, 1983; Dulberger, 1992); similarly, the spore wall disintegrates

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[2] Biology Department, Lafayette College, Easton, Pennsylvania 18042 USA;
[3] The James Franck Institute and Department of Chemistry, University of Chicago, 929 East 57th Street, Chicago, Illinois 60637 USA;
[4] Biology Department, Spelman College, 350 Spelman Lane, Atlanta, Georgia 30314 USA;
[5] Biostatistics Department, Rollins School of Public Health, Emory University, Atlanta, Georgia 30322 USA; and
[6] Molecular Genetics and Cell Biology Department, University of Chicago, Chicago, Illinois 60637 USA
[7] Author for correspondence (e-mail: edlunda@lafayette.edu), phone 610-330-5465, fax 610-330-5705
[8] Present address: Chromatin Inc., 10 S. LaSalle St. #2100, Chicago, IL 60603 USA
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during germination for the protist *Chlamydomonas monoica* (Malmberg and VanWinkle-Swift, 2001). Here, we examine pollen germination in *Arabidopsis thaliana* and report that despite the presence of three slit-shaped or colpate apertures (whose stiffness is detectably less than that of the surrounding walls), *A. thaliana* pollen tubes polarize toward the stigma, not the aperture closest to the stigma, and are able to penetrate any exine obstructing the most direct path into that stigma. In other words, germinating *A. thaliana* pollen tubes can break out right where the pollen grain touches the stigma and are not obligated to detour through a nearby aperture.

Pollen germination has been characterized in detail for several species, including rye, narcissus, hazel, eucalyptus, and tobacco (Heslop-Harrison, 1979b; Heslop-Harrison and Heslop-Harrison, 1982, 1991, 1992; Cresti et al., 1985), all of which release pollen tubes only through apertures and show clear, structural preorientations toward the apertures before their use. In a classic model of hydration and germination, the aperture lying closest to the pollen–stigma contact point allows fluid influx, which provides both the directional cue and the turgor pressure necessary for pollen tube efflux from that same aperture (Heslop-Harrison, 1979a; Heslop-Harrison et al., 1986). Pollen grains of *Nicotiana alata*, immersed in purified lipids or in stigma exudates, with a nearby aqueous interface, produce tubes out of the aperture closest to the interface, and the tubes elongate toward the aqueous medium. Thus, the water source helps to establish pollen tube cell polarity (Feijó et al., 1995; Lush et al., 1998; Wolters-Arts et al., 1998, 2002), although it still remains unclear how pollen from species with multiple, closely positioned apertures (or with stigmas wet enough to submerge pollen grains) restrict tube emergence to a single site, despite simultaneous entry of water across multiple apertures (Heslop-Harrison, 1976, 1979a).

In addition to mechanisms for establishing cell polarity, there must be mechanisms for forceful cell escape from the exine wall (at an aperture or not). Evidence for preset biomechanical mechanisms is found in the ability of some dead pollen grains to hydrate to the same extent as live pollen and even to generate short tubes (Heslop-Harrison, 1979b). Germination forces are commonly credited to turgor pressure (Heslop-Harrison, 1979a), but deformation from this turgor pressure must somehow be focused at one site (Burgert, 2006; Schopfer, 2006) to avoid the ineffective development of multiple pollen tubes from a single grain. Hydraulic conductivity and rapid swelling have been described in barley pollen (Rehman et al., 2004) and in the *bursting pollen* mutant of *A. thaliana* (Hoedemaekers et al., 2015), and turgor pressure has been measured in elongating pollen tubes after germination (Benkert et al., 1997). Pectin is visible in the intine layers of pollen grains from many species, especially near apertures (Kress and Stone, 1983; Heslop-Harrison and Heslop-Harrison, 1991; Mogami et al., 1999; Lacoux et al., 2003; Noher de Halac et al., 2003; Abreu and Oliveira, 2004), where pectin expansion through chemical change and hydration likely ruptures the pollen grain wall. The chemical modification and hydrogel properties of pectins at *Eucalyptus globulus* apertures have recently been proposed to control water movement during pollen germination (Vieira and Feijo, 2016). And expanding pectin in the oncus of *Eucalyptus rhodantha* pollen even lifts aside the hatch-like opercular exine (Heslop-Harrison and Heslop-Harrison, 1985, 1991).

Given the rich variety of pollen grain and stigma structures, a single aperture-based model of pollen germination is not likely to apply to all species (Erdtman, 1952; Heslop-Harrison and Shivanna, 1977; Lee, 1978; Plitmann and Levin, 1983; Edlund et al., 2004; Kesseler and Harley, 2004). Although many past studies have focused on pollen tube behavior after germination (Jauh and Lord, 1996; Park, et al., 2000; Parton et al., 2001; Gu et al., 2003; Palanivelu et al., 2003; Johnson and Preuss, 2002; Justus et al., 2004) and many others on surface interactions between pollen and stigma lipid- and protein-rich coatings (Elleman and Dickinson, 1986; Elleman et al., 1992; Lolle and Cheung, 1993; Preuss et al., 1993; Hülskamp et al., 1995; Fiebig et al., 2000; Mayfield et al., 2001), the details of structure and function during pollen germination remain unknown for all but a few species (Larson, 1965). We have observed pollen germination across some 200 species and more closely characterized germination events in *Arabidopsis thaliana*, using stains and confocal microscopy to examine subcellular pollen features during the earliest stages of pollen tube formation. We report here that, unlike previously described germination mechanisms, the pollen tubes of *A. thaliana,* and at least eight of 37 other Brassicaceae family members, take the shortest route into the stigma, even when this route necessitates breaching a substantial exine wall. Both local swelling of pectin and local weakening of the exine appear to be part of this mechanism. With atomic force microscopy (AFM), we characterized the stiffness of the pollen grain surface, highlighting the heterogeneous barriers encountered by emerging pollen tubes.

MATERIALS AND METHODS

Plant materials—*Arabidopsis thaliana* strains were from the *Arabidopsis* Biological Resource Center (Columbus, OH) and included wild-type Columbia (Col-4, CS933), Landsberg *erecta* (Ler, CS20), and male sterile (*ms1*, CS75). Seeds were sown in soil, stratified at 4°C for 2 d, and plants were grown under fluorescent light (100 μE) for 16 or 24 h/day at 40% humidity.

For all other surveyed plants, we collected pollinated stigmas from various sources, including the Atlanta Botanical Garden, New York Botanical Garden, roadside and disturbed field sites in Atlanta, Georgia (GA) and Easton, Pennsylvania (PA), plants shipped from colleagues around the United States, commercially purchased seeds or plants, and plants located in the field at GPS coordinates provided by the University of Georgia Herbarium, Athens, GA. Many specimens were pressed, labeled with Genus species, collector, collection site and date and are held in an herbarium cabinet in Kunkel Hall, Lafayette College Biology Department, Easton, PA (Appendix S1, see Supplemental Data with the online version of this article).

Observations of pollen grains and pollen tubes—For *A. thaliana*, unpollinated *ms1* pistils at stages 14–15 (0–8 h after flowering; Bowman et al., 1989) were embedded at their base in 1% agar; stigmas were densely hand-dusted with Col or Ler pollen, a process that required <1 min. Pollinated stigmas were incubated for 45 min at ambient humidity (40–70%) or were placed in a humid chamber (>90%) either immediately or 5 min after pollination (sample sizes were $n = 17$ and greater). These three treatments are hereafter referred to, respectively, as "control", "high-humidity", and "transferred". For all other taxa, stigmas were either collected already naturally pollinated or were hand-dusted with pollen from a neighboring plant of the same species and the pollen allowed to germinate overnight. All pollinated stigmas were fixed in fresh 0.5×

Karnovsky's solution (2% v/v methanol-free electron-microscopy grade formaldehyde and 2.5% v/v glutaraldehyde, 0.15 M HEPES, pH 7.4), and mounted on slides. Pollen components were stained with Congo red (cellulosic pollen tube walls), auramine O (exine), alcian blue (acid polysaccharides) or ruthenium red (de-esterified pectic acids); dyes were dissolved at 0.01% w/v in 0.15 M HEPES, pH 7.4 and filtered immediately before use. Pollen germinated in vitro on solidified pollen growth medium [18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, and 0.5% w/v Noble agar] were similarly stained. Samples were mounted in the presence of the staining solution, except when ruthenium red was used; samples were washed and observed in HEPES buffer after 5 min in ruthenium red. Stained samples were viewed with either an Olympus BX40 or SZX2 microscope or a Zeiss LSM-510 or Nikon Eclipse C-1 laser-scanning confocal microscope. Multiple images of germinating pollen grains were used to determine the fraction of tubes emerging from apertures or nonaperturate walls.

We assessed pollen tube orientation toward the stigma using two measures: (1) angle of emergence and (2) length of exposed pollen tube. For the angle of pollen tube emergence, a line perpendicular to the stigma surface, drawn directly through the pollen–stigma contact point, was defined as 0°. ImageJ image analysis software (http://rsb.info.nih.gov/ij/download.html) was used to measure the exposed length of each pollen tube before it invaded the stigma. Only those pollen tubes whose entire lengths were clearly exposed, without danger of optical foreshortening effects, were digitized.

Atomic force microscopy—Atomic force microscopy (AFM) uses a cantilever to scan across a sample surface, providing high-resolution details of surface topography (Binnig et al., 1986; Bolshakova et al., 2004; Dufrêne, 2004; Santos and Castanho, 2004; Alessandrini and Facci, 2005). Unlike electron microscopy, AFM imaging is done under physiological conditions and yields physical and mechanical information about the surface. We used AFM to characterize pollen surface properties at various sites, including the apertures, as well as exine lacuna, tectal joints, and tectal ridges. To measure the stiffness of each structure, we captured a topographic image, along with a spatially resolved force-volume image. These data describe the deflection of the cantilever as it approached and withdrew from a sample, with force curves recorded at multiple locations across the plane of the pollen surface. Tip deflection resulted in a more or less pronounced indentation of the cell wall; the stiffer the sample, the less the indentation, and the darker it appeared on the force map.

Pollen grains were embedded in thin layers of pollen growth medium containing 0.75% agarose and mounted on fragments of glass slides coated with poly L-lysine (Doktycz et al., 2003). Pollen grains exposed above the agar surface, but firmly anchored to the glass, were suitable for observation. Glass slide fragments were mounted with epoxy onto steel AFM sample pucks. Cantilevers with silicon nitride probes (Veeco Metrology, Santa Barbara, California [CA], USA) were calibrated for Z sensitivities using a clean silicon oxide substrate in liquid pollen growth medium. The cantilever and laser position were not changed after calibration; the force constant of the cantilever batch was calibrated from the measured resonance frequency and estimated to be 0.14 N/m.

AFM height and force-mapping images were captured in 25°C pollen growth medium using a MultiMode AFM with a NanoScope IV controller (Digital Instruments, Santa Barbara, CA). The Young's modulus for a variety of surface regions was compared among three separate pollen grains (captured on different days with different cantilevers), and 3–5 random points were measured within each region of interest (apertures, tectal walls, tectal joints, and lacunar floors). Procedures for extracting Young's modulus (stiffness) were detailed by Touhami et al. (2003), who used them to describe yeast cell wall properties. In brief, force curves (deflection vs. tip-sample distance curves) obtained for a hard silicon oxide surface (force curve slope equals 1) are compared with those for the softer sample. The loading force applied by the cantilever is computed from the cantilever deflection using Hooke's law. After force curves are converted to force–indentation curves (load force F vs. indentation depth δ), the force–indentation curves are fitted with the Hertz model (Hertz, 1882) for a conical indenter. Material stiffness, or the Young's modulus, for specific locations is then determined from the following equation:

$$F = \frac{2}{\pi}\tan\alpha\frac{E}{1-\upsilon^2}\delta^2,$$

where $\alpha = 18°$ is the half-opening angle of a conical tip, and $\upsilon = 0.5$ is Poisson's ratio for soft biological materials (Touhami et al., 2003).

Statistical analyses of pollen cell polarity and material property measurements—To compare pollen tube emergence angles, we used the extension of Fisher's exact test (Rosner, 1995) to determine whether there was an overall difference in the proportions with 0° angles across the three treatment groups (control, high-humidity, and transferred). After the overall test, we conducted the three planned pairwise comparisons of proportions (that is, control vs. transferred, control vs. high-humidity, and transferred vs. high-humidity), with a Bonferroni adjustment (Rosner, 1995) for multiple comparisons. Specifically, the three comparisons were done separately via Fisher's exact test, with significance of each judged at the 0.05/3 level. To compare median exposed pollen tube lengths across the three treatment groups, we used the Kruskal–Wallis test (Rosner, 1995), this time followed by the three corresponding pairwise Wilcoxon rank sum tests with the Bonferroni multiple comparisons adjustment. To statistically compare mean AFM measurements of material stiffness across different pollen surface features and across the three sampled pollen grains, we applied a two-way fixed-effects analysis of variance (ANOVA) for unbalanced data (Kleinbaum et al., 1988) after applying a base 10 log transformation of the stiffness values to better conform with the required normality assumptions. Potential interaction was first assessed between the two factors (surface feature and pollen grain), followed by inferences directed at the primary aim of comparing mean stiffness across surface features.

RESULTS

Arabidopsis thaliana *pollen tubes can breach any portion of the exine wall, not only apertures*—To characterize the early stages of pollen germination, we hand-pollinated *A. thaliana* stigmas and stained them 45 min later, viewing exine with auramine O (Fig. 1A) and emerging pollen tubes with Congo red (Fig. 1B). Of the first 100 germinated pollen grains we looked at (while scoring angle of emergence and exposed pollen tube length), greater than 90% had pollen tubes emerging at the point of contact with the stigma. Pollen

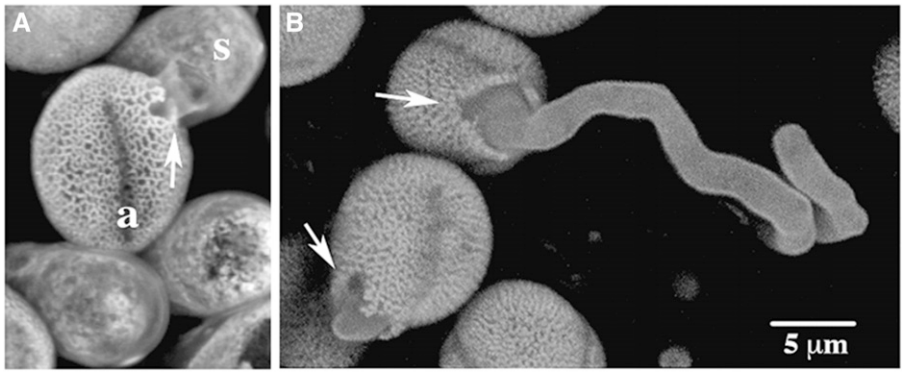

FIGURE 1 *Arabidopsis thaliana* pollen tubes emerge at sites of exine rupture (arrows). Pollen grains were germinated in situ (A) or in vitro (B); auramine O staining (A); Congo Red staining (B). a, apertures, s, stigma papillar cell.

grains do not always fall with an aperture in their interface with the stigma; thus, if the tube always emerges at the interface, then it must sometimes emerge through a nonaperturate wall. A more careful assay of tube escape sites revealed that the pollen tube passed through an aperture in only 59% of Col germinations (n = 391) and 33% of Ler germinations (n = 252; Table 1). Thus, *A. thaliana* pollen tubes took the shortest route into the stigma papilla, even when this route necessitated rupturing a substantial exine wall (Fig. 1, arrows). Using confocal microscope images of hydrated pollen, we measured the surface area of apertures relative to the remainder of the pollen grain and estimated that the three apertures occupy approximately 13% of the grain's surface. Although *A. thaliana* apertures are used for tube emergence more often than 13% of the time, it is difficult to account for the effects of pollen grain contours on grain positioning; that is, pollen grains may more commonly lie with an aperture fortuitously touching the stigma surface.

In another study, the behaviors of 1343 pollen tubes (in situ and in vitro and in Col and Ler accessions) were analyzed independently of each other. Surprisingly, even when the entire surface of the grain was uniformly exposed to growth medium in vitro, some pollen tubes still emerged through the nonaperture walls, rather than passing through the apertures (Figs. 1B, 2). The fraction breaking through walls in vitro was roughly one third less than in situ (Table 1), and statistically, the odds of breakout differed significantly between in situ and in vitro treatments in both Col and Ler accessions (estimated odds ratio [OR] = 1.73, $\chi^2_1 = 11.76$, $P = 0.001$ and OR = 3.66, $\chi^2_1 = 56.72$, $P < 0.001$, respectively). The two accessions differed significantly in their breakout behaviors in situ, with Ler being more likely to break out than Col (OR = 2.97, $\chi^2_1 = 41.51$, $P < 0.001$). Ler and Col behaviors in vitro, however, did not differ significantly after Bonferroni adjustment for four multiple comparisons (OR = 1.41, $\chi^2_1 = 4.34$, $P = 0.037$; Table 1).

TABLE 1. Pollen tube germination behaviors in situ and in vitro for *Arabidopsis thaliana* and eight other Brassicaceae family members, each with the ability to germinate through a nonaperture wall. Note that the frequency of breakout behavior differs between taxa and within a single species (two accessions of *A. thaliana* were assayed, Columbia [COL] and Landsberg *erecta* [LER]). Overall, the percentage breakout decreases (aperture use increases) when pollen is germinated in vitro on solidified pollen growth medium.

Species	In situ breakout / Total germinated	In situ % breakout	In vitro breakout / Total germinated	In vitro % breakout
Arabidopsis thaliana COL	159 / 391	41	93 / 328	28
Arabidopsis thaliana LER	169 / 252	67	133 / 372	36
Berteroa incana	20 / 53	38		
Capsella bursa-pastoris	35 / 295	12	26 / 528	5
Cardamine hirsuta	52 / 294	18	29 / 279	10
Draba verna	45 / 80	56	205 / 611	34
Draba brachycarpa	13 / 23	57		
Hesperis matronalis	41 / 75	55	464 / 792	59
Lunaria annua	123 / 271	45	294 / 566	52
Matthiola incana	40 / 40	100	35 / 35	100

***Pectin components act in pollen germination mechanism of* A. thaliana**—To characterize the stages of pollen tube emergence, we collected confocal micrographs over time during in vitro pollen tube germination. Within an hour of immersion in pollen growth medium, a number of grains displayed bulges in their exine walls (Fig. 2A). Shortly after this time, small tube tips could be seen emerging from the grains (not shown). We collected optical sections from auramine O-stained grains containing obvious bulges and found unstained areas lacking cytoplasm directly beneath the bulges (Fig. 2B, C). These clear areas stained with both alcian blue (Fig. 2D, E) and ruthenium red (data not shown), indicating that they contained pectin (Fig. 2D, E). Similar pectin staining was observed at the stigma–pollen-tube interface of grains germinated in situ (Fig. 2F). We observed apparent stretching of the exine lattice, just above the patches of swelling pectin (Fig. 3, arrow), and fragments of broken exine at sites where pollen tubes had emerged, both in situ and in vitro (Fig. 1). Our observations thus suggest that pectin expansion plays a role in nonaperture rupture of the exine. Although mechanisms involving expanding pectin are conserved across germinating pollen grains of other species (Heslop-Harrison and Heslop-Harrison, 1991; Mogami et al., 1999; Lacoux et al., 2003; Noher de Halac et al., 2003; Abreu and Oliveira, 2004), pectin in those species is localized to the apertures.

Pollen tubes germinate toward a localized fluid source—Because *A. thaliana* pollen tubes initiate precisely at the point of contact with the stigma papilla, regardless of aperture position, we investigated the role of localized water transfer in their initial orientation. We pollinated stigmas and monitored the angle of pollen tube emergence relative to the pollen–stigma interface and the length of pollen tube that was exposed on the stigma surface (Figs. 4, 5). When pollen were germinated at ambient humidity (40–70%), almost every tube emerged within 5° of the stigma contact site (Figs. 4A, 5A); in fact, 15 of 16 tubes emerged at a 0° angle under these conditions. Moreover, these tubes took the shortest possible path to the grain–papilla contact site, leaving a mean of 2.9 ± 0.5 μm of tube exposed on the stigma surface (Figs. 4A, 5B). Because higher humidity would be expected to disrupt directional water traffic, we also placed stigmas in a humid (>90%) chamber immediately after pollination. In these cases, the pollen tubes emerged at a

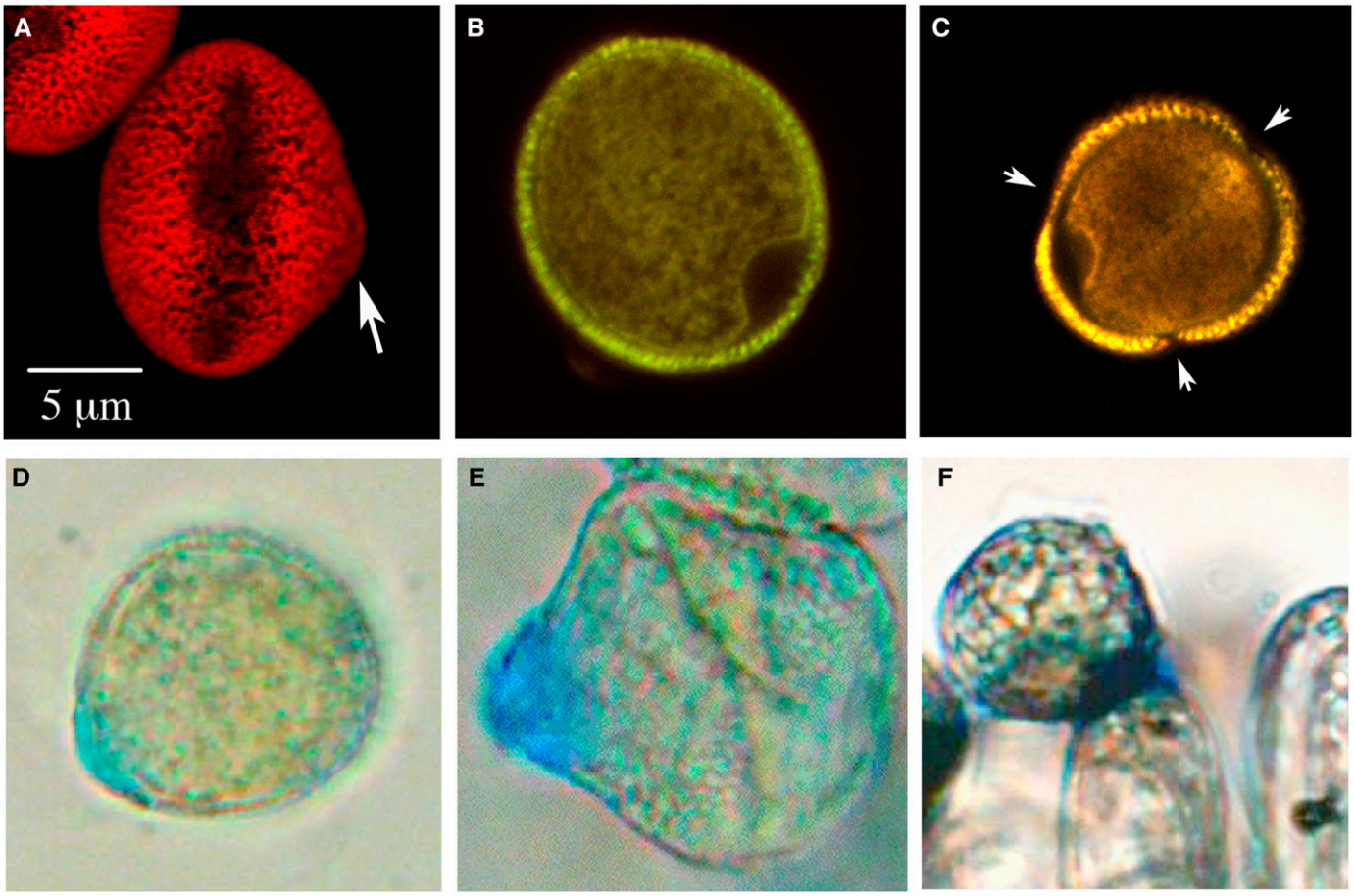

FIGURE 2 Exine walls can become stretched (arrow in A) and pectin-containing bulges predict the site of tube emergence. Arrows in C show aperture positions. *Arabidopsis thaliana* pollen emergence for grains germinated (A–E) in vitro or (F) on the stigma surface; (A) Congo red staining. (B, C) auramine O staining; (D–F) alcian blue staining reveals patches of pectin.

variety of angles relative to the stigma contact point and often wandered across the stigma surface rather than immediately invading the papilla (Figs. 4B, 5). Specifically, only 2 of 54 such tubes emerged at a 0° angle, and the mean of recordable exposed tube lengths was 23.2 ± 7.9 μm. In these humid conditions, emergence angles and exposed tube lengths were highly variable, suggesting random behavior with respect to the location of the stigma papillar cells. Emergence angles and exposed tube lengths were both significantly different between control (ambient humidity) and high-humidity treatments (Fisher's exact $P < 0.001$). Intriguingly, lowering the humidity for only the first 5 min after pollination was sufficient to provide the pollen tubes with a proper orientation toward the stigma, such that they emerged at efficient angles and exposed only short lengths. In this group, 32 of 32 tubes emerged at a 0° angle, and the mean exposed tube length of 4.3 ± 2.0 μm was similar to that of the ambient humidity control group. Once this polarity was established, transferring the stigmas to a humid chamber did not disorient tube growth (Fig. 5A), even though pollen tubes themselves did not emerge until 10–15 min after pollination. There was no significant difference between emergence angles in the control and 5 min transferred treatment groups (Fisher's exact $P = 0.33$).

Material properties of* A. thaliana *pollen grains—Conversion of the AFM images to force–indentation curves suggested that the materials covering the apertures are indeed less stiff than those of the exine wall; the aperture appears lightest in color (least stiff) in Fig. 6C. Descriptive statistics on the raw (untransformed) scale suggest that the mean stiffness of aperture regions (1.4 ± 1.0 MPa) was an order of magnitude lower than that of tectal ridges and joints (11.9 ± 6.5 MPa). However, the aperture covering is quite heterogeneous with soft spots (0.17 ± 0.12 MPa) and somewhat stiffer spots (2.0 ± 0.55 MPa); stiff spots colocalize with small accretions or clumps of sporopollenin scattered within the covering. The sculptured exine wall is also heterogeneous. Raw descriptive measures suggest that the stiffest region is found at the junctions within the reticulate pattern of exine, the tectal joints (15.4 ± 6.0 MPa); next are the tectal ridges (8.5 ± 5.2 MPa); the least stiff elements in the nonaperturate exine wall are the bottoms of the lacunae (1.7 ± 0.79 MPa).

In total, 55 measurements of stiffness were recorded across the three pollen grains. To be consistent with the statistical analysis based on logged stiffness measurements, Table 2 provides a summary of the sample size and mean base 10 log stiffness values for each combination of surface location and grain. The replicate numbers were unbalanced (number of measurements varied between grains), but at least three measurements were made for all but one combination of surface location and grain. Two-way ANOVA analysis revealed a statistical interaction between surface location and pollen grain

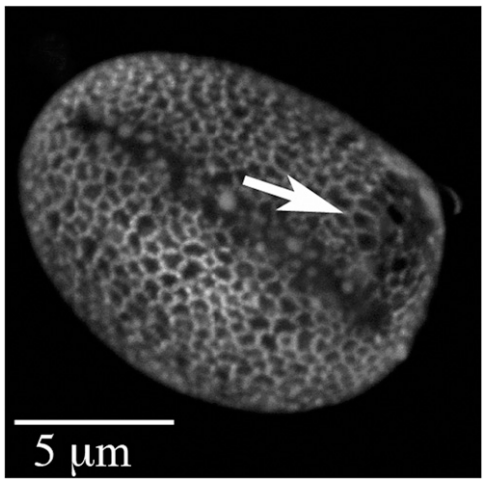

FIGURE 3 The exine wall appears altered (arrow) above the bulge of an emerging pollen tube, during *Arabidopsis thaliana* pollen germination in situ (this grain broke off the stigma surface). Auramine O stain.

($F_{8, 40} = 3.9$, $P = 0.002$; see in Table 2 that grain 2 reflected the lowest mean stiffness at the aperture and lacuna locations but not at tectal ridges and joints). Our separate analysis of the 37 aperture and lacuna observations, however, did not reveal significant statistical interactions, so a straightforward assessment of the main factor (grain and surface location) effects was feasible. Although the mean stiffness differed across pollen grains ($F_{2, 32} = 4.5$, $P = 0.020$), the test for a mean difference across surface regions was also highly significant ($F_{2, 32} = 56.3$, $P < 0.001$). Step-down tests adjusting for multiple comparisons revealed major differences in stiffness between soft and hard regions of the apertures ($F_{1, 32} = 103.8$, $P < 0.001$) and between soft aperture regions and lacunae floors ($F_{1, 32} = 84.1$, $P < 0.001$), but no difference between hard apertures and lacunae floors ($F_{1, 32} = 1.7$, $P = 0.21$).

To properly compare mean stiffness measures of apertures (soft and stiff surface locations combined) with nonaperturate walls (joints and ridges combined), we could not ignore grain by region interactions. We therefore created three linear contrasts to test the corresponding null hypothesis that aperture and wall regions exhibit the same mean stiffness, separately for each pollen grain. This analysis was conducted using all 55 observations, which are characterized descriptively in Table 2. The hypothesis was soundly rejected (reference distribution = $F_{1, 40}$, $P < 0.001$) for each of the three pollen grains, confirming the indications from Table 2 that "wall" regions (tectal ridges and joints) exhibit greater stiffness than apertures. We conclude that germinating *A. thaliana* pollen tubes are able to break through nonaperture exine walls, whose structural elements have material properties that are between one and two orders of magnitude stiffer than those covering the apertures.

***The nonaperture germination mechanism is rare but not unique to* A. thaliana**—We surveyed ca. 210 species from ca. 67 families in ca. 163 genera (Appendix S1). Of these, we found nine species (counting *A. thaliana*) with breakout germination. All nine are in the Brassicaceae family (and among the 37 taxa from this family that we sampled, the breakout behavior appeared in ~25% of the sampled Brassicaceae and 4% of the sampled total). Brassicaceae pollen form a group with intergrading morphological characters, but small differences between the grains were visible (Fig. 7). Intriguingly, the nine breakout species identified are not especially closely related phylogenetically (Fig. 8; Al-Shehbaz, 2012; Warwick et al., 2010; Couvreur et al., 2010) and were indeed scattered across seven tribes. The species also differed in the frequencies with which they used their apertures to escape the pollen grain, ranging from 12 to 67% breakout germination (Table 1).

DISCUSSION

Here we demonstrated that at ambient humidity, *A. thaliana* pollen tubes exit pollen grains at their interface with the stigma cell, even when emergence at this site requires rupture of nonaperture sporopollenin elements that are markedly stiffer than aperture coverings. This germination behavior is not unique to *A. thaliana*, although it is rare, as we found only nine of 210 species surveyed that had the same ability. These findings are surprising, given sporopollenin's special resistance to enzymatic breakdown (Wiermann and Gubatz, 1992) and persistence over geological time periods. The unusual and highly oriented germination process of *A. thaliana* is disrupted by transferring stigmas to humid conditions immediately after pollination and likely reduces overall pollination efficiency (Fiebig et al., 2000), but proceeds normally if the pollinated stigma is first incubated for only 5 min at ambient humidity. Our study thus defines a discrete stage in pollen development—tube cell polarization toward an emergence site, which occurs in the short window between pollen–stigma adhesion (within seconds of pollen–stigma contact; Zinkl et al., 1999) and complete pollen hydration (within 5 min of contact; Mayfield and Preuss, 2000; Rehman et al., 2004). Furthermore, our dissection of the pollen germination mechanism of *A. thaliana* revealed bulging grain walls, which are underlain by pectin-rich swellings.

We hypothesize that these pectin-rich sites exert pressure directly on the overlying exine as their volume increases and/or that they

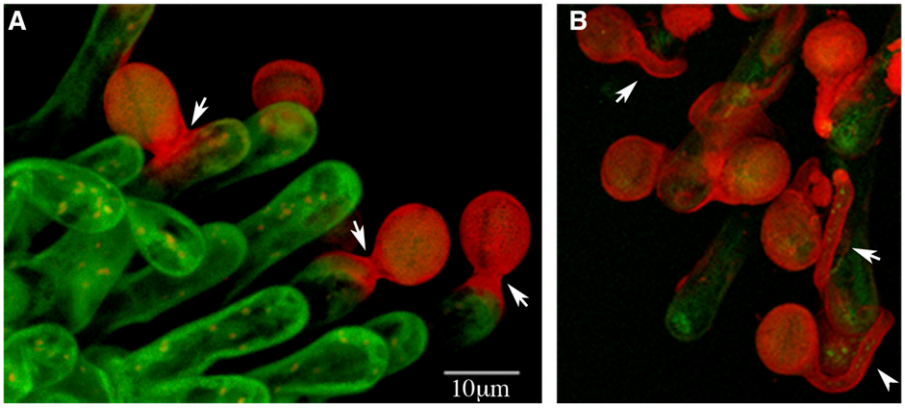

FIGURE 4 *Arabidopsis thaliana* pollen tube polarity varies with humidity. Pollination at ambient humidity leads to pollen tube emergence at the point of contact with the stigma (A), while incubation in humid conditions causes disoriented growth (B). Arrows point to Congo-red-stained pollen tubes, outside grain, and stigma walls.

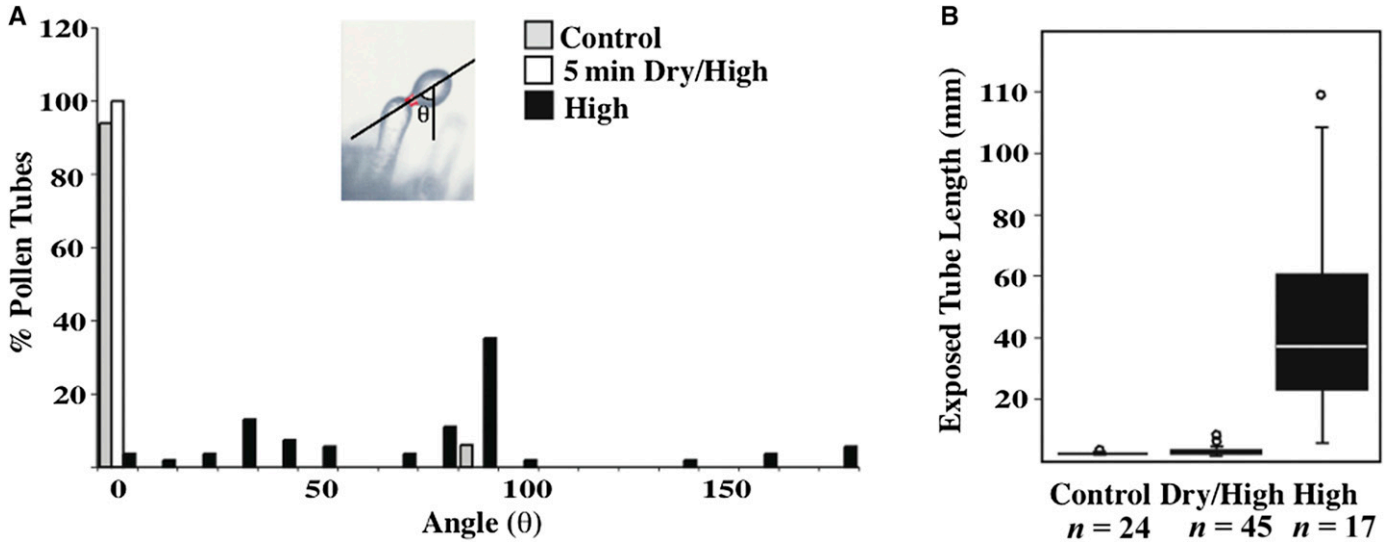

FIGURE 5 *Arabidopsis thaliana* pollen tube polarity quantified as the angle of emergence (A) relative to the stigma–pollen contact point (inset), and the length of exposed tube outside the stigma papilla (B). Pollen that were exposed 5 min to the polarizing influence of the stigma, then transferred to high humidity (A, white bars) behaved indistinguishably from low humidity controls (A, gray bars; $P < 0.0001$).

allow focused access of the cytoplasmic turgor pressure to the exine by weakening the intine cell wall (through insertion of soft material between existing cell wall polymers). Although we do not yet know the methylesterification state of the pectins in these swellings or whether the pectin is gellified, we now believe that localized alterations in the intine's pectin (similar to those described at apertures in *E. globulus*; Vieira and Feijo, 2016) provide the focused biomechanical forces required for pollen tube emergence between apertures.

Pectin swelling during pollen germination has been observed previously in other species, but prior reports have localized pectin swelling to the aperture region (Hyde, 1955; Heslop-Harrison, 1979a;

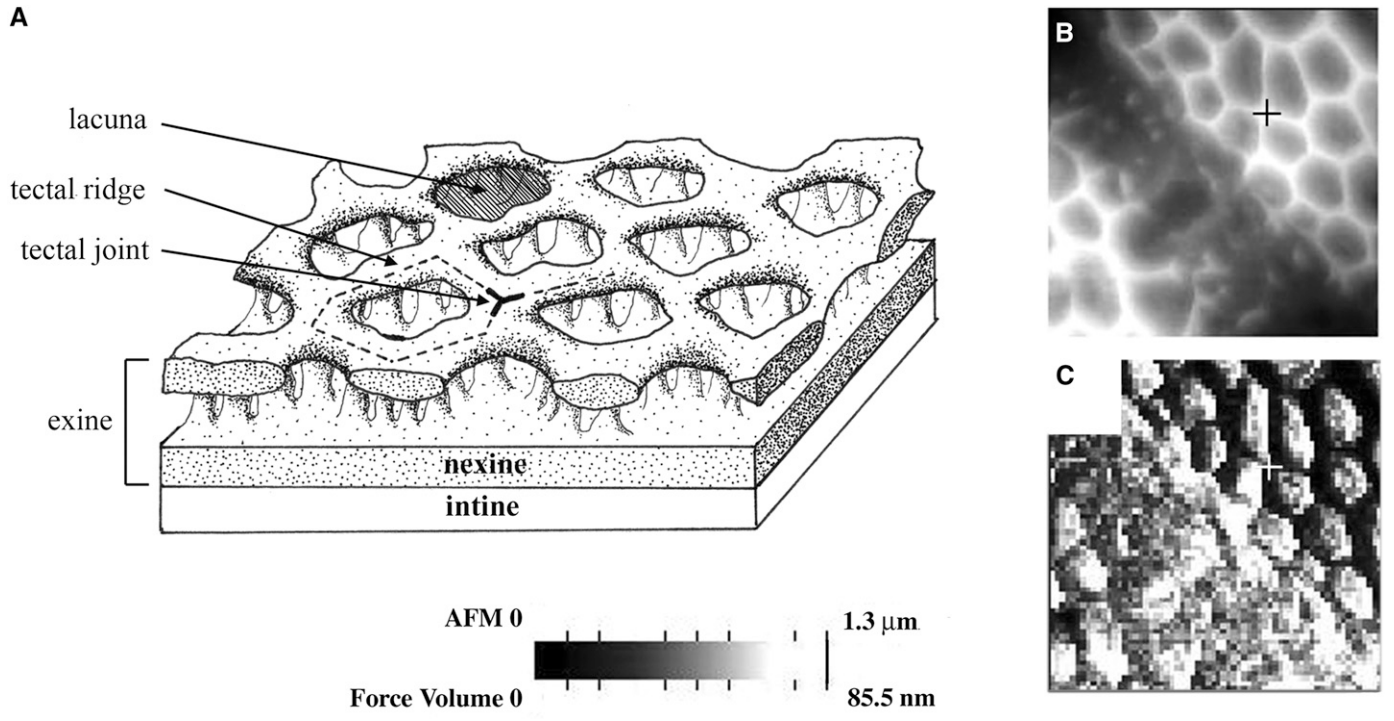

FIGURE 6 Pollen wall properties imaged by atomic force microscopy (AFM). (A) Diagram (~2 μm square) of *Arabidopsis thaliana* pollen wall structures. AFM height (B) and force–volume (C) images of a region of hydrated, living pollen grain. The scanned region contains an aperture, which appears darker in image B and lighter (less stiff) in C than the surrounding material at tectal ridges and tectal joints. Panels B and C are of the same 5 μm wide region (note "+" at the same location in both).

TABLE 2. Number of measurements and mean $\log_{10}$ stiffness according to surface category and pollen grain. Aperture coverings contain distinct soft and hard regions; hard regions are visible as sporopollenin accretions or clumps. The sculptured exine wall is also heterogeneous, being most stiff at tectal joints, intermediate in stiffness at tectal ridges, and least stiff (in some cases > 10-fold lower) in the lacunae. Log_{10} Pa units are reported here and used for statistical comparisons; MPa units are reported in the Results text.

	Mean $\log_{10}$ Stiffness in Pa (SD; *n*)		
Surface location	**Grain 1**	**Grain 2**	**Grain 3**
Apertures (soft)	5.35 (0.01; 3)	4.76 (0.14; 3)	5.56 (–; 1)
Apertures (hard)	6.31 (0.12; 5)	6.24 (0.16; 5)	6.31 (0.06; 5)
Lacunae	6.18 (0.14; 5)	6.03 (0.50; 5)	6.30 (0.18; 5)
Tectal ridges	6.52 (0.09; 3)	6.86 (0.05; 3)	7.17 (0.07; 3)
Tectal joints	6.95 (0.04; 3)	7.21 (0.12; 3)	7.31 (0.09; 3)

Heslop-Harrison et al., 1986; Dulberger, 1989; Noher de Halac, et al., 2003; Rehman et al., 2004; Vieira and Feijo, 2016). In eucalyptus, the swelling force generated by this pectin is sufficient to lift aside the aperture opercula (Heslop-Harrison and Heslop-Harrison, 1985). Indeed, gel hydration forces have been described as "explosive" (Verdugo, 1991) and are exploited mechanically in diverse biological systems, including respiratory mucosa (Verdugo, 1991), sea urchin embryos (Lane et al., 1993; Davidson et al., 1995), and migrating microbes (Wolgemuth et al., 2002). Flower pectin has been studied in developing pollen (Noher de Halac, et al., 2003; Aouali et al., 2001; Rhee and Somerville, 1998; Owen and Makaroff, 1995), flower styles, and well-extended pollen tubes (Mollet et al., 2000; Stepka et al., 2000; Lenartowska et al., 2001; Geitmann and Parre, 2004; Bosch and Hepler, 2005; Geitmann and Steer, 2006). Especially for elongating pollen tubes, studies have focused upon pectin's methylesterification state, structural roles of wall pectins, and the effects of treatment with pectinase or other cell-wall-hydrolyzing enzymes (Roggen and Stanley, 1969; Geitmann and Parre, 2004; Bosch and Hepler, 2005; Parre and Geitmann, 2005). Although functionally expanding pectin in the intine between pollen apertures is novel, constitutively and globally thickened intines in grains with globally thin exines are known (Walker, 1974; Stone, 1987; van der Merwe et al., 1990) as are constitutively, but locally thickened intines below continuous exines (Furness and Rudall, 1999). A hidden, single aperture (or functionally monoaperturate) grain, is distinct from those we described here, which have three clear, but possibly unused apertures (and which we term functionally omniaperturate). Because the orientation of the *A. thaliana* pollen on the stigma is unpredictable, the subsequent appearance of a localized pectin-filled bulge raises questions about the resting distribution of pectin around the perimeters of these pollen grains, their pectin chemistry and mobilization, and the fluid entry sites into the grains.

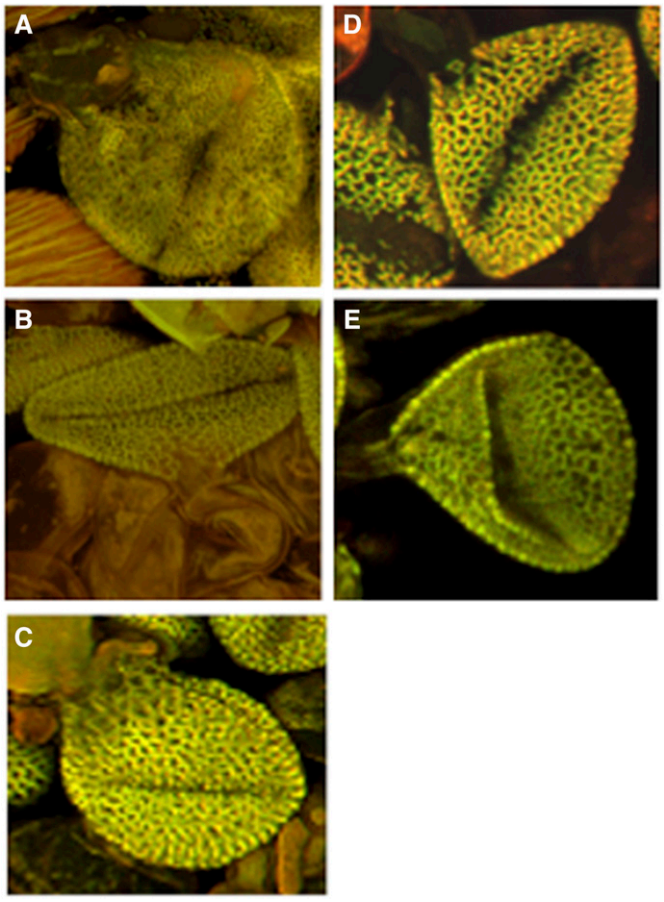

FIGURE 7 Pollen germination through nonaperture wall in the Brassicaceae. Of over 35 Brassicaceae species surveyed to date, we find obligate aperture use in most, with the exception of nine: (A) *Draba verna*, (B) *Berteroa incana*, (C) *Hesperis matronalis*, (D) *Lunaria annua*, (E) *Draba brachycarpa* and (not shown) *Capsella bursa-pastoris*, *Matthiola incana*, *Cardamine hirsute*, and *Arabidopsis thaliana*.

Although swelling pectin gel could exert pressure on the exine, such pressure would likely be projected into the cytoplasm, if the overlying sporopollenin lattice were not concomitantly weakened (Fig. 2B, C, note indentations in cytoplasm). Weakening could be enzymatic or chemical (from the inside and/or the outside), or it could simply be due to tensile, mechanical strain of a deformable substance (Wainwright et al., 1976). Sporopollenin is chemically resistant in the laboratory, but can corrode from the exterior, when left for long periods in leaf mold (Havinga, 1971; Rowley et al., 1990; Skvarla et al., 1996) and is occasionally breached by fungal hyphae (Goldstein, 1960; Elsik, 1966, 1971). We found only one report of exine degradation in the gut of an arthropod (Scott and Stojanovich, 1963). In a few plant species, exine structure also alters dramatically after contact with the stigma and during hydration and germination (Gherardini and Healey, 1969; Dickinson and Lewis, 1974; Rowley and Rowley, 1983; Dulberger, 1992); perhaps in these species, and in *A. thaliana* and other breakout Brassicaceae, materials derived from the stigma contribute to exine degradation. Stigma contact is not required by *A. thaliana* pollen, which can germinate in vitro, however, their in vitro germination frequency is especially low (even when the germination medium is optimized; Boavida and McCormick, 2007). In addition, *A. thaliana* pollen tubes use apertures more frequently during germination in vitro than in situ (Table 1), and the exine bulges we observed were most prominent in pollen germinated in vitro (Fig. 2D, E), where bulges (and indentations of the cytoplasm) may represent prolonged pectin swelling without the aid of wall degradation. Finally, pollen from the Ler accession (infamous for poor germination in vitro) use nonaperture breakout germination more often than do pollen from Col (in situ, Ler breaks out 67% vs. 41% in Col). When in situ and in vitro germination behaviors are compared for the two accessions, the in vitro increase in aperture use is

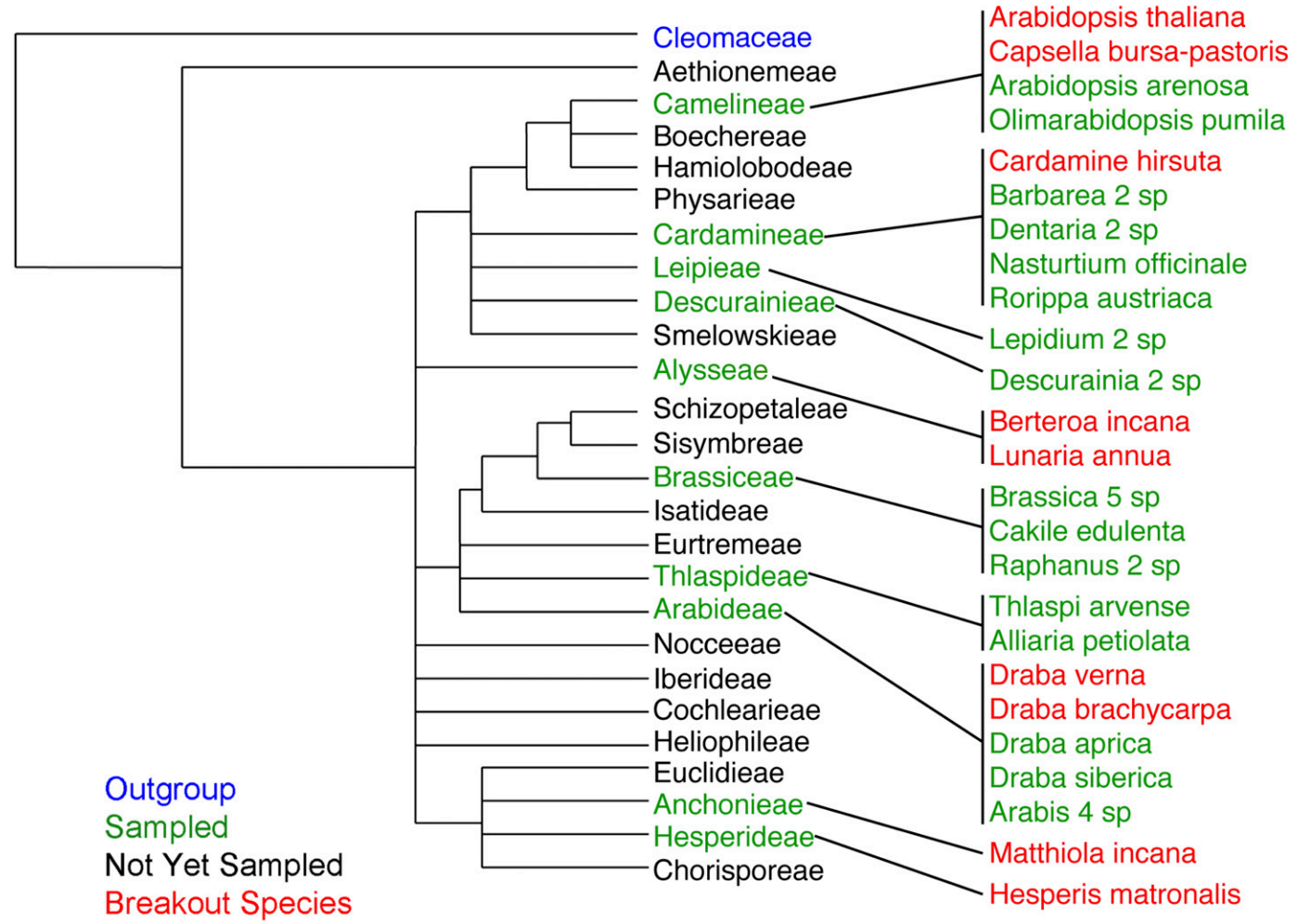

FIGURE 8 Phylogenetic relationships in the Brassicaceae color-coded to show those we scored for germination behavior (adapted from Couvreur et al., 2010; Warwick et al., 2010; Al-Shehbaz, 2012). Note that the nine species with breakout (nonaperture) germination are not closely clustered phylogenetically, but are instead scattered across seven tribes.

also more marked for Ler than it is for Col (46% increase in aperture use by Ler vs. 32% increase in aperture use by Col; Table 1).

Given that *A. thaliana* pollen tubes apparently take the shortest route outward toward the stigma, it is likely that liquid entry is by the shortest route inward, marking the site for pectin expansion by passing directly across the exine wall, rather than through an aperture opening; in other words, the *A. thaliana* exine wall is likely porous or permeable to water. Porosity of the wall could also increase following contact with the stigma. Porous exine has been reported in several species, especially in developing pollen grains (Rowley et al., 2003; Pettitt, 1976), and has also been studied in mature pine pollen (Bohne et al., 2005). Even though water's availability across the entire pollen surface (i.e., humid conditions) is sufficient to disorient germinating *A. thaliana* pollen tubes, checkpoints must exist to prevent the formation of multiple pollen tubes, as only a single tube emerges from grains germinated in vitro or in humid environments. This "single-pollen tube" requirement applies to pollen grains of all architectures, including those grains described as omniaperturate, which have globally thin or absent exine, similar to the thin coverings of apertures (Skvarla and Rowley, 1970; Thanikaimoni, 1984; Furness and Rudall, 1999).

Our survey of other taxa reveals that *A. thaliana* is not alone in its germination abilities, although the behavior appears to be rare overall. Within the Brassicaceae, however, roughly 25% of those species sampled display the ability, so we could expect to find many more examples, if more members of this family were surveyed. All nine breakout species, so far, are not only in the Brassicaceae, but have dry stigmas (as do all Cruciferae in Heslop-Harrison and Shivanna, 1977), tricellular pollen (Williams et al., 2014) and pollen grains of similar size, shape, tricolpate apertures, and reticulate tectal pattern (Fig. 7). We predict that further comparisons across taxa will reveal other cases of pollen tube breakout through the nonaperture exine, especially among plants with dry stigma surfaces, where fortuitous contact between an aperture and the stigma surface does not always occur, and perhaps also among tricellular pollen, known for their similarly rapid germination and low viability (Williams et al., 2014). By definition, the subset of pollen grains that lack apertures entirely (including many in the Lauraceae, Araceae, and Orchidaceae) will exhibit efficient mechanisms for exine rupture. Of the six Brassicaceae (Cruciferae) family members described by Erdtman (1952) as inaperturate or nonaperturate, we looked only at *Matthiola incana* (Table 1). An entire further survey

of species with inaperturate pollen is needed. "Breakout" pollen grains like those of *A. thaliana* may experience unique selective pressures favoring thin, or at least rapidly penetrable, exine walls (to increase pollen tube exit speed and advantage in the race to fertilize ovules; Winsor et al., 2000). Not surprisingly, *A. thaliana* exine walls between apertures are thin; mature *A. thaliana* exine is only ~1 μm across (Owen and Makaroff, 1995; Suzuki et al., 2008), in contrast to the majority of pollen exines described by Erdtman (1952); a cursory comparison of these found nearly all reported to be thicker than 1.7 μm and many to be far (even 10-fold) thicker (Erdtman, 1952). Selection for thin exine would be balanced by the necessity for pollen wall durability and protection (Walker, 1974).

Evolutionary retention of the apertures in *A. thaliana* may be attributed to their persistent *occasional* use for escape during germination. Regardless of their utility during germination, apertures likely remain valuable during the large volume changes accompanying pollen desiccation and hydration. Aperture retention could still reduce pollen longevity from over-desiccation and/or provide sites for fungal or bacterial invasion; some fungi are, in fact, known to breach the nonaperture sporopollenin wall, leaving holes similar to those made by exiting *A. thaliana* pollen tubes (Elsik, 1966, 1971; Goldstein, 1960; Skvarla et al., 1996). More comparative studies are needed of site selection, in situ, during pollen tube emergence, both between pollen grains of various species and wall thicknesses, and between *A. thaliana* mutants—for example, those with thin and mispatterned sporopollenin (Jackson et al., 2000; Zinkl and Preuss, 2000; Paxson-Sowders et al., 2001; Nishikawa et al., 2005; Suzuki et al., 2008; Dobritsa and Coerper, 2012), or those with disrupted pectin biosynthesis (Sterling et al., 2006).

Our AFM measurements of *A. thaliana* pollen exine are of local material stiffness and are not to be confused with measurements of the breaking strengths of complex structures (Wainwright et al., 1976; Gordon, 1978; Bolick and Vogel, 1992). In addition, we applied a localized force perpendicular to and from the exterior of a curved surface; the cell wall's responses to the AFM probe are therefore unlike its responses to turgor-induced stresses running parallel with the cell wall and forces applied from the grain's interior. Material properties measured using different instruments and designs can differ markedly (Geitmann, 2006); for example, two reports of the elastic modulus of the chitinous cell wall of the yeast *Saccaromyces cerevisiae* differed by 100-fold (Touhami et al., 2003; Smith et al., 2000). Nevertheless, the elastic moduli we measured across *A. thaliana*'s heterogeneous pollen wall (of 11.9 ± 6.5 MPa at the stiffest tectal joint and 0.17 ± 0.12 MPa at the softest aperture location) are in the range of those measured by Touhami et al. (2003), who used a similar AFM technique to describe *S. cerevisiae* wall heterogeneity (6.1 ± 2.4 MPa at the budding scar and 0.6 ± 0.4 MPa elsewhere). Optical trapping measurements yielded an elastic modulus of 50 MPa for the wall of the bacterium *Bacillus subtilis* (Mendelson et al., 2000). Microindenter measurements of cell wall stiffness in extended pollen tubes, though difficult to compare with our AFM measurements, do reveal that tube wall stiffness is heterogeneous within 20 μm of the tube apex (Geitmann and Parre, 2004; Bolduc et al., 2006). When entire pollen grains are subjected to mechanical stress (instead of local AFM nanoindentation), the majority of the tested pollen grains have 50% breaking strengths between 2 and 55 MPa (see the full list of Bolick and Vogel, 1992; no Brassicaceae family members were studied). Such strength measurements can also be adjusted, based on pollen grain radii and wall thickness, to measurements of stress at 50% breaking (Bolick and Vogel, 1992). Together, stiffness and strength are good quantitative descriptors of pollen exine's properties and compliment more qualitative descriptions of pollen exine as durable and elastic (Rowley and Skvarla, 2000).

Atomic force microscopy allows sampling of living pollen during germination (not possible with electron microscopy) and could be used in the future to reveal dynamic mechanical changes in the exine during development and after pollination, either globally or at the site of tube emergence during germination. Indeed, AFM has already been used successfully on various bacterial surfaces (Camesano et al., 2000; van der Mei et al., 2000; Bolshakova et al., 2003, 2004) including the spore coat of germinating *Bacillus atrophaeus* (Chada et al., 2003; Plomp et al., 2007) and with yeast, to chronicle real time changes in the composition of living cell walls during growth and after addition of protease solutions (Touhami et al., 2004; Ahimou et al., 2002). Similar assays in germinating pollen (over time, across species, and in mutant or experimentally altered grains) could be very fruitful. AFM has allowed visualization of the substructures of pollen exine (Wittborn et al., 1996, 1998; Rowley et al., 1995; Xing et al., 2000), the heterogeneity of microfibrillar plant cell walls (van der Wel, et al., 1996; Hanley et al., 1997; Thimm et al., 2000; Geitmann, 2006; Kirby, 2010; Radotić et al., 2012), and extensive study of animal cell elasticity (Cross et al., 2007; Kuznetsova et al., 2007; Thomas et al., 2013). Close AFM examinations of pollen wall structure and material properties in multiple species have the potential to reveal phylogenetic patterns or correlations. For instance, pollen that are obligate aperture users may be found to have larger relative differences in stiffness between nonaperture pollen walls and apertures than in *A. thaliana* or other "breakout" species.

CONCLUSIONS

Palynological and biomechanical studies of exine structure are best united with cell biological studies of exine function during germination, early cell orientation, and pollen tube escape. We cannot yet know whether the wide variety in pollen grain (and stigma) structures is accompanied by similar variety in pollen tube germination mechanisms. However, our findings with *A. thaliana* (and the eight other Brassicaceae family members whose pollen germinate similarly) lead us to expect other examples of pollen cells receiving stigma fluid, polarizing, and breaching the heterogeneous and dynamic barrier of sporopollenin where the pollen wall touches the stigma.

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