The pollen tube is a cellular protuberance formed by the pollen grain, or male gametophyte, in flowering plants. Its principal metabolic activity is the synthesis and assembly of cell wall material, which must be precisely coordinated to sustain the characteristic rapid growth rate and to ensure geometrically correct and efficient cellular morphogenesis. Unlike other model species, the cell wall of the Arabidopsis (Arabidopsis thaliana) pollen tube has not been described in detail. We used immunohistochemistry and quantitative image analysis to provide a detailed profile of the spatial distribution of the major cell wall polymers composing the Arabidopsis pollen tube cell wall. Comparison with predictions made by a mechanical model for pollen tube growth revealed the importance of pectin deesterification in determining the cell diameter. Scanning electron microscopy demonstrated that cellulose microfibrils are oriented in near longitudinal orientation in the Arabidopsis pollen tube cell wall, consistent with a linear arrangement of cellulose synthase CESA6 in the plasma membrane. The cellulose label was also found inside cytoplasmic vesicles and might originate from an early activation of cellulose synthases prior to their insertion into the plasma membrane or from recycling of short cellulose polymers by endocytosis. A series of strategic enzymatic treatments also suggests that pectins, cellulose, and callose are highly cross linked to each other.

Upon contact with the stigma, the pollen grain swells through water uptake and develops a cellular protrusion, the pollen tube. During its growth in planta, the pollen tube invades the transmitting tissue of the pistil and finds its way to the ovary to deliver the male gametes for double fertilization to happen (Heslop-Harrison, 1987). Depending on the species, pollen tubes can grow extremely rapidly both in planta and in in vitro conditions. To fulfill its biological function, the pollen tube has to (1) adhere to and invade transmitting tissues (Hill and Lord, 1987; Lennon et al., 1998), (2) provide physical protection to the sperm cells, and (3) control its own shape and invasive behavior (Parre and Geitmann, 2005b; Geitmann and Steer, 2006). For all of these functions, the pollen tube cell wall plays an important regulatory and structural role. Although the pollen tube does not form a conventional secondary cell wall layer, its wall is assembled in two phases. The “primary layer” is mainly formed of pectins and other matrix components secreted at the apical end of the cell. The “secondary layer” is assembled by the deposition of callose in more distal regions of the cell (Heslop-Harrison, 1987). Depending on the species, cellulose microfibrils have been found to be associated either with the outer pectic or with the inner callosic layer. Unlike most other plant cells, cellulose is not very abundant representing only 10% of total neutral polysaccharides in Nicotiana alata pollen tubes, whereas callose accounts for more than 80% in this species (Schlüpmann et al., 1994).

The biochemical composition of the pollen tube cell wall has been well characterized in many species such as Lilium longiflorum (Lancelle and Hepler, 1992; Jauh and Lord, 1996), tobacco (Nicotiana tabacum; Kroh and Kruiman, 1982; Geitmann et al., 1995; Ferguson et al., 1998; Derksen et al., 2011), Petunia hybrida (Derksen et al., 1999), Pinus sylvestris (Derksen et al., 1999), and Solanum chacoense (Parre and Geitmann, 2005a). But for Arabidopsis (Arabidopsis thaliana), the model for plant molecular biology studies (Arabidopsis Genome Initiative, 2000), there is a striking lack of quantitative information concerning the composition of the pollen tube cell wall as well as the spatial distribution of its components. This is all the more surprising because numerous mutants defective in enzymes involved in cell wall synthesis exhibit a pollen tube phenotype (for
example, Jiang et al., 2005; Nishikawa et al., 2005; Wang et al., 2011). Two studies have characterized the Arabidopsis pollen germinating in vitro (Derksen et al., 2002) and in vivo (Lennon and Lord, 2000), but both are qualitative rather than quantitative. A biochemical study by Dardelle and coworkers investigated the cell wall sugar composition in a more quantitative way but does not provide any detailed spatial information (Dardelle et al., 2010; Lehner et al., 2010). This lack of information is not surprising given that until recently Arabidopsis pollen was known to be rather challenging to germinate is not surprising given that until recently Arabidopsis to its pollen (Pina et al., 2005). Therefore, the Arabidopsis pollen tube has become a valuable system for the link between intracellular signaling, biochemistry, cell mechanical properties, and morphogenesis in plant cells. Because of its typically fast growth rates, it responds quickly to any environmental triggers such as pharmacological, hormone, or enzymatic treatments. Adding Arabidopsis to the group of commonly studied pollen tube species is particularly timely, because one-third of the approximately 800 cell wall synthesis genes identified in this species are expressed in or are specific to its pollen (Pina et al., 2005). Therefore, the Arabidopsis pollen tube has become a valuable system for cell wall studies, especially with the increasing availability of cell wall mutant lines (Liepman et al., 2010).

Here we describe the biochemical composition of the Arabidopsis pollen tube cell wall grown in vitro conditions using immunocytochemical labeling coupled with epifluorescence and electron microscopic techniques. Rather than relying on imaging alone, we developed a quantitative strategy to assess the precise spatial distribution of cell wall components. This quantitative approach will provide an important tool and baseline dataset for the investigation of mutant phenotypes and for the interpretation of pharmacological studies. Furthermore, we used selective and strategically combined enzymatic digestions to determine the degree of connectivity between the individual types of cell wall polysaccharide networks.

RESULTS

Cytoarchitecture of the Arabidopsis Pollen Tube

In our in vitro growth conditions, Arabidopsis pollen tubes germinated at 2 h after contact with the growth medium and grew at a rate of 25 to 50 μm h⁻¹ as calculated from pollen tube length at 6 h after imbibition. Arabidopsis pollen tubes had an average diameter of 5 μm with small variations between individual tubes. They were characterized by an apical clear zone extending approximately 2 to 4 μm back from the pole of the cell. For clarity, we will refer to the following cellular regions in this paper: the “tip” or “apex” is characterized by the clear, vesicle-filled zone and comprises the first 3 μm extending from the pole of the cell along the longitudinal axis; the “subapical region” is considered to be the region between 3 and 10 μm; the “distal region” begins at 10 μm. The “pole” of the tube is the outermost tip of the cell (Fig. 1A). We use two different measures for the distance: “longitudinal” indicates the distance from the pole measured on the long axis (symmetry axis) of the cell and “meridional” distance is measured along the periphery of the tube (Fig. 1A').

High and Low Esterified Pectins Show Steep, Opposite Gradients at the Same Distance from the Tube Pole

Pectins were labeled with JIM7 and JIM5 (for John Innes Monoclonal) antibodies that specifically recognize pectin molecules with high and low degrees of methyl esterification, respectively (Knox et al., 1989; Van den Bosch et al., 1989). Pectins with high degree of esterification were primarily found at the pollen tube tip, along the first 5 μm of meridional length. The amount of highly esterified pectins decreased by two-thirds in the first 10 to 12 μm, where it reached a stable value that was maintained along the entire distal region. By contrast, the label for low esterified pectins was weak at the tip (around 10% of the maximum label intensity determined in an individual cell) and increased 4-fold in the first 10 to 12 μm, where it reached a plateau (Fig. 1B, B’, C, and C’). Low esterified pectins showed a somewhat irregularly patterned deposition, a phenomenon that has been linked to changes in the growth rate (Li et al., 1994, 1996; Derksen et al., 2011). These irregularities are not reflected in the graph (Fig. 1B’), because the values represent an average of several tubes.

Electron micrographs showed that the cell wall in the distal region of the Arabidopsis pollen tube had an average thickness of 0.2 μm (n = 10; Fig. 2). The pectin label was associated with the outer wall layer that had a fibrillar appearance. No immunogold label for pectins was found in the inner, translucent layer. In sections of the distal region, pectins with a low degree of esterification seemed more abundant than pectins with a high degree of esterification, although direct quantitative comparison was not possible because different antibodies were used (Fig. 2, D and G). Some cytoplasmic vesicles were labeled for highly esterified pectins (Fig. 2G). For comparison, we also examined the distribution of immunolabel in pollen grains. Here, both types of pectins were found in the intine, but the label for pectins with a high degree of esterification was predominant (Fig. 2F). In the grain, a high number of vesicles in the pollen grain cytoplasm were labeled for
Figure 1. Organization of the cytoarchitecture and relative spatial distribution of cell wall components in the Arabidopsis pollen tube. DIC micrograph of a chemically fixed Arabidopsis pollen tube (A) and nomenclature of distance measurements.
highly esterified pectins, whereas none were labeled for low esterified pectins (Fig. 2, C and F).

Callose Is Only Detected in the Distal Part of the Tube

To localize callose, we performed immunofluorescent labeling with an antibody specific for (1→3)-β-glucan. No callose deposition was found in the first 8 µm (meridional) of the pollen tube. Callose deposition began at a 10 µm meridional distance from the tip and increased steadily until 40 µm, where it reached a plateau (Fig. 1D'). Occasionally, peaks of fluorescence were observed at intervals of approximately 20 µm, corresponding to a high deposition of callose at the cell wall. Such intense depositions of callose were also typical for cell wall regions about to form a callose

Figure 1. (Continued.)
(A'). The first 3 µm (longitudinal distance) correspond to the tip or apex, the subapical region is located between 3 and 10 µm, and the distal region starts at 10 µm longitudinal distance from the pole. The border between the apex and the subapical region is the transition region (arrow). Fluorescence micrographs (B–I) of median optical sections (left) and corresponding quantification of relative label intensities along the meridional cell surface (right). Specific labeling was performed using antibodies or histochemical stains for pectins with low (B) and high (C) degree of esterification, callose (D), microfibrillar polysaccharides (E), crystalline cellulose (F and G), and fucosylated xyloglucans (H). The xyloglucan label was absent or barely detectable at locations where the pollen tube diameter changed (I and J; arrows). In the graphs, the black curve represents the mean relative fluorescence of all analyzed tubes, gray areas represent the SD, the dashed line represents the transition region between the hemisphere shaped apex of the pollen tube, and the cylindrical shank of the tube. The arrowhead in D indicates the pole of the pollen tube. The label for crystalline cellulose revealed the presence of two distinct populations of tubes: approximately 70% of the tubes were intensely labeled at the apex (F and F'), whereas 30% were labeled only weakly at the tip (G and G'). n ≥ 30 for each sample. A to J are at the same scale (bar = 10 µm). [See online article for color version of this figure.]
This layer was formed between the pectic deposited in the inner layer of the pollen tube cell wall. Electron micrographs showed that callose was the grain (Fig. 2H).

In pollen transparent (Fig. 2I), similar to observations made in and the plasma membrane, and appeared electron grown Arabidopsis pollen tubes, calcofluor white is a commonly used stain for cellulose at the very tip (Chebli et al., 2010). Scanning electron micrographs of these digested although calcofluor white has high affinity for cellulose microfibrils, it is known to stain a variety of other polysaccharides such as callose and chitin (Hughes and McCully, 1975; Herth and Schnepf, 1980; Wood and Fulcher, 1983; Falconer and Seagull, 1985). In in vitro grown Arabidopsis pollen tubes, calcofluor staining revealed a very high amount of microfibrillar polysaccharides at the very tip (first meridional 3 μm), which decreased drastically to one-half the maximal label intensity in the subsequent 6 μm and reached a plateau in the shank of the tube (Fig. 1, E and E′). Some tubes showed a fluorescent cone in the apical cytoplasm. The plug and for the collar region between emerging pollen tube and pollen grain (Fig. 1, D and D′). Transmission electron micrographs showed that callose was deposited in the inner layer of the pollen tube cell wall. This layer was formed between the pectic fibrillar layer and the plasma membrane, and appeared electron transparent (Fig. 2I), similar to observations made in tobacco pollen tubes (Derksen et al., 2011). In pollen grains, the callose label was only found in the intine of the grain (Fig. 2H).

**Crystalline Cellulose Is More Abundant in the Apical Region of the Tube**

Calcofluor white is a commonly used stain for cellulose detection in different plant systems. However, although calcofluor white has high affinity for cellulose microfibrils, it is known to stain a variety of other polysaccharides such as callose and chitin (Hughes and McCully, 1975; Herth and Schnepf, 1980; Wood and Fulcher, 1983; Falconer and Seagull, 1985). In in vitro grown Arabidopsis pollen tubes, calcofluor staining revealed a very high amount of microfibrillar polysaccharides at the very tip (first meridional 3 μm), which decreased drastically to one-half the maximal label intensity in the subsequent 6 μm and reached a plateau in the shank of the tube (Fig. 1, E and E′). Some tubes showed a fluorescent cone in the apical cytoplasm. The shape and location of the cytoplasmic label seemed to correspond to the apical aggregation of vesicles that is typical for growing angiosperm pollen tubes, as revealed by the label with the styryl dye FM4-64 (Fig. 3, A and B).

For more specific localization, crystalline cellulose was labeled using Cellulose Binding Module3a (CBM3a; Blake et al., 2006) combined with an anti-poly-His antibody and a tertiary antibody coupled to Alexa Fluor 594 (Tormo et al., 1996; Moller et al., 2007; Alonso-Simón et al., 2010). Two populations of tubes could be clearly distinguished; 70.7% ± 1.8% of the tubes showed intense label at the tip (Fig. 1F), whereas 29.3% ± 1.8% showed no or very low fluorescence intensity at the apex (Fig. 1G; n = 2,857). In tubes with weakly labeled apex, label intensity increased away from the apex to reach a maximum at 20 μm. Remarkably, in all tubes the CBM3a label decreased very gradually from this point backward in the remaining distal region (Fig. 1, F, G and G′).

Transmission electron micrographs showed that crystalline cellulose labeled with CBM3a was deposited in the inner layer of the pollen tube cell wall (Fig. 2A). The label colocalized with that for callose between the pectic layer and the plasma membrane. Some crystalline cellulose label was also visible in cytoplasmic vesicles both in chemically and rapid freeze-fixed samples (Figs. 2 and 4). To ascertain that this was not the background label, we quantified label density on various cytoplasmic components and the cell wall (Fig. 2J). No label was visible inside mitochondria, whereas most of the gold particles were found in vesicles and in the cell wall. The CBM3a label was also found to be associated with the trans-Golgi network (Fig. 4A). An occasional label in the cytosol can be explained with the presence of peripherally sectioned vesicles that were not recognizable as such. Similar results were obtained on samples fixed conventionally and by rapid freeze fixation (Figs. 2 and 4).

**Microfibrils and CESA6 Complexes Are Arranged near Parallel to the Longitudinal Axis of the Tube**

Microfibril orientation is a crucial parameter that influences the mechanical behavior of the cell wall (Baskin, 2005; Geitmann and Ortega, 2009; Geitmann, 2010). To be able to determine the principal direction of cellulose microfibril orientation in the Arabidopsis pollen tube, we digested the outer pectic layer of the cell wall with pectinase after chemical fixation of the cells (Aouar et al., 2010). Scanning electron micrographs of these digested pollen tubes showed that the Arabidopsis pollen tube cell wall comprised a fibrous component whose main orientation was nearly parallel to the longitudinal axis of the cell (Fig. 5B). Given that cellulose microfibrils are the only known fibrous components in the pollen tube wall, this suggests that the net orientation of microfibrils is near parallel with the longitudinal axis of the cell.

To corroborate this further, we observed the spatial arrangement and motion pattern of cellulose synthases (CESAs) in the plasma membrane of Arabidopsis pollen
tubes. Cellulose synthesis is performed by several CESAs grouped into rosette complexes (Somerville, 2006) and in other plant cell types the motion pattern of CESA was found to be correlated with the orientation of cellulose microfibrils (Paradez et al., 2006). Variable angle epifluorescence microscopy (VAEM) of Arabidopsis pollen tubes expressing GFP-CESA6 (Desprez et al., 2007) revealed that the punctate label for GFP-CESA6 was localized at and uniformly distributed along the pollen tube plasma membrane including the apex (Fig. 3C). Tangential optical sections of the tube showed that the GFP-CESA6 puncta were frequently aligned in a near-parallel manner to the longitudinal axis of the tube (Fig. 5C), and time-lapse imaging revealed that they move along these lines (Fig. 5D; Supplemental Movie S1).

Fucosylated Xyloglucans Are Uniformly Deposited in the Cell Wall

Cellulose microfibrils alone cannot confer much tensile resistance to the cell wall unless they are cross-linked into a network. Well known cross-linkers of cellulose are xyloglucans, hemicelluloses formed of a backbone of β-1,4-linked β-D-Glc with α-D-Xyl branching (Obel et al., 2007). CCRC-M1 antibody was used to label fucosylated epitopes that are found principally in xyloglucans (Freshour et al., 2003). The label for these fucosylated epitopes was uniform along the whole cell wall of straight growing pollen tubes (Fig. 1, H and H'). Lower label intensity was observed at the distal region. No label was found at the collar region between the base of the pollen tube and the pollen grain. Contrary to crystalline cellulose, no distinction between two different types of apical distribution patterns could be made. Remarkably, at locations corresponding to changes in pollen tube diameter or growth direction, xyloglucan label was barely detectable (Fig. 1, I and J).

Highly Esterified Pectins Are Tightly Embedded into the Cellulose Network

Contrary to our expectations, the fluorescence intensity of label for crystalline cellulose decreased with increasing distance from the apices of most pollen tubes, in particular after the first 10 μm (Fig. 1F). Because this spatial gradient was mirrored by an increasing degree of deesterification in cell wall pectins, we hypothesized that crystalline cellulose epitopes in the shank might be partially masked to the antibody by the increasingly gelled pectins. To verify this hypothesis, we devised two strategies: (1) we enzymatically removed the pectic outer layer after fixation to expose all putatively masked crystalline cellulose epitopes before labeling with CBM3a, and (2) we treated living pollen tubes with pectin methylesterase (PME) to deesterify the apically located pectins and thus facilitate their gelation (Parre and Geitmann, 2005a). If the presence of gelled pectin in the shank was the reason for a longitudinal gradient in the cellulose label, both treatments should result in evening out the gradient, but overall label intensity should be higher after treatment 1 than after treatment 2. A combination of both treatments was tested as an additional control. In addition, immunolabeling for pectins was carried out in parallel to test the efficiency of the enzyme treatments.

Control samples (not treated with PME nor digested with pectinase) showed a significant label at the tip for highly esterified pectins and weak labeling for low esterified pectins, whereas in the distal region labeling was more intense for highly esterified pectins (Figs. 1, B and C, and 6). Tubes that were treated with PME but not digested with pectinase showed a very poor label for highly esterified pectins confirming the efficiency of PME (Figs. 6 and 7, E and F). Tubes that were digested with pectinase, with or without prior treatment with PME, did not show any label for pectins, suggesting the pectinase activity was fully efficient even on chemically fixed cells (Figs. 6 and 7, A, B, I, and J).

After treatment with PME only (Fig. 7, E, F, G, and H), the crystalline cellulose staining pattern was identical to that of the nontreated tubes (Fig. 1F), suggesting that the degree of esterification and gelation of the pectins was not responsible for the longitudinal gradient in cellulose label in the distal region. After pectin digestion, more than 80% of the pollen tubes showed less or no label for crystalline cellulose at the apex (Figs. 6 and 7, C and D). In these cells, crystalline cellulose was only detectable after the first 10 μm. This removal of apical cellulose by pectin-specific digestive
action suggests that the cellulose microfibrils in this region do not form an independently stable network. They seem to form links to the highly esterified pectins that are dominant in this region, and when these are enzymatically removed they are washed away as well. Alternatively, the cellulose network may be stably embedded in the solid pectin matrix and not necessarily bound to it. Treatment with PME prior to fixation and pectin digestion was able to restore the crystalline cellulose labeling pattern observed in tubes that were not treated with pectinase (Figs. 6 and 7, G and K). This implies that (1) pectin, independently of its degree of esterification, does not mask cellulose epitopes, and (2) that the cellulose network is less embedded or bound to low esterified pectins than to highly esterified pectins.

Calcofluor staining confirmed this as the staining pattern was identical to CBM3a labeling. Tubes that were not treated with pectinase displayed a high amount of calcofluor staining at the apex and less in the shank, whereas tubes treated with pectinase showed the same pattern of staining as tubes stained for crystalline cellulose (Fig. 7, H and L).

The Cellulose Layer Is Removed When Pectin and Callose Are Digested

The enzymatic removal of the pectin matrix clearly resulted in the (partial) removal of the apical population of cellulose microfibrils during specimen preparation, suggesting that microfibrils are either rather short in this region or not well cross-linked, or both. Given these results, we hypothesized that cellulose microfibrils might be physically stabilized by the surrounding pectin matrix. However, at the transmission electron microscopy (TEM) level we had also observed the colocalization of crystalline cellulose with the callose layer. Therefore, we wanted to test the effect of enzymatic removal of callose on cellulose label. We subjected pollen tubes to digestion by lyticase (an enzyme that specifically digests callose) or to a combination of lyticase and pectinase. When fixed pollen tubes were treated with lyticase alone, the distribution profile of crystalline cellulose was not affected. After combined pectinase and lyticase digestion, no crystalline cellulose was detected in the pollen tube cell wall by immunolabeling or calcofluor white stain. The only labeling was visible inside the pollen tube cytoplasm (Fig. 8), confirming that

| Enzymatic Treatment | PME | Pectinase | JIM5 | JIM7 | CBM3a | Calcofluor |
|---------------------|-----|-----------|------|------|-------|-----------|
|                     | -   | -         | +    | -    |       | 03%       |
|                     | -   | +         |      | +    | 32%   | 40%       |
|                     | -   | -         | 32%  | -    | 60%   | 03%       |
|                     | -   | +         | 60%  |    | 80%   | 40%       |
|                     | +   | -         | 20%  | 20%  | 71%   | 17%       |
|                     | -   | -         | 29%  | 29%  | 50%   | 60%       |

Figure 5. Scanning electron micrographs (A and B) of the surface of Arabidopsis pollen tubes and VAEM images of pollen tube expressing GFP-CESA6 (C and D). A, The control tube displays a smooth surface with shallow undulations representing the outer pectin layer. B, Tubes digested with pectinase after chemical fixation reveal that the wall contains a fibrous component that detaches in longitudinal direction. C, A maximum projection of a time-lapse series of variable angle epifluorescence micrographs taken over 50 s shows that GFP-CESA6 punctae move primarily in longitudinal direction. D, Still images of the same image series at 0, 7, and 15 s. Arrowhead indicates one moving GFP-CESA6 complex. Bars = 2 μm (A–C), 1 μm (D). [See online article for color version of this figure.]

Figure 6. Relative fluorescence intensity after enzymatic treatments. Pollen tubes were subjected to different combinations of enzymatic treatments before (PME) and after chemical fixation (pectinase), and subsequently labeled for pectins and cellulose. The figure summarizes the spatial profiles of relative fluorescence intensities. Black stands for highest label intensity, white for absence of label. Percentage values indicate the size of the two populations if distinct label patterns were observed within individual samples.
cellulose microfibrils are interconnected with callose polymers as well as with pectins. Finally, the residual label of small cytoplasmic organelles after enzyme treatment corroborates the presence of cellulose in vesicles.

The Mechanical Properties of the Pollen Tube Display a Longitudinal Gradient

Our quantitative assessment of the spatial distribution pattern of cell wall components in the Arabidopsis pollen tube indicates a dramatic change in the biochemical composition at the transition region between the hemisphere-shaped apex and the cylindrical shank of the tube. Computational modeling has shown that this transition region must also display a significant change in mechanical properties to ensure stability of the cylindrical shank against tensile stress caused by the turgor pressure (Fayant et al., 2010). To demonstrate that the observed changes in biochemical composition translate into a change in mechanical properties, we used microindentation to measure the local cellular stiffness of the Arabidopsis pollen tube along its longitudinal axis. The spatial profile revealed that the pollen tube apex was significantly softer than the cylindrical region of the cell (Fig. 9). This is consistent with data from other plant species (Geitmann and Parre, 2004; Zerzour et al., 2009).

DISCUSSION

Pectin Deposition in Arabidopsis Pollen Tubes Takes Place at the First 5 μm

The main components of the pollen tube cell wall are pectins that are deposited at the pollen tube tip by exocytosis in high methyl-esterified form (Bosch et al., 2010).
This also seems to be true in Arabidopsis pollen tubes because immunolabeling at TEM level showed that cytoplasmic vesicles were only labeled for highly esterified pectins but not for low esterified pectins. The presence of only highly esterified pectins in vesicles of the pollen grain shows that a pool of pectin is already present before pollen germination, allowing pollen to rapidly initiate germination before the cellular machinery required for de novo pectin synthesis is fully activated (Geitmann, 2010). During germination, exocytosis events delivering pectin to the elongating cell wall seem to occur only in the first 5 μm (meridional), similar to other species (Li et al., 1994; Geitmann et al., 1995; Jauh and Lord, 1996; Geitmann and Parre, 2004; Parre and Geitmann, 2005a; Dardelle et al., 2010; Fayant et al., 2010; Lehner et al., 2010).

The Spatial Distribution of Pectin Deesterification Determines the Pollen Tube Diameter

The spatial distribution of the different configurations of pectin suggests that de-esterification takes place in the apical region between the first 3 and 10 μm (meridional distance), implying that PME is active in this cell wall region. This spatial profile coincides with that of Arabidopsis PME inhibitor (At3g17220) transiently expressed in tobacco pollen tubes, which is specifically localized at the pole of the tube and likely endocytosed in the flanks (Röckel et al., 2008; Fig. 10B). Mechanical modeling of pollen tube growth has shown that the transition point between curved apex and cylindrical shank coincides exactly with a steep gradient in the Young’s modulus of the cell wall necessary to achieve self-similar growth with a strain distribution typical for pollen tubes (Fayant et al., 2010). The gelation of pectins into a stiffer material in the transition region stabilizes the cell wall and prevents any further expansion of the cell wall in the subapical part of the tube (Fig. 10B; Parre and Geitmann, 2005a). Micromechanical tests confirmed that the cellular stiffness in the shank of the Arabidopsis pollen tube is increased compared with the apex.

Callose Distribution Is Consistent with Its Role in Resisting Tension Stresses

Callose (1,3-β-D-glucan with 1,6-linked branches) is synthesized at the plasma membrane by the callose synthase complex. Callose deposits are visible 30 μm from the tip in tobacco (Ferguson et al., 1998) and 20 μm in Lilium orientalis (Fayant et al., 2010). Micro-indentation combined with enzymatic treatments have shown that callose plays a role of reinforcement against compression and tension stresses (Parre and Geitmann, 2005b). The spatial profile of our callose label is consistent with previous observations in other species (Ferguson et al., 1998; Geitmann and Parre, 2004; Parre and Geitmann, 2005b; Fayant et al., 2010; Derksen et al., 2011). Callose deposition in Arabidopsis only began after the first 10 μm and reached a plateau in the distal region. Callose plug formation could be seen in older pollen tubes beginning at a distance of 100 μm from the tip. This differs from observations made in vitro (Derksen et al., 2002) and in vivo (Lennon and Lord, 2000) germinating Arabidopsis pollen tubes, where callose was found very close to the tip (as close as 5 μm meridional from the pole), and callose plugs were observed to begin to form as close as 40 μm to the tip. These differences in spatial distribution may be the result of a higher in vitro growth rate obtained here (30 μm h⁻¹ compared with 10.4 μm h⁻¹ in Derksen et al., 2002). Because callose synthase seems to be inserted into the apical plasma membrane (Cai et al., 2011), the distance...
Figure 10. Conceptual model of the assembly and structure of the pollen tube cell wall. Spatial profiles of the relative abundance of cell wall components and cellular stiffness in pollen tubes (A) related to the spatial distribution of assembly and

Extensibility predicted by FE model
De-esterified pectin
Callose
Esterified pectin

Meridional distance from the pole

Pole Transition region

B

Secretion of methyl-esterified pectin; PME inhibitor active
Abundance of PME inhibitor reduced; PME de-esterifies pectins
Acidic pectins are cross-linked by Ca²⁺
Putative re-uptake of cellulose microfibrils

Initiation of microfibril synthesis in secretory vesicles
Deposition of microfibrils and cellulose synthases at the surface
Deposition of microfibrils by membrane-located cellulose synthases
Ongoing deposition of cellulose microfibrils
Callose layer thickens
at which the synthesis of callose accumulates to visible amounts may simply result from the ratio between the synthesis rate of the enzymes and the growth rate of the tube. Stress is likely to increase callose production in pollen tubes, in Arabidopsis in particular, because in our hands in vitro culture conditions, other than those we optimized (Bou Daher et al., 2009), resulted in the presence of the callose label at the tube apex (Y. Chebli, F. Bou Daher, and A. Geitmann, unpublished data). This is consistent with callose synthesis being a known response to mechanical stress and wounding in plant tissues (Jacobs et al., 2003; Geitmann and Steer, 2006).

**Fucosylated Xyloglucans Are Secreted in Their Final Form**

Xyloglucans are synthesized and processed in the Golgi apparatus before being exported via secretory vesicles (Edelmann and Fry, 1992). Once inserted into the cell wall, they are associated to cellulose via hydrogen bonds, thus contributing to the formation of a tight network (Hayashi, 1989; Acebes et al., 1993; Hayashi et al., 1994). Information about the role and the presence of xyloglucans in pollen tubes is scant. No significant amount of xyloglucan was detected in pollen tubes of tobacco (Schlüpmann et al., 1994), whereas in Arabidopsis, fucosylated xyloglucans were found in pollen grains of the *mur1* mutant (Freshour et al., 2003). Transmission electron microscopy showed that fucosylated xyloglucans were only associated with the inner layer of the Arabidopsis pollen tube wall (Dardelle et al., 2010), suggesting that they are cross linked to cellulose microfibrils.

CCRC-M1 labeling demonstrated that fucosylated xyloglucans were distributed evenly along the Arabidopsis pollen tube cell wall. This is consistent with the fact that they are secreted in their final fucosylated form at the apical plasma membrane (Obel et al., 2007). In locations where the tubes seem to change direction or diameter, fucosylated xyloglucans were not detected. We speculate that a temporary failure to deliver these linker molecules to the cellular surface may lead to a transient widening of the tube diameter because of a lack of cellulose cross linking, similar to the phenomenon observed after treatment with cellulase (Aouar et al., 2010) and predicted by finite element simulations of the growth process (Fayant et al., 2010).

**Cellulose Synthesis Might Be Initiated in Vesicles**

Unlike most other plant cell types in which cellulose (1,4-β-D-glucan) is typically the major cell wall component, pollen tubes have a very low amount of cellulose in their cell wall; less than 10% of the tobacco (Schlüpmann et al., 1994) and approximately 6% to 7% dry weight of the *L. longiflorum* pollen tube cell wall is composed of cellulose (Van der Woude et al., 1971). In most plant cells, cellulose synthesis is initiated once cellulose synthase complexes have been deposited into the plasma membrane (Somervelle, 2006). Both our TEM and fluorescence data suggest that crystalline cellulose is present in cytoplasmic vesicles in the Arabidopsis pollen grain and tube. It is unclear whether these vesicles derive from endocytosis and thus represent recycled cellulose or whether they carry cellulose synthases and newly assembled, short microfibrils. However, their association with the trans-Golgi network and with vesicles newly assembled, short microfibrils. However, their association with the trans-Golgi network and with vesicles located in the apical cytoplasm indicates that at least a portion of these vesicles is destined for exocytosis. This putative cellulose synthesis activity ahead of surface deposition is not unique because β-glucan synthetase activity has been detected in Golgi vesicle fractions isolated from *P. hybrida* pollen tubes (Helsper et al., 1977). Furthermore, cellulose residues have been found in the vesicles of pollen tubes from *L. longiflorum* (Van der Woude et al., 1971) and *P. hybrida* (Engels, 1973, 1974a, 1974b) and in the alga *Pleurochrysis scherffeli* (Brown, 1969). The activation of cellulose synthases prior to their insertion into the plasma membrane may give pollen tubes a head start in assembling the cell wall necessary to sustain rapid elongation.

**Spatial Distribution and Orientation of Cellulose Microfibrils Suggest Particular Mechanical Functions**

Whereas the apex of tobacco pollen tubes is devoid of cellulose (Ferguson et al., 1998), microfibrils were present in the apex of the Arabidopsis pollen tube according to both calcofluor and CBM3a label. However, label intensity at the very tip of the tube was variable and two populations of pollen tubes displaying either intense or very weak label at the pole could be distinguished with CBM3a label. The differences in the cellulose pattern within the population may result from the temporal changes in growth behavior of individual pollen tubes and/or from the abundance of active cellulose synthases.

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**Figure 10.** (Continued.)

Modification processes (B). Highly esterified pectins synthesized in the Golgi apparatus are delivered to the cell wall forming the apical dome (green spheres and arrows). Pectin deesterification takes place in the shoulders of the apical dome, generating steep, opposite gradients in the abundance of high and low esterified pectins (gradient of green) before reaching a stable level of esterification in the shank (dark green). Curves for pectin and callose are taken from Figure 1. The gradient in the abundance of PME inhibitor in tobacco pollen tubes (blue curve) quantified from figure 4A in Röckel et al. (2008), matches the position of the change in pectin configuration observed in Arabidopsis and the increase in Young’s modulus (black) predicted by a finite element model for tip growing walled cells (Fayant et al., 2010) after normalization for cell size. Crystalline cellulose is found in secretory vesicles (red) and is likely deposited together with cellulose synthases in form of short microfibrils. The abundance of crystalline cellulose in the wall (red lines) decreases slightly toward the distal region of the tube suggesting that they may be recycled by endocytosis (dashed red arrow). Callose (gray) is synthesized at the plasma membrane and is detectable in the distal region only with a steadily increasing abundance. Objects in B are not to scale.
at the pollen tube tip (Fig. 3C). Temporal changes in growth rate are associated with modifications in cell wall thickness at the pole (Li et al., 1996; McKenna et al., 2009; Derksen et al., 2011).

The presence of cellulose in the apex is corroborated by the fact that cellulose synthases are abundant at the apical plasma membrane of the Arabidopsis (Fig. 3C) and tobacco (Cai et al., 2011) pollen tubes, and it has been proposed that cellulose microfibrils play a role in regulating tube diameter (Aouar et al., 2010). Cellulose may confer tensile resistance to the most crucial position on the cellular surface, the transition region between the apical dome and the cylindrical shank (Fayant et al., 2010). A direct comparison between the spatial distribution profiles of high and low esterified pectins points at the potential reason for the need of cellulose in the transition region (Fig. 10B). Although pectin gelation begins before the transition point, the crossover point between the two pectin configurations in Arabidopsis pollen tubes is further distal, and complete gelation is only achieved at approximately 10 μm. This is strikingly different from *L. longiflorum* pollen tubes in which maximal gelation is reached exactly at the transition point (Fayant et al., 2010). Therefore, additional reinforcement by cellulose in this region may be a crucial determinant of tube diameter in Arabidopsis pollen tubes.

In the tubular portion of the tube, starting at the transition region, all tubes displayed a gradient with a steady decrease of label intensity for crystalline cellulose with increasing distance from the apex. This longitudinal gradient is in agreement with observations made by others (Derksen et al., 2002; Fayant et al., 2010). However, the orientation of the gradient—lower abundance of cellulose in more mature portions of the wall—is puzzling. Our control experiments showed that this phenomenon was not a labeling artifact or a result of the masking of epitopes by other cell wall components. This raises the question how the amount of cellulose decreases during cell wall maturation if the maturing wall does not expand? It is possible that cellulose may be reincorporated into the cytoplasm following digestion by endogenous cellulases (Lane et al., 2001; Römling et al., 2005). A recycling mechanism may allow the fast growing pollen tube to sustain growth over long distances by ensuring a constant supply of material to its tip region, but further research is warranted to provide proof for this hypothesis.

The cellulose in the tubular portion of the tube displays another unexpected property. Scanning electron microscopy and fluorescence based localization and dynamics of CESA6 suggest that microfibrils are produced and oriented almost parallel to the longitudinal axis of the cell. This primarily longitudinal orientation is very different from the circumferential orientation of microfibrils in other plant cell types with cylindrical shape (Baskin, 2005; Geitmann and Ortega, 2009). However, it is similar to the low pitch of cellulose orientation observed in other pollen tube species (*P. hybrida*: 45° (Sassen, 1964); *Lilium* spp.: 20° to 25°; *S. chacoense*: 15° to 20° (Aouar et al., 2010)). Furthermore, immunohistochemical localization of CESA in tobacco pollen tubes also suggests a low pitched helical arrangement (Cai et al., 2011). In the root and shoot cells, the circumferential arrangement of cellulose microfibrils is thought to prevent lateral expansion of the cell and forces cell growth to occur in longitudinal direction. A helical or near longitudinal orientation of the reinforcing fibrous component suggests a different function in pollen tubes. Helical curling would be consistent with a role in stabilizing the tube against buckling and against collapse during curved growth and compression stress in axial direction. Similarly to a helically reinforced catheter, the pollen tube has to invade a mechanically resistant tissue and to ensure that despite its winding journey through the pistil the male germ unit passes unimpeded. The cellulose microfibrils in the cylindrical portion of the tube may thus play an important role in the pollen tube fulfilling its biological function as a catheter-like delivery system for the sperm cells.

### The Cellulosic Network Is Stabilized by the Pectic Gel and Callose

Treatment with PME prior to pectinase digestion prevented the loss of crystalline cellulose from the tube tip that occurred in tubes only treated with pectinase. This suggests that the degree of pectin esterification plays a role in stabilizing the cellulosic network. When pectins are low esterified, they tend to gelate in the presence of Ca2+, thus forming a tight gel and losing the ability to bind to the cellulose microfibrils. This can explain why after pectin digestion, the label for crystalline cellulose disappeared entirely from the pollen tube apical region but was only slightly reduced in the distal region. Because pectins are known to bind to cellulose in vitro (Zykwinska et al., 2005) and in vivo (Iwai et al., 2001; Oechslin et al., 2003; Vignon et al., 2004), we hypothesize that the two types of polymers are closely linked in pollen tube walls and that cellulose was washed off together with the highly methyl-esterified pectins following pectin digestion, despite the chemical fixation. Formaldehyde fixation cross links mostly proteins, and the polysaccharidic cell wall moiety may not be stable if one of the components is digested.

The stability of cellulose in the distal region of the tube during pectinase digestion may also be explained by the presence of callose in this portion of the tube. The colocalization of cellulose and callose in the inner, electron translucent cell wall layer is consistent with observations made in tobacco (Ferguson et al., 1998) and suggests a close connection between the two polymers in this maturing region of the cell wall. This notion is also supported by the finding that double digestion with pectinase and lyticase removed the entire label for crystalline cellulose, leaving only cytoplasmic organelles displaying residual cellulose label. Together, the observations indicate that cellulose is linked to or tightly embedded into both pectins (apex).
and callose (shank), and that the cell wall polymers form a tight network.

CONCLUSION

During pollen tube growth, cell wall precursors and enzymes are deposited at the tip by exocytosis. During wall maturation, the cell wall components undergo a structural reorganization through different chemical changes and recycling events. Figure 9 summarizes our model for the Arabidopsis pollen tube cell wall organization and the intracellular transport of the major cell wall precursors. In the cell wall region comprised between 3 and 10 μm (meridional), the pectin deesterification (through the regulation of the PME activity), the beginning of callose deposition and the regulation of cellulose deposition take place, thus changing drastically the mechanical properties of the cell wall as predicted by finite element modeling (Fig. 10A). This suggests that in Arabidopsis pollen tubes, the regulation of the cell wall chemistry in this transition region is crucial for determining the shape of the pollen tube.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 plants were grown in trays in a greenhouse as described earlier (Bou Daher et al., 2009). Pollen was collected every day from the time flowers bloomed using a modified vacuum cleaner as described by (Johnson-Brousseau and McCormick, 2004). Pollen grains were then dehydrated over silica gel for 2 h and stored at -20°C until use.

Pollen Culture

Pollen grains were hydrated for 30 min. For fluorescence microscopy and scanning electron microscopy, hydrated pollen was incubated at 22.5°C for 4 to 5 h under continuous shaking in a 25-mL Erlenmeyer flask containing 3 mL of liquid growth medium containing 0.49 mM H3BO3, 2 mM Ca(NO3)2, 4H2O, 5 mM CaCl2, 1 mM KCl, 1 mM MgSO4,7H2O, pH 7, and 18% (w/v) Suc (Bou Daher et al., 2009). For transmission electron microscopy, hydrated pollen was incubated at 22.5°C for 6 h in a solidified growth medium made of 1.62 mM H2BO3, 5 mM Ca(NO3)2, 4H2O, 5 mM CaCl2, 1 mM KCl, 1 mM MgSO4,7H2O, pH 7, and 18% (w/v) Suc (Bou Daher et al., 2009). For immunolabeling, pollen tubes were then washed three times with phosphate-buffered saline (PBS; 135 mM NaCl, 6.5 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.3) with 3.5% (w/v) bovine serum albumin (BSA). All subsequent washes were done with PBS buffer with 3.5% (w/v) BSA for 40 s. All antibodies were diluted in PBS buffer with 3.5% (w/v) BSA, and incubations were done for 10 min followed by three washes in buffer. Controls were performed by omitting incubation with the primary or the secondary antibody. Pectins with a high degree of esterification were labeled with JIM5 and JIM7, respectively (diluted 1:50; Paul Knox, University of Leeds, UK) followed by Alexa Fluor 594 anti-rat IgG (diluted 1:100; Molecular Probes).

Selective Digestion of Cell Wall Components

After 6 h of contact with the growth medium, 1.28 mg mL-1 pectin methyl esterase (334 units/mg protein; Sigma) was added to the germination medium, and pollen tubes were incubated for 45 min. Controls were performed with an enzyme that had been denatured by boiling for 15 min. Pollen tubes were then fixed as described above, washed three times, and subsequently incubated in PBS buffer containing 5 mg mL-1 pectinase (Sigma) or 1 mg mL-1 lyticase (Sigma) or a mix of the two enzymes for 3 h with constant shaking. Pollen tubes were then washed three times with PBS buffer and three times with PBS buffer with 3.5% (w/v) BSA prior to labeling for fluorescence microscopy as described above.

Fluorescence Microscopy

Differential interference contrast (DIC) and fluorescence imaging of immunohistochemical labeling were done with a Zeiss Imager-Z1 microscope equipped with structured illumination setup (ApoTome Axio Imager), a Zeiss AxioCam MRm Rev.2 camera, and AxioVision Release 4.5 software. For confocal labeling, a filter set with excitation BP 450-490 nm, beam splitter FT 510 nm and emission BP 515/565 nm was used. For Alexa Fluor 594 detection, the filter set comprised an excitation filter BP 590/22 nm, beam splitter FT 420 nm, and emission filter BP 460/50 nm. Exposure times were adjusted for each image, so that only 1 or 2 pixels were saturated prior to insertion of the ApoTome Imager into the light pathway. The ApoTome Imager was then inserted and Z-stacks of 1-μm intervals were acquired. Image reconstruction was performed using the AxioVision software by the maximum projection of the stacks. Localization of vesicles was done using a Zeiss LSM 510 META/LSM S LIVE/Axiovert 200M system, the 561-nm laser was used with an emission filter LP 650.

Live cell imaging of pollen tubes expressing GFP-CESA6 was done using VAEM (lab of Sebastian Bednarek, University of Wisconsin, Madison, WI) as described in (Konopka and Bednarek, 2008). Briefly, we used a Nikon Eclipse TE2000-U equipped with the Nikon 1-FL-TIRF attachment. GFP fluorescence was excited with the 488-nm argon laser, and an emission filter 535/30 nm was used. Images were captured with a Photometrics Evolve 512 camera at 4 frames s-1.

Image Processing and Fluorescence Quantification

ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://r.ij.nibd.nih.gov/ij/, 1997-2008) was used for quantification.

Immunohistochemistry

All steps were carried out in a microwave oven (Pello BioWave 34700 equipped with a Pello Cold Spot) operating at 150 W under 21 in of H2 gas at a controlled temperature of 30°C ± 1°C. For fluorescence labeling, pollen tubes were filtered and subsequently fixed in 3.5% (w/v) freshly prepared formaldehyde inPIPES buffer (50 mM PIPES, 1 mM EGTA, 5 mM MgSO4, 0.5 mM CaCl2, pH 7) for 40 s followed by three washes in PIPES buffer. For immunolabeling, pollen tubes were then washed three times with phosphate-buffered saline (PBS; 135 mM NaCl, 6.5 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.3) with 3.5% (w/v) bovine serum albumin (BSA). All subsequent washes were done with PBS buffer with 3.5% (w/v) BSA for 40 s. All antibodies were diluted in PBS buffer with 3.5% (w/v) BSA, and incubations were done for 10 min followed by three washes in buffer. Controls were performed by omitting incubation with the primary or the secondary antibody. Pectins with a high degree of esterification were labeled with JIM5 and JIM7, respectively (diluted 1:50; Paul Knox, University of Leeds, UK) followed by Alexa Fluor 594 anti-rat IgG (diluted 1:100; Molecular Probes).

Callose was labeled with a monoclonal IgG antibody to (1-3)-β-glucan (diluted 1:200; Biospecs Australia Pty Ltd.) followed by Alexa Fluor 594 anti-mouse IgG (diluted 1:100; Molecular Probes). Xyloglucans were labeled with CCRC-M1 antibody directed against fucosylated epityopes (diluted 1:50; Michael Hahn, Athens, GA) followed by Alexa Fluor 594 anti-mouse IgG (diluted 1:100; Molecular Probes). Labeling for crystalline cellulose was performed with CBM5a (diluted 1:200; Paul Knox, University of Leeds, UK) followed by a monoclonal mouse anti-poly-His antibody (diluted 1:12; Sigma), and subsequent incubation with Alexa Fluor 594 anti-mouse IgG (diluted 1:100; Molecular Probes). Cellulose was also stained directly after fixation and washes by incubating for 10 min with 1 mg mL-1 calcofluor white (Fluorescent Brightener 28; Sigma) in double-distilled water.

Following the labeling procedure and final washes, all samples were mounted on glass slides in a drop of Citifluor (Electron Microscopy Sciences) for microscopical observations. Each experiment was repeated at least four times. To label cytoplasmic vesicles, growing pollen tubes were incubated for 10 min with 6 μg mL-1 FM4-64 (Molecular Probes) prior to observation.

ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://r.ij.nibd.nih.gov/ij/, 1997-2008) was used for quantification.
of fluorescence intensity based on maximum projections of Z-stacks. Pixel intensity was measured along the peripheral of each pollen tube, beginning from the pole. Values for fluorescence intensity were normalized to the highest value present on an individual tube before averaging over all tubes (n > 10 for each sample). Values on the x axis in the graphs represent the meridional distance from the pole of the cell. Given an average tube diameter of 5 μm and an approximately hemisphere-shaped apex for Arabidopsis, a distance of 10 μm on the meridional curvature corresponds to a tube length of 8.6 μm measured along the longitudinal axis.

Sample Preparation for Transmission Electron Microscopy

Rapid Freeze Fixation and Freeze Substitution

Pollen tubes grown in liquid medium were filtered on 5 μm mesh nylon filters. The filters were plunged into liquid ethane at ~173°C in a Leica EM CPC cryo-preparation device. Freeze substitution was performed in anhydrous acetone containing 0.5% (v/v) glutaraldehyde by gradually increasing the temperature to 0°C over a period of 96 h. Resin infiltration was performed using LR-White hard grade resin (Electron Microscopy Sciences) in four steps with increasing resin concentration up to 100%. These samples were then left in 100% resin overnight. Resin polymerization was performed using LR-White hard grade resin (Electron Microscopy Sciences) in four steps with increasing resin concentration up to 100%. Resin polymerization was done at 50°C for 48 h.

Conventional Sample Preparation for Transmission Electron Microscopy

All fixation, washing, and dehydration steps were conducted using a microwave oven (Pelco BioWave 34700 equipped with a Pelco Cold Spot) at 150 W and 21 in of Hg vacuum for 40 s each. Pollen tubes grown in 0.5% (w/v) agar medium were fixed in freshly prepared 2% (w/v) formaldehyde and 2.5% glutaraldehyde in 0.05 M PBS buffer, pH 7.2. Samples were subsequently washed three times with PBS buffer and three times with double-distilled water. Dehydration was done using an increasing ethanol gradient ranging from 25% to 100%, with the last step repeated three times. Resin infiltration was performed using LR-White hard grade resin (Electron Microscopy Sciences) in four steps with increasing resin concentration up to 100%. These steps were conducted three times at 300 W for 2 min each, under 21 in of Hg vacuum. Samples were then left in 100% resin overnight. Resin polymerization was done at 50°C for 48 h.

Following both freeze fixation and conventional fixation, ultrathin sections were cut with a Leica Ultracut and collected on 150-mesh formvar coated nickel grids. Samples were observed with a JEOL JEM 100S transmission electron microscope operating at 80 kV.

Immunogold Labeling

Grids with ultrathin sections were floated with the sections facing down on drops of PBS buffer containing 4% (w/v) BSA for 1 h. Grids were then incubated for 30 min with 1:200 anti-callose, 1:200 CBM3a, or 1:100 JIM5 or JIM7. Samples were subsequently incubated for 30 min with 1:200 anti-callose, 1:200 CBM3a, or 1:100 JIM5 or JIM7. Samples were then rinsed several minutes with deionized, decarbonated water. Dehydration was done using an increasing ethanol gradient ranging from 25% to 100%, with the last step repeated three times. Resin infiltration was performed using LR-White hard grade resin (Electron Microscopy Sciences) in four steps with increasing resin concentration up to 100%. These steps were conducted three times at 300 W for 2 min each, under 21 in of Hg vacuum. Samples were then left in 100% resin overnight. Resin polymerization was done at 50°C for 48 h.

Sample Preparation for Scanning Electron Microscopy

Samples for scanning electron microscopy were fixed in freshly prepared 2% (w/v) formaldehyde and 2.5% glutaraldehyde in 0.05 M PBS buffer, pH 7.2. Samples were subsequently washed three times with PBS buffer. Dehydration was done using an increasing ethanol gradient ranging from 30% to 100% with the last step repeated three times. Samples were then critical point dried, gold-palladium coated, and observed with a FEI Quanta 200 3D microscope operating at 20 kV.

Microindentation

Pollen was incubated as described and after germination had occurred cover slips were submerged in the growth medium containing experimental chamber of the microindenter. The design and principles of operation of the microindenter have been described previously (Petersen et al., 1982; Elson et al., 1983). The microindentation assemblies used here were mounted on a Nikon TE2000 inverted microscope and used as described earlier (Geitmann and Parre, 2004). The motor was programmed to execute a single triangular waveform with a velocity of 4 μm s⁻¹ and a total amplitude of 10 μm.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_125870 (CesA).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Movie S1. VAEM of Arabidopsis pollen tube expressing GFP-CESA6. Images were acquired in a focal plane located tangentially at 4 frames s⁻¹ over a period of 50 s and shown here 10× accelerated. The accelerated representation allows to appreciate the movement of the GFP-CESA6 punctae in an orientation near parallel to the longitudinal axis of the tube. Width of the frame equals 21.6 μm.

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