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Abstract	To further knowledge on cell wall composition in early land plants, we localized cell wall constituents in placental cells of the liverwort *Marchantia polymorpha* L. using monoclonal antibodies (MAbs) in the transmission electron microscope and histochemical staining. The placenta of *M. polymorpha* is similar to the majority of bryophytes in that both generations contain transfer cells with extensive wall ingrowths. Although the four major cell wall polymers, i.e., cellulose, pectins, hemicelluloses, and arabinogalactan proteins, are present, there are variations in the richness and specificity across generations. An abundance of homogalacturonan pectins in all placental cell walls is consistent with maintaining cell wall permeability and an acidic apoplastic pH necessary for solute transport. Although similar in ultrastructure, transfer cell walls on the sporophyte side in *M. polymorpha* are enriched with xyloglucans and diverse AGPs not detected on the gametophyte side of the placenta. Gametophyte wall ingrowths are more uniform in polymer composition. Lastly, extensins and callose are not components of transfer cell walls of *M. polymorpha,* which deviates from studies on transfer cells in other plants. The difference in polymer localizations in transfer cell walls between generations is consistent with directional movement from gametophyte to sporophyte in this liverwort.

Keywords (separated by '-')	AGP - Cell wall - Hemicellulose - *Marchantia polymorpha* - Pectin - Transfer cell - Wall ingrowth

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REGULAR PAPER – PHYSIOLOGY/BIOCHEMISTRY/MOLECULAR AND CELLULAR BIOLOGY

Differential localization of cell wall polymers across generations in the placenta of *Marchantia polymorpha*

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Abstract

To further knowledge on cell wall composition in early land plants, we localized cell wall constituents in placental cells of the liverwort *Marchantia polymorpha* L. using monoclonal antibodies (MAbs) in the transmission electron microscope and histochemical staining. The placenta of *M. polymorpha* is similar to the majority of bryophytes in that both generations contain transfer cells with extensive wall ingrowths. Although the four major cell wall polymers, i.e., cellulose, pectins, hemicelluloses, and arabinogalactan proteins, are present, there are variations in the richness and specificity across generations. An abundance of homogalacturonan pectins in all placental cell walls is consistent with maintaining cell wall permeability and an acidic apoplastic pH necessary for solute transport. Although similar in ultrastructure, transfer cell walls on the sporophyte side in *M. polymorpha* are enriched with xyloglucans and diverse AGPs not detected on the gametophyte side of the placenta. Gametophyte wall ingrowths are more uniform in polymer composition. Lastly, extensins and callose are not components of transfer cell walls of *M. polymorpha,* which deviates from studies on transfer cells in other plants. The difference in polymer localizations in transfer cell walls between generations is consistent with directional movement from gametophyte to sporophyte in this liverwort.

Keywords AGP · Cell wall · Hemicellulose · *Marchantia polymorpha* · Pectin · Transfer cell · Wall ingrowth

Abbreviations

AGP	Arabinogalactan protein
HG	Homogalacturonan
MAb	Monoclonal antibody

Introduction

In bryophytes (mosses, liverworts, and hornworts), water and nutrients are transported from the gametophyte to the dependent sporophyte across a persistent apoplastic junction known as the placenta (Gunning et al. 1974; Ligrone and Gambardella 1988; Pate and Gunning 1972; Regmi and Gaxiola 2017). As the bridge between two generations that have different needs and environmental and genetic constraints, the placenta plays a critical role in the lifecycle of these plants, namely, to ensure nourishment during the production of meiotic spores (Ligrone et al. 1993, 2012a, b).

Considerable variability occurs in the organization and structure of the placenta of bryophytes, especially in the location of transfer cells. Transfer cells are specialized cells with elaborate wall ingrowths that maximize transport potential by vastly increasing cell membrane surface (Browning and Gunning 1979a; Ligrone et al. 1993; Offler et al. 2003). In most mosses and liverworts, transfer cells occur on both sides of the placenta, but in some taxa, they are restricted to one generation or are absent. Only in hornworts is the placenta comprised of transfer cells that are exclusive to the gametophyte side and are intermingled with elongated haustorial cells of the sporophyte foot (Gambardella and Ligrone 1987; Vaughn and Hasegawa 1993). Although much is known about placental diversity across bryophytes, there are no comprehensive studies of cell wall composition in the specialized cells that make up the gametophyte-sporophyte junction.

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The properties of cell walls are dictated by the composition and arrangement of their constituent polymers (Humphrey et al. 2007). The prevailing model of the primary plant cell wall is a cellulose and hemicellulose foundation integrated with an interacting and abundant pectin network (Broxterman and Schols 2018; Cosgrove 2005). In general, pectins are responsible for porosity, flexibility, and adhesion, while cellulose and hemicelluloses serve a supportive structural role. Although macromolecules of primary walls in plants are assembled to impart structural integrity, cell adhesion, and facilitate signal transduction, they play an additional role in the walls of transfer cells of the placenta. Here, macromolecules of the apoplast are the matrix in which nutrients, including sugars and amino acids, flow across generations (Browning and Gunning 1979b; Graham and Wilcox, 2000; Regmi and Gaxiola 2017; Renault et al. 1992). Given the significance and diversity of the bryophyte placenta, this tissue complex provides a unique opportunity to correlate cell wall architecture with carbohydrate/ proteoglycan composition and to assess these variations in light of the known functions of specific cell wall polymers.

In an effort to gain a deeper understanding of the carbohydrate composition of placental cell walls in early land plants, we conducted a study of cell wall composition in the model liverwort *Marchantia polymorpha* L. (Bowman et al. 2017; Shimamura 2016). The occurrence of transfer cells in both generations of *M. polymorpha* provides a platform on which to explore the variability of wall composition in architecturally distinct walls that have a common function, i.e., directional transport (Ligrone et al. 1993). We addressed the following fundamental question: How do cell wall constituents differ in the two generations of the placenta of this bryophyte? Specifically, if cells with similar wall ingrowths are present on both sides of the path of transport, is there a difference in cell wall composition?

Using immunogold labeling at the TEM level, we probed with 16 monoclonal antibodies to cell wall carbohydrates and arabinogalactan proteins (AGPs) to identify the major polymers present in the placenta of this liverwort. We demonstrate that all the major polymers in plant primary cell walls (cellulose, pectin, hemicellulose and AGPs) are differentially localized in gametophyte and sporophyte transfer cell walls, and that callose and extensins are absent in both. The difference in relative abundance and localization of polymers in transfer cell walls between generations is consistent with the directional flow of nutrients from gametophyte to sporophyte.

Materials and methods

Gametophyte culture

Marchantia polymorpha was purchased from Carolina Biological Supply Company, North Carolina. Plants were placed in a growth chamber and maintained under a constant temperature of 15 °C with 12 h light and 12 h dark each day until archegoniophores and sporophytes were mature.

Light microscopy

Specimens were thick sectioned (1–1.5 µm) on an ultramicrotome, placed on glass slides, and stained with toluidine blue. Sporophytes were screened for developmental stages. Further examination was restricted to placentae with post-meiotic developing spores with the expectation that the wall ingrowths at this stage are fully developed and fully functional.

Preparation for transmission electron microscopy

Plants were prepared for TEM observation using the standard fixation protocol outlined in Renzaglia et al. (2017). Excised portions of gametophytic tissue with embedded feet were fixed in 2.5% v/v glutaraldehyde in 0.05 M Sorenson's buffer (pH 7.2) for one h at room temperature and overnight at 4 °C. Following 2–3 rinses in same buffer for 15 min each, plants were post-fixed in 2% buffered osmium tetroxide and rinsed in autoclaved, distilled water. The specimens were dehydrated in progressively higher ethanol to water concentrations and rinsed twice in anhydrous ethanol. Infiltration was achieved by progressive placement of specimens in higher concentrations of LR White resin diluted with ethanol. Once specimens reached 100% LR White and exchanged twice, they were placed in gel capsules and heated in an oven at 60 °C for 48 h. The samples were sectioned on an ultramicrotome until the placenta was located. Either thin Sects. (90–100 nm) were collected on 200 mesh nickel grids for immunogold labeling, or thick Sects. (1000 to 1500 nm) were collected on glass slides for histochemical fluorescence staining.

Fluorescence staining

To visualize cellulose, resin-embedded thick-Sects. (1 µm) were placed on glass slides and incubated for 3–5 min in a drop of Calcofluor White (Sigma-Aldrich) and a drop of 10% KOH buffer in the dark. Calcofluor White is a fluorescent dye specific for fibrillar β(1 → 4) glucans of plant cell walls such as cellulose (Maeda and Ishida 1967). In order to

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localize callose, 1–1.5 µm sections were collected on slides, covered by 1% aniline blue in 0.067 M Na_2HPO_4 (pH 8.5), placed in the dark at 4 °C for 3–5 days, and rinsed in buffer. Controls were made using the respective buffers without aniline blue or Calcofluor White. Three replicates were made for each treatment and controls. All stained material was viewed with a Leica DM500B compound microscope (excitation filter equipped with ultraviolet fluorescence between 360 and 400 nm). Images were collected digitally using a Q-Imaging Retiga 2000R digital camera.

Immunogold labeling

Specimens were processed as follows and outlined in (Lopez et al. 2017). Grids were placed in BSA/PBS overnight at 4 °C and then overnight on a primary antibody specific to the desired wall epitope. Samples were then rinsed 4 × 4 min each in 0.05 M BSA/PBS. Samples were treated with a secondary antibody with a gold tag attached for 30 min at room temperature. Samples were then rinsed in PBS 4 × 4 min per each and rinsed with a jet of sterile H_2O.

Samples were observed before and after post-staining using lead citrate and uranyl acetate. These stains result in better contrast but may obscure the immunogold labels in the transmission electron microscope. Control grids were prepared by excluding the primary antibodies. For each treatment, 3–5 replicates were examined. Samples were viewed, and digital micrographs were collected in a Hitachi H7650. The monoclonal antibodies (MAbs) used in this study are listed in Table 1.

Scoring label abundance

Images were opened in the PhotoScapeX (Mooii Tech) editing app. Three counting frames 100 × 100 pixels in size were randomly placed onto the wall in the image and labels within the frames were counted. This process was repeated three times per 10–15 images for each MAb. The average of all counts was calculated. Averages of 1 to 4 labels per frame

Table 1 Primary antibodies used to immunogold label carbohydrates and arabinogalactan proteins in transfer cell walls of placentae in *Marchantia polymorpha*

Antibody	Antigen (s)/epitope	Reference/source
Anticallose	Callose/ (1,3)-β-linked penta-to-hexa-glucan	Meikle et al. 1991/Biosupplies Australia
LM15	XXXG motif of xyloglucan	Marcus et al. 2008/J. P. Knox PlantProbes University of Leeds, UK
LM21	Mannan/ β-(1,4)-manno-oligosaccharide	Marcus et al. 2010/J. P. Knox PlantProbes, University of Leeds, UK
LM25	Galactoxylated xyloglucans	Pedersen et al. 2012/J. P. Knox PlantProbes, University of Leeds, UK
LM28	Glucuronoxylan	Cornuault et al., 2015/ J. P. Knox PlantProbes, University of Leeds, UK
LM19	Homogalacturonan/ Un-esterified	Verhertbruggen et al. 2009a/J. P. Knox PlantProbes, University of Leeds, UK
LM20	Homogalacturonan/ Methyl-esterified	Verhertbruggen et al. 2009a/J. P. Knox PlantProbes, University of Leeds, UK
JIM5	Homogalacturonan/ Un-esterified	Knox et al. 1990/J. P. Knox PlantProbes, University of Leeds, UK
JIM7	Homogalacturonan/ Methyl-esterified	Willats et al. 2000/M. Hahn, Complex Carbohydrate Research Center, University of Georgia, USA
LM5	Galactan, rhamnogalacturonan-I/ (1–4)-β-D-galactan	Jones et al. 1997/J. P. Knox PlantProbes, University of Leeds, UK
LM6	Arabinan, rhamnogalacturonan-I/ (1–5)-α-L-arabinan (also labels AGP)	Willats et al. 1998; Verhertbruggen et al. 2009b/J. P. Knox PlantProbes, University of Leeds, UK
LM13	Arabinan, rhamnogalacturonan-I/ (1–5)-α-L-arabinan (linear)	Moller et al. 2007/J. P. Knox PlantProbes, University of Leeds, UK
JIM12	Extensin	Smallwood et al. 1994/J. P. Knox PlantProbes, University of Leeds, UK
LM2	Arabinogalactan protein (AGP)/ β-D-GlcA (glucuronic acid)	Smallwood et al. 1996/J. P. Knox PlantProbes, University of Leeds, UK
JIM8	Arabinogalactan protein (AGP)/ unknown	Pennell et al. 1991/J. P. Knox PlantProbes, University of Leeds, UK
JIM13	Arabinogalactan protein (AGP)/ β-D-GlcA-(1,3)-α-D-GalpA-(1,2)-L-Rha (glucuronic acid-galacturonic acid-rhamnose)	Yates et al. 1996/J. P. Knox PlantProbes, University of Leeds, UK

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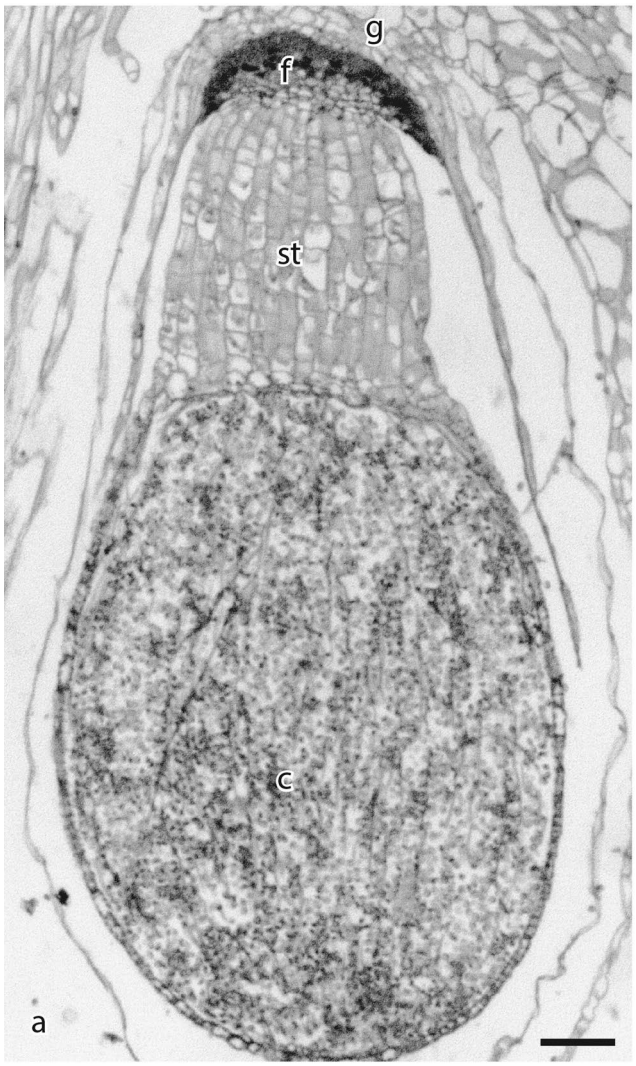

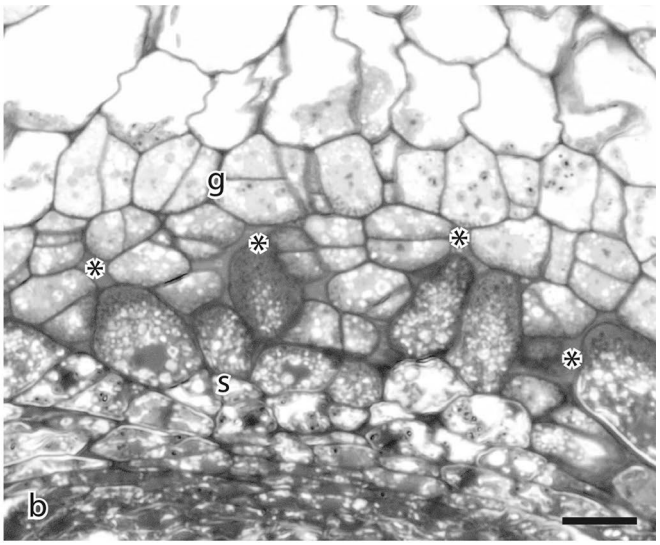

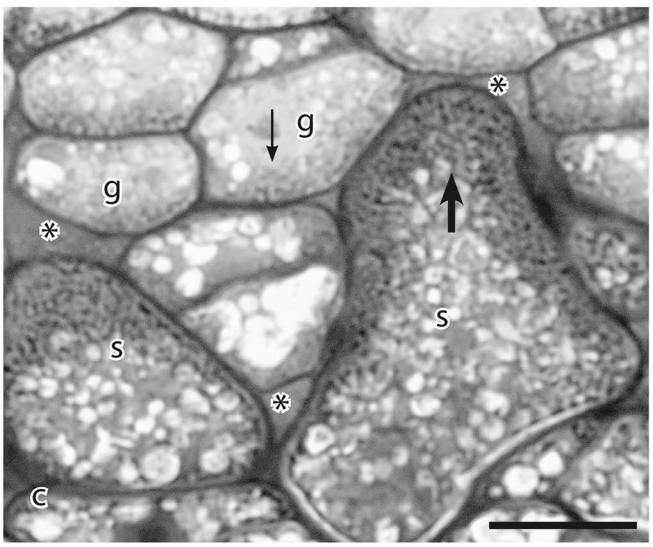

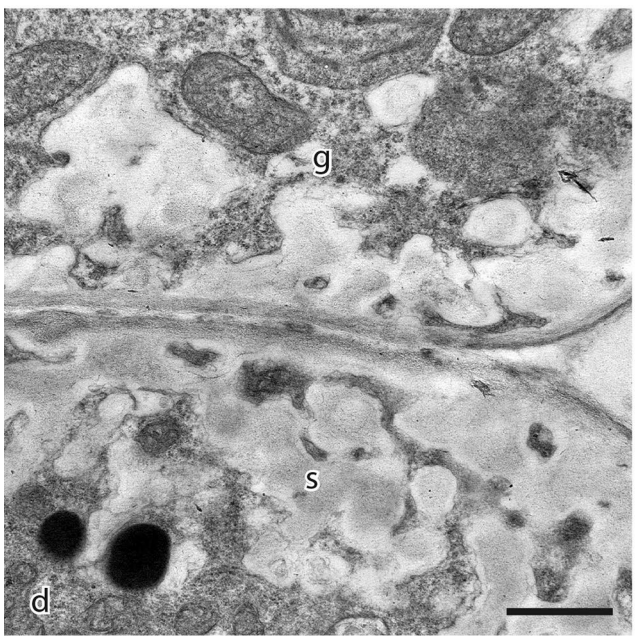

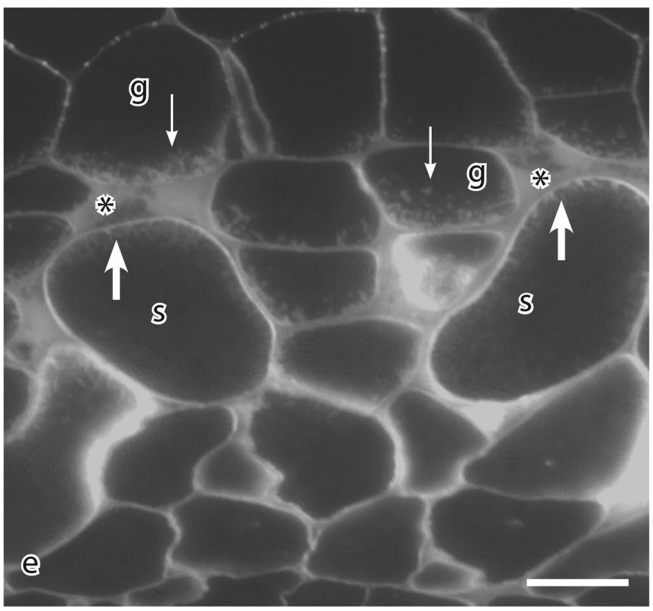

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◂**Fig. 1** *Marchantia polymorpha* sporophyte and placenta. a Light micrograph longitudinal section of **a** sporophyte composed of a capsule (c), short seta (st), and anchor-shaped foot (f) embedded in gametophyte tissue (g) on the underside of the archegoniophore. **b** Higher magnification light micrograph of the placental region showing the arrangement of sporophyte (s) and gametophyte (g) cells and intergenerational space (*). Cell wall ingrowths are particularly prominent in sporophyte cells. **c** Light microscope showing the extensive wall ingrowths in sporophyte cells (s) (large arrow) adjacent to intergenerational space (*) and gametophyte cells with less prominent wall ingrowths (g) (small arrow). **d** TEM showing gametophyte (g) and adjacent sporophyte (s) transfer cells with robust wall ingrowths. **e** Calcofluor White fluorescence identifies the presence of cellulose in the primary walls and in wall ingrowths in gametophyte cells (small arrow) but is visible only in wall ingrowths near the primary walls in sporophyte (s) cells (large arrow) (cf., 1b, c). Scale bars: 10 µm for **a**; 0.5 µm for **d**; 5.0 µm for **b**, **c**, **e**

were assigned a single plus (+), and two pluses (++) were assigned to averages between 5 and 9 labels. Any averages that were greater than 10 labels per frame received a triple plus (+++). Antibodies with average label density between 0 and 1 and were assigned a plus/ minus (±).

Results

The sporophyte of *M. polymorpha* is composed of a capsule, short seta and anchor-shaped foot that is embedded on the underside of the elongated archegoniophore (Fig. 1a). The placenta consists of gametophyte and sporophyte transfer cells that are intermingled (Fig. 1b). A narrow irregular space separates the generations (Fig. 1b, c). The labyrinth apparatus is remarkably larger in sporophyte than gametophyte transfer cells (Fig. 1c). In both generations, the wall labyrinth lies above a thin cell wall, henceforth referred to as the basal wall (Fig. 1d). As expected, Calcofluor White staining showed that cellulose is a constituent of all cell walls, including the labyrinth apparatus of gametophyte transfer cells and the underlying cell wall; interestingly, however, no Calcofluor White staining was observed in the labyrinth apparatus of sporophyte transfer cells (Fig. 1e), denoting a significant reduction in the cellulose content relative to the other cell walls in the same tissue complex.

Table 2 summarizes the location and relative abundance of the 16 MAbs used to probe cell wall constituents. Homogalacturonan pectins (MAbs JIM7, JIM5, LM20 and LM19) occur in cell walls on both sides of the placenta of *M. polymorpha,* but no RG-I pectins (MAbs LM5, LM6, and LM13) are present (Table 2, Fig. 2). The JIM7 epitope is moderately detected in the wall ingrowths of both generations, with labels more concentrated in the electron dense regions of gametophyte wall ingrowth (Fig. 2a, b). The JIM5 MAb labels both generations, and this epitope is abundant in primary walls and in older portions of wall ingrowths near the primary wall with scattered labels throughout older ingrowth regions (Fig. 2c, d). Labeling with the LM20 MAb is aggregated in the electron dense cores of the cell wall ingrowths in both generations with fewer labels in the sporophyte (Fig. 2e, f). Sparse and scattered labeling with the LM19 MAb is seen in both generations and is most notable in the electron dense regions of the basal wall layer (Fig. 2g, h).

Four MAbs (LM15, LM25, LM21, LM28) were used to target hemicellulose epitopes (Fig. 3). LM15, LM25, and LM28 identify xyloglucans. LM15 abundantly labels the intergenerational zone while wall ingrowths lightly label in the electron dense regions (Fig. 3a). Labels with the LM25 MAb are abundant throughout sporophytic wall ingrowths and are scattered along electron dense regions in wall ingrowths of the gametophyte (Fig. 3b). The LM21 MAb that binds to mannan epitopes sparsely labels throughout wall ingrowths in both generations (Figs. 3c, d), with some concentration in outer regions. LM28 MAb (glucuronic xylans) is not detected in any cell walls (not shown).

Considerable diversity in AGP localizations (MAbs JIM13, LM2, JIM8, LM6) exists between placental cell walls in the two generations (Fig. 4). Heavy labeling with JIM13 occurs throughout sporophyte placental cells, especially wall ingrowths, while gametophyte cells are sparsely labeled, mostly near the plasma membrane along wall ingrowths (Fig. 4a). LM2 epitopes are concentrated in the electron dense areas in the original wall layer of the sporophyte generation, with fewer labels visible in the wall ingrowths (Fig. 4b). JIM8 AGP epitopes show similar distribution in both generations in *M. polymorpha*, occurring along the outside of wall ingrowths (shown in the sporophyte only) (Fig. 4c). The LM6 MAb does not label the placental cells of either generation (not shown).

No detection of the extensin epitope was seen with JIM12 localization. Callose, as visualized with the anti-callose MAb, is also lacking.

Parenchyma cell walls adjacent to transfer cells show a similar difference between generations with LM25, LM2 and JIM13 but labeling is much less abundant than in wall ingrowths (Fig. S1).

Discussion

All four major types of cell wall constituents, i.e., cellulose, pectins, hemicellulose and arabinogalactan proteins (AGPs), are present in the transfer cell walls in *M. polymorpha*. As such, these cell walls are comparable to the primary cell walls of bryophytes (Mansouri 2012; Roberts et al. 2012) and tracheophytes in composition, but with notable variations in the abundance and specificity of each polymer type across generations. As evidenced by Calcofluor White fluorescence, cellulose is the structural foundation of primary

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Table 2 Relative intensity of immunogold labeling of placental cells in *Marchantia polymorpha* with the following monoclonal antibodies: JIM7, JIM5, LM19, LM20, LM13, LM5, LM15, LM21, LM25, LM28, JIM8, JIM13, LM2, and anti-callose

Primary antibody	*Marchantia sporophyte*	*Marchantia gametophyte*
JIM7 partially methyl-esterified HG	+	+ +
LM20 methyl-esterified	+	+ +
JIM5 partially de-esterified HG	+ +	+ +
LM19 de-esterified HG	+	+
LM5 RG-I galactan	–	–
LM6[b]RG-I arabinan	–	–
LM13 RG-I arabinan	–	–
LM15 xyloglucan	+[a]	+[a]
LM21mannan	+	+
LM25 galactoxyloglucan	+ + +	+
LM28 glucoxyloglucans	–	–
JIM8 AGP	±	±
JIM13 AGP	+ + +	±
JIM12 extensin	–	–
Callose	–	–

Notes: + + +, strong; + +, moderate; +, weak; ±, present; –, absent

[a]Intergenerational zone

[b]LM6 binds to arabinan residues in RG-I pectins and AGPs

AQ2 cell walls, but is less prominent in wall ingrowths on the sporophyte side. Homogalacturonan (HG) pectins are abundant in all cell walls while rhamnogalacturonan (RG-I) pectins are undetected (Table 2). Xyloglucans are plentiful in the matrix between generations, in sporophytic wall ingrowths, and primary cell walls but are scattered around the periphery of gametophyte wall ingrowths. AGP epitopes are abundant in sporophyte wall ingrowths in *M. polymorpha* with little detection of these epitopes in the gametophyte.

Pectins account for 30% of polysaccharides found in the primary cell walls of dicots, gymnosperms, and non-*Poales* monocots (10% in *Poales*) (Carpita 1996; O'Neill and York 2018; Ridley et al. 2001). The content of specific pectic domains and their arrangement within the cell wall play significant roles in the cell wall properties and hence their function (Caffall and Mohnen 2009) (Table 3). Homogalacturonan pectins (HG) are laid down in an esterified form (Clausen et al. 2003), and de-esterification happens *in muro.* Methyl-esterified HGs are stretchable, influence the porosity of cell walls and have a lower apoplastic pH, all of which would support nutrient uptake and movement (Clausen et al. 2003). These properties explain the high levels of methyl-esterified HG pectins and their relatively even distribution across generations in the placental of *M. polymorpha.* Methyl-esterified HGs also occur in the wall ingrowths in transfer cells of the fern *Ceratopteris richardii* (Johnson 2008), epidermal transfer cells of *Vicia faba* (Vaughn et al. 2007), and in meristematic cells of the developing gametophore of *Physcomitrium (Physcomitrella) patens* (Berry et al. 2016; Mansouri 2012), supporting similar roles in wall extension and porosity across plant tissues and groups. Interestingly, HG pectins were not detected in the wall ingrowths of *Elodea canadensis* leaf transfer cells but were present in their outer wall layers (Ligrone et al. 2011).

De-esterified HG pectic domains localize in the placental cells of both the sporophyte and gametophyte generations of *M. polymorpha* and are more abundant in older portions of wall ingrowths, a finding that is consistent with the concept that a de-esterified pectin layer provides a rigid platform upon which additional walls are constructed (Liners et al. 1989; Xia 2018) (Table 3). De-esterified pectins play a similar structural role in undifferentiated cells and protonemata in *P. patens* (Berry et al. 2016; Lee et al. 2005; Mansouri 2012).

In general, RG-I pectins are not major polymers in the primary cell walls of bryophytes and ferns as detected by comprehensive microarray polymer profiling (CoMPP) (Möller et al. 2007), and glycan microarrays analysis (Eeckhout et al. 2014). RG-I pectins are absent from the extensive wall ingrowths in placental cells of *M. polymorpha*, as they are in the fern *C. richardii* (Johnson 2008). They are sparse in epidermal transfer cells in *Vicia faba* (Vaughn et al. 2007), *Pisum sativum* (Dahiya and Brewin 2000) and *Elodea canadensis* (Ligrone et al. 2011). In some mosses, RG-I pectins are abundant in water conducting cells but they are less abundant and non-specific in liverworts (Ligrone et al. 2002; Mansouri 2012).

Although hemicelluloses associate with both cellulose networks and acidic pectins across land plants, they occur at much lower concentrations in bryophyte primary walls than in seed plants (Cornuault et al. 2018; Popper and Fry 2003; Sarkar et al. 2009). In spite of the report that xyloglucans in liverworts and mosses have different motifs and structures than those of hornworts and tracheophytes (Peña et al. 2008), the angiosperm MAbs used in this study (LM15, and LM25) reveal the abundance of these polymers in the *M. polymorpha* placenta. Hemicelluloses targeted with polyclonal antibodies also show high levels of labeling in epidermal transfer cells of *V. faba* (Vaughn et al. 2007). The location of xyloglucans in the sporophyte wall ingrowths and intergenerational zone of *M. polymorpha,* the latter location is consistent with the muco-adhesive nature of these hemicelluloses (Madgulkar et al. 2016) (Table 3). The differential pattern of labeling of xyloglucans differs from gametophyte and sporophyte vegetative cell walls that evenly label with the LM15 MAb (Fig. S1a). Mannans occurs in both generations of the placenta in small amounts and because they occur in protonemata and rhizoids in *P. patens* these polymers have been speculated to facilitate nutrient uptake, water

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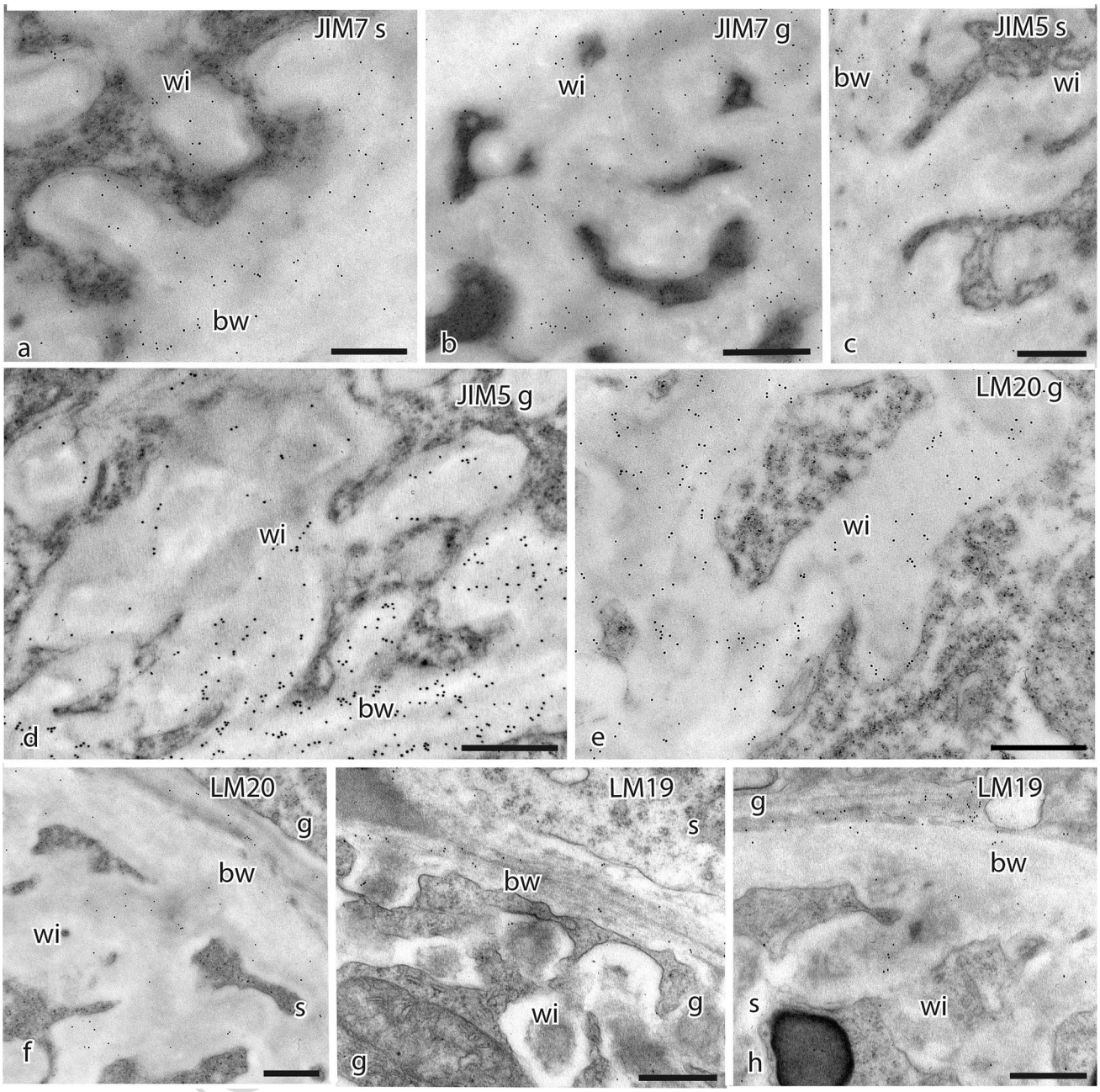

Fig. 2 TEM immunogold labeling with monoclonal antibodies to pectin epitopes. S = sporophyte transfer cell and G = gametophyte transfer cell. **a** JIM7 labels throughout the wall ingrowths (wi) and less so in the basal wall (bw) of sporophyte cells. **b** JIM7 labels the electron dense regions of the wall ingrowths (wi) in gametophyte cells. **c** JIM5 labels the basal wall (bw) and wall ingrowths (wi) in sporophyte cells. **d** In gametophyte placental cells, JIM5 labels the electron dense regions of the basal wall (bw) and wall ingrowths (wi) with decreased labeling away from the original wall. **e** LM20 labeling occurs in gametophyte transfer cell throughout wall ingrowths (wi). **f** LM20 labeling is sparse in the sporophyte basal wall (bw) and in sporophyte (s) cell walls and wall ingrowths (wi). **g** LM19 sparsely labels the electron dense material of the basal wall (bw) in gametophyte cells with fewer labels in wall ingrowth (wi). **h** LM19 labels the basal wall (bw), wall ingrowths (wi), and middle lamella (ml) of sporophyte cells. scale bars: 0.5 µm for (**a**–**h**)

sensing and cell wall reinforcement (Dehors et al. 2019; Moore 2009; Plancot et al. 2019) (Table 3).

Arabinogalactan proteins (AGPs) are suspected to be involved in several vital processes in plants, such as differentiation, cell to cell recognition, embryogenesis, programmed cell death, and tip-growth (Gaspar et al. 2001; Majewska-Sawka and Nothnagel 2000; Nguema-Ona et al. 2012) (Table 3). AGPs are also speculated to function as pectin plasticizers in cell walls. When AGPs separate from their GPI anchors in the plasmalemma and are released into the

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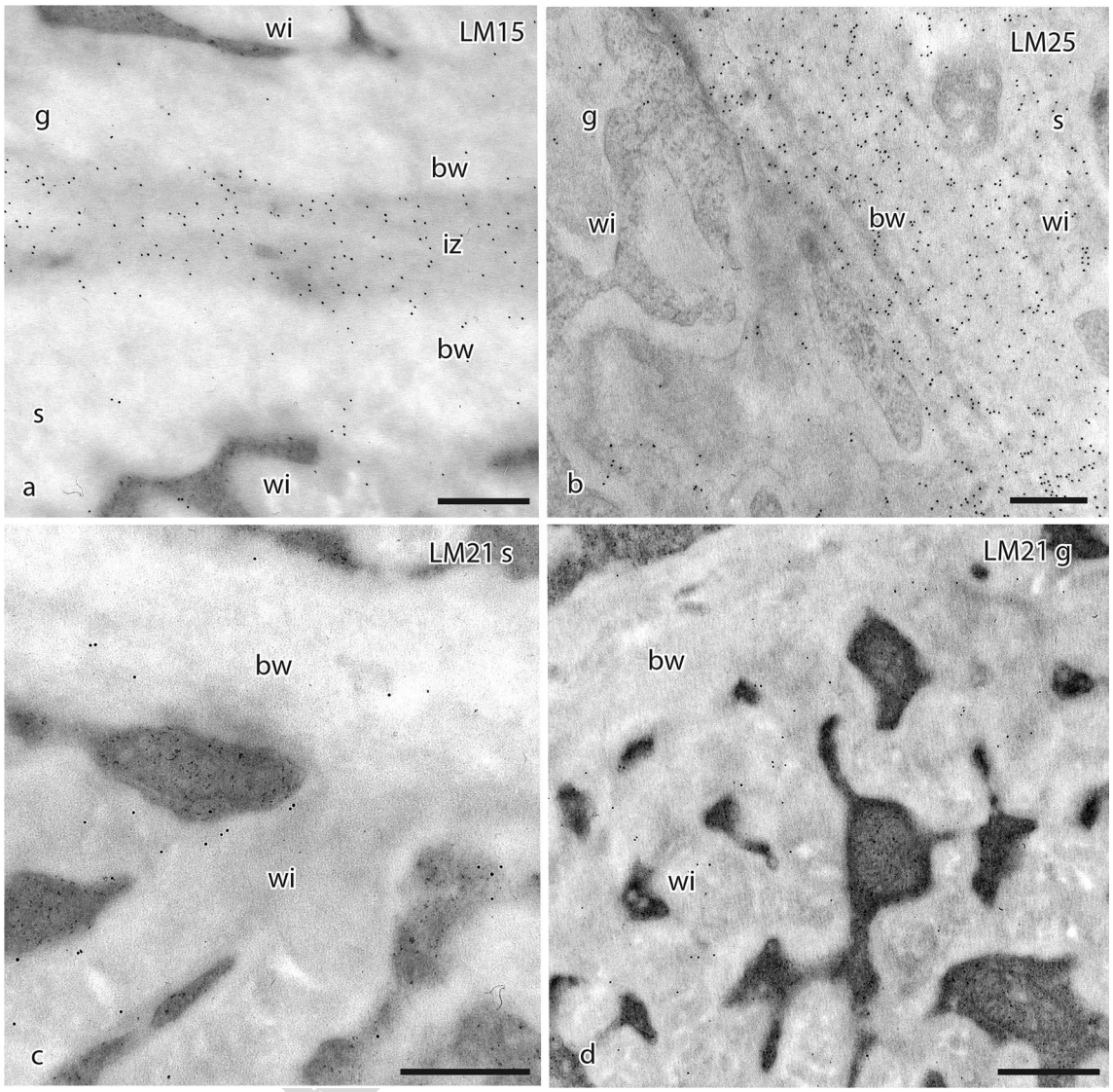

Fig. 3 TEM immunogold labeling with monoclonal antibodies to hemicellulose epitopes. **a** LM15 is abundant in the intergenerational zone (iz) and scattered in the basal wall (bw) and wall ingrowths (wi) of both sporophyte (s) and the gametophyte (g) cells. **b** LM25 is abundant in the basal wall (bw) in both the gametophyte (g) and sporophyte (s) and in the electron dense regions of wall ingrowths (wi) in sporophyte cells. Sparse labeling occurs in gametophyte wall ingrowths. **c** LM21 lightly labels the basal wall (bw) and wall ingrowths (wi) of sporophyte (s) cells. **d** LM21 lightly labels the basal wall (bw) and wall ingrowths (wi) of gametophyte (g) cells. Scale bars: 0.5 µm for (**a**–**d**)

cell wall, they increase porosity and keep HG domains from crosslinking (Lamport et al. 2006). AGPs are also involved in pH-dependent signaling by releasing Ca^{2+} as a secondary messenger that regulates development (Lamport and Várnai 2013; Lamport et al. 2014). As evidenced by immunolabeling with MAbs, AGPs are common in the placenta of *M. polymorpha* but are variable across generations (Table 2). Sporophyte transfer cell walls abundantly contain both JIM13 and LM2 epitopes, while these epitopes are sparse to absent in gametophyte transfer cells. Johnson (2008) documented intense labeling for AGPs in the placental cells of *C. richardii*; the outer regions of wall ingrowths in the gametophyte label and the electron-lucent regions of wall ingrowths label in both generations. Small amounts of AGP epitopes are detected in wall ingrowths of transfer cells of the cotyledon epidermis in *V. faba,* and cells treated with the AGP inhibitor ß-D-glucosyl Yariv, showed roughly a 50% reduction in the density of wall ingrowths, which points to structural and developmental roles in these walls (Vaughn et al. 2007).

The structure, location, and role of AGPs in bryophytes are slowly emerging (Happ and Classen 2019). Locations of these proteoglycans include water conducting cells in both mosses and liverworts (Ligrone et al. 2002) and

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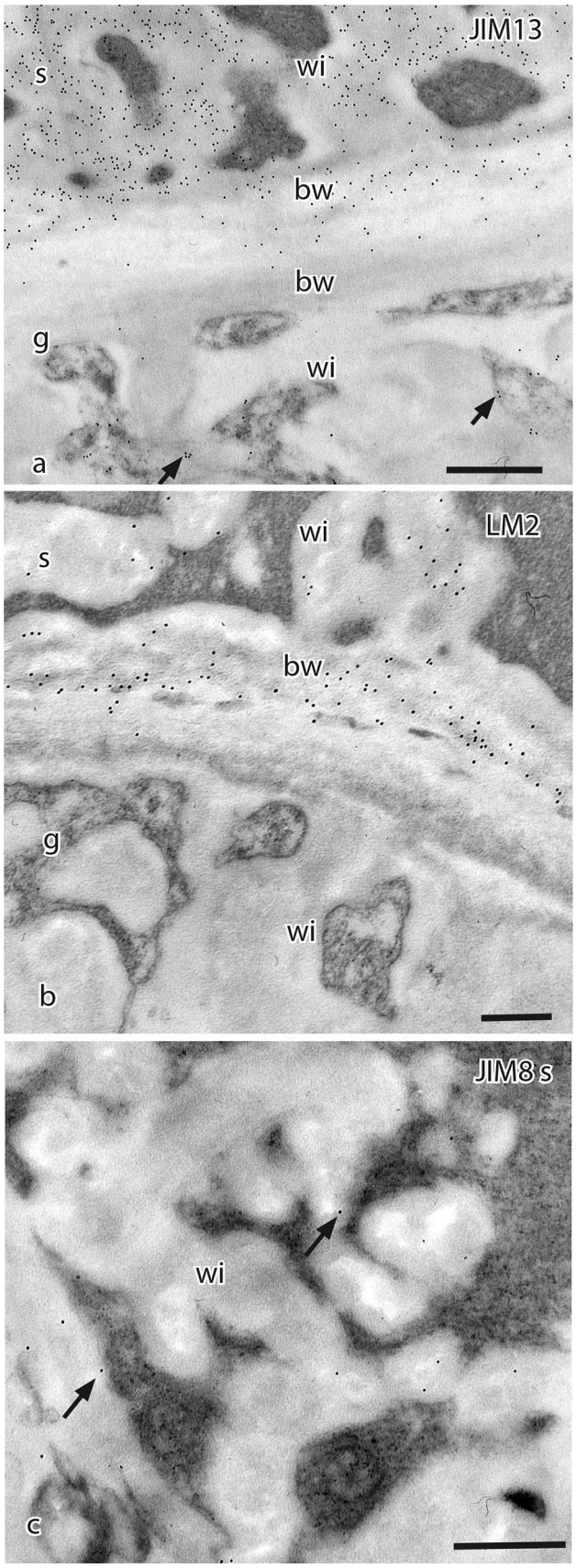

Fig. 4 TEM immunogold labeling with monoclonal antibodies ▶ to AGPs. **a** JIM13 strongly labels sporophyte (s) cells throughout the basal wall (bw) and wall ingrowths (wi) while labels are sparse (arrows) around the dark fibrillar region of gametophyte (g) ingrowths (wi).**b** LM2 labels are restricted to the basal wall (bw) and wall ingrowths (wi) in the sporophyte (s). **c** Few JIM8 labels occur (arrows) along the outer edges of the wall ingrowth (wi) in sporophyte transfer cells (s). Scale bars: 0.5 µm for (**a**–**d**)

hyaline cell walls in *Sphagnum novo-zelandicum* (Kremer et al. 2004). In *M. polymorpha,* AGPs have been implicated in protonemata differentiation (Shibaya et al. 2005), cell wall regeneration of cultured protoplasts (Shibaya and Sugawara 2007) and cell plate formation (Shibaya and Sugawara 2009). They are involved with tip cell extension of protonemata and water balance in *P. patens* (Kobayashi et al. 2011; Lee et al. 2005). AGPs are also abundant during spermatogenesis and oogenesis in *C. richardii* (Lopez and Renzaglia 2014, 2016) and spermatogenesis in the moss *Aulacomnium palustre* (Lopez-Swalls 2016).

Extensins were not detected in the placenta of *M. polymorpha* in sharp contrast with wall ingrowths of the transfer cells in root nodules of *Pisum sativum,* where abundant labeling with extensin antibodies suggests that these hydroxyproline-rich glycoproteins are involved in nodule development (Dihaya and Brewin 2000). Because extensins are highly diverse, probing with additional antibodies to other epitopes may well identify this constituent in the transfer cell walls of bryophytes. The absence of callose in *M. polymorpha* placental cells also contrasts with studies on transfer cells in tracheophytes. In *V. faba,* callose is a prominent constituent of the translucent outer layer of transfer cell wall ingrowths, suggesting this polymer is involved in a "spreading" process similar to that in cell plate formation (Samuels et al. 1995; Vaughn et al. 1996). Callose was also detected in “channel-like” structures found in transfer cell wall ingrowths that form next to plasmodesmata in *P. sativum* root nodules (Dihaya and Brewin 2000).

A key finding in this study is the differential localization of cell wall polymers in the transfer cells on either side of the placenta in *M. polymorpha*. Although similar in ultrastructure, cell walls on the sporophyte side are cellulose poor and enriched with xyloglucans (LM25) and diverse AGPs not found or absent on the gametophyte side. Both xyloglucans and AGPs would impart strength while maintaining flexibility in the absence of cellulose. These wall constituents play a similar role in male gamete maturation in both mosses and ferns (Lopez and Renzaglia 2014; Lopez-Swalls 2016). AGPs are likely involved in signaling through calcium binding and release in *M. polymorpha* sporophyte placental cells as has been speculated in sperm cell differentiation. This is consistent with directional signaling from gametophyte through the apoplast into foot cells.

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Table 3 Cell wall polymers and their associated properties in the wall

Cell wall polymer		Associated wall properties	References
Esterified HG		Porosity Expansibility Elasticity	Braybrook and Jönsson (2016); Cornuault et al. (2017); Verhertbruggen (2009)
De-esterified HG		Ca^{2+} binding increases rigidity Resistance to mechanical stress Cell adhesion	Cornuault et al. (2017); Verhertbruggen et al. (2013); Verhertbruggen (2009)
RG-I Pectin	Arabinan	Spatially buffer HG Flexibility/elasticity Expansibility Porosity Water holding capacity Signaling	Cornuault et al. (2017); McCartney et al. (2003); Jones et al. (2003); Verhertbruggen et al. (2013)
	Galactan	Rigidity Tip growth in some cells	McCartney et al. (2003); Cornuault et al. (2017)
Hemicellulose	Xyloglucan	Expansibility Cell to cell adhesion Cross-linkage/ tethering Regulating yield threshold Nutrient supply	Braybrook and Jönsson (2016); Whitney et al. (2006); Chanliaud et al. (2002); Ordaz-Ortiz et al. (2009); Bunterngsook et al. (2015)
	Mannan	Anchorage to substrate Interaction with soil particles and/or microorganisms Nutrient uptake Water sensing Strengthening Hydrated/de-hydrated cycles Cross-link with cellulose Nutrient supply	Marcus et al. (2010); Scheller and Ulvskov (2010); Dehors et al. (2019); Plancot et al. (2019)
AGPs		Developmental processes Cell identity Structural integrity Galactan turnover Ca^{2+} regulation/signal transduction Plasticity – unidirectional deformation Desiccation tolerance Membrane integrity Tip growth Sexual reproduction	Torode et al. (2018); McCartney et al. (2003); Lamport et al. (2014); Lamport et al. (2018); Lee et al. (2005); Lopez et al. (2014)
Extensins		Cell wall assembly and growth Tip growth in pollen tubes/root hairs Cell wall/cytoplasm communication	Diet et al. 2006; Ringli 2010; Velasquez et al. 2012; Bascom et al. 2018
Callose		Stress response Sieve plate/ sieve areas Cell plate formation Plasmodesmata Developmental processes Tip growth/ Pollen tube Spore wall development Sperm cell differentiation Desiccation tolerance	Samuels et al. 1995; Vaughn et al. 1996; Renzaglia et al. 2000; Lopez et al. 2017; Schuette et al. 2009; Cao et al. 2014; Moller et al. 2007; Tang 2007; Berry et al. 2016; Bopp et al. 1991; Renzaglia et al. 2015; Renzaglia and Garbary 2001; Radford 1998

Characterization of the carbohydrate and protein constituents of cell walls is the first step in understanding the interactions and specific functions of wall polymers in bryophyte cell walls. As evidenced in this study of the labyrinth cell walls of placental transfer cells, similarities in architecture are not necessarily reflective of common wall composition and organization. Clearly, additional studies of cell walls across tissue types and across the diversity of bryophytes are now required to assess variability and changes in cell wall architecture through evolution. With this fundamental information, targeted genetic studies can be conducted to identify the function of individual genes involved in the

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manufacturing of cell wall polymers and their effects on wall properties. Because of their abundance and differential expression, AGP genes are of particular interest in the placenta of *Marchantia polymorpha*. In particular, the GT31 gene subfamilies hyp-galactosyltransferases (Hyp-GALTs and HPGTs) that are involved in AGP glycosylation (Showalter and Basu 2016) are widespread across land plants (Harholt et al. 2012; Ogawa-Ohnishi and Matsubayashi 2015) and are good candidates for studying the role AGPs play in multiple tissues and processes. Such work would not only advance understanding of the genetic mechanisms involved in the construction and function of special cell walls such as those in transfer cells but also primary cell walls in general.

Author contributions Jason S Henry and Karen S Renzaglia designed the study and wrote the manuscript. Material preparation and data collection were performed by Jason S Henry. All authors analyzed data, edited the writing and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors affirm there is no conflict of interest.

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