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SHORT COMMUNICATION

A cytochemical and immunocytochemical analysis of the wall labyrinth apparatus in leaf transfer cells in *Elodea canadensis*

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• *Background and Aims* Transfer cells are plant cells specialized in apoplast/symplast transport and characterized by a distinctive wall labyrinth apparatus. The molecular architecture and biochemistry of the labyrinth apparatus are poorly known. The leaf lamina in the aquatic angiosperm *Elodea canadensis* consists of only two cell layers, with the abaxial cells developing as transfer cells. The present study investigated biochemical properties of wall ingrowths and associated plasmalemma in these cells.
• *Methods* Leaves of *Elodea* were examined by light and electron microscopy and ATPase activity was localized cytochemically. Immunogold electron microscopy was employed to localize carbohydrate epitopes associated with major cell wall polysaccharides and glycoproteins.
• *Key Results* The plasmalemma associated with the wall labyrinth is strongly enriched in light-dependent ATPase activity. The wall ingrowths and an underlying wall layer share an LM11 epitope probably associated with glucuronoarabinoxylan and a CCRC-M7 epitope typically associated with rhamnogalacturonan I. No labelling was observed with LM10, an antibody that recognizes low-substituted and unsubstituted xylan, a polysaccharide consistently associated with secondary cell walls. The JIM5 and JIM7 epitopes, associated with homogalacturonan with different degrees of methylation, appear to be absent in the wall labyrinth but present in the rest of cell walls.
• *Conclusions* The wall labyrinth apparatus of leaf transfer cells in *Elodea* is a specialized structure with distinctive biochemical properties. The high level of light-dependent ATPase activity in the plasmalemma lining the wall labyrinth is consistent with a formerly suggested role of leaf transfer cells in enhancing inorganic carbon inflow. The wall labyrinth is a part of the primary cell wall. The discovery that the wall ingrowths in *Elodea* have an antibody-binding pattern divergent, in part, from that of the rest of cell wall suggests that their carbohydrate composition is modulated in relation to transfer cell functioning.

Key words: Aquatic plants, ATPase activity, cell walls, *Elodea*, immunogold labelling, transfer cells.

INTRODUCTION

Transfer cells are specialized plant cells functioning in solute transport across the apoplast/symplast interface (Pate and Gunning, 1972; Gunning and Pate, 1974; Offler *et al.*, 2002). Ubiquitous from liverworts to angiosperms, transfer cells probably evolved ancestrally in land plants as a means to enhance nutrient translocation at the interface between the sporophyte and parental gametophyte (Ligrone *et al.*, 1993); in angiosperms, transfer cells have been recruited in numerous other histological locations and participate in a diversity of processes including phloem loading, secretion and nutrient uptake besides intergenerational nutrient relationships (Offler *et al.*, 2002).

Transfer cells are not a specific cell type but rather a pattern of structural and physiological specialization that is expressed in different tissues either constitutively or in response to external signals. The most distinctive feature of transfer cells is a wall labyrinth apparatus, consisting of cell wall ingrowths and the outlining plasmalemma. It is generally maintained that this apparatus enhances polarized solute transport because of local amplification of active plasmalemma surface areas, but the details of the underlying processes, notably the nature and distribution of membrane transporters and the possible role of the wall matrix, are still largely unknown (Offler *et al.*, 2002).

The wall labyrinth of transfer cells is referred to as a secondary cell wall, essentially because it develops as an addition to the primary wall following the completion of cell enlargement (Offler *et al.*, 2002). Current knowledge of the chemical composition of the wall labyrinth in transfer cells is still extremely scanty. Early histo- and cytochemical studies show that the wall ingrowths contain cellulose and acid polysaccharides (Dashek *et al.*, 1971; Gunning and Pate, 1974). No quantitative difference, however, was detected in cell-wall composition between a maize endosperm cell line displaying transfer-cell morphology and a control cell line (DeWitt *et al.*, 1999). Immunogold cytochemistry, a technique potentially capable of providing detailed information on cell wall topochemistry (Knox, 2008), has so far been applied to vascular transfer cells of *Pisum sativum* root nodules (Dahiya and Brewin, 2000) and epidermal transfer cells of *Vicia faba* cotyledons (Vaughn *et al.*, 2007). In both studies the wall ingrowths displayed much the same reactivity as the associated primary cell wall except for the presence of areas positive for antibodies against callose.

In the present study, biochemical properties of the wall labyrinth apparatus of epidermal transfer cells in the leaves of the aquatic monocot *Elodea canadensis* have been investigated using a cytochemical procedure for ATPase localization and a battery of monoclonal antibodies against carbohydrate epitopes, in part coincident with those tested in previous immunocytochemical studies. The differentiation of transfer cells at the water/plant interface is a widespread feature of freshwater angiosperms and is interpreted as an adaptation enhancing nutrient uptake and inorganic carbon inflow in growth-limiting environmental conditions (Rascio *et al.*, 1994, 1999, and references therein). The leaves of *Elodea* and other aquatic angiosperms constitutively form transfer cells that are directly accessible to experimental manipulation and therefore appear to be a favourable alternative model system for investigating transfer cell properties.

MATERIALS AND METHODS

Samples of *Elodea canadensis* (Hydrocharitaceae, Liliopsida) were collected in spring from a population growing in a pond in the Botanical Gardens of Padua (Italy).

For standard light and electron microscopy, mature leaves were fixed overnight at 4 °C in 3 % (v/v) glutaraldehyde in 0·1 M Na-cacodylate buffer, pH 6·9, post-fixed with 1 % (w/v) osmium tetroxide in the same buffer for 2 h at room temperature, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Araldite. For electron microscopy, thin sections (80 nm) cut with a diamond knife were collected on 200-mesh uncoated copper grids, stained with uranyl acetate and lead citrate and observed with a TEM300 transmission electron microscope (Hitachi, Japan). For light microscopy, 0·5-μm-thick sections were cut with a diamond histo-knife, mounted on glass slides coated with BioBond (EMS, USA) to enhance section adhesion, stained with 0·5 % (w/v) toluidine blue in 0·5 % (w/v) Na-tetraborate and photographed with a Zeiss Axioskop light microscope equipped with a Sensicam QE (Applied Scientific Instrumentations, USA) digital photocamera.

For ATPase cytochemical localization, the procedure described in detail by Price and Whitecross (1983) was followed. In brief, whole leaves of *Elodea* were incubated in a reaction medium containing 2 mM ATP, 2 mM $Pb(NO_3)_2$, 2 mM $Mg(NO_3)_2$ and 5 mM $KHCO_3$ in 0·1 M Tris maleate buffer (pH 7·2), for 2 h at 20 °C under a light source of about 1400 μmol photons m^2 s^{-1} PAR. As for other aquatic plants (Price and Whitecross, 1983; Rascio *et al.*, 1999), the presence of light was essential for ATPase activity. Control samples were incubated in light in a medium lacking ATP or in the dark in complete medium. After incubation, the leaf pieces were briefly rinsed with distilled water and prepared for electron microscopy as described above. The ATPase activity was demonstrated by the formation of electron-dense deposits of leaf phosphate, due to the lead ion (trap ion) reaction with phosphate ions liberated during enzymatic hydrolysis of ATP.

For immunogold electron microscopy, the procedure described in detail in Vaughn *et al.* (2007) was followed. Briefly, leaves fixed only with glutaraldehyde and embedded in LR White resin were cut with a diamond knife at 100 nm and the sections mounted on 600-mesh uncoated nickel grids. The grids were treated as follows: 1 % (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 30 min; primary monoclonal antibodies diluted 1 : 20 (v/v) with PBS-BSA, 4 h; three rinses with PBS–BSA; gold-conjugated goat-anti-rabbit secondary antibody (BB International, UK) diluted 1 : 20 (v/v) in PBS–BSA, 30 min; three rinses with PBS. The grids were then stained for 2 min in 2 % (w/v) aqueous uranyl acetate and 30 s in Reynold's lead citrate. Negative controls were routinely made by omitting the incubation step with the primary antibody. The monoclonal antibodies tested in this study, their specificities and sources are reported in Table 1. For each antibody, three separate tests were run, each on serial sections from a different block, and 10–14 pictures were produced for each test. The number of gold particles per square micrometre was determined using the gravimetrical method described by Vaughn *et al.* (2007). The length of the plasmalemma and cuticle was evaluated using a manual map reader.

TABLE 1. *Monoclonal antibodies utilized for immunocytochemical characterization of cell walls in* Elodea *leaves*

Antibody	Epitope/target antigen	Reference/source
LM1	Undefined/hydroxyproline-rich glycoproteins	Smallwood *et al.*, 1995/J. P. Knox*
LM2	β-Glucuronyl residues/arabinogalactan proteins	Smallwood *et al.*, 1996/J. P. Knox*
LM5	Tetra-(1 → 4)-β-galactan/galactan, rhamnogalacturonan-I	Jones *et al.*, 1997/ J. P. Knox*
LM6	Penta-(1 → 5)-α-arabinan/arabinan, rhamnogalacturonan-I	Willats *et al.*, 1998/ J. P. Knox*
LM8	Undefined/xylogalacturonan	Willats *et al.*, 2004/ J. P. Knox*
LM10	Undefined/unsubstituted and low-substituted (1 → 4)-β-xylan	McCartney *et al.*, 2005/J. P. Knox*
LM11	Undefined/(1 → 4)-β-xylan substituted by arabinose and glucuronic acid	McCartney *et al.*, 2005/J. P. Knox*
JIM5	Undefined/low- or non-methyl-esterified (1 → 4)-α-galacturonan	Willats *et al.*, 2000/ J. P. Knox*
JIM7	Undefined/partially methyl-esterified (1 → 4)-α-galacturonan	Willats *et al.*, 2000/ J. P. Knox*
CCRC-M1	Fucosylated side group/xyloglucan, rhamnogalacturonan-I	Puhlmann *et al.*, 1994/M. Hahn†
CCRC-M2	Undefined/rhamnogalacturonan-I	Puhlmann *et al.*, 1994/M. Hahn†
CCRC-M7	Arabinosylated (1 → 6)-β-galactan/ arabinogalactanproteins, rhamnogalacturonan-I	Puhlmann *et al.*, 1994; Steffan *et al.*, 1995/M. Hahn†

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RESULTS

As already described in a former study (Rascio *et al.*, 1994), the leaf lamina in *Elodea* consists of only an adaxial (upper) and abaxial (lower) cell layer, with small intercellular spaces between them (Fig. 1). Both cell types contain numerous well-differentiated chloroplasts, but the adaxial cells are much larger and more vacuolate than the abaxial ones. The periclinal walls of abaxial cells are particularly thick and, in toluidine-blue-stained sections, they display a bilayered structure,

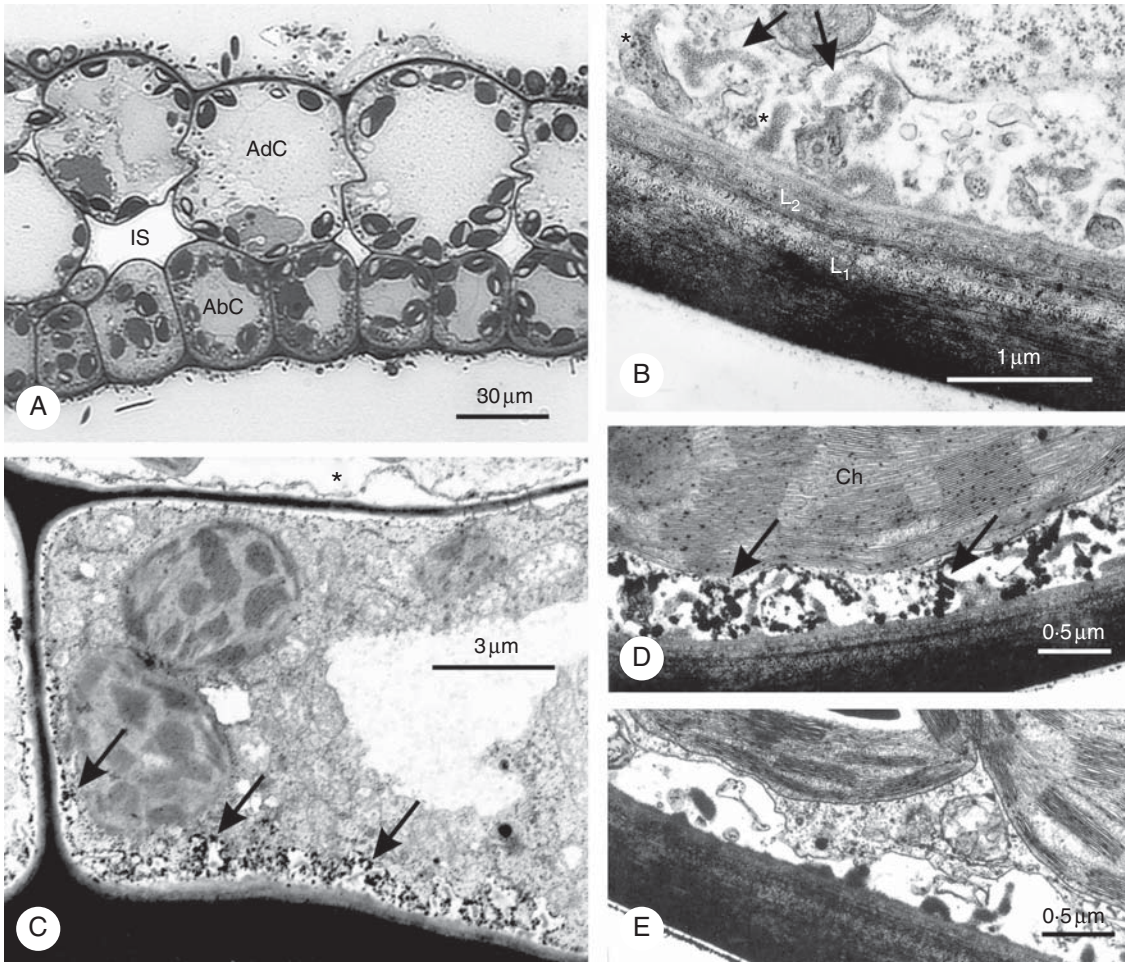

FIG. 1. Leaf anatomy and ATPase cytochemistry in *Elodea*. (A) Light microscopy of leaf lamina showing adaxial (AdC) and abaxial cells (AbC) and small intercellular spaces (IS). Note the bilayered structure of outer periclinal walls in abaxial cells. (B–E) Electron microscopy of abaxial cells. (B) Detail of outer periclinal wall, showing the L_1 and L_2 layer and wall ingrowths consisting of a fibrillar core (arrows) and an electron-transparent area (asterisks). (C–E) Cytochemical localization of ATPase. (C and D) Massive lead precipitates mark the plasmalemma in the wall labyrinth area (arrows). Less abundant precipitates are visible on the plasmalemma along the rest of the walls. Note the virtual absence of reaction in the adaxial cell on the top (asterisk). Scattered lead precipitates are also visible along the thylakoid membrane in chloroplasts (Ch). (E) Control from a sample incubated in a medium lacking ATP, showing absence of reaction.

with a densely stained outer layer and a less dense inner layer (Fig. 1A). In sections prepared for electron microscopy, the periclinal walls appear to consist of an outer layer (henceforth referred to as the L_1 layer), externally covered by a thin cuticle, and an inner layer (henceforth referred to as the L_2 layer) (Fig. 1B). Both cell wall layers display a distinct multilamellar texture but the L_1 layer is more heterogeneous and generally more opaque to electrons than the L_2 layer. Associated with the inner layer is a a typical labyrinthine apparatus of the reticulate type (Offler *et al.*, 2002), consisting of relatively small wall ingrowths with an amorphous fibrillar core and a electron-transparent outer area outlined by the plasmalemma (Fig. 1B). The L_2 layer, but not L_1, extends to form the bulk of anticlinal and inner periclinal walls of abaxial cells. The periclinal cell walls in adaxial cells exhibit a similar bilayered structure as in abaxial cells, but they are thinner and never form a wall labyrinth.

After incubation in a medium containing ATP and lead salts and in the presence of light, a strong reaction was observed at the level of plasmalemma areas associated with the wall labyrinth (Fig. 1C, D). A weaker reaction was also visible on plasmalemma areas facing the inner, non-labyrinthine walls in abaxial cells, whereas virtually no reaction was detected in adaxial cells (Fig. 1C). Some reaction, probably due to CF_1-ATPase activity, was observed on thylakoid membranes in chloroplasts (Fig. 1D). No reaction was detected in controls incubated in a medium lacking ATP (Fig. 1E) and a very low reaction in those incubated in complete medium in the dark (not shown).

Of the 12 monoclonal antibodies tested, only five gave a positive reaction (Table 2). LM1, an antibody that binds to hydroxyproline-rich glycoproteins, labelled the electron-transparent area of wall ingrowths and the outlining plasmalemma (Fig. 2A). This antibody also specifically labelled the interface between the cuticle and the L_1 layer in both abaxial and adaxial cells (Fig. 2A). Of the two antibodies against $(1 \rightarrow 4)$-β-xylan, LM10 produced no labelling whereas LM11 strongly labelled the fibrillar core of wall ingrowhts (Fig. 2B). LM11 also labelled the L_2 layer in both periclinal

TABLE 2. *Results of immunogold electron microscopy of cell walls in* Elodea *leaves*

Antibody	Wall ingrowths in abaxial (transfer) cells	Rest of cell walls in abaxial (transfer) cells	Cell walls in adaxial cells
LM1	Electron-transparent area (14 ± 4)	Wall/cuticle interface in periclinal outer walls (8 ± 3)	Wall/cuticle interface in periclinal outer walls (8 ± 3)
	Associated plasmalemma (8 ± 3)		
LM2	–	–	–
LM5	–	–	–
LM6	–	–	–
LM8	–	–	–
LM10	–	–	–
LM11	Electron-opaque area (50 ± 12)	L_2 layer (24 ± 9)	L_2 layer (22 ± 7)
JIM5	–	L_2 layer (28 ± 9)	L_2 layer (26 ± 7)
		L_1 layer (26 ± 4)	L_1 layer (18 ± 4)
JIM7	–	L_2 layer (22 ± 6)	L_2 layer (20 ± 5)
		L_1 layer (11 ± 3)	L_1 layer (12 ± 4)
CCRC-M1	–	–	–
CCRC-M2	–	–	–
CCRC-M7	Electron-opaque area(25 ± 8)	L_2 layer (28 ± 8)	L_2 layer (27 ± 7)

The average numbers of gold particles per square micrometre are given in parenthesis. The density of labelling of the wall/cuticle interface and plasmalemma/wall ingrowth interface by LM1 is expressed on a linear micrometre basis. The values are means ± s.d. –, no labelling.

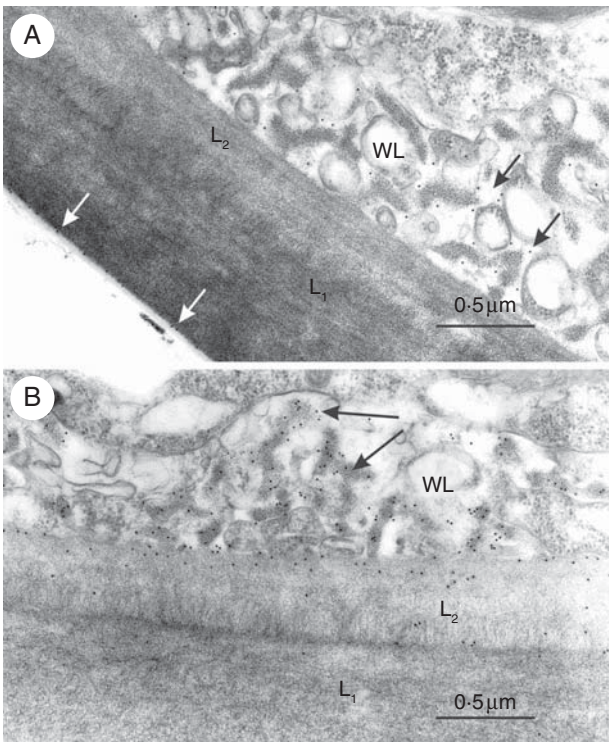

FIG. 2. Immunogold labelling of outer periclinal walls in leaf abaxial cells of *Elodea*. (A) LM1 labelling of the electron-transparent area of wall labyrinth (black arrows) and the interface between the L_1 layer and cuticle (white arrows). (B) LM11 labelling of the electron-opaque area of wall labyrinth (arrows) and of L_2 layer. WL, Wall labyrinth.

and inner cell walls. JIM5 and JIM7, two antibodies against epitopes associated with pectins with diverse degrees of methylation, labelled both the L_2 layer and less intensely the L_1 layer, but neither of them bound to the wall ingrowths (Fig. 3A, B). CCRC-M7 specifically labelled the fibrillar core of wall ingrowths and the L_2 layer (Fig. 4).

DISCUSSION

The results of the cytochemical test performed in this study reveal that the plasmalemma area associated with the wall labyrinth in leaf transfer cells of *Elodea* is highly enriched with light-dependent ATPase activity. Similar results have been reported for epidermal transfer cells in *Ranunculus trichophyllus* (Rascio *et al.*, 1999) and charasomes in *Chara corallina* (Price and Whitecross, 1983). The ATPase activity demostrated in *Elodea* transfer cells in the present study most likely coincides with the light-dependent proton extrusion activity reported by earlier studies in the same species (Elzenga and Prins, 1989; Marré *et al.*, 1989). It has been suggested that the resulting acidification of the wall labyrinth might enhance CO_2 inflow to leaf cells by promoting pH-dependent conversion of HCO_3^- to CO_2 in the periplasmic space (Rascio *et al.*, 1999, and references therein). Known precedents for transporters preferentially associated with the plasmalemma in the wall labyrinth area are of an H^+-ATPase, a sucrose-binding protein and a H^+-sucrose symporter in cotyledonary transfer cells of *Vicia faba* (Harrington *et al.*, 1997) and *Pisum sativum* (Tegeder *et al.*, 1999).

In line with cytochemical tests, the immunogold labelling experiments demonstrate that the wall labyrinth in *Elodea* transfer cells presents distinctive traits relative to the rest of the cell walls. The lack of reactivity of wall ingrowths to both JIM5 and JIM7 suggests that these lack non-methylated and low-methylated homogalacturonan. In contrast, both the L_1 and L_2 layers bind JIM5 and JIM7. Since JIM5 recognizes both methyl-esterified and un-esterified galacturonan whereas JIM7 requires a certain degree of methylation (Willats *et al.*, 2000), the labelling pattern observed indicates that the homogalacturonan in the L_1 and L_2 layers is, at least in part, in the methyl-esterified form. In contrast to *Elodea* transfer cells, the wall ingrowths and associated primary wall in *Pisum* nodule transfer cells were positive to JIM7 (Dahiya and Brewin, 2000). Likewise, the wall ingrowth inner core and associated cell wall in cotyledonary transfer cells of *Vicia* were strongly positive to JIM7 (Vaughn *et al.*, 2007). Neither in *Pisum* nor in *Vicia* transfer cells were the wall ingrowths labelled by JIM5, suggesting that galacturonan was present here only in the methyl-esterified form.

LM11 strongly labelled the fibrillar core of wall ingrowths in *Elodea*, indicating the presence of xylan-associated epitopes. LM11 also bound to the L_2 layer, though with a lower intensity than in wall ingrowths, whereas no labelling was observed in the L_1 layer with this antibody (Table 2). The high level of xylan-associated epitope and the lack of JIM5

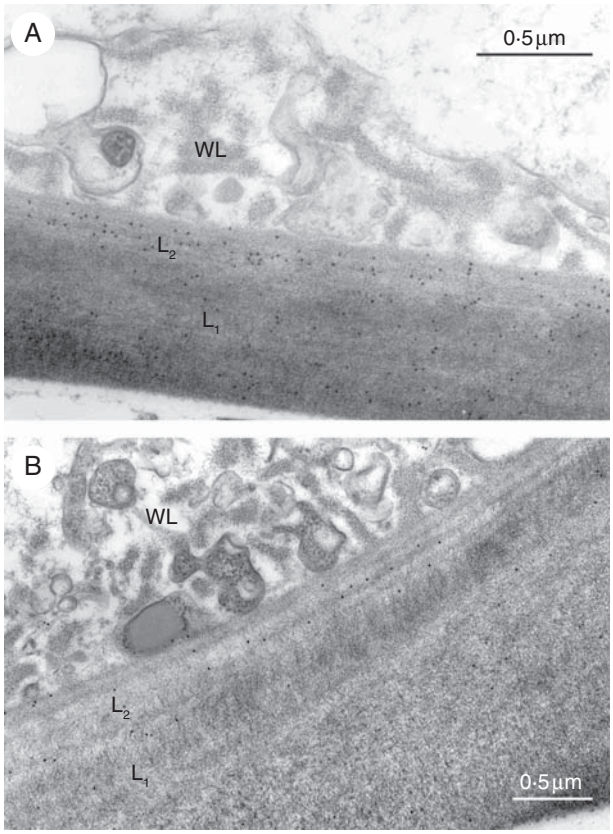

FIG. 3. Immunogold labelling of outer periclinal walls in leaf abaxial cells of *Elodea*: (A) JIM5 and (B) JIM7 labelling of L_1 and L_2 layers. Note absence of labelling on wall labyrinth (WL). The numbers on bars are micrometers.

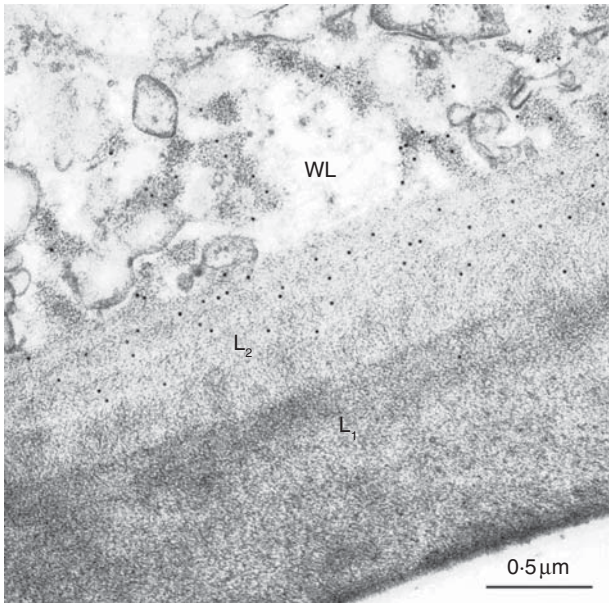

FIG. 4. Immunogold labelling of outer periclinal walls in leaf abaxial cells of *Elodea* by CCRC-M7, showing antibody binding to the electron-opaque area of the wall labyrinth (WL) and the L_2 layer.

and JIM7 epitopes, typically abundant in primary cell walls but absent or scarce in secondary cell walls (O'Neill and York, 2003; McCann and Carpita, 2008; Knox, 2008), might be taken as an indication that the wall ingrowths in *Elodea* are a secondary cell wall Nevertheless, the absence of labelling with LM10, an antibody that binds to un-substituted or low-substituted xylan (McCartney *et al.*, 2005) and consistently labels secondary cell walls in higher plants (Carafa *et al.*, 2005), suggests that LM11 labelling in *Elodea* is likely to signal a relatively highly substituted xylan that is not exclusive to secondary cell walls, especially in monocots (Carafa *et al.*, 2005, and references therein). Likewise, the L_2 layer is labelled by LM11 but not LM10 and, moreover, it binds both JIM5 and JIM7 and forms the bulk of the inner cell walls that clearly are primary cell walls. We conclude that most likely the L_2 layer and the wall labyrinth in epidermal transfer cells of *Elodea* both belong to a primary cell wall. Recent research on cotton bolls (J. E. Mellon and K. C. Vaughn, unpubl. res.) has revealed that xylan can mask the pectin epitopes recognized by JIM5 and JIM7. This, however, is unlikely to hold for *Elodea* transfer cells as here the L_2 layer binds both LM11 and JIM5/JIM7. A distinct cell wall layer comparable to the 'uniform layer' underlying the wall labyrinth in *Vicia* transfer cells (Vaughn *et al.*, 2007) and possibly functioning as a foundation layer in wall labyrinth development (Farley *et al.*, 2000) appears to be absent in *Elodea*. The L_2 layer is unlikely to be an equivalent of the uniform layer because it has a distinct multilamellar structure, unlike the wall labyrinth it binds JIM5 and JIM7, and it extends to forms the inner cell walls where no wall labyrinth is present.

The positive reaction to CCRC-M7 by the L_2 layer and the fibrillar core of wall ingrowths is a major commonality of these two wall areas. CCRC-M7 recognizes an arabinosylated $(1 \rightarrow 6)$-β-galactan motif associated with rhamnogalacturonan I (RG-I) and arabino-galactan proteins (AGPs) (Puhlmann *et al.*, 1994; Steffan *et al.*, 1995). RG-I is a major pectic polysaccharide in primary cell walls, whereas AGPs are typically localized at the plasmalemma/cell wall boundary (Carpita and Gibeaut, 1993; Freshour *et al.*, 1996). Interestingly, CCRC-M7 specifically labelled the boundary between the electron-transparent area and plasmalemma in cotyledonary transfer cells of *Vicia* (Vaughn *et al.*, 2007), whereas this area was not labelled in *Elodea.* CCRC-M7 binding in *Vicia* was interpreted as an indication of the presence of arabinogalactan proteins, possibly involved in wall ingrowth development (Vaughn *et al.*, 2007). The localization of CCRC-M7 labelling in the fibrillar core in *Elodea* transfer cells appears more likely to signal an epitope associated with RG-I. The lack of reaction to LM5 and LM6, two antibodies that recognize galactan and arabinan motifs, suggests that the RG-I present in wall ingrowths and L_2 layer lacks galactan and arabinan side branches.

LM1, an antibody that detects hydroxyproline-rich glycoproteins belonging to the extensin family (Smallwood *et al.*, 1995), produced closely similar results in *Elodea* and pea nodule transfer cells, in both cases labelling the electron-transparent areas of wall ingrowths and associated plasmalemma (Dahiya and Brewin, 2000). Extensins are thought to be self-assembling amphiphiles that form cross-linking networks or sheets at hydrophobic/hydrophilic interfaces in cell walls (Rapaport, 2006). The observed labelling pattern of LM1 in *Elodea* transfer cells, including the labelling of the cuticle/wall interface, is in good agreement with this notion.

CONCLUSIONS

The wall-membrane apparatus of leaf transfer cells in *Elodea* is a specialized structure with distinctive biochemical properties. The high level of light-dependent ATPase activity associated with the plasmalemma in the wall labyrinth area supports the hypothesis that these cells promote photosynthetic activity by enhancing inorganic carbon inflow (Rascio *et al.*, 1994).

The discovery that the wall ingrowths present specific biochemical traits relative to the rest of cell walls, notably the absence or inaccessibility of JIM5 and JIM7 epitopes (associated with homogalacturonan) and the high level of the LM11 epitope (probably associated with glucuronoarabinoxylan), suggests that the composition of the extracellular matrix in the wall-membrane apparatus in *Elodea* is specifically modulated and may be relevant to transfer cell functioning.

Immunocytochemical analysis is to be extended to other types of transfer cells in order to understand whether the molecular architecture of the wall apparatus varies with specific functional specialization or, more simply, according to the histological location and taxonomy.

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