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Cell wall polymers in the *Phaeoceros* placenta reflect developmental and functional differences across generations

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Abstract

The placenta of hornworts is unique among bryophytes in the restriction of transfer cells that are characterized by elaborate wall labyrinths to the gametophyte generation. During development, cells around the periphery of the sporophyte foot elongate, forming smooth-walled haustorial cells that interdigitate with gametophyte cells. Using immunogold labeling with 22 antibodies to diverse cell wall polymers, we examined compositional differences in the developmentally and morphologically distinct cell walls of gametophyte transfer cells and sporophyte haustorial cells in the placenta of *Phaeoceros*. As detected by Calcofluor White fluorescence, cellulose forms the cell wall scaffolding in cells on both sides of the placenta. Homogalacturonan (HG) and rhamnogalacturonan I (RG-I) pectins are abundant in both cell types, and haustorial cells are further enriched in methyl-esterified HGs. The abundance of pectins in placental cell walls is consistent with the postulated roles of these polymers in cell wall porosity and in maintaining an acidic apoplastic pH favorable to solute transport. Xyloglucan hemicellulose, but not mannans or glucuronoxylans, are present in cell walls at the interface between the two generations with a lower density in gametophytic wall ingrowths. Arabinogalactan proteins (AGPs) are diverse along the plasmalemma of placental cells and are absent in surrounding cells in both generations. AGPs in placental cell walls may play a role in calcium binding and release associated with signal transduction as has been speculated for these glycoproteins in other plants. Callose is restricted to thin areas in cell walls of gametophyte transfer cells. In contrast to studies of transfer cells in other systems, no reaction to the JIM12 antibody against extensin was observed in *Phaeoceros*.

Keywords: AGPs, bryophytes, hemicelluloses, immunocytochemistry, pectins, transfer cells, wall ingrowths

Introduction

Although the hornwort sporophyte is capable of carbon assimilation sufficient for maintenance, carbon uptake for continued growth requires transport of sugar and nutrients from the gametophyte through the placenta, a specialized tissue at the interface between the two generations (Thomas *et al*. 1978). The hornwort sporophyte elongates from a persistent basal meristem and produces and disperses spores for an extended timespan. Consequently, the placenta is persistent and active throughout the growing season. The sporophyte develops a bulbous foot before emerging from the gametophyte involucre and initiating spore production. During development, cells at the foot periphery elongate to form smooth-walled haustorial cells that penetrate the adjoining gametophyte tissue (Gambardella & Ligrone 1987, Ligrone & Renzaglia 1990, Ligrone *et al*. 1993). Concomitant with the intrusive growth of haustorial cells, gametophyte cells develop elaborate cell wall labyrinths that are diagnostic of transfer cells and vastly increase the surface area of the plasmalemma (Gambardella & Ligrone 1987, Ligrone & Gambardella 1988, Ligrone & Renzaglia 1990, Ligrone *et al*. 1993). Transfer cells are widespread across land plants where they are found in strategic locations and facilitate enhanced membrane-mediated nutrient transport from symplast to apoplast or vice versa (Pate & Gunning 1972, Renault *et al.* 1992, Thompson *et al*. 2001, Offler *et al.* 2002, Regmi *et al.* 2017). In the hornwort placenta, transfer

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cells are restricted to the gametophyte generation, but are highly variable in location in the placenta of mosses and liverworts (Renzaglia 1978, Browning & Gunning 1979, Ligrone & Renzaglia 1990, Ligrone *et al*. 1993, Vaughn & Hasegawa 1993, Villarreal & Renzaglia 2006).

The degree of integration of placental cells varies across hornwort taxa. In *Phaeoceros* Proskauer (1951: 346) and most other genera, gametophytic transfer cells and sporophytic haustorial cells are interdigitated with random intergenerational spaces between. Placental spaces in some genera including *Phaeoceros* contain prominent crystals of proteinaceous material (Gambardella & Ligrone 1987). On the other extreme, exemplified by *Anthoceros* Linnaeus (1753: 1139), the foot contains an orderly palisade of peripheral cells and a zone of crystal-free mucilage separates the foot from transfer cells on the gametophyte side (Renzaglia 1978, Ligrone *et al*. 1993, Frangedakis *et al*. 2020).

A key to understanding the complexity of biochemical and cellular processes at the gametophyte-sporophyte junction lies in the composition and organization of the apoplastic interface between the generations. In this zone, the composition and arrangement of constituent cell wall polymers largely prescribe the transport of materials and cellular interactions between generations (Humphrey *et al.* 2007). In the liverwort *Marchantia* Linnaeus (1753: 1137), which has well-developed transfer cells in both generations, differential expression of cell wall polymers is consistent with gametophyte-to-sporophyte unidirectional movement of materials (Henry *et al*. 2020). Whereas pectins are widespread on both sides of the placenta in this liverwort, hemicelluloses (xyloglucans) and arabinogalactan proteins (AGPs) show restricted occurrences. Notably, sporophyte transfer cell walls are relatively poor in cellulose and enriched with xyloglucans and a diversity of AGPs not found in the gametophyte counterpart, a finding that is consistent with directional movement. With sparse data on cell wall composition in the placenta of other taxa and transfer cells in general, it is not clear whether there is a common cell wall configuration that supports the function of placental cells. The organization of the placenta of *Phaeoceros* provides a platform on which to explore the variability of wall composition in morphologically distinct cells that co-operate in unidirectional transport.

In this second study of placental cell walls in bryophytes, we probed two species of *Phaeoceros, P. carolinianus* (Michaux 1803a: 280) Proskauer (1951: 347) and *P. laevis* (Linnaeus 1753: 1139) Proskauer (1951: 347), using immunogold labeling at the TEM level with 22 monoclonal antibodies to cell wall polysaccharides and AGPs. Because they are closely related genetically, these morphologically similar species are recognized by some as subspecies of the genus *P. laevis* sensu latu (Duff *et al.* 2007, Bisang *et al.* 2010). The two cell types of interest in this study of the hornwort placenta are haustorial cells and intermingling gametophyte cells with transfer cell morphology. Focusing on this system, we addressed the following questions: What are the compositional differences and/or commonalities between the labyrinthine cell walls in gametophyte transfer cells and the smooth walls in sporophyte haustorial cells*?* How does polymer composition in these cells compare with transfer cell wall composition in other systems?

Materials and Methods

Fertile plants of *Phaeoceros carolinianus* from Makanda, IL, USA and *Phaeoceros laevis* from Campania, Italy were collected and processed immediately for light and electron microscopy. Histochemistry and immunolabeling were conducted on fully developed placentae with mature cells and developed cell walls.

Preparation for transmission electron microscopy

Plants were prepared for transmission electron microscopy (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 1 h at room temperature and overnight at 4 °C. Following 2–3 rinses in the same buffer for 15 min each, the samples were post-fixed in 2% buffered osmium tetroxide for 30 min and rinsed in autoclaved, distilled water. The samples were dehydrated in progressively higher ethanol to water concentrations and rinsed twice in 100% ethanol. Infiltration was achieved by progressive placement of material in higher concentrations of LR White resin diluted with ethanol. Once the samples reached 100% LR White, 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. Thin sections (90–100 nm) were collected on 200 mesh nickel grids for immunolabeling.

Thick sections (800–1500 nm) were collected on glass slides and stained with 1% toluidine blue in 1% sodium tetraborate to assess the stage of development and the quality of the tissue for histochemical staining and immunogold labeling. Only sporophytes with fully developed feet and good structural preservation were probed further as follows.

Histochemical staining

Cellulose was detected by incubating sections on glass slides for 1 h in a drop of Calcofluor White (Sigma-Aldrich) and a drop of 10% KOH in the dark. Controls were made using only 10% KOH solution. Materials were viewed under a Leica DM5000 B compound microscope using UV fluorescence.

For silver intensification of immunohistochemical tests, 0.5 μm-thick sections were mounted on glass slides coated with BioBond (EMS) to enhance sticking. The slides were placed in a chamber with high relative humidity for the following incubation steps: 1) 30 min in 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS); 2) 4 h in primary antibodies diluted 1:40 with PBS-BSA; 3) three 30 min exchanges of PBS-BSA; gold-conjugated goat-anti-rabbit secondary antibody (BB International) diluted 1:20 in PBS-BSA; 4) three washes of PBS. After a rinse with double-distilled water, the slides were incubated in a freshly prepared solution from the Amersham InstenSe (Amersham Bioscience) silver enhancement kit, which reacts with gold particles and makes them visible under the light microscope as a fine black precipitate. After the silver developed (30 min), slides were washed with distilled water, dried and coverslip-mounted with Permount (Polysciences). Serial sections were stained for 1 min with 1% toluidine blue in 1% sodium tetraborate for general histology. The sections were photographed using a Zeiss Axioskop microscope (Carl Zeiss).

Immunogold labeling

Samples were processed as detailed in Lopez *et al*. (2017) and Renzaglia *et al*. (2017). In short, thin sections on nickel grids were floated on drops of BSA/PBS overnight at 4 °C, and then overnight on a primary antibody specific to the desired wall epitope. The grids were then rinsed 4×4 min each in 0.05 M BSA/PBS, treated with a secondary antibody with a gold tag attached for 30 min at room temperature, rinsed again in PBS 4×4 min each, and then with a jet of sterile H_2O.

Grids were observed before and after post-staining with lead citrate and uranyl acetate (these stains allow for better contrast but may obscure the immuno-gold labels in the transmission electron microscope). Control grids were prepared by excluding the primary antibodies. Samples were viewed and digital micrographs were collected with a Hitachi H7650 transmission electron microscope. The monoclonal antibodies used in this study are listed in Table 1.

Quantification of label abundance

Images were analyzed in PhotoScapeX (Mooii Tech) by counting the number of labels in three randomly placed 100 Å~ 100-pixel frames in the cell wall of interest. This process was repeated on 10–15 images for each monoclonal antibody (MAb). The average of all counts was calculated and scored as follows: averages of 1 to 4 labels per frame were assigned a single plus (+), and two pluses (+ +) were assigned to averages between 5 and 9 labels. Any average greater than 9 labels per frame received a triple plus (+ + +). Antibodies with average label density between 0 and 1 were assigned a plus/ minus (±).

TABLE 1. Primary antibodies used to immunogold label carbohydrates and arabinogalactan proteins in placental cell walls of *Phaeoceros*.

Antibody	Antigen (s)/ Epitope	Reference/ Source
CCRCM38	homogalacturonan (HG)	Pattathil (2010). Souce: Carbosource Service, University of Georgia
JIM5	Homogalacturonan/ Un-esterified	Knox *et al.* (1990)/ J. P. Knox PlantProbes, University of Leeds, UK
LM19	Homogalacturonan/ Un-esterified	Verhertbruggen *et al.* (2009)/ J. P. Knox PlantProbes, University of Leeds, UK
JIM7	Homogalacturonan/Partial Methyl-esterified	Willats *et al.* (2000)/ M. Hahn, Complex Carbohydrate Research Center, University of Georgia, USA
LM18	Homogalacturonan/ Methyl-esterified	Verhertbruggen *et al.* (2009)/ J. P. Knox PlantProbes, University of Leeds, UK

...Continued on the next page

TABLE 1. (Continued)

Antibody	Antigen (s)/ Epitope	Reference/ Source
LM20	Homogalacturonan/ Methyl-esterified	Verhertbruggen *et al.* (2009)/ J. P. Knox PlantProbes, University of Leeds, UK
CCRCM36	Rhamnogalacturonan-I	Carbosource Service, University of Georgia
CCRCM2	Rhamnogalacturonan-I MeBSA complex	Pattathil (2010). Souce: Carbosource Service, University of Georgia
LM5	Galactan, rhamnogalacturonan-I/ (1-4)-β-d-galactan	Jones *et al.* (1997)/ J. P. Knox PlantProbes, University of Leeds, UK
LM16	galactosyl residues on rhamnogalacturonan I (RGI)	Vehrertbruggen *et al.* (2009). Source: PlantProbes, Leeds
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
CCRCM1	Xyloglucan alpha-Fuc-(1,2)-beta-Gal	Pattathil (2010). Souce: Carbosource Service, University of Georgia
CCRCM7	Rhamnogalacturonan I trimer or larger of beta-(1,6)-Gal carrying one or more Ara residues of unknown linkage	Pattathil (2010). Souce: Carbosource Service, University of Georgia
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
LM2	Arabinogalactan protein (AGP)/ β-d-GlcA (glucuronic acid)	Smallwood *et al.* (1996)/ J. P. Knox PlantProbes, University of Leeds, UK
LM6	Arabinan, rhamnogalacturonan-I/ (1-5)-α-L-arabinan, also labels arabinogalactan protein	Willats *et al.* (1998); Verhertbruggen *et al.* (2009)/ J. P. Knox PlantProbes, University of Leeds, UK
JIM12	Extensin	Smallwood *et al.* (1994)/ J. P. Knox PlantProbes, University of Leeds, UK
Anticallose	Callose/ (1,3)-β-linked penta-to-hexa-glucan	Meikle *et al.* (1991)/ Biosupplies Australia

Results

The placenta of *Phaeoceros* consists of peripheral foot cells that lack wall ingrowths and elongate to form haustorial cells that interdigitate with gametophyte transfer cells (Fig. 1). Haustorial cells develop early in embryology and penetrate the gametophytic tissue, which in response initiates cell division followed by the development of cell wall ingrowths (Fig. 1A). Gametophyte cells in the fully developed placenta display extensive wall labyrinths that vastly increase the surface area of the cell membrane (Fig. 1B). The wall ingrowths consist of a peripheral electron-lucent area and an inner fibrous core that is continuous with the basal primary wall (Fig. 1B). Haustorial cells have thin walls throughout (Fig. 1B–D). The placenta includes a network of intergenerational spaces locally expanding into wide lacunae, which originate from the separation of gametophyte cells and intrusive growth of haustorial cells. The intergenerational zone contains loose fibrillar/granular material and protein crystals of varying size in *P. laevis* but not *P. carolinianus* (Fig. 1C). Calcofluor white staining demonstrates that cellulose is a key constituent of placental cell walls including the smooth wall of haustorial cells and the wall labyrinth apparatus of gametophyte cells (Fig. 1D).

In general, the haustorial cells and the fibrous core of wall ingrowths are enriched in pectins relative to neighboring non-placental tissues (Fig. 2). Labeling with monoclonal antibodies targeted to un-esterified HG epitopes (CCRC-M38, JIM5, LM19) gives a positive reaction in placental cells (Fig. 2A–G). Silver enhancement of sections incubated in CCRC-M38 shows even labeling of both gametophyte and sporophyte cells as well as intercellular spaces (Fig. 2A, B). Immuno-silver labeling with JIM5 demonstrates the presence of un-esterified HGs in all placental cells as

well as adjacent parenchyma cells on both sides of the placenta (Fig. 2C). TEM imaging of CCRC-M38 labeling shows a strong reaction in the core of wall ingrowths (Fig. 2D), the haustorial cell walls and the fibrillar material in intercellular spaces (Fig. 2E). Un-esterified HG epitopes targeted by JIM5 are also aggregated along haustorial cell walls and concentrated in the cores of wall ingrowths (Fig. 2F). LM19 (targeting un-esterified HG) abundantly labels haustorial cell walls and also produces scattered labeling of wall ingrowth cores (Fig. 2G). Placental cells also react with antibodies against methyl-esterified HG pectins (LM18, JIM7, LM20) (Fig. 2H-K). LM18 labels the haustorial cell walls throughout (Fig. 2H) and the wall ingrowth cores (Fig. 2I). JIM7 gives a similar labeling of haustorial cells as LM18 but produces only a sparse labeling of wall ingrowths (Fig. 2J). The LM20 epitope is scattered and restricted to sporophyte cells (Fig. 2K).

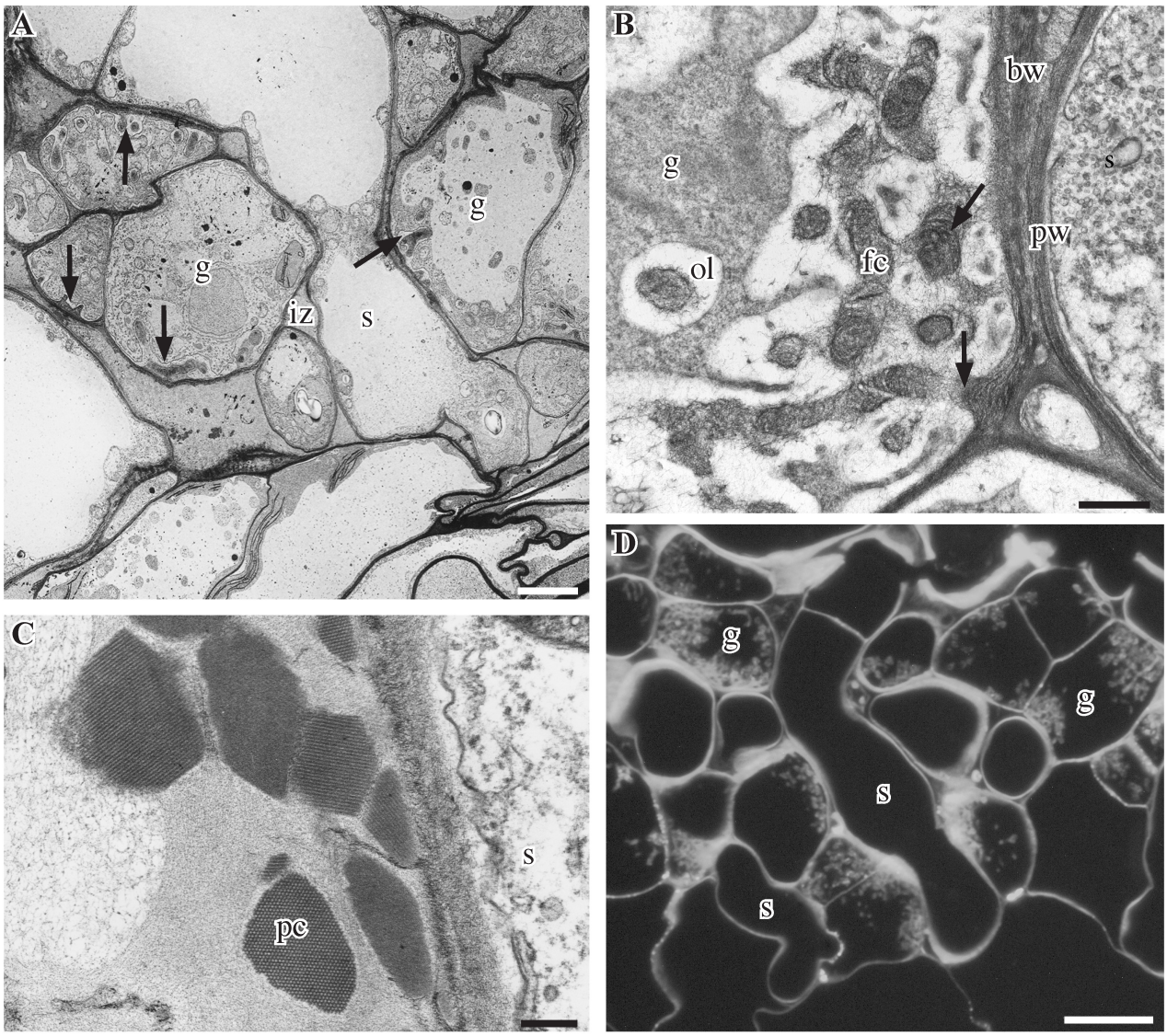

FIGURE 1. Sporophyte-gametophyte junction in *Phaeoceros*. A. TEM image of developing placenta in *P. laevis* showing an elongated sporophyte haustorial cell (s) penetrating differentiating gametophytic (g) transfer cells with nascent wall ingrowth (arrows) separated by an intergenerational zone (iz). B. *P. carolinianus* showing smooth thin primary cell wall (pw) of a sporophyte haustorial cell (s) adjacent to a gametophyte cell (g) with extensive wall ingrowths that are continuous with the basal wall (arrows) and are composed of an electron-lucent outer layer (ol) and fibrous inner core (fc). C. *P. laevis.* TEM image showing sporophyte (s) haustorial cell and adjacent protein crystals (pc) found in the granular to fibrous material between generations. D. *P. carolinianus.* Calcofluor white fluorescence showing cellulose in cell walls of elongated haustorial sporophyte (s) cells, and in basal wall and extensive wall ingrowths of intermingled gametophyte cells (g) in thick section. Scale bars: 0.5 µm (A), 0.2 µm (B), 0.25 µm (C), 2 µm (D).

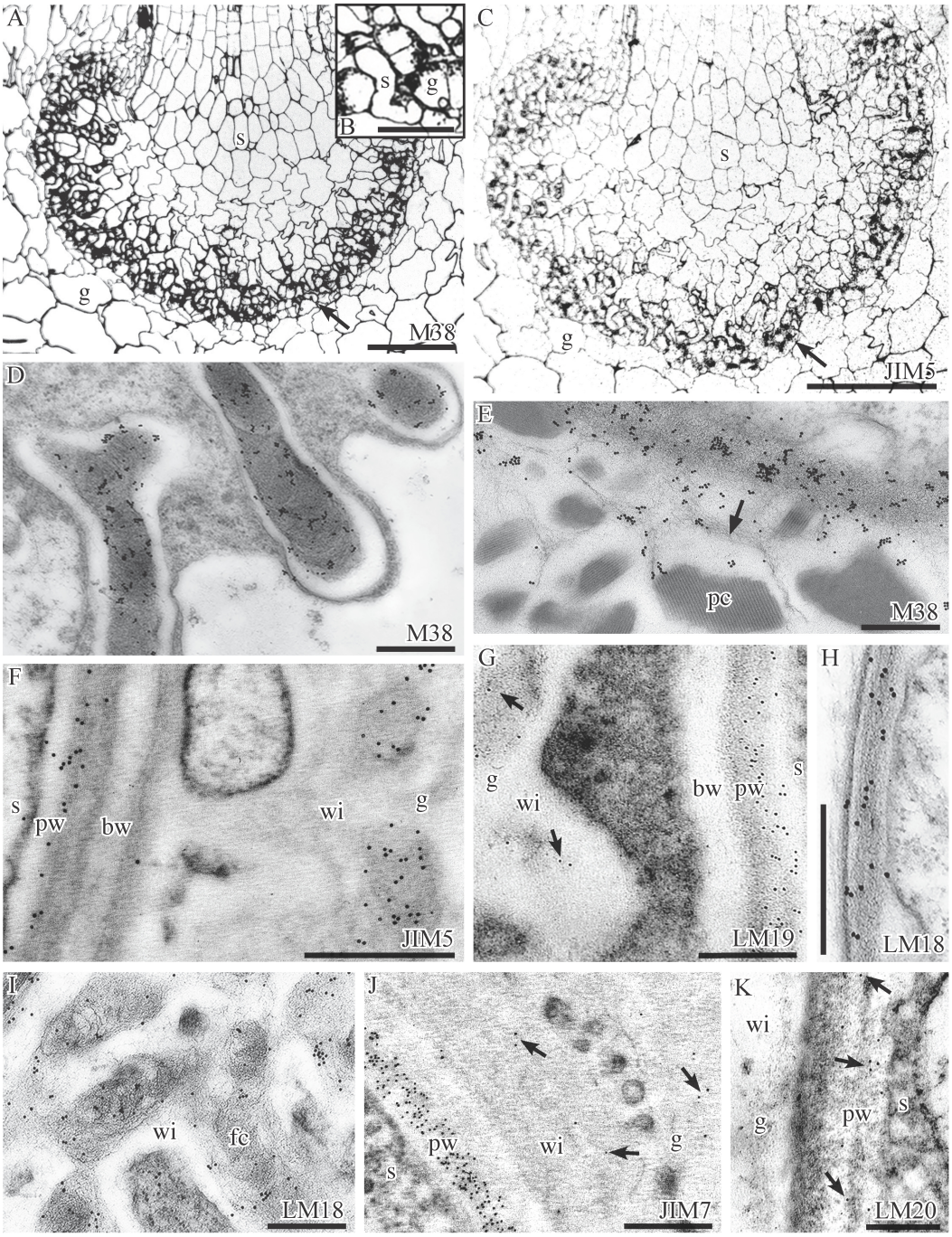

FIGURE 2. Immunolabeling with monoclonal antibodies to pectin epitopes in *Phaeoceros.* A–C *P. laevis* silver-enhanced immunogold labeling light micrographs. A. CCRC-M38 labels all cell walls in sporophyte (s) and gametophyte (g) and is particularly intense in the placenta (arrow). B. Higher magnification of CCRC-M38 labeling showing silver in haustorial cells of the sporophyte (s), the wall labyrinth in gametophyte (g) cells and intergenerational zone. C. JIM5 labels gametophyte (g) and sporophyte (s) cells with particular intensity in the wall ingrowths of gametophyte cells (arrow). D–K Immunogold labeling TEM micrographs. D, E. *P. laevis.* CCRC-M38 labeling yields abundant aggregates of gold particles in placental walls. (D) Fibrous core of gametophyte wall ingrowths, (E) Haustorial cell wall and associated fibrous material (arrow) around protein crystals (pc) in the intergenerational zone. F. *P. carolinianus.* JIM5 labels inner primary wall (pw) of sporophyte (s) haustorial cells and the fibrous core of gametophyte (g) wall ingrowths (wi), but not the basal wall (bw). G. *P. carolinianus.* LM19 abundantly labels the sporophyte (s) primary wall (pw) but in the gametophyte (g), labeling is sparse (arrows) in the electron dense region of wall ingrowths (wi) and absent in the basal wall (bw). H, I. *P. laevis.* Strong labeling with LM18. H. Sporophyte haustorial cell wall. I. Fibrous core (fc) of gametophyte wall ingrowths (wi). J. *P. carolinianus.* JIM7 labels throughout the sporophyte (s) primary cell wall (pw) but is sparse (arrows) in gametophyte (g) wall ingrowths (wi). K. *P. carolinianus.* LM20 lightly labels the sporophyte (s) primary wall (pw) (arrows) but does not label gametophyte (g) wall ingrowths (wi). Scale bars: 200 μm (A, C), 100 μm (B), 0.1 μm (D, E, G, I, J), 0.5 μm (F, H).

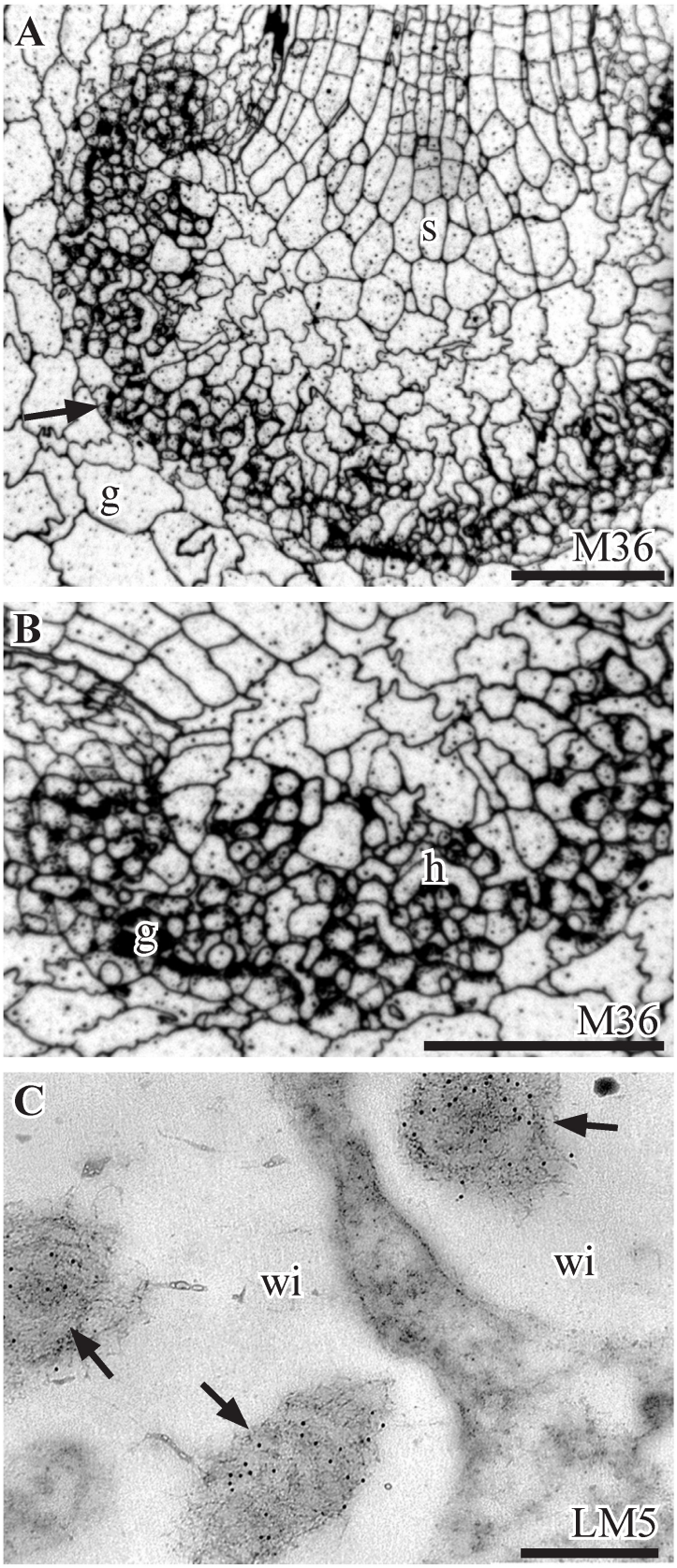

FIGURE 3. Immunolabeling with monoclonal antibodies to rhamnogalacturonan-I epitopes in *Phaeoceros.* A, B. *P. laevis.* Silver-enhanced immunogold labeling with CCRC-M36. A. Labeling occurs in all cell walls in the gametophyte (g) and sporophyte (s) and is particularly intense in the placenta (arrow). B. Higher magnification of the placenta showing abundant staining in haustorial cells (h), gametophyte wall ingrowths (g) and zone between generations. C. *P. carolinianus.* TEM micrograph of abundant LM5 labels (arrows) in the fibrous cores of gametophyte wall ingrowths (wi). Scale bars: 200 µm (A, B), 0.1 µm (C).

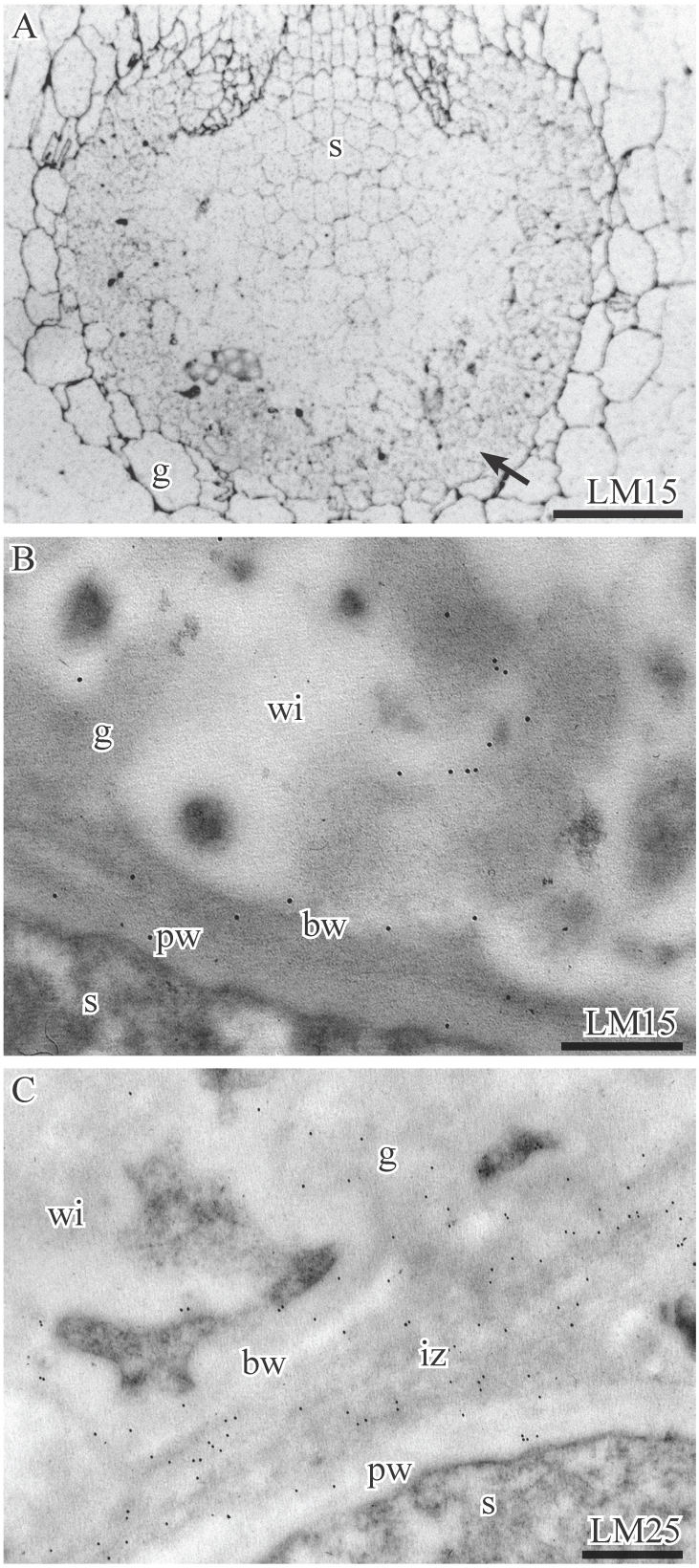

FIGURE 4. Immunolabeling with monoclonal antibodies to hemicellulose epitopes in *Phaeoceros.* A. *P. laevis*. Silver-enhanced immunogold labeling with LM15 shows strong labeling of gametophyte (g) surrounding the placenta and weak labeling of sporophyte (s) and placental cells (arrow). B, C. *P. carolinianus* TEM micrographs. B. LM15 labeling is scattered in the basal wall layer (bw) and along the periphery of the fibrous core of wall ingrowths (wi) in gametophyte (g) cells and is sparse in the sporophyte (s) primary wall (pw). C. LM25 weakly labels the basal walls (bw) and wall ingrowths (wi) in the gametophyte (g) and the primary cell wall (pw) of the sporophyte (s) but more strongly labels the intergenerational zone (iz). Scale bars: 200 µm (A), 0.1 µm (B, C).

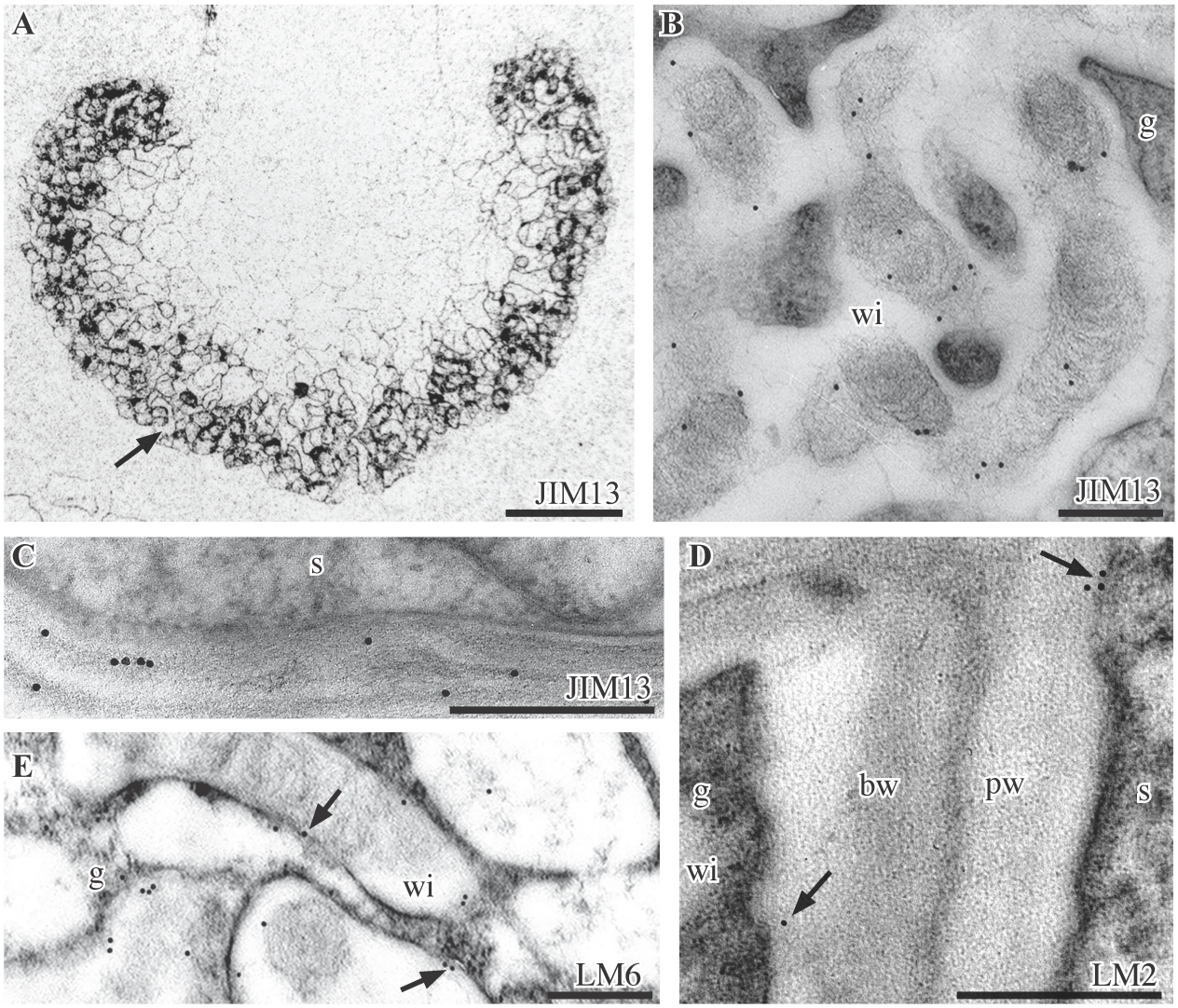

FIGURE 5. Immunolabeling with monoclonal antibodies to AGPs in *Phaeoceros.* A–C. *P. laevis.* JIM13 immunolabeling. A. Silver-enhanced immunogold labeling shows labeling in the placenta only (arrow). B. TEM micrograph shows JIM13 labels the fibrous core of gametophyte (g) wall ingrowths (wi). C. TEM image of sporophyte (s) haustorial cell wall labeled. D, E. *P. carolinianus.* TEM micrographs. D. Few LM2 labels (arrows) occur in the primary cell wall (pw) of the sporophyte (s) and basal wall layer (bw) of gametophyte (g) cells. E. LM6 labels on and near the plasmalemma (arrows) around gametophyte (g) wall ingrowths (wi). Scale bars: 200 µm (A), 0.1 µm (B, E), 0.5 µm (C, D).

RG-I pectins, labeled with CCRC-M36 are found in all cells and are abundant in wall ingrowths of gametophyte cells and interdigitating haustorial cells (Fig. 3A, B). In the placenta, RG-I pectin epitopes recognized by LM5 are restricted to the fibrous core of wall ingrowths on the gametophyte side (Fig. 3C).

Four antibodies (LM15, LM21, LM25, LM28) were used to target epitopes associated with xyloglucan and glucuronoxylan hemicelluloses, with only LM15 and LM25 epitopes located in the *Phaeoceros* placenta (Fig. 4). Immunosilver labeling with LM15 identifies xyloglucan epitopes clearly in non-placental gametophyte cells but not in placental cells (Fig. 4A). However, TEM imaging of LM15-treated sections reveals scattered labeling of wall ingrowths (Fig. 4B). Galactosylated xyloglucan epitopes recognized by LM25 are relatively abundant at the interface between the two generations and less so in wall ingrowth cores (Fig. 4C). Tests with LM21 (mannan) and LM28 (glucuronoxylan) produced no appreciable labeling of placental cells (not shown).

Gametophyte and sporophyte display considerable diversity in the use of AGPs (targeted by JIM13, LM2, LM6, JIM8) in the placenta of *Phaeoceros* (Fig. 5). AGP epitopes recognized by JIM13 are specific to the placenta (Fig. 5A), where they are localized in the fibrous core of wall ingrowths (Fig. 5B) and in haustorial cell walls (Fig. 5C). Very weak labeling is observed with LM2 in both generations (Fig. 5D). LM6 arabinan epitopes are expressed in the

gametophyte along the plasma membrane outlining wall ingrowths (Fig. 5E) and, to a lower extent, in haustorial cells (not shown). JIM8 AGP epitopes are absent from *Phaeoceros* (not shown). No expression of the extensin protein was detected with JIM12.

Callose, as visualized with an anti-callose monoclonal antibody, occurs in clusters associated with thin cell wall areas away from wall ingrowths in gametophyte transfer cells (Fig. 6).

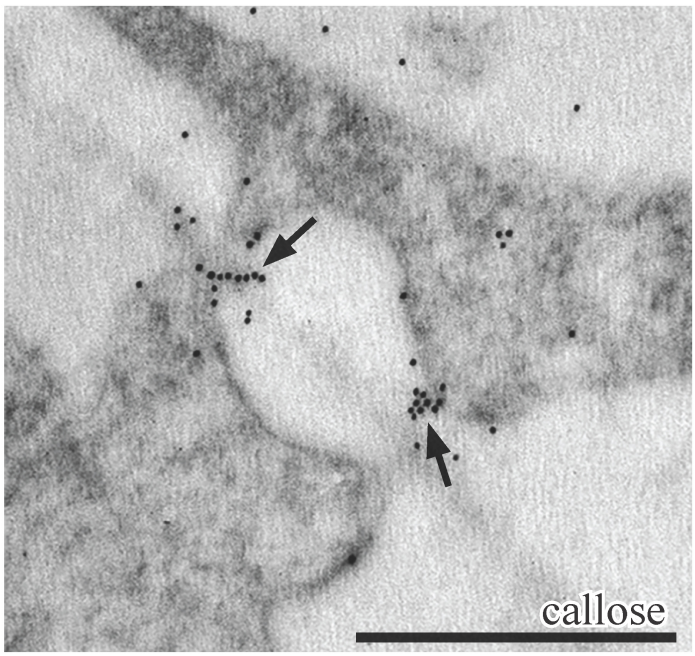

FIGURE 6. *P. carolinianus.* TEM micrograph of immunogold labeling with anti-callose in *Phaeoceros* gametophyte cells of placenta show labels are clustered (arrows) in the thin areas of the primary wall at the former site of plasmodesmata. Scale bar: 0.5 µm.

TABLE 2. Relative intensity of immunogold labeling of placental cells in *Phaeoceros.* * *P. carolinianus*, ** *P. laevis. Notes*: +++, very strong; ++, strong; +, weak; ±, present; −, absent; LM6 binds to arabinan residues in RG-I pectins and AGPs. Primary antibody colors indicate polymers targeted: grey = HG pectins, orange = RG-I pectins, green = hemicelluloses, blue = AGPs, yellow = extensin, and red = callose.

Primary Antibody	Sporophyte	Gametophyte
CCRCM38 **	++	++
JIM5 *, **	++	+
LM19 *	++	+
JIM7 *	+++	++
LM18 **	++	++
LM20 *	+	–
CCRCM36 **	++	++
LM5 *	+	++
LM16 **	–	–
LM15 *, **	±	+
LM21 *	–	–
LM25 *	+	+
LM28 *	–	–
JIM8 *	–	–
JIM13 *, **	+	+
LM2 *	±	±
LM6 *, **, [1]	±	++
JIM12 *	–	–
Callose *, **	–	+

Discussion

The primary difference between the placenta of the two hornwort species examined in this study is the occurrence of protein crystals in *P. laevis* and their absence in *P. carolinianus*. Gambardella & Ligrone (1987) found that placental crystals in *P. laevis* are not stained with the PATAg test for carbohydrates, but are readily digested by proteases, suggesting a sugar-free protein composition. In line with these results, the crystals did not label with any of the antibodies used in this study, whereas the fibrillar material around them gave positive response to CCRC-M38. This MAb recognizes a number of HG epitopes and the reaction of material around crystals may indicate middle lamellae material that has become detached during the formation of the placenta. Alternatively, because CCRC-M38 was raised against *Arabidopsis* Heynhold (1842: 538) seed mucilage, the fibrillar material may represent compositionally similar mucilage in the intercellular spaces of the gametophyte/ sporophyte junction. Placental crystals are restricted to hornworts among bryophytes and have been reported to be taxonomically diagnostic and consistent within genera (Vaughn & Hasegawa 1993, Renzaglia *et al*. 2009), yet this does not appear to be the case in *Phaeoceros*. It is possible that differences in the abundance of symbiotic cyanobacteria and the consequent differential availability of assimilated nitrogen in the two taxa may account for this discrepancy, but this requires further investigation.

A cellulosic scaffolding is the foundation of cell walls in the placenta of *Phaeoceros*. This contrasts with findings in *Marchantia*, where cellulose is sparse in wall ingrowths, especially on the sporophytic side of the placenta (Henry *et al*. 2020). As typical of the primary plant cell walls (Cosgrove 2005, Broxterman & Schols 2018), cellulose in *Phaeoceros* placental cells is embedded in a diverse polysaccharidic network rich in pectins and hemicelluloses, and interspersed with AGPs (Table 2).

Pectins are the most diverse and abundant non-cellulosic polymers in the *Phaeoceros* placenta, as is true in the primary cell walls of seed plants (O'Neill & York 2018). The arrangement and composition of pectins are hypothesized to control cell wall properties such as porosity and flexibility (Caffall *et al*. 2009) (Table 3), thus providing a window into their role in placental cells. The antibodies against homogalacturonans epitopes (JIM7 and LM20) label haustorial cell walls more intensely than gametophyte wall ingrowths (Table 2). Cell walls enriched in methyl-esterified HGs have a lower apoplastic pH, and may facilitate nutrient uptake by membrane transport proteins (Willats *et al*. 2001, Clausen *et al*. 2003), all properties arguably of primary importance in placental cells. The abundance of methyl-esterified HGs in haustorial cells, which conspicuously elongate during intrusive growth, is consistent with the role suggested for these polymers in priming cell wall flexibility. In gametophyte placental cells, the bulk of HGs occurs in wall ingrowth cores, a trait consistent with their proposed role in defining cell wall permeability properties. Methyl-esterified HGs, which bind Ca^{2+} ions to form ridged gels, might enhance cell wall strength while maintaining a high level of porosity (Liners *et al*. 1989). Methyl-esterified HGs also occur in transfer cell wall ingrowths on both sides of the placenta in the fern *Ceratopteris richardii* Brongniart (1821: 184) (Table 3) and in the epidermis of *Vicia faba* Linnaeus (1753: 737) (Vaughn *et al*. 2007). In contrast, both methyl-esterified and de-esterified HG pectins are absent from the wall labyrinth apparatus in *Elodea* Michaux (1803b: 20) leaf cells, but are abundant in the rest of the cell walls (Ligrone *et al*. 2011). Both types of HG pectins are widespread in vegetative tissues of *Physcomitrium patens* (Hedwig) Mitten (1851: 363), including apical meristem cells (Mansouri 2012), rhizoids and protonemata (Lee *et al*. 2005, Berry *et al*. 2016).

Of the four tested antibodies that target rhamnogalacturonan-I pectic domains, two (LM5 and CCRC-M36) label the *Phaeoceros* placenta, with slightly higher levels of LM5 epitopes on the gametophyte side. Because LM5 targets a pectin domain that is thought to interact with HGs and AGPs to enhance the firmness and porosity of the wall, this pectin might ensure the maintenance of nutrient transport following cessation of growth, when cells must be rigid but porous. The location of the CCRC-M36 epitope (associated with RG-I) in the *Phaeoceros* placenta may indicate a signaling function as reported in *Arabidopsis* roots (Cornuault *et al*. 2018, McCartney *et al*. 2003). RG-I pectins are variable in occurrence in transfer cells of other plants. They are absent from the placenta of *Marchantia* (Henry *et al.* 2020) and *C. richardii* (Johnson 2008), and present in transfer cells of *Vicia* (Vaughn *et al*. 2007) and *Elodea* (Ligrone *et al*. 2011). In bryophytes, RG-I pectins are more restricted in occurrence than HG pectins. For example, LM5 does not label primary cell walls in the gametophyte apex of *P. patens* but it does bind to water-conducting cells, rhizoids and protonemal cells (Ligrone *et al*. 2002, Mansouri 2012), as well as disparate tissues in liverworts (Möller *et al*. 2007, Berry *et al*. 2016, Dehors *et al*. 2019).

Xyloglucan hemicelluloses (LM15, LM25) occur in both species of *Phaeoceros* on both sides of the placental junction, but they are not abundant (Table 2). Hemicelluloses are commonly complexed with cellulose and form the structural framework for primary walls across land plants but are generally less abundant in bryophytes than

tracheophytes (Popper & Fry 2003, Sarkar *et al*. 2009, Cornuault *et al*. 2018). Xyloglucans in hornworts and tracheophytes have similar motifs (Peña *et al*. 2008) and are known to increase cell wall expansibility by weakening the cellulose network (Chanliaud *et al*. 2002, Whitney *et al*. 2006). High levels of xyloglucans in sporophyte wall ingrowths of *Marchantia* suggest a role in binding pectin and AGPs for ensuring sufficient stiffness despite the low cellulose content (Henry *et al*. 2020). The low abundance of xyloglucan and the absence of LM21 and LM28 epitopes associated with other hemicelluloses in *Phaeoceros* suggests that here pectins may play a more significant role in the regulation of wall extensibility and porosity. Hemicelluloses showed high levels of labeling in epidermal transfer cells of *Vicia* when targeted with polyclonal antibodies (Vaughn *et al*. 2007), and xyloglucans were found to be evenly distributed in transfer cell walls in *Pisum* Linnaeus (1753: 727) root nodules (Dahiya & Brewin 2000).

Arabinogalactan proteins (AGPs) are common in the placenta of the three land plants hitherto probed but are variable across taxa and across generations (Table 3). Most notable is the restriction of JIM13 AGP epitopes to the placental cells in *Phaeoceros*. AGPs are proteoglycans made of a heavily O-glycosylated (90% of the overall mass) protein backbone (Dehors *et al*. 2019). They are ubiquitous cell wall components in land plants, speculated to be involved in a diversity of vital processes such as differentiation, cell to cell recognition, embryogenesis, programmed cell death, and tip-growth (Happ & Classen 2019). AGPs are hypothesized to be involved in pH-dependent signaling by releasing Ca^{2+} as a secondary messenger that regulates development (Lamport & Várnai 2013, Lamport *et al*. 2014). Accumulation of cytosolic Ca^{2+} is important in the development of reticulate wall ingrowths in angiosperms that are similar to those in bryophytes (Offler & Patrick 2020). AGPs are thought to act as positional markers that aid in directing the polarized growth of wall ingrowths (Seifert & Roberts, 2007). The regulated signaling by AGPs might also be critical in the placenta, where the two generations have to interact and transport nutrients in a coordinate way. AGPs are released into the cell wall when they separate from their glycosylphosphatidylinositol anchors in the plasmalemma. In the cell wall, they are believed to function as pectin plasticizers by preventing HG domain crosslinking (Lamport & Kieliszewski 2005).

Blocking of AGP function using β-D-glycosyl Yariv reagents in Tobacco BY-2 cells inhibits cell expansion (Lamport & Kieliszewski 2005). The control of cell expansion by AGPs might be a key-function to the development of the hornwort placenta. The LM2 epitope is the only AGP epitope detected in the placenta of all three taxa so far investigated, being located in sporophyte cell walls and abundantly in *Marchantia* (Table 3) (Johnson 2008, Henry *et al*. 2020). Sporophyte transfer cell walls of *Marchantia* are rich in the JIM13 epitope (Henry *et al*. 2020), which is also widespread in *Phaeoceros* but has not yet been searched in *Ceratopteris*. In contrast, Johnson (2008) documented intense labeling for JIM8 AGPs in gametophyte placental cells, but not in the sporophyte in *Ceratopteris*. JIM8 weakly labels both generations in *Marchantia* but gives no signal in *Phaeoceros*. LM6 specifically binds to (1,5)-α-L-arabinan residues, thus signaling the occurrence of either RG-I pectins or AGPs (Willats *et al.* 1998). The specific localization of LM6 labeling at the plasmalemma/cell wall interface in *Phaeoceros* points to an AGP, not pectin. The LM6 epitope is expressed in gametophyte and sporophyte placental cell walls of *Phaeoceros* and *Ceratopteris* but not in either generation in *Marchantia*. Small amounts of LM6 and JIM8 epitopes are expressed in wall ingrowths of cotyledon epidermal cells in *Vicia* (Vaughn *et al*. 2007). Treatment of expanding *Vicia* cotyledons with the AGP inhibitor ß-D-glucosyl Yariv, caused roughly a 50% reduction in the size of the wall labyrinth apparatus in epidermal cells (Vaughn *et al*. 2007).

AGPs in bryophytes are reported to be associated with water conducting cells (Ligrone *et al*. 2002), apical cell extension and differentiation of protonemata, (Lee *et al*. 2005, Kobayashi *et al*. 2011), water balance (Shibaya *et al*. 2005), cell wall regeneration in cultured protoplasts (Shibaya & Sugawara 2007), cell plate formation (Shibaya & Sugawara 2009) and hyaline cell wall deposition in *Sphagnum* Linnaeus (1753: 1106) (Kremer *et al*. 2004). Consistent with a role in signaling, AGPs are abundantly expressed during spermatogenesis and oogenesis in *Ceratopteris* (Lopez & Renzaglia 2014, 2016) and spermatogenesis in *Aulacomnium palustre* (Hedwig) Schwägrichen (1827: 216) (Lopez-Swalls 2016).

Extensins, as probed with JIM12, are not present in the placenta of the two hornwort species (Table 3). Extensins are hydroxyproline-rich glycoproteins that add strength to cell walls and are involved in cell wall assembly and growth, e.g., in tip growth in pollen tubes and root hairs of *Arabidopsis* (Diet *et al*. 2006, Ringli 2010, Velasquez *et al*. 2012), and in interaction between the cell wall and cytoplasm (Bascom *et al*. 2018).

Callose is widely used in bryophytes in processes such as tip growth, spore wall development and sperm cell differentiation (Bopp *et al.* 1991, Renzaglia & Garbary 2001, Möller *et al.* 2007, Tang 2007, Schuette *et al.* 2009, Cao *et al.* 2014, Renzaglia *et al*. 2015, Berry *et al.* 2016). Callose occurrence in thin areas of gametophyte cell walls of *Phaeoceros*, which were presumably crossed by plasmodesmata before cell separation, suggests employment in the closing of plasmodesmatal pores. Callose was also found at the base of newly formed cell wall ingrowths and adjacent

plasmodesmata in transfer cells in *Pisum* nodule vascular tissues (Dahiya & Brewin 2000). In contrast, callose was not found in the placenta of *Marchantia* (Henry *et al*. 2020). Callose is a regular occurrence in *Vicia* transfer cells (Samuels *et al.* 1995, Vaughn *et al.* 1996) and has been suggested to provide a scaffolding for the assembly of cell wall polymers during the development of wall ingrowths (Meikle *et al.* 1994, Vaughn *et al.* 2007). Because we immuno-probed only fully-developed placentae, it is possible that callose was present during the initial phases but was removed during maturation.

TABLE 3. Summary of cell wall constituents in the placenta of three land plants: two bryophytes, *Phaeoceros* and *Marchantia* and a fern, *Ceratopteris*. *Notes:* P = present, A = absent, - = not tested. Antibodies targeting HG in light blue. MAb colors indicate polymers targeted: grey = HG pectins, orange = RG-I pectins, green = hemicelluloses, blue = AGPs, yellow = extensin, and red = callose.

Taxon MAb	*Phaeoceros* Sporophyte	*Phaeoceros* Gametophyte	*Marchantia* Sporophyte	*Marchantia* Gametophyte	*Ceratopteris* Sporophyte	*Ceratopteris* Gametophyte
CCRCM38	P	P	-	-	-	-
JIM5	P	P	P	P	A	A
LM19	P	P	P	P	-	-
JIM7	P	P	P	P	P	P
LM18	P	P	-	-	-	-
LM20	P	A	P	P	-	-
CCRCM36	P	P	-	-	-	-
CCRCM2	-	-	-	-	A	A
LM5	P	P	A	A	A	A
LM16	A	A	-	-	-	-
LM15	P	P	P	P	-	-
LM21	A	A	P	P	-	-
LM25	P	P	P	P	-	-
LM28	A	A	A	A	-	-
CCRCM1	-	-	-	-	P	P
CCRCM7	-	-	-	-	A	A
JIM8	A	A	P	P	A	P
JIM13	A	P	P	P	-	-
LM2	P	P	P	A	P	P
LM6	P	P	A	A	P	P
JIM12	A	A	A	A	-	-
Callose	A	P	A	A	-	-

Conclusions

Compared with tracheophytes, the primary cell walls of bryophytes are not well characterized (Mansouri 2012, Roberts *et al*. 2012, Berry *et al*. 2016). Although the major wall polymers, namely cellulose, pectins, hemicelluloses and AGPs, are present in bryophytes, there are fewer cell wall-related genes in the genomes of bryophytes compared with seed plants (Yokoyama 2020). Special cell walls such as those around sperm cells, spores and in placental cells have modifications in the type, abundance and arrangement of cell wall constituents relative to normal primary walls, which probably reflect specific functional modulation (Cosgrove 2005, Lopez Swalls 2016, Broxterman & Schols 2018). The present study shows that placental cell walls in *Phaeoceros* are enriched with cellulose that imparts strength, and pectins that may enhance porosity and permeability. Cell wall compositional differences between sporophyte and gametophyte placental cells probably reflect modulation functional to cellular interaction and directional nutrient transport. Notably, the abundance of methyl-esterified HGs in sporophytic haustorial cells likely underpins the intrusive growth of these cells. Likewise, the occurrence of a diversity of AGPs restricted to placental cells suggests a role in calcium binding and release associated with signal transduction and solute movement across generations.

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