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***Corresponding author:** Jean-Claude Mollet, Laboratoire Glyco-MEV, UPRES EA 4358, IFRMP 23, Université de Rouen, 76821 Mont Saint-Aignan, Cedex - France.

Fax: + 33235146535. E-mail address: jean-claude.mollet@univ-rouen.fr

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Biochemical and immunocytological characterizations of *Arabidopsis thaliana* pollen tube cell wall ¹[W].

Flavien Dardelle, Arnaud Lehner, Yasmina Ramdani, Muriel Bardor, Patrice Lerouge, Azeddine Driouich, Jean-Claude Mollet*

Laboratoire de Glycobiologie et Matrice Extracellulaire Végétale, UPRES EA 4358, IFRMP 23, Université de Rouen, 76821 Mont Saint-Aignan Cedex, France.

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* Corresponding author; e-mail: jean-claude.mollet@univ-rouen.fr

^[W] The online version of this article contains Web-only data.

Abstract

During plant sexual reproduction, pollen germination and tube growth require a development under tight spatial and temporal control for the proper delivery of the sperm cells to the ovules. Pollen tubes are fast growing tip-polarized cells able to perceive multiple guiding signals emitted by the female organ. Adhesion of pollen tubes *via* cell wall molecules may be part of the battery of signals. In order to study these processes, we investigated the cell wall characteristics of *in vitro*-grown *Arabidopsis thaliana* pollen tubes using a combination of immunocytochemical and biochemical techniques. Results showed a well-defined localization of cell wall epitopes. Low esterified homogalacturonan epitopes were found mostly in the pollen tube wall back from the tip. Xyloglucan and arabinan from rhamnogalacturonan I epitopes were detected along the entire tube within the two wall layers and the outer wall layer, respectively. In contrast, highly esterified homogalacturonan and arabinogalactan-protein epitopes were found associated predominantly with the tip region. Chemical analysis of the pollen tube cell wall revealed an important content of arabinosyl residues (43%) originating mostly from (1→5)- α -L-arabinan, the side chains of rhamnogalacturonan I. Finally, MALDI-TOF MS analysis of *endo*-glucanase-sensitive xyloglucan showed mass spectra with two dominant oligosaccharides (XLXG/XXLG and XXFG), both being mono *O*-acetylated, and accounting for over 68% of the total ion signals. These findings demonstrate that *Arabidopsis* pollen tube wall has its own characteristics compared to other cell types in the *Arabidopsis* sporophyte. These structural features are discussed in terms of pollen tube cell wall biosynthesis and growth dynamics.

INTRODUCTION

Fertilization of flowering plants requires the delivery of the two sperm cells, carried by a fast growing tip polarized pollen tube, to the egg cell. In plants with dry stigma and solid style such as *Arabidopsis thaliana*, this process begins with the deposition and specific adhesion of the pollen grains on the stigmatic tissue, subsequent hydration of the pollen grains and germination of pollen tubes (Palanivelu and Preuss, 2000). Pollen tubes invade the papillae cell wall of the stigma, enter the short style, and grow through the apoplast of the specialized transmitting tract (TT) cells that is filled with a nutrient-rich extracellular matrix (Kandasamy et al., 1994; Lennon et al., 1998). During this invasive growth, pollen tubes are guided to the ovules *via* signals that need to pass through the cell wall to reach their membrane-associated or intracellular targets (Lord and Russell, 2002; Kim et al., 2003; McCormick and Yang, 2005; Boavida et al., 2005; Johnson and Lord, 2006). In plant species with wet stigma and hollow style such as lily, adhesion between the pollen tube wall and the TT epidermis extracellular matrix is important for the growth of the pollen tubes toward the ovules (Mollet et al., 2000; Park et al., 2000; Chae et al., 2007; Mollet et al., 2007). In addition of being the interface between the tube cells and the surroundings (female sporophyte or culture medium), the pollen tube wall also controls the cell shape, protects the generative cells and allows resistance against turgor pressure (Geitmann and Steer, 2006; Geitmann 2010).

Most of our knowledge on cell wall polymers of higher plants comes from investigations on vegetative organs in which cells have diffuse growth. The cell wall is mainly composed of polysaccharides (cellulose, hemicellulose, pectin and occasionally callose, depending on the tissue) and proteoglycans (*e.g.* extensin and arabinogalactan proteins) forming a complex network with processing enzymes.

Pectins are complex wall macromolecules with uncertain supramolecular organization (Vincken et al., 2003) consisting of homogalacturonan (HG) that can be methyl- and acetyl-esterified, rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (Carpita and McCann, 2000). HG is a polymer of repeated units of (1→4)- α -D-galacturonic acid that can be cross-linked with calcium upon block wise action of pectin methylesterases on methylesterified HG (Micheli, 2001). RG-II has the same homopolymer backbone as HG but is substituted with four different oligosaccharides composed of unusual sugars such as apiose, aceric acid, 3-deoxy-D-manno-2-octulosonic acid (Kdo) of unknown function (reviewed by Caffall and Mohnen, 2009). RG-I consists of the repeating

disaccharide, (1→4)- α -D-galacturonic acid-(1→2)- α -L-rhamnose, unit with a wide variety of side chains attached to the rhamnosyl residues, ranging from monomers to large oligosaccharides such as (1→4)- β -D-galactan, (1→5)- α -L-arabinan, and/or type I arabinogalactan (Caffall and Mohnen, 2009).

Xyloglucan (XyG) is the major hemicellulosic polysaccharide of the primary wall of flowering plants. Classic XyG consists of a (1→4)- β -D-glucan backbone substituted with xylose, galactose-xylose or fucose-galactose-xylose motifs which correspond, according to the one letter code proposed by Fry et al. (1993), to X, L and F, respectively, G being the unsubstituted glucosyl residue of the glucan backbone. The main XyG fragments released after *endo*-glucanase treatment of the cell wall from wild type *Arabidopsis* vegetative organs are generally XXXG, XXLG/XLXG, XXFG and XLFG (Zablackis et al., 1995; Lerouxel et al., 2002; Nguema-Ona et al., 2006; Obel et al., 2009). In addition, *O*-acetylation of XyG can occur, most generally on the galactosyl residues, but its biological function is unknown (Cavalier et al., 2008). In the primary wall, XyG interacts with cellulose microfibrils *via* hydrogen bonds and participate in the control of cell expansion (Cosgrove, 1999).

Arabinogalactan-proteins (AGPs) and extensin belong to the hydroxyprolin-rich glycoproteins (HRGPs) superfamily with very high level of type II arabinogalactan glycosylation (Nothnagel, 1997; Showalter, 2001). These proteoglycans have been implicated in many aspects of plant development including cell expansion, cell signaling and communication, embryogenesis, wound response and pollen tube guidance (Wu et al., 1995; Nothnagel, 1997; Seifert and Roberts, 2007; Driouich and Baskin, 2008).

Despite the importance of pollen tubes for the delivery of the sperm cells to the egg, little is known about the underlying molecular mechanisms that regulate the mechanical interaction of pollen tubes with female floral tissues. There are very scarce data concerning the different components of the pollen tube cell wall. Past approaches to characterize the pollen tube cell wall are limited to few plant species including *Camellia* (Nakamura and Suzuki, 1981), *Lilium* (Jauh and Lord, 1996; Mollet et al., 2002), *Nicotiana* (Rae et al., 1985; Li et al., 1995; Ferguson et al., 1998; Qin et al., 2007), *Pinus* (Derksen et al., 1999) and *Zea* (Rubinstein et al., 1995) and are mostly based on immunocytochemistry. These studies revealed that, depending on the species, the pollen tube cell wall contains epitopes that are found in the polymers described above including HGs with varying levels of methylesterification, AGPs, extensin-like proteins, and low amounts of cellulose. Unlike most other plant cells, callose, a (1→3)- β -glucan, is predominant and it is deposited in the wall

back from the tip. Moreover, it is deposited in regular intervals to form callose plugs that maintain the tube cell in the apical expanding region of the tube and separate the viable from the degenerating region of the tube (reviewed by Geitmann and Steer, 2006). Only few reports have investigated the pollen tube of the model plant, *Arabidopsis thaliana*. They have focused either on *in vivo* or on *in vitro* grown pollen tubes using monoclonal antibodies (MAbs) directed against a subset of cell wall epitopes present in HG, XyG and AGPs (Lennon and Lord, 2000; Freshour et al., 2003; Pereira et al., 2006) but quantitative chemical analyses are lacking. This lack of information is most likely due to the fact that substantial amounts of pollen tube material is needed for chemical analysis and a reproducible and efficient method for liquid culture of *Arabidopsis* pollen tubes had not been established until recently (Boavida and McCormick, 2007; Bou Daher et al., 2009).

Here, we report the composition and localization of different cell wall polymers of *in vitro*-grown wild-type *Arabidopsis* pollen tubes based on biochemical analyses coupled to immunocytochemical investigations both at light and transmission electron microscope (TEM) levels using recently developed MAbs. Our results show distinct patterns of labeling (tip, whole tube, and shank of the tube) depending on the recognized epitope. The most striking observations are (1) the abundance of (1→5)- α -L-arabinan in the tube wall (> 40 mol% of arabinose), mostly localized, with LM6 and LM13, in the outer wall layer of the tube and (2) an atypical XyG MALDI-TOF MS profile with over 68% of the oligosaccharide fragments being *O*-acetylated.

RESULTS

In vitro pollen tube growth and β -glucan localization

In order to study the cell wall of *Arabidopsis* pollen tubes, sufficient material was necessary to perform the biochemical analysis. To achieve this, a large number of flowers, a good *in vitro* pollen germination rate with sufficient pollen tube length were required. As shown in Fig. 1A, high rates of pollen germination were obtained ($67\% \pm 12$) with an average of pollen tube length of $1248 \mu\text{m} \pm 374$ and a growth rate of $0.46 \mu\text{m}\cdot\text{min}^{-1} \pm 0.05$ after 16h of culture in liquid medium. Moreover, 16h-old pollen tubes displayed typical tip organization (Fig. 1A insert) without any plasmolysis event. These results showed that using these *in vitro* conditions, pollen tube length is approaching what we should expect in *in vivo* condition given the distance between stigma and ovules. By comparison, 6h-old pollen tubes were four-fold shorter with an average length of $307 \mu\text{m} \pm 102$ and an estimated growth rate of $1.6 \mu\text{m}\cdot\text{min}^{-1} \pm 0.17$.

Cytochemical staining of β -glucans with aniline blue (for callose) and calcofluor white (mainly callose and cellulose) was performed in order to visualize the distribution of these β -glucans along the tube wall and within the tube. Aniline blue staining was not detectable in the tip region and was mainly localized in the wall, back from the tip, and within the tube as callose plugs that are periodically synthesized (Fig. 1B; Table I). In contrast, staining with calcofluor white was uniform along the whole tube and the tip. The bright regions within the tubes revealed several plugs (Fig. 1C). At the ultrastructural level, the Arabidopsis pollen tube wall back from the tip appeared as a bi-layered structure consisting of a fibrillar outer and a weakly electron-dense inner wall layer (Fig. 2A). Immunogold localization of callose resulted in dense labeling in the inner wall layer back from the tip (Supplemental data, Fig. S1A).

Immunolocalization of Arabidopsis pollen tube cell wall epitopes

Immunolabeling pattern of pectin domains (Table I). Probing with JIM7 and JIM5 that recognize partially and highly methylesterified HGs revealed that JIM7 epitopes were dominantly localized in the tip region of the pollen tubes (Fig. 1D). Similar observations were made at tube tips emerging from pollen grains (Fig. 1D insert). In contrast, JIM5 staining was observed mostly back from the tip with brighter ring-like deposits presumably originating from successive temporary slow-downs in the growth of the tubes (Fig. 1E). LM8, specific for xylogalacturonan epitopes, uniformly labeled the whole pollen tube wall (Figs. 1F, 1F insert). (1 \rightarrow 5)- α -L-arabinan epitopes, localized with LM6 and LM13, were clearly detected in the entire pollen tube wall (Figs. 1G, 1I) with stronger fluorescent signal at the pollen tube tip (Figs. 1H, 1J). A ring-like structure was also noticeable as a collar at the emergence of the pollen tube from the pollen grain (Fig. 1G). Immunogold labeling of epitopes recognized by LM6 was mostly found in the fibrillar outer wall layer (Figs. 2B-2C). A strong concentration of gold particles was also observed over densely stained vesicles possibly associated with the *trans* Golgi network (Fig. 2B). Epitopes recognized by LM6 were also detected in the intine wall of the pollen grain (Supplemental data, Fig. S1B).

Immunolabeling of xyloglucan motifs. To investigate the distribution of XyG, pollen tubes were probed with the MAbs LM15 and CCRC-M1 that recognize non-galactosylated (XXXG motif) and fucosylated XyG domains, respectively. As shown in Figs. 1K and 1L, both MAbs bound to the entire pollen tube wall.

TEM observations showed that fucosylated XyG epitopes probed with the MAb CCRC-M1 were mainly found in the inner pollen tube wall (Fig. 2D), whereas the non-galactosylated XyG epitopes tagged with LM15 were detected in both the inner and outer layers of the wall (Figs. 2E-2F; Table I). Immunolabeling of pollen grains with the LM15 and CCRC-M1 MAbs resulted in a strong detection of XyG epitopes, mostly in the intine wall and at the vicinity of the plasma membrane (Supplemental data, Figs. S1C-S1D).

Immunolabeling of arabinogalactan-proteins. Epitopes of AGPs recognized by the MAb LM2 were found slightly labeled throughout the pollen tube wall with a stronger signal in the tip region (Figs. 1M-1N; Table I). Similar results were observed with MAC207 whereas JIM13 did not label the tube cell wall (Table I).

Immunolabeling at fluorescent microscopy level was carried out on 6h and 16h-old pollen tubes and did not show any difference in labeling pattern between the two time points (data not shown). Controls for immunofluorescent labeling did not show any fluorescence of the pollen tubes and a weak autofluorescence of the pollen grain exine (data not shown). Controls for immunogold detection did not exhibit any significant non specific gold particles on the resin, pollen tubes and pollen grains (Supplemental data, Figs. S1E-S1H). A summary of immunolocalization data on the Arabidopsis pollen tube wall is shown in Table I.

Contribution of pollen grain wall *versus* pollen tube wall for biochemical analysis

We estimated the relative abundance of the pollen tube cell wall *versus* the pollen grain cell wall by measuring the cell wall thickness based on MET observations, pollen germination rates and pollen tube length (Supporting Table SI). The estimated ratio of the pollen grain cell wall on the pollen tube cell wall was about 1:7.4. Similarly, we also quantified by gas chromatography, the amount of carbohydrates present in non-germinated pollen grains and 16h-old germinated pollen tubes isolated from 520 flowers (Supplemental data, Fig. S2). Monosaccharide composition of the pollen grain and the pollen tube cell walls was similar and the amount of carbohydrates contained in the pollen grain cell wall was eight-fold lower than the amount of carbohydrates contained in the pollen tube cell wall. These data indicate that the quantity of pollen grain cell wall material would interfere only weakly in the cell wall analysis and were used as a baseline for the monosaccharide composition of Arabidopsis pollen tubes.

Monosaccharide composition of the Arabidopsis pollen tube wall

Monosaccharide analysis of Arabidopsis pollen tube cell wall (Table II) displayed a high level of glucose (19.8 mol%) originating probably mostly from callose and to a lesser extent from XyG polymers. The most abundant monosaccharide, arabinose (43.6 mol%), and galactose (8.4 mol%) may come from AGP glycans and/or the pectic RG-I domain. Indeed, both sugars composing the backbone of RG-I (*i.e.* rhamnose and galacturonic acid) were present in the extract in equimolar amounts (~5 mol%). The remaining galacturonic acid (~5 mol%) composes undoubtedly the pectic HG backbone. XyG polymers generally consist of Xyl and Fuc residues that are also found in this analysis although in low amounts (1.2 mol% Fuc and 6.2 mol% Xyl) suggesting that XyG is not a major cell wall component of pollen tube cell wall.

The sequential extraction of the Arabidopsis pollen tube cell wall with ammonium oxalate and KOH aimed at yielding pectin- and hemicellulose-enriched fractions. Both extracts were analyzed for monosaccharide composition by gas chromatography (GC) and linkage/substitution sites by GC-mass spectrometry (MS) (Table II). The oxalate extract showed similar levels of uronic acid content by colorimetric assay (21.5 mol%) and GC (24.9 mol%) analysis. This extract was enriched in galacturonic acid (21.5 mol%) indicating the presence of the pectin domains HG and highly branched RG-I, as shown by the detection of 4.2 mol% of rhamnose, 24.7 mol% of arabinose and 18.3 mol% of galactose. These data are supported by the immuno-dot assay (Fig. 3) showing a strong interaction of the oxalate extract with JIM5 for partially methylesterified HG and LM6 for (1→5)- α -L-arabinan. This labeling was consistent with the presence of 28.2% of 5-Araf (Table II). Labeling with LM5 was weak which suggests a low amount of (1→4)- β -D-galactan. This observation was confirmed by the detection of only 1.5% of 4-linked galactosyl residues (Table II). Instead, galactosyl residues detected in the monosaccharide analysis originate probably from type II arabinogalactans as shown by the detection of 3-Gal, 6-Gal and 3,6-Gal (Table II).

In the KOH extract, levels of HG (galacturonic acid) and RG-I backbone (rhamnose and galacturonic acid) decreased significantly compared to the oxalate extract but the remaining RG-I domains were probably highly substituted by arabinans and/or arabinogalactans, as both arabinosyl and galactosyl residues counted for 37 mol% of the total sugars (Table II). The dot-blot immuno-assay (Fig. 3) revealed a significant reduction of the

signal in the KOH extract with JIM5 compared to the oxalate extract indicating that KOH treatment had substantially removed methylester groups from the partially methylesterified HG leaving low amount of non-esterified HG. In contrast, labeling for (1→5)- α -L-arabinan with LM6 was still strong (Fig. 3) and was confirmed with the detection of 14.6% of 5-Araf (Table II). The different components of XyG (*i.e.* Glc, Xyl, Gal and Fuc) were also present in the KOH extract (Table II), with a large increase in the glucose level (40.1 mol%) compared to the oxalate extract. As revealed by methylation analysis, the latter originated from XyG (2.2% 4,6-Glc), starch and/or amorphous cellulose (15.4% 4-Glc) but dominantly from callose with the presence of 3-Glc (27.6%) and possibly 2,3-Glc (7%) which may suggest that callose can be substituted on the C2 of the glucosyl residues (Table II). The MAb LM15 reacted strongly with both oxalate and KOH extracts (Fig. 3) suggesting that XyG were present in both extracts.

Matrix-Assisted Laser Desorption/Ionisation (MALDI) Time Of Flight (TOF) Mass Spectrometry (MS) analysis of pollen tube cell wall xyloglucan fragments

Analysis by MALDI-TOF MS of XyG fragments released after xyloglucan *endo*-glucanase treatment of Arabidopsis pollen tube cell wall (Fig. 4A) showed the typical ions, XXXG, XLXG/XXLG and XXFG (14.7%, 5.7% and 8.4%, respectively), found in XyG (Table III). However, these oligosaccharides were not the main motifs. Instead, the two principal ions identified (XLXG/XXLG and XXFG) present a shift of $m/z = 42$, characteristic of an *O*-acetyl group, branching presumably the galactosyl residue. These two *O*-acetylated fragments count for over 68% of the total oligosaccharide released. Overall, galactosylated and fucosylated xyloglucan oligosaccharides showed a relative abundance of 85% and 52%, respectively. By comparison, the main XyG fragment released after *endo*-glucanase treatment of Arabidopsis leaf cell wall was XXXG (Supplemental data, Fig. S3) and the total level of *O*-acetylated fragments was 33%. Moreover, based on the signal intensity, the ratio of Fuc to Xyl can be estimated to 1:5.7 similar to what was found in the compositional analysis of the pollen tube wall extract (1:5.2) which suggests that other xylose-containing polymers such as xylan may not be present or in very low amount in the pollen tube cell wall. Finally, monosaccharide analysis of the *endo*-glucanase-resistant pollen tube cell wall residue (Fig. 4B) revealed, despite a clear decrease in the carbohydrate content of Xyl, Gal and Fuc compared to the untreated cell wall material, that all the XyGs have not been completely

degraded by the enzyme, perhaps due to their tight association with other cell wall components.

DISCUSSION

Since the AGI (2000), increasing genomic, transcriptomic and proteomic data on *Arabidopsis* have become available (Becker et al., 2003; Honys and Twell, 2004; Tung et al., 2005; Qin et al., 2009; Zou et al., 2009). The use of male and female gametophyte or sporophyte mutants has allowed major insight into pollen formation, pollen tube growth, guidance and signaling (Rhee et al., 2003; Dong et al., 2005; Escobar-Restrepo et al., 2007), but the biosynthesis, the remodeling and the overall role of the cell wall of the tip growing pollen tube cell is far from being fully understood. Using the *in vitro* pollen tube method developed by Boavida and McCormick (2007), we obtained high levels of pollen germination ranging from 55% to 79% with pollen tubes that can expand over 1.5 mm after 16h of culture. Based on this, we decided to investigate one of the main components, the cell wall, of this tip-growing cell by means of immunolocalization at light and electron microscopy levels and analytical polysaccharide biochemistry. Our results showed that (1) the pectin wall is enriched in HG and (1→5)- α -L-arabinan, and (2) *O*-acetylation of XyG fragments released by *endo*-glucanase is high compared to other sporophytic tissues.

Most of the precursor studies on pollen tube wall of the so-called “pollen tube model plants” (*i.e.* *Nicotiana* or *Lilium*) employed MAbs recognizing the HG pectin domains (JIM5 and JIM7) and a subset of carbohydrate epitopes of the cell surface AGPs (mainly JIM13, MAC207, LM2 and JIM8). To date, only scarce reports on pollen tube wall have been published using other well-defined MAbs such as the anti-XyG (CCRC-M1; Freshour et al., 2003) and, to our knowledge, none with the more recently produced MAbs on pollen tube wall.

Arabidopsis pollen tube wall is composed of an inner layer enriched in callose and an outer fibrillar layer

TEM observations of *in vitro* rapid frozen *Arabidopsis* pollen tubes showed the typical two wall layers at the shank of the tip (a fibrillar outer and a weakly electron-dense inner wall layer) as observed in *in vitro*-grown tobacco pollen tube (Li et al., 1995; Ferguson et al., 1998) and *in vivo*-grown pollen tube from lily (Roy et al., 1997) and *Arabidopsis* (Lennon and Lord, 2000; Derksen et al., 2002). Our data and those of others have shown that callose is the

main component of the inner wall layer but it is not detectable at the tip whereas cellulose is weakly detected (Ferguson et al., 1998).

Arabidopsis pollen tube wall contains a subset of AGPs recognized by LM2

We found that AGPs were labeled in the whole tube with a stronger signal at the tip with LM2 whereas JIM13 labeling was almost absent. In contrast, JIM13 labeled lily pollen tube at the tip (Mollet et al., 2002), uniformly or in a ring-like deposition along the tube of tobacco (Li et al., 1992, Qin et al., 2007). Pereira et al. (2006) also noted a lack of labeling of Arabidopsis pollen tube with JIM13 unlike MAC207, which labels uniformly the whole tube (Coimbra et al., 2008). These divergent data depend on the MAb used (despite the apparent identical epitope, β -D-GlcPA-(1 \rightarrow 3)- α -D-GalpA-(1 \rightarrow 2)-L-Rha, recognized by MAC207 and JIM13) and the species investigated. These differences may suggest that the carbohydrate moieties of AGPs are species and/or cell type specific (vegetative and sperm cells) or that the cell wall organization is different in these species which may alter the accessibility of the epitopes.

HG pectin domains are present in different location in Arabidopsis pollen tube wall

Immunofluorescent labeling with JIM5 and JIM7 shows a pattern similar to that observed in pollen tubes from species possessing a solid style such as the *Solanaceae* (potato, tobacco, petunia), *Oleaceae* (jasmine) and *Poaceae* (corn) (Li et al., 1994; Qin et al., 2007). A dominant localization of highly methylesterified HG, recognized by JIM7, was present at the tip and a periodic patterning of low methylesterified HG epitopes, labeled with JIM5, was visible behind the tip region. Pollen tube wall contains 10 mol% of GalA, half of it being presumably dedicated to HG building, which explains the weak labeling by JIM5 along the wall compared to other species (Jauh and Lord, 1996; Qin et al., 2007) even though reduced accessibility of the MAb to the epitopes may occur due to other polymers. In fact, the pectin-enriched extract isolated from the pollen tube wall showed a strong interaction with JIM5 (Fig. 3). Highly methylesterified HG and other pectic motifs are deposited at the growing tip by Golgi-derived vesicles and de-esterified *via* pectin methylesterases (PMEs) in the wall back from the tip, to allow calcium cross linking of the carboxyl groups (Geitmann and Steer, 2006). In the tobacco pollen tube, two PME isoforms have been localized in the Golgi, secretory vesicles and at the outer surface of the plasma membrane where they may deesterify HG (Li et al., 2002). Two Arabidopsis mutants, *vanguard1* (Jiang et al., 2005) and *Atppme1* (Tian et al., 2006), defective in two different pollen specific PMEs, showed slight reduction

(about 20%) of overall PME activity, reduced growth compared to wild type pollen tubes but different pollen tube phenotypes. *Vanguard1* pollen tubes were unstable *in vitro* resulting in tip bursting and *in vivo* *vanguard1* plants show reduced male fertility. In contrast, *atppme1* pollen tubes showed *in vitro* branching pattern with numerous tips but did not show male sterility *in vivo*. These reports suggest that these mutant pollen tubes have modified cell wall mechanical properties, despite a lack of either immunolocalization or biochemical data showing a modification in the methylesterification level of the HG motif. It is noteworthy to point out, as suggested by Jiang et al. (2005), that these PMEs may also have a role in modifying the female TT wall to facilitate pollen tube progression through the female tissue. *AtPPME1* and *VANGUARD1* are among a group of 15 other pollen specific genes that are separated in two groups (I and II) and encode putative PMEs containing either a catalytic domain only (5 genes), or containing a catalytic domain and a putative PME inhibitor (PMEI) domain (10 genes) (Pelloux et al., 2007; Chen and Ye, 2007). More studies on these important protein members are required to understand the fine-tuning of demethylesterification of HG, to determine the interaction between PME and PMEI and their roles in modifying the pollen tube mechanics and sensing (Bosch et al., 2005). Low esterified HG have been implicated in important physiological processes such as cell attachment in vegetative cells or organs (Bouton et al., 2002; Leboeuf et al., 2005; Durand et al., 2009) and, associated with a stigma/style cysteine-rich adhesin (SCA), a secreted plant lipid transfer protein (LTP), in lily pollen tube adhesion (Mollet et al., 2000; Park et al., 2000). Several LTPs are present in the TT of the *Arabidopsis* pistil along the pollen tube path (Tung et al., 2005). Recently, the LTP5, produced in *Arabidopsis* pollen tube and in the pistil TT, has been proposed to play important roles in maintaining cell polarity at the tube tip and adhesion-mediated guidance perhaps by interaction with pectins (Chae et al., 2009).

***Arabidopsis* pollen tube wall is enriched in pectin Arabinan**

In addition to HG motifs, *Arabidopsis* pollen tube wall contains highly branched RG-I with arabinan. Our results based on immunolocalization using LM6 and LM13, both recognizing (1→5)- α -L-arabinan epitopes, and biochemical analysis indicate firstly, a strong and evenly distributed labeling in the shank of the tube with a slight increase of the signal in the tip region. Secondly, the most striking point is the high level of arabinosyl residues present in the pollen tube wall, oxalate and KOH extracts, which counts for over 40 mol% of the total analyzed sugar. Chemical analysis of the pollen tube cell wall from tobacco (Rae et al., 1985), revealed also a high level of arabinose (between 15.4 and 26.4 mol% depending on

the hydrolysis method) mostly 5-linked and to a lesser extent 5,2-linked. Moreover, tobacco pollen tube wall displayed low level of galactosyl residues and uronic acids (Rae et al., 1985). Similarly, *Lilium*, *Camellia* and *Tulipa* pollen tube wall showed also high levels of arabinosyl residues (Nakamura and Suzuki, 1981). Analysis of Golgi vesicles from *Camellia* pollen tubes were also enriched in arabinose, galactose and uronic acid (Hasegawa et al., 1998). All together, our data suggest that *Arabidopsis* pollen tube wall contains short RG-I backbone harboring long chains of (1→5)- α -L-arabinans in the outer wall layer. The significance of this common feature (high level of arabinan) between the pollen tubes originating from non-related species is not known but may suggest an important role of this polymer in pollen tube biology. The role of arabinan side chains of RG-I is not clear. Indeed, mutation in *ARABINAN DEFICIENT 1* gene (*ARADI*), a putative arabinosyltransferase involved in the biosynthesis of pectic arabinan in *Arabidopsis*, showed high reduction of arabinan side chains of RG-I without any compensation by other polymers. However, no obvious phenotype was detected, suggesting that a loss of arabinan in RG-I does not affect vegetative growth (Harholt et al., 2006). On the other hand, plants exhibiting mutations in two *Reversibly Glycosylated Peptides* (*RGP1* and *RGP2*) that may be involved in cell wall biosynthesis showed a strong defect in the inner pollen wall and pollen lethality (Drakakaki et al., 2006). Interestingly, rice RGPs show strong amino acid sequence identity (~80%) with UDP-arabinopyranose mutase implicated in UDP-Arap and UDP-Araf interconversion (Konishi et al., 2007). We might speculate that RGP may be part of the arabinan biosynthesis network but further biochemical studies are required to validate this as many other molecules contain arabinosyl residues such as arabinoxylans and arabinogalactans. In *Commelina communis*, arabinan side chains of RG-I have been implicated in guard cell opening and closing. The authors suggested that they may prevent HG polymers from forming tight associations (Jones et al., 2003). A similar effect may occur between the pollen tube wall and the TT cell wall. Finally, pectic arabinans have also been implicated in cell attachment (Iwai et al., 2001; Orfila et al., 2001; Leboeuf et al., 2004, 2005; Peña and Carpita, 2004). Adhesion of pollen tubes to the TT cells may be an important cue for guiding the tube cell within the female tissue as was shown in the hollow style plant, lily (Mollet et al., 2007). Investigation on mutant pollen defective in arabinosyltransferase will undoubtedly give insight into the function of arabinan side chains of RG-I in pollen/pollen tube biology.

***Arabidopsis* pollen tube wall endo-glucanase-sensitive XyG is highly acetylated**

MAbs directed against two different XyG epitopes labeled the whole tube mostly in the outer wall layer and the in the tip. Oligosaccharide fragments containing these two epitopes (XXXG and XXFG) were also detected with MALDI-TOF MS after pollen tube cell wall digestion. Interestingly, XyG analysis from vegetative organs (leaf, stem or root), revealed consistently that the main fragments are XXXG and XXFG (Zablackis et al., 1995; Pauly et al., 2001; Lerouxel et al., 2002). Arabidopsis pollen tube XyG shows distinct mass spectra with two major fragments detected (XLXG/XXLG and XXFG) both being substituted by one acetyl group, presumably linked to the galactosyl residue. The signal intensity of these two fragments consistently accounted for over 68%. Recent investigations on XyG from leaf tissues isolated after laser microdissection has allowed a more detailed analysis showing subtle modification of XyG (Obel et al., 2009). As an example, the level of acetylation of XLFG fragments in the mesophyll tissue was significantly higher than in the vascular tissue but the overall acetylation level in these tissues did not exceed 35-40% (Obel et al., 2009). Together, this indicates that subtle changes in the acetylation level of XyG can occur but the function of these hydrophobic groups in cell wall biology is still unknown.

XyG are known to interact with cellulose microfibrils. Despite the low amount of cellulose in the pollen tube wall (Doblin et al., 2001), cellulose is present at the tip and plays an important role in stabilizing the pollen tube tip wall. Cellulase treatment on growing pollen tubes resulted in tip swelling and eventually bursting (Aouar et al., 2010). Cellulose at the tip region may also serve as an interacting partner for XyG cross-linking. Acetylation of XyG does not seem to play a major role in the interaction with cellulose. *In vitro* experiments have shown that native (acetylated) and de-acetylated XyG were able to cross-link cellulose microfibrils in similar manner (Whitney et al., 2006). In contrast, the high molecular weight of the XyG (880 kDa) and the presence of galactosyl residues appeared to be necessary to promote the interaction with cellulose. A lack of galactose in the XyG resulted in the self-association of XyG polymers instead of the interaction between XyG and cellulose (Whitney et al., 2006). Similarly, Arabidopsis mutants with XyG lacking xylosyl, galactosyl or fucosyl residues showed reduction of tensile strength (Peña et al., 2004) or abnormal bulging in the tip growing root hairs possibly due to impaired cellulose-XyG assembly (Nguema-Ona et al., 2006; Zabolina et al., 2008). A similar phenotype was also noticed in plants exhibiting double mutations in the two XyG xylosyltransferases 1 and 2 genes (*XXT1* and *XXT2*) that are apparently lacking XyG (Cavaliere et al., 2008). In the moss *Physcomitrella*, AGPs were found to be enriched in 3-*O*-Me-rhamnosyl residues compared to AGPs from more evolved plant species. This suggests a possible hydrophobic interaction of AGPs, which for a long time had

been considered to have large capacity of interacting with water (Fu et al., 2007). All together, acetyl groups of XyG may promote hydrophobic interactions with other molecules within the pollen tube cell wall but also with other female components of the TT extracellular matrix during the intrusive growth of pollen tubes. Moreover, acetylation may modulate water uptake due to the non-polar property of these groups, but this requires further investigation.

In summary, this study provides a solid foundation for the use of *Arabidopsis* pollen tubes in cell wall biology and for the investigation of the male gametophyte cell elongation with mutants impaired in proteins implicated in the biosynthesis of specific cell wall polymers (XyG, HG, RG-I, RG-II and AGPs), cell wall deposition and cell wall remodeling. It may also help to dissect the mechanisms controlling the interaction between the pollen tube wall with the female counterpart. The challenge in this field is to overcome the generally observed phenotypes such as arrest of pollen formation, lack of pollen viability, absence of pollen tube germination or growth resulting in reduced male fertility when mutations are located in important male gametophyte genes (Lalanne et al., 2004; Iwai et al., 2006; Drakakaki et al., 2006; Delmas et al., 2008; Boavida et al., 2009). Chemical genetic screens on *in vitro*-grown pollen tubes may be an alternative. Nevertheless, studies on the two distinct features of *Arabidopsis* pollen tube wall (high levels of pectic arabinan side chains and XyG acetylation) require closer attention and investigation to better understand their possible roles in pollen tube growth, signaling, guidance and adhesion.

MATERIAL AND METHODS

Plant materials

Arabidopsis thaliana ecotype Columbia seeds stored at 4°C were spread on the surface of a sterile soil and cultured in a growth chamber with a photoperiod of 16h light/8h dark cycle at 20°C during the light phase and at 16°C in the dark phase with 60% humidity and daily watering. Only recently open flowers were collected.

***In vitro* pollen tube growth**

Pollen was grown *in vitro* in a liquid medium according to the method described by Boavida and McCormick (2007). Briefly, flowers (forty per 1.5 mL tube) were submerged in 1 mL of germination medium (GM) containing 5 mM CaCl₂, 2H₂O, 0.01% (w/v) H₃BO₃, 5 mM KCl, 1 mM MgSO₄, 7H₂O and 10% (w/v) sucrose, pH 7.5. Tubes were shaken with a vortex to release the pollen grains from the anthers. Flowers were removed with a pair of

tweezers and the pollen suspension was then pelleted down at 3200g for 6 min. New GM (250 μ L) was added to the pellet and pollen grains were transferred into glass vials (14 x 45 mm) and grown in a growth chamber, in the dark at 22°C. For large scale culture, pollen from 200 flowers was grown in glass vials (25 x 50 mm) in 1.25 mL of GM. Using this harvesting method, the number of pollen grains collected per flower was estimated to 291 ± 63.3 ($n = 10$ independent experiments). Pollen tubes were grown for 6h for TEM sample preparation, 6h and 16h for immunolabeling at fluorescent microscopy level and 16h for biochemical analysis. Before any further manipulation, pollen germination and pollen tube growth were assessed under an inverted microscope.

Cytochemical staining

Calcofluor white (0.01% w/v) and decolorized aniline blue (0.1% w/v) in 100 mM K_3PO_4 pH 11 (Johnson-Brousseau and McCormick, 2004) were used to localize β -glucans (cellulose and callose) and callose, respectively.

Monoclonal antibodies

JIM13, MAC207 and LM2 MAbs recognize a carbohydrate moiety of AGPs (Yates et al., 1996). Extensins were probed with LM1 (Smallwood et al., 1995). The MAbs JIM5 and JIM7 recognize different levels of esterification of HG regions of pectins (Clausen et al., 2003). (1 \rightarrow 4)- β -D-galactans were probed with LM5 (Jones et al., 1997) and (1 \rightarrow 5)- α -L-arabinans with LM6 (Willats et al., 1998) and LM13 (Verhertbruggen et al., 2009). The MAbs directed against XyG were CCRC-M1 which recognizes an α -Fuc-(1 \rightarrow 2)- β -Gal epitope (Puhlmann et al., 1994) and LM15 that binds to XXXG motif (Marcus et al., 2008). MAbs were kindly provided by P. Knox (Univ of Leeds, UK) and M. Hahn (CCRC, Univ of Georgia, USA) or purchased at Plant Probes (Leeds, UK). Finally, callose was localized with a mouse anti-(1 \rightarrow 3)- β -glucan (Biosupplies Australia).

Immunolocalization of Arabidopsis pollen tube wall epitopes

Pollen tubes in GM were mixed v/v with a fixation medium containing 100 mM PIPES buffer, pH 6.9, 4 mM $MgSO_4 \cdot 7H_2O$, 4 mM EGTA, 10% (w/v) sucrose, 5% (w/v) formaldehyde and incubated for 90 min at room temperature. Pollen tubes were rinsed 3 times by centrifugation with 50 mM PIPES buffer, pH 6.9, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM EGTA and 3 times with phosphate-buffered saline (PBS) (100 mM potassium phosphate, 138 mM NaCl,

2.7 mM KCl, pH 7.4) supplemented or not with 3% fat free milk. Primary antibodies were diluted at 1/5 or 1/10 as previously described (Mollet et al., 2002) with PBS (with or without 3% milk). Pollen tubes were rinsed with the buffer and incubated overnight at 4°C in the dark with the secondary antibody combined with fluorescein isothiocyanate (FITC, Sigma) diluted at 1/50 with the appropriate buffer for 3h at 30°C. For JIM, LM and MAC antibody detection, goat anti-rat (immunoglobulin G, whole molecule)-FITC was used, for CCRC antibody detection, sheep anti-mouse (immunoglobulin G, whole molecule)-FITC was used. Controls were carried out by incubation of the pollen tubes with the secondary antibody only.

Microscope observation and acquisition of pollen tube pictures

Pollen tubes were observed under Nomarski differential interference contrast (DIC) optics or fluorescence illumination on a Leica DLMB microscope equipped with FITC (absorption, 485–520 nm; emission, 520–560 nm wavelength) or calcofluor white filter sets. Pictures were acquired with a Leica DFC300FX camera.

Pollen count and germination rate were estimated with a Nageotte chamber. A pollen grain was considered germinated if the pollen tube length was greater than the pollen grain diameter. Pollen tube length was measured from the picture using the nih program ImageJ (Abramoff et al., 2004). At least 350 pollen tubes were measured in three independent experiments.

Electron microscopy preparation of *in vitro* grown pollen tubes

High pressure freezing/Freeze substitution (HPF/FS) sample preparation. Centrifuged 6h-old pollen tubes were transferred into the cavity of gold cupules (200 µm in depth and 1.2 mm in diameter) coated with soybean lecithin (100 mg.mL⁻¹ in chloroform). Excess medium was removed using a filter paper. Then, samples were frozen using a high-pressure freezing EM-PACT (Leica), according to a maximum cooling rate of 10000°C/s, incoming pressure of 7.5 bars and working pressure of 4.8 bars. Cupules containing frozen samples were stored in liquid nitrogen until the freeze substitution procedure was initiated.

After high-pressure freezing, samples were transferred to a freeze substitution automate (EM-AFS, Leica) precooled to -140°C. Freeze substitution conditions followed a modified procedure from D. Studer (personal communication). Substitution media were composed of 2% osmium in anhydrous acetone. Samples were substituted at -90°C for 72h. The temperature was gradually raised (2°C.h⁻¹) to -60°C and stabilized during 12h, then gradually raised (2°C.h⁻¹) to -30°C (12h) and gradually raised again (2°C.h⁻¹) to 0°C for 2h.

Samples were washed at room temperature with fresh anhydrous acetone. Infiltration was done at +4°C in acetone-Spurr resin (ratio 2:1; 1:1; 1:2, 8h each step) and with pure resin for at least 2 days. Polymerization was performed at 60°C for 16h. Using an ultracut EM-UC6 (Leica), thin sections (90 nm) were mounted on formvar-coated nickel grids.

Immunogold labeling and TEM observation. Grids were rehydrated in a TBS + BSA 0.2% buffer and blocked in a TBS/BSA 0.2%/milk 3% solution for 30 min. After 3 brief rinses in TBS/BSA 0.2% solution, grids were incubated 3h at 25°C in primary antibodies: non-diluted for LM15 and CCRC-M1 or diluted (LM6: 1/2; anti-callose: 1/100) in TBS/BSA 0.2% buffer. Then, grids were washed (6 x 5 min) in TBS/BSA 0.2% and incubated for 1h at 25°C in a 1/20 secondary antibody (goat anti-rat for LM6 and LM15 or goat anti-mouse for CCRC-M1 and anti-callose) conjugated to 10 nm gold particles (British Biocell Intl). Finally, grids were washed 6 x 5 min in a TBS + BSA 0.2% buffer, 1 min in TBS, 10 min in TBS + glutaraldehyde 2%, 5 min in TBS then 2 x 5 min in ddH₂O.

The sections were stained with 0.5% (w/v) uranyl acetate in methanol for 10 min in the dark, rapidly rinsed 10 times with water and stained with lead citrate for 10 min and briefly rinsed 10 times with water.

Grids were observed at 80 kV with a TEM (Tecnai 12 Bio-Twin, Philips, FEI) and pictures were acquired with an Erlangshen ES500W camera (Gatan).

Cell wall extraction of Arabidopsis pollen tubes

16h-old pollen tubes from pollen grains from 8500 flowers collected manually were pooled after addition of three volumes of EtOH 95% to the GM. This experiment was performed three times to allow three independent replicates. Pollen tubes were centrifuged at 5000g and rinsed three times with EtOH 70% to remove salts and sucrose from the GM. The insoluble material (3 x 12 mg) was ground and then treated three times with EtOH 70% at 70°C for 15 min followed by incubation with a mixture of chloroform:methanol (1:1, v/v). The remaining insoluble material was then dried to yield the cell wall fraction (3 x 10 mg). As baseline control, cell wall from non-germinated pollen grains was extracted. A similar extraction method was applied to Arabidopsis mature rosette leaves to yield a leaf wall extract except that an acetone incubation was added after the chloroform:methanol treatment for pigment removal. This extract was used to compare the XyG fragments analyzed by MALDI-TOF MS with the pollen tube cell wall extract.

As described by Ray et al., (2004), a pectin enriched fraction was obtained by extraction with boiling ammonium oxalate 0.5% (w/v) for 1h. After centrifugation, the supernatant was dialyzed against ddH₂O and freeze-dried. The ammonium oxalate insoluble residue was then treated with 4M KOH supplemented with 20 mM NaBH₄ at room temperature for 12h. The alkaline soluble fraction was acidified to pH 5.5 with acetic acid, dialyzed against water and freeze-dried, to yield a hemicellulose-enriched fraction.

Analysis of cell wall polysaccharides

Photometric assays. Total uronic acid and neutral sugars were estimated with the *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973) and the phenol sulfuric method (Dubois et al., 1956) with galactose and galacturonic acid as standards. Estimation of uronic acid and neutral sugar contents were calculated after correction of the interference of galacturonic acid with the phenol sulfuric and galactose with the mHDP assays.

Immuno-dot blot assays. Ammonium oxalate and KOH extracts (8, 4, 1 and 0.5 µg) were blotted onto nitrocellulose membrane. Immuno-dot blots were processed according to Jauh and Lord (1996). Arabinan from sugar beet (Megazyme), gum arabic from acacia (Fisher), pectin with 8.6% *O*-methylation from citrus fruits (Sigma) and XyG from tamarind seeds (Megazyme) were used as controls. MAb binding was revealed with a goat anti-rat IgG (whole molecule) antibody conjugated with alkaline phosphatase (Sigma) diluted 1:1000 and developed with the NBT-BCIP kit (Promega).

Gas chromatography mass spectrometry. Monosaccharide compositions of the total cell wall, pectin and hemicellulose enriched fractions were determined by gas liquid chromatography according to York et al. (1985) using inositol as internal standard. Briefly, each fraction (500 µg) was treated with 1 M methanolic-HCl at 80°C for 16h and the free monosaccharides were converted to their methyl glycosides. After silylation at 110°C for 20 min, samples were dried, dissolved in cyclohexane, and analyzed using a GC 3800 Varian gas chromatography system equipped with a DB1 capillary column and a flame ionization detector. A temperature program optimized for separation of the most common cell wall monosaccharides (Ara, Fuc, Gal, GalA, Glc, GlcA, Man, Rha, Xyl) was used. Data were analyzed and integrated using Varian GC Star Workstation software with the quantity of each monosaccharide corrected according to its response factor.

Preparation and linkage analysis of the partially methylated alditol acetates of pectin-

and hemicellulose-enriched fractions were performed according to Smith et al. (1994) except that the ultrasonic bath was performed for 1h instead of 15 min, dichloromethane was used instead of chloroform and a stream of air was used to dry samples instead of nitrogen. The resulting partially methylated alditol acetates were separated by GC (Hewlett-Packard 6890) on a Optima 5-MS capillary column (length 30 m, i.d. 0.25 mm, Macherey Nagel) and analysed by electron impact mass spectrometry using an Autospec mass spectrometer (Micromass) equipped with a Opus 3.1 data system.

Preparation of XyG oligosaccharides. 0.5 mg of pollen tube cell wall or leaf cell wall extracts were incubated under agitation for 16h at 37°C with 500 μ L of an endo-(1 \rightarrow 4)- β -D-glucanase (5 Units, EC 3.2.1.4, Megazyme) prepared in 10 mM ammonium acetate buffer pH 5.0. Glucanase-resistant material was removed by centrifugation after addition of EtOH to reach a final concentration of 80%. The EtOH soluble XyG oligosaccharides were concentrated by evaporation under a stream of air.

MALDI-TOF mass spectrometry analysis of xyloglucan derived oligosaccharides. MALDI-TOF mass spectra were acquired on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems) equipped with a 337 nm nitrogen laser. Mass spectra were performed in the reflector delayed extraction mode using 2,5-dihydroxybenzoic acid (Sigma) as matrix. The matrix, freshly dissolved at 5 mg.mL⁻¹ in a 70:30 acetonitrile/ 0.1% TFA, was mixed with the water solubilized oligosaccharides in a ratio 1:1 (v/v). These spectra were recorded in a positive mode, using an acceleration voltage of 20,000 V with a delay time of 100 ns and above 50% of the laser energy. They were externally calibrated using commercially available mixtures of peptides and proteins (Applied Biosystems). In this study, the spectra were calibrated using des-Arg¹-bradykinin (904.4681 Da), angiotensin I (1296.6853), Glu¹-fibrinopeptide B (1570.6774 Da), ACTH₁₈₋₃₉ (2465.1989 Da). Laser shots were accumulated for each spectrum in order to obtain an acceptable signal to noise ratio (sum of 10 spectra of 1000 shots per spectrum).

Supplemental data

Supplemental Table SI. Estimation of the amount of pollen grain wall versus pollen tube cell wall.

Supplemental Figure S1. Immunogold of cell wall epitope labeling in Arabidopsis pollen tube, hydrated pollen grain and controls.

Supplemental Figure S2. Monosaccharide composition and carbohydrate content of the cell wall of non-germinated pollen grains and 16h-*in vitro* grown pollen tubes isolated from 520 flowers.

Supplemental Figure S3. MALDI-TOF MS of XyG fragments of Arabidopsis cell wall leaf released by *endo*-glucanase treatment.

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Table I. Summary of cytochemical staining and immunolocalization of cell wall polymers in *in vitro* grown Arabidopsis pollen tubes.

Based on observations at fluorescent microscopy (FM) and electron microscopy (EM) levels.

Target	Probe	Back from the tip		Tip
		FM level	EM level	FM level
β -glucan	calcofluor white	+	na	+
Callose	aniline blue anti-callose	+	na	-
		nd	iw	nd
Extensin	LM1	-	nd	-
AGP	LM2	+/-	nd	+
	MAC207	+/-	nd	+
	JIM13	-	nd	-
HG	JIM5	+	nd	+/-
	JIM7	+/-	nd	+
Galactan	LM5	+/-	nd	+/-
Arabinan	LM6	+	ow	+
	LM13	+	nd	+
Xylogalacturonan	LM8	+	nd	+
XyG	CCRC-M1	+	iw/ow	+
	LM15	+	iw/ow	+
Controls	-	-	-	-

AGP, arabinogalactan-protein; HG, homogalacturonan; XyG, xyloglucan. -, no labeling detected (at over 500 ms exposure time) to + labeling detected. na, not applicable; nd, not determined; iw, inner wall layer; ow, outer wall layer.

Table II. Monosaccharide composition and linkage analysis of total pollen tube cell wall, pectin enriched (ammonium oxalate) and hemicellulose enriched (KOH) extracts.

	Pollen tube wall	Oxalate extract	KOH extract
NS (%)	-	78.5	97.6
UA (%)	-	21.5	2.4
Monosaccharide composition ^a			
Ara	43.6 (± 4.5)	24.7 (± 1.9)	23 (± 7.4)
Fuc	1.2 (± 0.2)	1.0 (± 0.5)	0.9 (± 0.7)
Gal	8.4 (± 1.0)	18.3 (± 0.6)	14 (± 3.2)
Glc	19.8 (± 5.1)	19.3 (± 2.8)	40.1 (± 6.6)
GalA	10.9 (± 0.7)	21.5 (± 1.4)	6.8 (± 0.2)
GlcA	2.2 (± 3.0)	3.4 (± 0.8)	1.5 (± 0.4)
Rha	5.3 (± 0.8)	4.2 (± 0.2)	2.4 (± 0.7)
Man	2.2 (± 1.5)	1.7 (± 3.0)	4.9 (± 0.1)
Xyl	6.2 (± 1.2)	5.8 (± 0.6)	6.5 (± 0.4)
Linkage analysis ^b			
<i>t</i> -Araf	-	27.4	8.5
5-Araf	-	28.2	14.6
2,5-Araf	-	3.7	2.2
3,5-Araf	-	3.9	2.7
<i>t</i> -Xyl	-	1.4	1.1
2-Xyl	-	1	2.4
<i>t</i> -Gal	-	1.7	6
3-Gal	-	2	1.2
4-Gal	-	1.5	nd
6-Gal	-	4	1.8
3,6-Gal	-	5.6	7.3
4,6-Gal	-	1.2	2.2
3-Glc	-	10	27.6
4-Glc	-	8.4	15.4
2,3-Glc	-	nd	7

NS, neutral sugar; UA, uronic acid (determined by the phenol sulphuric and mHDP colorimetric assays). ^aDetermined by GC and expressed as mol%. Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; Rha, rhamnose; Man, mannose; Xyl, xylose; nd, not detected. Values are the mean ± SD from two independent experiments. ^bDetermined by GC-MS of partially methylated alditol acetates and expressed as % of total area of the identified peaks. *t*-Araf denotes 1,4-di-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-methyl-D-arabinitol, etc...., - not determined, nd. not detected.

Table III. Relative quantification of XyG oligosaccharides obtained after *endo*-glucanase digestion of *Arabidopsis* pollen tube wall.

Mass ^a	Composition ^b	Structure ^c	Relative abundance ^d (%)
1085	Hex ₄ Pent ₃	XXXG	14.7 ± 3.3
1247	Hex ₅ Pent ₃	XLXG/XXLG	5.7 ± 0.6
1289	Hex ₅ Pent ₃ OAc ₁	XLXG/XXLG + 1 OAc	27.6 ± 3.9
1393	Hex ₅ Pent ₃ Dox ₁	XXFG	8.4 ± 0.5
1435	Hex ₅ Pent ₃ Dox ₁ OAc ₁	XXFG + 1 OAc	41.2 ± 6.6
1555	Hex ₆ Pent ₃ Dox ₁	XLFG	2.5 ± 0.1 ^e

^aMass of the fragments [M+Na⁺]⁺. ^bHex, hexose; Pent, pentose; Dox, deoxyhexose; OAc, O-acetyl substituent. ^cBased on XyG oligosaccharide structures found in Pauly et al. (2001). ^dValues are means ± SD from MALDI spectra obtained after *endo*-glucanase digestion from three different *Arabidopsis* pollen tube cell wall extracts. ^eRelative abundance of this oligosaccharide corresponds to the total of the K⁺ and Na⁺ adduct fragments.

Figure legends

Figure 1. *In vitro* pollen tube growth, β -glucan staining and immuno-localisation of Arabidopsis pollen tube wall epitopes. **A**, 16h-old *in vitro* pollen tubes grown in liquid medium (arrows show callose plugs). Insert is a close up of 16h-old *in vitro* pollen tube tip. **B**, Cytochemical staining of β -glucan (callose) with aniline blue showing a callose plug (arrow) and a lack of staining in the pollen tube tip region (arrowhead). **C**, Cytochemical staining with calcofluor white showing callose plugs (arrow) and the localization of β -glucans in the wall. **D-N**, Immuno-fluorescence labeling of cell wall polymer epitopes at the surface of pollen tubes. Arabidopsis pollen tube wall epitopes probed with anti-pectin MAbs JIM7 (**D**), JIM5 (**E**) and LM8 (**F**), specific for low and highly methylesterified HG and xylogalacturonan, respectively. **D**, JIM7 tag showed a strong signal at the tip in well-developed pollen tube and emerging tube tip from the pollen grain (insert). **E**, pollen tube stained with JIM5 showing a very weak intensity of labeling at the tip (arrowhead) compared to the wall back from the tip. **F**, LM8 labeled evenly the entire pollen tube wall. Insert is a close up of *in vitro* pollen tube tip. **G-J**, Detection of (1 \rightarrow 5)- α -L-arabinan epitopes with LM6 (**G**, **H**) and LM13 (**I**, **J**). Labeling was evenly distributed along the entire tube wall with a strong signal at the pollen tube tip. Note a collar-like structure (*) labeled at the emergence of the pollen tube from the pollen grain in **G**. **K-L**, Immuno-fluorescence staining of the non galactosylated (XXXG motif) and fucosylated XyG with LM15 (**K**) and CCRC-M1 (**L**), respectively. Labeling with both MAbs was not evenly detected in the wall and displayed periodic deposition of the epitopes containing polysaccharides during pollen tube growth. **M-N**, LM2 which recognizes β -D-GlcpA-(1 \rightarrow 3)- α -D-GalpA-(1 \rightarrow 2)-L-Rha moieties of AGPs labeled weakly the whole tube with the strongest signal at the tip. In **M**, a more intense labeling (*) was observed at the emergence of the tube from the pollen grain. pg, pollen grain. Pollen tubes were grown for 6h except in **A** and **C** (16h). Scale bars = 50 μ m (**A-C**) and 5 μ m (**A** insert; **D-N**).

Figure 2. Electron micrographs showing the ultrastructure (**A**) and immunogold labeling of cell wall epitopes (**B-F**) of high pressure frozen/freeze substituted Arabidopsis pollen tube grown *in vitro* for 6h. **A**, Cross section of pollen tube showing the cell wall (cw) composed of two distinct layers: a fibrillar outer wall (ow) and a weakly electron-dense inner wall (iw). Well preserved organelles are also clearly distinguishable including endoplasmic reticulum (er), Golgi stacks (g), mitochondria (m) and vacuoles (v). pm, plasma membrane. **B-C**, Immunogold labeling of (1 \rightarrow 5)- α -L-arabinan epitopes with LM6. Gold particles (arrowheads) are mostly localized in the outer wall (ow) layer. In **B**, possible *trans* Golgi network (tgn) and secretory vesicles (sv) labeled with LM6. **D**, Immunogold labeling of fucosylated xyloglucan motif recognized by CCRC-M1 in the inner (iw) and outer wall (ow) layers. Note the presence of gold particles in vesicles in the vicinity of the plasma membrane (white arrow). **E-F**, Immunogold labeling of non fucosylated xyloglucan motif (XXXG) with LM15. Gold particles (arrowheads) are visible in the inner (iw) and mainly in the outer walls (ow). cw, cell wall; er, endoplasmic reticulum; g, Golgi stacks; iw, inner wall layer; m, mitochondria; ow, outer wall layer; pm, plasma membrane; sv, secretory vesicles; tgn, *trans* Golgi network; v, vacuoles. Scale bars = 1 μ m (**A**), 0.5 μ m (**B**, **D**, **E**) and 100 nm (**C** and **F**).

Figure 3. Immuno-dot blot assay of pectin (oxalate extract) and hemicellulose (KOH extract) enriched fractions isolated from Arabidopsis pollen tube cell wall.

Oxalate and KOH extracts (8, 4, 1 and 0.5 μg) were probed with JIM5 for partially methylesterified HG, LM5 for (1 \rightarrow 4)- β -D-galactan, LM6 for (1 \rightarrow 5)- α -L-arabinan and LM15 for non galactosylated XyG (XXXG). Controls (8 μg): a, arabinan from sugar beet; b, pectin with 8.6% methylesterification from citrus; c, XyG from tamarind seed; d, gum arabic from acacia.

Figure 4. Analysis of Arabidopsis pollen tube XyG. **A.** MALDI-TOF mass spectrum of *endo*-glucanase-generated XyG fragments from the cell wall of 16h-grown Arabidopsis pollen tubes. The structures of the XyG fragments are shown according to the nomenclature proposed by Fry et al. (1993). Underlined and bold structures represent *O*-acetylated side chains (+ 1 OAc). *, indicates the signal of XLFG fragment with K^+ adduct ion instead of Na^+ . **B.** Monosaccharide composition of 16h-grown Arabidopsis pollen tube cell wall before (untreated) and after (treated) *endo*-glucanase treatment. Only the monosaccharides composing XyG are shown: Fuc, fucose; Gal, galactose; Glc, glucose; Xyl, xylose. Others include arabinose, galacturonic acid, glucuronic acid, mannose and rhamnose.

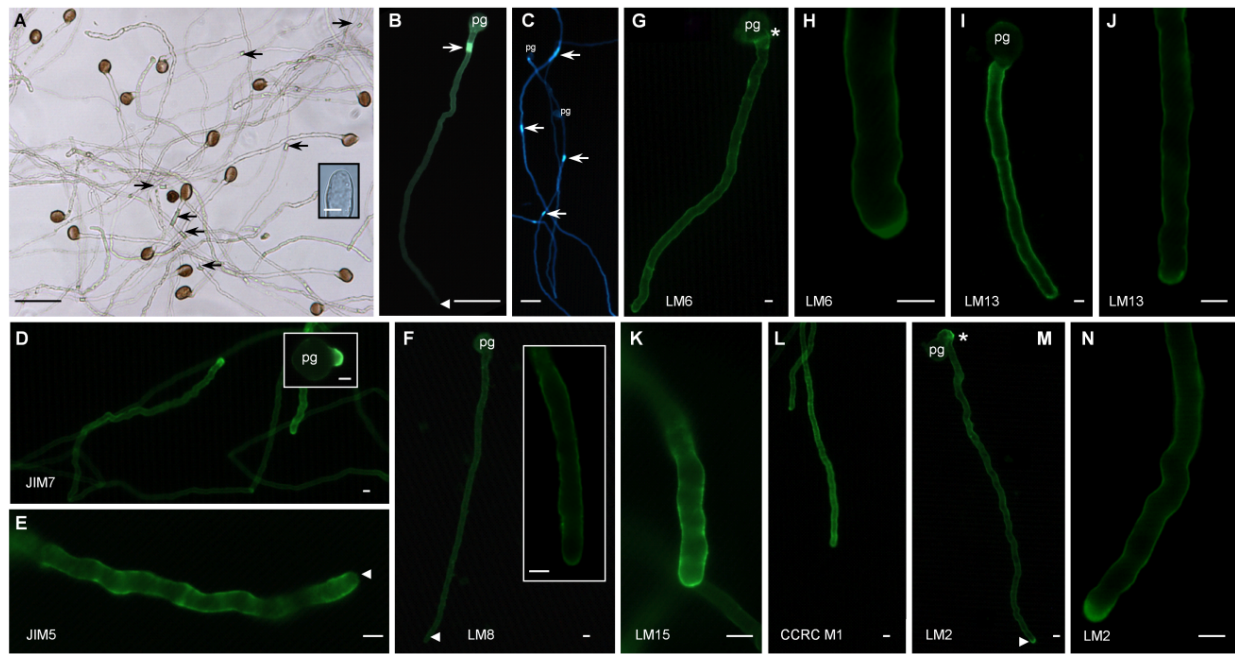


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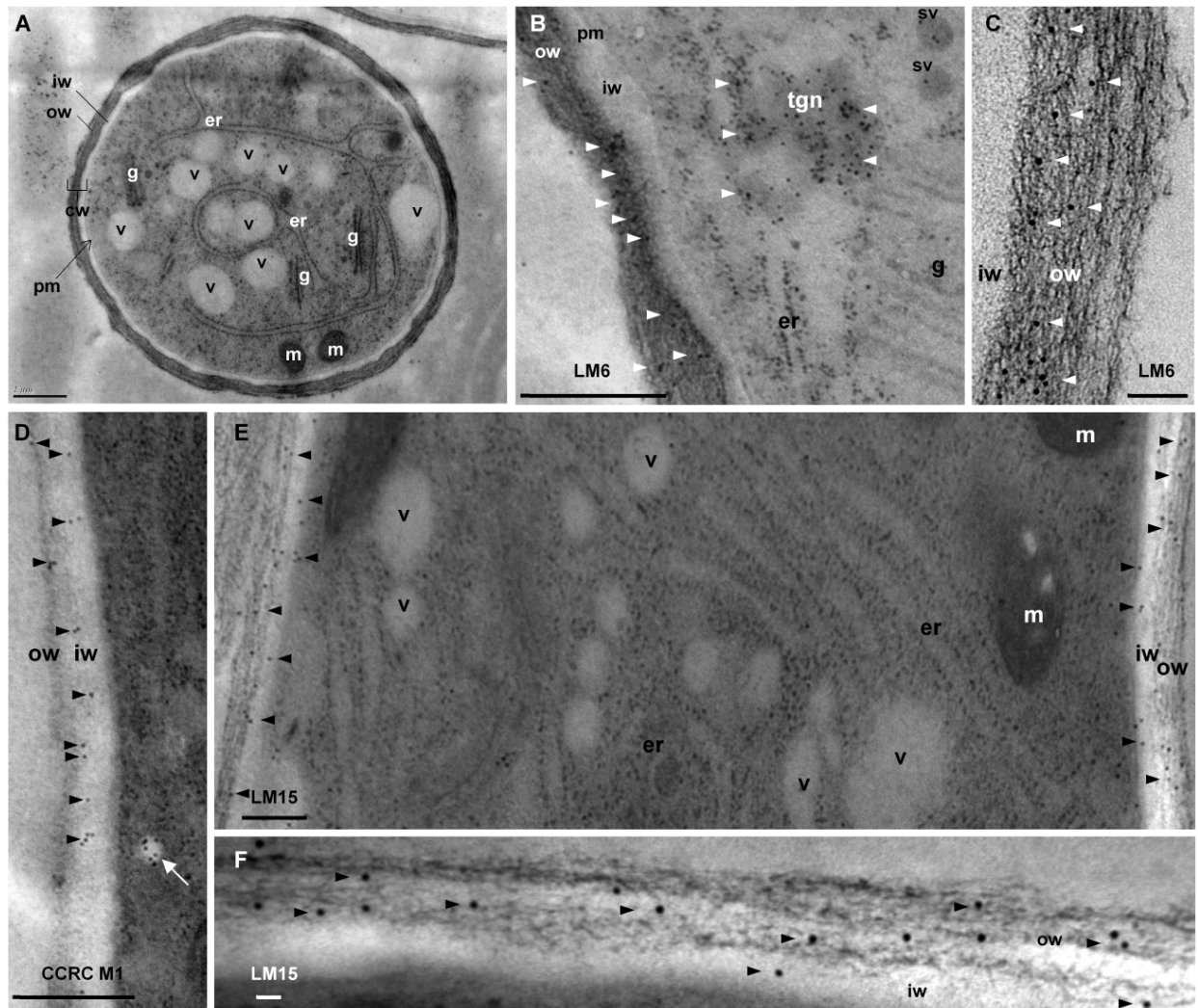


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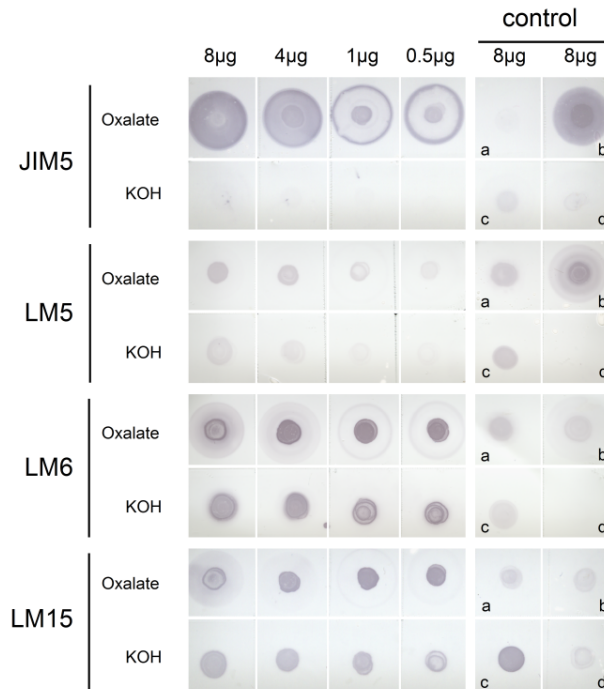


Figure 3. Immuno-dot blot assay of pectin (oxalate extract) and hemicellulose (KOH extract) enriched fractions isolated from *Arabidopsis* pollen tube cell wall.

Oxalate and KOH extracts (8, 4, 1 and 0.5 μ g) were probed with JIM5 for partially methylesterified HG, LM5 for (1 \rightarrow 4)- β -D-galactan, LM6 for (1 \rightarrow 5)- α -L-arabinan and LM15 for non galactosylated XyG (XXXG). Controls (8 μ g): a, arabinan from sugar beet; b, pectin with 8.6% methylesterification from citrus; c, XyG from tamarind seed; d, gum arabic from acacia.

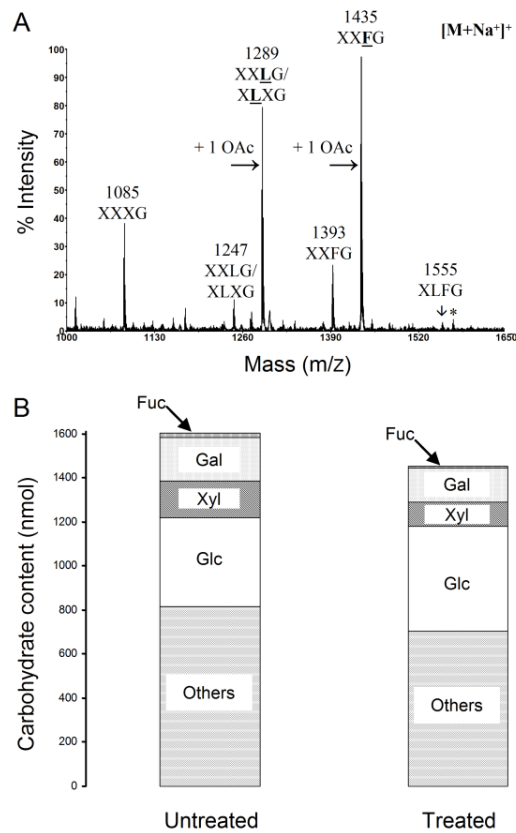


Figure 4. Analysis of Arabidopsis pollen tube XyG. **A.** MALDI-TOF mass spectrum of *endo*-glucanase-generated XyG fragments from the cell wall of 16h-grown Arabidopsis pollen tubes. The structures of the XyG fragments are shown according to the nomenclature proposed by Fry et al. (1993). Underlined and bold structures represent *O*-acetylated side chains (+ 1 OAc). *, indicates the signal of XLFG fragment with K^+ adduct ion instead of Na^+ . **B.** Monosaccharide composition of 16h-grown Arabidopsis pollen tube cell wall before (untreated) and after (treated) *endo*-glucanase treatment. Only the monosaccharides composing XyG are shown: Fuc, fucose; Gal, galactose; Glc, glucose; Xyl, xylose. Others include arabinose, galacturonic acid, glucuronic acid, mannose and rhamnose.