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Immuno-Glyco-Imaging in Plant Cells: Localization of Cell Wall Carbohydrate Epitopes and Their Biosynthesizing Enzymes

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1. Introduction

Plant cells are surrounded by a carbohydrate-rich compartment called the cell wall. This compartment plays a central role in growth, development, sexual reproduction and defence (Vicré et al., 2005; Mollet et al., 2007; Hématy et al., 2009; Seifert & Blaukopf, 2010). It is a carbohydrate-based structure composed mostly of polysaccharides (~90%) and glycoproteins (~10%). Polysaccharides include cellulose, pectin domains such as homogalacturonans (HG) with different levels of methylesterification, xylogalacturonan, rhamnogalacturonan-I (RG-I) and rhamnogalacturonan- II (RG-II) and hemicelluloses (depending on the species, xyloglucan [XyG] or arabinoxylan) (Liepman et al., 2010), whereas cell wall proteins are either enzymatic such as glucanases (Gilbert, 2010) or non-enzymatic such as arabinogalactan proteins (AGP) (Ellis et al., 2010, Driouich & Baskin, 2008).

Unlike cellulose microfibrils, complex polysaccharides (pectins and hemicelluloses) are assembled within the Golgi apparatus (GA) and are shuttled by GA-derived secretory vesicles to the cell wall (Driouich et al., 1993; Chevalier et al., 2010). The synthesis of these glycomolecules requires the action of a set of Golgi glycosyltransferases, in addition to nucleotide sugar transporters and nucleotide sugar interconversion enzymes (Sandhu et al., 2009).

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In this chapter, we describe a number of sample preparation and immunostaining procedures devoted to i) localization of cell wall heteropolysaccharides and proteoglycans within the cell wall domains and endomembrane system (Golgi stacks and associated vesicles) in various plant cell types and to ii) localization/mapping of Golgi-enzymes (glycosyltransferases) responsible for complex polysaccharide assembly within Golgi cisternae. We present procedures and examples of epitope localization using epifluorescence/confocal microscopy as well as immunogold electron microscopy.

2. Material

2.1 Plant materials

2.1.1 Arabidopsis and flax cultures

Arabidopsis (*Arabidopsis thaliana*) and flax (*Linum usitatissimum*) seedlings were grown as described by Durand et al. (2009). Briefly, for root tip or border-like cell studies, seeds were surface sterilized with 35% (v/v) ethanol and with 35% (v/v) bleach. After several washes in sterile distilled water, the seeds were sown on agar-solidified nutrient medium (Baskin et al., 1992). Growth conditions were identical to those described by Vicré et al. (2005). Seeds were grown in vertically orientated square Petri dishes in 16-h-day/8-h-night cycles at 24°C for 7 to 15 days. For Arabidopsis pollen and pistil studies, seeds were spread on the surface of sterile soil and cultured in a growth chamber as described by Dardelle et al. (2010) and Coimbra et al., (2007). Pollen was collected and grown *in vitro* in a liquid medium in the dark at 22°C (Boavida & McCormick, 2007). The flowers were collected at different stages of pistil development, according to the stages of early flower development set by Smyth *et al.* (1990), starting at stage 8, up to stage 13, when buds open and anthesis occurs.

2.1.2 Tobacco suspension-cultured cells

Wild type or transformed suspension-cultured cells of *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) expressing Arabidopsis protein fused to the Green Fluorescent Protein (GFP) (Chevalier et al., 2010) were cultured in the Murashige and Skoog (MS) liquid medium. Cells were subcultured every week by transferring 10 ml of suspension cells in 150 ml of fresh medium.

2.2 Monoclonal antibodies

A set of monoclonal antibodies (MAb) directed against the carbohydrate moiety of cell wall polysaccharides (Table 1) such as xyloglucan (XyG) and pectin domains including homogalacturonan (HG), xylogalacturonan, and side chains of rhamnogalacturonan-I (RG-I) were used. In addition, the cell surface glycoproteins such as arabinogalactan proteins (AGP) were localized with four MAbs (JIM8, JIM13, LM2 and MAC207).

2.3 Microscopes

For fluorescence studies, samples were examined using a confocal laser-scanning microscope (Leica TCS SP2 AOBS). Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) and Fluorescein isothiocyanate (FITC) fluorescences were imaged using an excitation wavelength of 543 nm or 488 nm, with the emission wavelength at 565–585 nm or 500–600

MAb	Epitope recognized	Polymer	References
CCRC-M1	α-Fuc-(1,2)-β-Gal	FucogalactoXyG	Puhlmann et al., (1994)
CCRC-M86	unknown	XyG	Pattathil et al., (2010)
JIM5	MeGalA-(1,4)-[(GalA)]$_4$-(1,4)-MeGalA	Weakly methylesterified HG	Clausen et al., (2003)
JIM7	GalA-(1,4)-[(MeGalA)]$_4$-(1,4)-GalA	methylesterified HG	Clausen et al., (2003)
JIM8	unknown	AGP	Pennell et al., (1991)
LM2	β-linked GlcA	AGP	Yates et al., (1996)
LM5	[(1-4)-β-D-Gal]$_{>3}$	Galactan of RG-I	Jones et al., (1997)
LM6	[(1-5)-α-L-Ara]$_{5/6}$	Arabinan of RG-I	Willats et al., (1998)
LM8	unknown	Xylogalacturonan	Willats et al., (2004)
LM13	linear (1-5)-α-L-arabinan	RG-I	Moller et al., (2008)
LM15	XXXG	XyG	Marcus et al., (2008)
MAC207	β-GlcA-(1,3)-α-GalA-(1,2)-Rha	AGP	Yates et al., (1996)

Table 1. List of monoclonal antibodies directed against cell wall carbohydrate epitopes used in this report. Fuc, fucose; Gal, galactose; MeGalA, 6-*O*-methyl-galacturonic acid; GalA, galacturonic acid; GlcA, glucuronic acid; Ara, arabinose; Rha, rhamnose. AGP, arabinogalactan protein; HG, homogalacturonan; MAb, monoclonal antibody; RG-I, rhamnogalacturonnan-I; XyG, xyloglucan. For more information see www.ccrc.uga.edu/~mao/wallMAb/Antibodies/antib and www.plantprobes.net

nm, respectively. Arabidopsis pollen tubes and pistils were observed under fluorescence illumination on a Leica DLMB microscope or a Zeiss Axio Imager Z1 inverted epifluorescence microscope equipped with FITC (absorption, 485–520 nm; emission, 520–560 nm wavelength) or filter UV filter (365/445 nm) for callose and β-glucan detection with aniline blue and calcofluor white, respectively. Images were acquired with a Leica DFC300FX camera or an Axiocam MR in automatic exposure mode, and processed with Axiovision 4.4 software.

For immunogold observations, grids were observed with a transmission electron microscope (TEM) apparatus (Tecnai 12 Bio-Twin; Philips) at 80 kV and images were acquired with an Erlangshen ES500W camera.

For all samples, control experiments were performed by omission of the primary antibody.

3. Spatial distribution of non cellulosic polysaccharides and proteoglycans in the cell wall and endomembrane compartments by fluorescence microscopy

These immunostaining methods were applied to various cell types including flax root cap and associated border-like cells, arabidopsis pollen tube or female sporophyte and tobacco suspension-cultured cells.

3.1 Cell surface immunostaining

Cell surface imaging was carried out in order to have an overview of plant cell wall composition. While the plant surface is the first structure involved during various biotic or abiotic stresses, the plant surface tissues are also implicated in plant growth and development. Therefore information on the composition of cell wall is important and can be obtained by immunocytochemistry and Fourier transform infrared microspectroscopy (Durand et al., 2009). We describe below several protocols dedicated to polysaccharides immuno-detection and cytochemical localization at the cell surface of flax root border-like cells (BLC), Arabidopsis pollen tube and pistil.

3.1.1 Root border-like cells

Root tips of most plant species produce a large number of cells programmed to separate from the root cap and to be released into the external environment named the border cells (Hawes et al., 2003). As described in Brassicaceae family plants (Driouich et al., 2007), flax doesn't produce isolated border cells but it does produce and release cells that remain attached to each other, forming a block of several cell layers called border-like cells (Vicré et al., 2005). We investigate the role of cell wall pectins in cell attachment and the organization of border-like cells in flax.

BLC from 5 days-old flax seedlings were put down gently on the clean surface of 10 wells glass slides (teflon printed diagnostic slide, Fischer Scientific). BLC were then fixed for 1 hour in 4% (w/v) paraformaldehyde (PAF) and 1% (v/v) glutaraldehyde in 50 mM piperazine-N,N'-bis(2-ethanesulfonic) acid buffer (PIPES), pH 7, and 1 mM $CaCl_2$ (adapted from Willats et al., 2001). BLC were washed in 50 mM PIPES, 1 mM $CaCl_2$, pH 7, and incubated for 30 min in a blocking solution of 3% (w/v) low-fat dried milk in phosphate-buffered saline (PBS), pH 7.2. After being carefully rinsed in PBS containing 0.05% (v/v) Tween 20 (PBST), BLC were incubated overnight at 4°C with the primary antibody (LM8 dilution 1:5 in 0.1% [v/v] PBST; Willats et al., 2004). After five washes with 0.05% PBST, roots were incubated with the secondary antibody, goat IgG anti-rat FITC conjugated (dilution 1:50 in 0.1% PBST) for 1 h at 37°C in the dark. BLC were rinsed in 0.05% PBST, mounted in anti-fade solution (Citifluor AF2; Agar Scientific). Between each immunostaining step, incubation medium was cautiously removed with filter paper to prevent the loss of BLC.

Labelling with the MAb LM8 specific for xylogalacturonan occurred in the outer surface of the border-like cells (Fig. 1). These results are similar to those already obtained for Arabidopsis (Vicré et al., 2005; Durand et al., 2009) that have revealed the importance of pectins in the organization of BLC.

3.1.2 Arabidopsis pollen tubes

Pollen tubes are fast growing tip-polarized cells carrying the two sperm cells. After landing on compatible stigmatic cells, pollen germinates and the tube grows deep inside the pistil, penetrating different tissues following guidance cues from both the sporophytic tissues and from the female gametophyte to arrive precisely at the micropylar end of the ovule and deliver sperm. Pollen grains can germinate *in vitro* and produce pollen tubes in liquid and on solid media (Fig. 2A). Arabidopsis pollen tubes are small (5 µm diameter) but can reach over 1mm long.
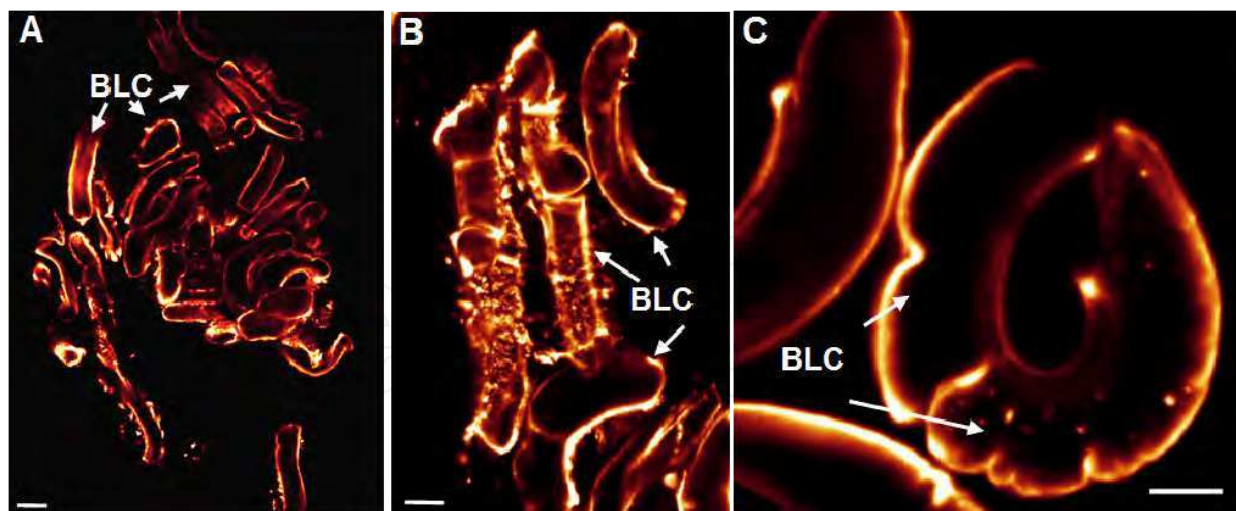
Fig. 1. Immunofluorescence labelling of flax root border-like cells (BLC) with the MAb LM8 specific for xylogalacturonan. Bars = 20 μm.

Pollen tubes grown in liquid germination medium were mixed (v/v) with a fixation medium containing 100 mM PIPES buffer, pH 6.9, 4 mM $MgSO_4$ $7H_2O$, 4 mM ethylene glycol tetraacetic acid (EGTA), 10% (w/v) Suc, and 5% (w/v) PAF and incubated for 90 min at room temperature. Pollen tubes were rinsed three times by centrifugation with 50 mM PIPES buffer, pH 6.9, 2 mM $MgSO_4$ $7H_2O$, and 2 mM EGTA and three times with phosphate-buffered saline (100 mM potassium phosphate, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) supplemented or not with 3% fat-free milk. Primary antibodies were diluted at 1:5 or 1:10 as described previously (Mollet et al., 2000) with PBS (with or without 3% milk). Pollen tubes were rinsed with the buffer and incubated overnight at 4°C in the dark with the secondary antibody combined with FITC (Sigma) diluted at 1:50 with the appropriate buffer for 3 h at 30°C. For JIM, LM, and MAC antibody detection, goat anti-rat IgG (whole molecule)-FITC was used; for CCRC antibody detection, sheep anti-mouse IgG (whole molecule)-FITC was used.

Arabidopsis pollen tubes display specific cell wall organization with a strong detection of epitopes associated with arabinogalactan proteins in the tip region (Fig. 2B-C) as observed in other species such as lily (Mollet et al., 2002) or tobacco (Li et al., 1992). Moreover, pectic domains such as the the arabinan side chains of RG-I (Fig. 2D-E) and methylesterified HGs (Fig. 2F) are also present in the cell wall of the pollen tubes. The cell surface immunolabeling gives important information on the accessibility of the epitopes. The enzyme or chemical treatments of the sample can sometimes be informative on the presence or not of a set of epitopes. As observed in Arabidopsis pollen tube, lily pollen tube was strongly labelled at the tip with the MAb directed against AGPs. Removal of the pectin with 0.1% pectinase extended the labelling for AGP to the entire pollen tube cell wall (Jauh & Lord, 1996).

In Arabidopsis, the female sporophyte is composed of a stigma with papillae, the receptive surface of the pollen grains, a short style and the ovary containing the ovules (Fig. 3B). Within the style and ovary, the pollen tubes grow in a specialized tissue, the transmitting tract guided toward the ovules.

Pollen tubes can also be observed *in vivo* (i.e. within the female tissues) by staining callose, a specific polysaccharide found in the intine wall of the pollen grain and pollen tube cell wall

and within the pollen tube as callose plugs deposited periodically by the vegetative cell to maintain the expanding cell in the tip region (Fig. 3D-E). This method is widely used to assess the pollen tube growth between wild type pollen and mutant. Pollinated pistils were fixed with a mixture of acetic acid: ethanol (1:3 vol/vol) at least 12h. Rinsed 5 times in water, the pistils were softened by incubation with 8M NaOH for 12h at room temperature. The pistil were rinsed 5 times with water, placed on a glass slide and stained with 0.1% buffered decolorized aniline blue, pH 11 according to Johnson-Brousseau & McCormick (2004) for 4h and observed.
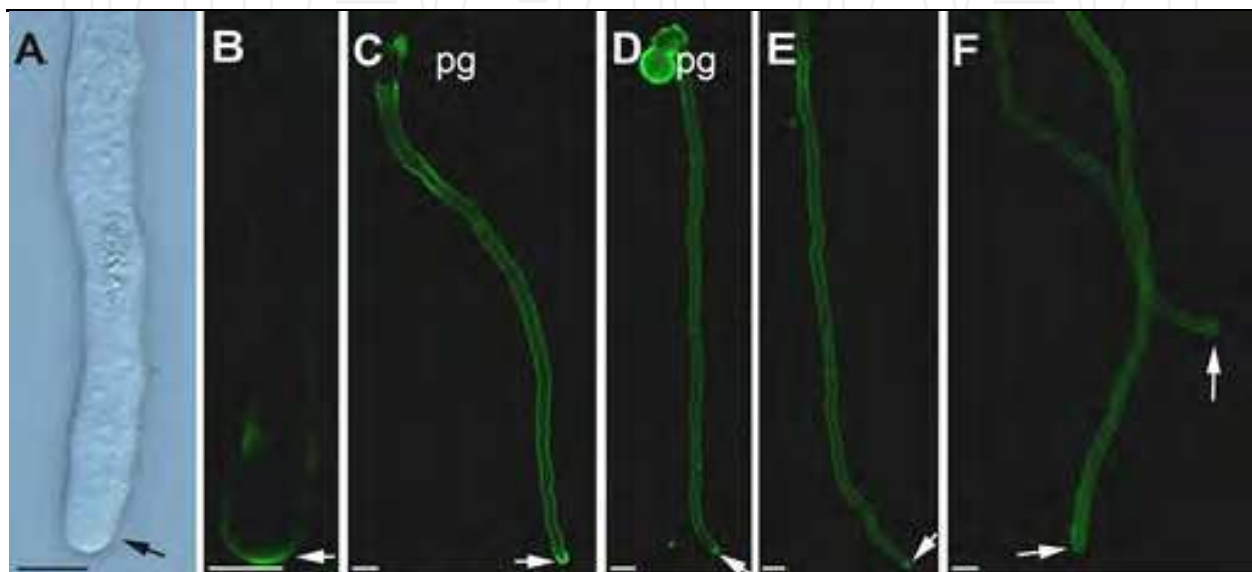

Fig. 2. Cell surface immunolocalization of cell wall epitopes in *Arabidopsis thaliana* pollen tube. A. Transmitted light of 6h-old *in vitro* grown pollen tubes, B-C. Localization of AGP epitopes at the tip of the pollen tubes using MAC207 and LM2 MAbs, respectively. D-E. Localization of arabinan epitopes found in the pectic polysaccharide RG-I in the entire pollen tube cell wall using LM6 and LM13 MAbs, respectively. F. Localization of highly methylesterified HG epitopes using JIM7 MAb. Arrow, pollen tube tip; pg, pollen grain. Bars = 5 μm.

After pollination, pollen grains germinate on the stigma and produce pollen tubes that grow within the cell wall of the papillae and in the transmitting tract of the style and ovary. Pollen grains are clearly detected on the stigma surface and the pollen tubes in the transmitting tract of the style and the ovary (Fig. 3D). Callose plugs are also visible. With higher magnification, the pollen tubes were observed in the ovary, emerging from the transmitting tract, growing on the funiculus toward the micropyle of the ovules (Fig. 3E).

3.2 Immunostaining of cell wall components on sections

The localization of cell wall polymers on sections is very instructive in particular to identify specific labelling of a given cell or tissue. When possible, the use of embedding medium is often avoided in order to increase the sugar epitope accessibility (Willats et al., 2001; Harholt et al., 2006). However, given the small sizes of Arabidopsis root tip, pollen tube or pistil, it is impossible to prepare hand sections. We currently use a procedure including fixation, dehydration and embedding to perform the sectioning step. We use methylmetacrylate

(Baskin, 1992) or London Resin White (LR White) resins as embedding media which do not disturb the detection of polysaccharides.

Pistils and anthers were fixed either with 4% PAF and 1% glutaraldehyde in 50 mM PIPES pH 7 for 2h or with 2% PAF and 2.5% glutaraldehyde in phosphate buffer (0.025 M, pH 7, with one micro drop of Tween 80), placed under vacuum for 1 h and then at 4 °C overnight. After dehydration in a graded ethanol series, the material was embedded in methylmetacrylate (Fig. 3A-C) or LR White (Fig. 3F-I). Sections (2 to 5 μm) were obtained with the Leica Reichart Supernova or EM UC6 microtome and placed on coated glass slides. Cytochemical staining with 0.05% toluidine blue showed that the cell wall of the transmitting tract cells is enriched in acidic polymers such as HG (Fig. 3B). The immunolocalization was performed as described in Coimbra et al., (2007) and Lehner al., (2010). Slides can be further counterstained with calcofluor white (fluorescent brightener; Sigma) for β-glucans (e.g., cellulose) detection as shown in Fig. 3F-I.

The arabinan epitopes associated with RG-I are strongly labelled in the style but very weakly in the cell wall of the papillae in the stigma (Fig. 3A). Weakly methylesterified HGs are also present in the cell wall of the ovary cells including the transmitting tract tissue (Fig. 3C). Glycosylated proteins, such as AGP, prevail in many stigma exudates, style transmitting tissues and pollen itself, and are believed to provide recognition signals and directional guidance for the pollen tube (Cheung et al., 1995; Wu et al., 2001). AGP represent a large group of highly glycosylated cell surface proteins, many of which contain glycosylphosphatidylinositol (GPI) anchors. They are suggested to act in signalling as soluble signals or as modulators and co-receptors of apoplastic morphogens. These immunolocalization studies, have shown a specific labelling of the central transmitting tissue of the style and also of the tracheary elements present all over the ovary, with MAbs JIM8 and JIM13 (Fig. 3F).

At the beginning of ovule development, when sections of Arabidopsis ovules were treated with MAbs JIM8 or JIM13, the specific labelling of the entire megaspore surface was striking. This labelling was specific to the first cell with a haploid constitution, marking the beginning of the gametophytic generation (Coimbra et al., 2007). As the development continues, the labelling with the same two MAbs was observed in the embryo sac wall, and was particularly intense in the synergid cells and in their filiform apparatus (Fig. 3H). At this more advanced stage of development, the labelling obtained with JIM8 and JIM13 extended to the integument micropylar cells and to the micropylar nucellus (Fig. 3H). The prevalence of AGPs in these reproductive tissues along with their possible role in signalling makes them excellent candidate molecules involved in guided pollen tube growth. This type of labelling was also shown for *Nicotiana tabacum* (Qin & Zhao, 2006) for *Amaranthus hypochondriacus* (Coimbra & Salema, 1997) and for *Actinidia deliciosa* (Coimbra & Duarte, 2003). The labelling obtained with the MAbs MAC207 and LM2, was found to be extensive and scattered throughout most cell types as shown in the style (Fig. 3G) and in the ovule, although excluded from the embryo sac (Fig. 3I).

The results obtained with this immunolabeling work were important not only from a developmental point of view, but also because they lay down the basis for the characterization of the expression of individual AGP genes in each of the developmental stages considered. As shown with cell surface labelling, removal of pectic polymers by enzymes (pectate lyase) or chemical treatments of the sections can effectively unmask other
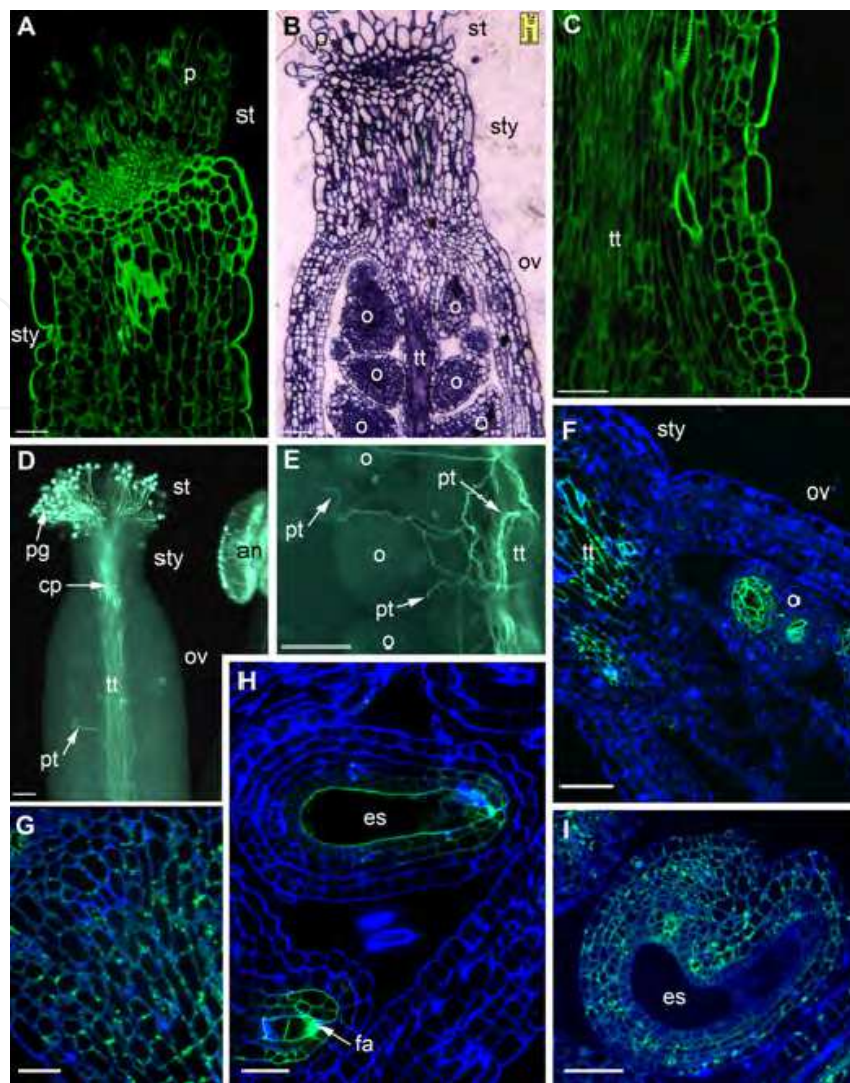
Fig. 3. Cytochemical staining and immunolocalization of cell wall epitopes in *Arabidopsis thaliana* pistil. A. Immunolocalization using the LM6 MAb specific for arabinan epitopes associated with RG-I in the cell wall of the stigma, style and ovary. B. Cytochemical staining of sectioned pistil with toluidine blue showing the acidic cell wall polysaccharides including the HG. C. Immunolocalization using the JIM5 MAb of epitopes associated with weakly methylesterified HG in the epidermal layer of the ovary and in the transmitting tract tissue. D-E. Cytochemical staining of callose with decolorized aniline blue on whole mount softened pollinated pistil showing the strong fluorescence of the pollen grains on the stigma surface, the pollen tube cell wall and the callose plugs within the female transmitting tissue in the style and ovary. E. A close up of D shows the pollen tubes emerging from the transmitting tract directed toward the ovules for the release of the sperm cells. F. The labelling with JIM8 is present in the centrally located transmitting tissue of the style, in the ovules embryo sac wall and extends into the integument micropylar cells. G. LM2 labelling all the style cells. H. The specific labelling by JIM8 of the embryo sac wall, the filiform apparatus and micropylar integuments are evident. I. This well developed ovule is showing all cell walls labelled by LM2, except the embryo sac cells and wall. an, anther; cp, callose plug; es, embryo sac; fa, filiform apparatus; o, ovule; ov, ovary; p, papillae; pg pollen grain; pt, pollen tube; st, stigma; Sty, style; tt, transmitting tract. Bars = 20 μm.

cell wall epitopes such those found on XyG as shown on tobacco stem sections by Marcus et al., (2008) and recently reviewed by Lee et al., (2011).

3.3 Immunostaining of cell wall polymers within the endomembrane compartment using protoplast

In higher plants, the endomembrane system and more precisely the Golgi apparatus plays a major role in the synthesis of the cell wall (Staehelin et al., 1990; Driouich et al., 1993). Involvement of plant Golgi stacks in the cell wall biosynthesis was revealed by cytochemical staining (Harris & Northcote, 1971) and biochemical experiments after fractionation (Ray, 1980; Gibeaut & Carpita, 1994). Hemicellulosic and pectic polymers are synthesized in the endomembrane system before their final assembly and deposition into the cell wall. Tobacco suspension-cultured cells (*Nicotiana tabacum*, Bright yellow-2 cells) are widely used in the study of endomembrane system (Pagny et al., 2003; Follet-Gueye et al., 2003; Toyooka et al., 2009; Langhans et al., 2011). Immunolabeling of N-glycan epitopes associated with Golgi membranes was easily achieved after a slight cell wall permeabilization (Fitchette et al., 1999). Here, we show that the production of protoplasts is necessary for performing immunodetection of cell wall polymer precursors in Golgi stacks.

3.3.1 Cell permeabilization and protoplast preparation

For tobacco cells immunolabeling, in 1.5 ml ependorf vials, four-day-old BY-2 cells (300 µl) were fixed with 800µl of 4% PAF in PBS (PBS: 8 g L^{-1} NaCl, 0.29 g L^{-1} KCl, 1.45 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4) for 1 h at room temperature. After six washes for 5 min with PBS buffer, fixed tobacco cells were incubated 30 min in 1 ml enzymatic mixture of 1% cellulase (Sigma) and 1% pectinase (Sigma) diluted in PBS buffer. Cells were then washed four times in PBS. For the protoplast preparation, four-day-old BY-2 cells (400 µl) were put in 1.5 ml ependorf vials, and plasmolyzed in 800 µl plasmolysis medium (20 mM MES buffer pH 5.6; 0.6 M mannitol; 0.5 mM $CaCl_2$; 0.25 mM $MgCl_2$) for 15–20 min. Then the cell wall was digested overnight at 25°C by incubation in 1 ml enzymatic mixture of 1% cellulase (Sigma) and 1% pectinase (Sigma) diluted in plasmolysis medium containing 0.1% bovine serum albumin (BSA). After three washes with plasmolysis medium, protoplasts were fixed 1 h with 4% PAF in PBS at room temperature. Then protoplasts were washed four times with PBS. Then, 25 µl of cells or protoplasts were squashed onto 1:10 poly-L-Lysine (sigma) coated 10 wells slides (Fischer Scientific) and air dried.

3.3.2 Immunostaining

Protoplasts and tobacco cells were sequentially incubated 10 min with 0.5% Triton X100 in PBS, with 1% BSA and 3% half-milk in PBS, and washed three times with 3% BSA in PBS. Samples were incubated overnight at 4°C, with the primary antibody CCRC-M86 diluted at 1/10 in PBS containing 1% BSA, 3% normal goat serum (NGS). After six washes in PBS containing 1% BSA, samples were incubated 1 h at 25°C with a secondary antibody (goat IgG anti-rat TRITC conjugate from Sigma) diluted at 1/50 in PBS containing 1% BSA, 3% NGS. Finally cells or protoplasts were washed three times in PBS containing 1% BSA.

When the cell wall is only partially digested (Fig. 4A), the density of XyG epitope remaining in the cell wall limits the MAbs penetration inside the cell. So even under confocal

microscopy observations, labelling of Golgi units is hardly detectable. In contrast, the complete removal of the cell wall in protoplasts allows the access of the MAb CCRC-M86 to the Golgi-associated epitopes (Fig. 4B). Moreover, it is well recognized that cell wall polymer synthesis is stimulated in protoplasts thus contributing to the increase of labelling of the endomembranes. With this procedure, we have clearly shown that sugar motifs associated with XyG (Fig. 4) or pectins (data not shown) are present in the Golgi units. These are numerous and dispersed throughout the cytoplasm (see numerous fluorescent spots on Fig. 4B).
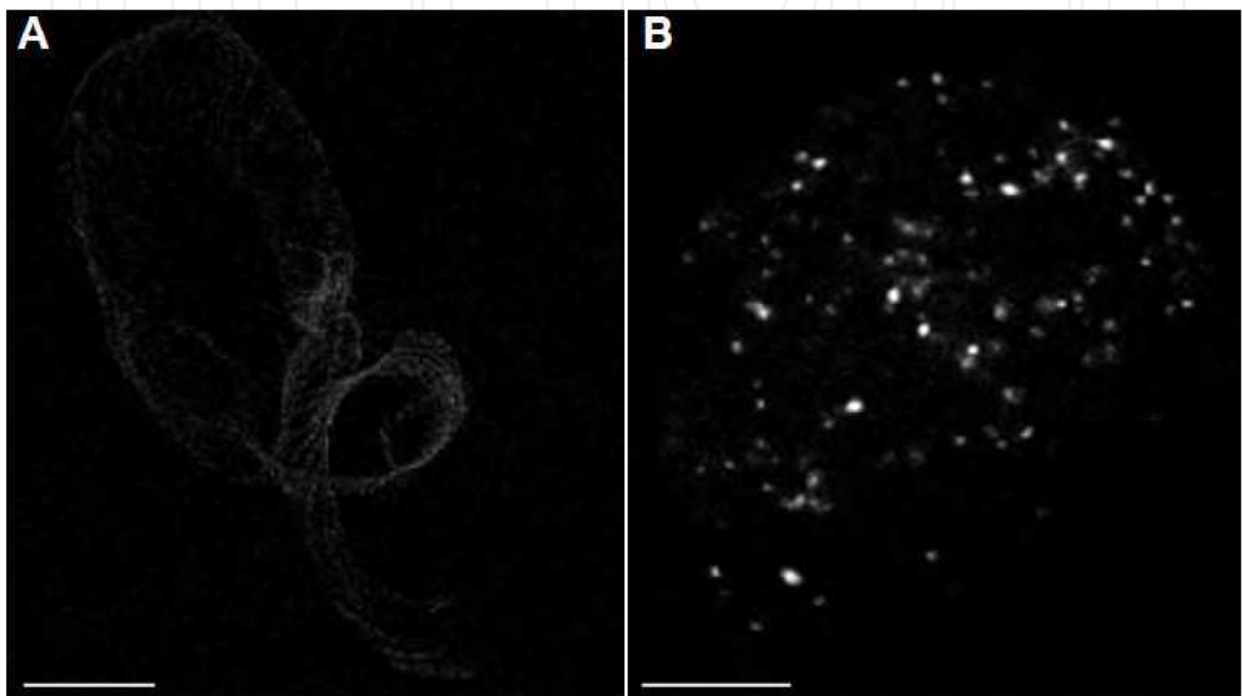

Fig. 4. Immunocytochemical localization of XyG epitopes in partially-digested tobacco BY-2 suspension-cultured cells and protoplasts with the MAb CCRC-M86. A- Confocal microscope images showing fluorescent staining of the cell surface and B- Golgi bodies. CCRC-M86 epitopes localize to both the cell wall and Golgi stacks of tobacco cell or protoplasts, respectively. Bars = 8 μm.

4. Spatial organization of non-cellulosic cell wall polysaccharides at the ultrastructural level

Due to the unability to subfractionate the plant Golgi stacks into *cis*, medial and *trans* cisternae, our knowledge, concerning the assembly of complex polysaccharides within the Golgi subcompartments, remains scarce. Progress towards the understanding of compartmentalization of matrix cell wall polysaccharide biosynthesis has come from immuno-electron microscopical analyses (Staehelin et al., 1990, Zhang & Staehelin, 1992; Driouich et al., 1993). These immunolabeling studies have been performed on samples prepared by HPF, a cryofixation technique that provides excellent preservation of Golgi stacks thereby allowing different cisternal subtypes to be easily distinguished. For ultrastructural and immunocytochemical investigations, we used the HPF coupled to freeze substitution (FS) technologies to prepare Arabidopsis root tips and pollen tubes, or tobacco

suspension-cultured cells. We present and discuss here protocols allowing epitopes detection associated with polysaccharides and membrane proteins (e.g., glycosyltransferases responsible for polysaccharide biosynthesis).

4.1 TEM sample preparation

4.1.1 High-Pressure Freezing/Freeze Substitution (HPF/FS)

4.1.1.1 Pre-cooling treatment

Prior to freezing, 3 day-old tobacco (BY-2) suspension-cultured cells were concentrated on 50 μm nylon membrane and incubated successively in MS + 5% glycerol, then in MS+10% glycerol for 1h each, at +4°C and under light stirring. Cells were then transferred to MS+20% glycerol and immediately frozen (see § 4.1.1.2). Root tips of seven day-old arabidopsis seedlings were first immersed in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (20mM, CaCl$_2$.2H$_2$O 2mM, KCl 2mM, Sucrose 0.2M; pH 5.5) containing a small drop of red ruthenium (to allow visualisation of the roots) for 5 min. Roots were then incubated with 0.2M sucrose in MES buffer for 5 min before freezing. Six hour-old pollen tubes were kept in the germination medium (GM) before freezing.

4.1.1.2 High pressure freezing

High-pressure freezing was performed with the freezer HPF–EM PACT I Leica-microsystems (Studer et al., 2001). Tobacco cells were concentrated on 50μm nylon membrane and transferred into the cavity of a copper ring used for cryofracture which is 100μm in depth and 1.2 mm in diameter. Excess medium was lightly absorbed with filter paper. Using a horizontal loading station, the specimen carriers were tightened securely into the pod of specimen holder. After fixation on the loading device, specimens were frozen according to a maximum cooling rate of 13000°C/s, incoming pressure of 7.5 bars and working pressure of 4.8 bars. Rings containing frozen samples were stored in liquid nitrogen until the freeze substitution procedure was initiated.

Root tips of *Arabidopsis thaliana* (1 to 2 mm) were excised with a fine razor blade in freezing media (0.2M sucrose in MES buffer). Three or four roots were placed in a gold platelet (200 mm in depth and 1.2 mm in diameter) carrier pre-filled with the freezing medium and coated with soybean lecithin (100 mg mL^{-1} in chloroform). Specimens were immediately frozen in the EM PACT freezer under similar conditions of pressure and cooling rate as for the tobacco cells (see above). Time between excision of the root tips and freezing was <1 min.

Like root tips, centrifuged 6-h-old pollen tubes were transferred into the cavity of gold cupules coated with soybean lecithin (100 mg mL^{-1} in chloroform). Excess medium was removed using a filter paper and were frozen as describe above.

4.1.1.3 Freeze substitution and resin embedding

After HPF, pollen tubes were transferred to a freeze substitution automate (EM-AFS; Leica) precooled to -140°C. Freeze substitution conditions followed a modified procedure from D. Studer (personal communication). Substitution media were composed of 2% osmium in anhydrous acetone. Samples were substituted at -90°C for 72 h. The temperature was gradually raised (2°C h^{-1}) to -60°C and stabilized during 12 h, then gradually raised (2°C $^{-1}$)

to -30°C (12 h) and gradually raised again (2°C h^{-1}) to 0°C for 2 h. Samples were washed at room temperature with fresh anhydrous acetone. Infiltration was done at +4°C in acetone-Spurr resin (2:1, 1:1, 1:2, 8 h each step) and with pure resin for at least 2 days. Polymerization was performed at 60°C for 16 h. Samples destined for protein immunocytochemistry (Arabidopsis root tips and BY-2 cells) were substituted in anhydrous acetone + 0.5% uranyl acetate (UA) using similar program as that described above except that the final substitution step was performed at -15°C. Samples were rinsed twice with anhydrous ethanol. Infiltration was processed at -15°C in a solution containing Ethanol : LR White (ratio 2:1; 1:1; 1:2, 8h each step) and with pure resin for 2x24 h. Polymerisation was performed into the AFS apparatus at -15°C under ultra violet light for 48h. Using an ultracut EM-UC6 (Leica), thin sections (90 nm) were mounted on formvar-coated nickel grids.

4.2 Immunogold staining

4.2.1 Single immunogold labelling

4.2.1.1 Cell wall immunogold labeling on epoxy sections

Grids, with pollen tube sections, were rehydrated in a Tris-buffered saline buffer (TBS) + 0.2% BSA and blocked in a TBS/BSA 0.2%/milk 3% solution for 30 min. After three brief rinses in TBS/BSA 0.2% solution, grids were incubated 3 h at 25°C in primary antibodies: non diluted for LM15 and CCRC-M1 or diluted (LM6, 1:2; anti-callose, 1:100) in TBS/BSA 0.2% buffer. Then, grids were washed (six times for 5 min each) in TBS/BSA 0.2% and incubated for 1 h at 25°C in a 1:20 secondary antibody (goat anti-rat for LM6 and LM15, goat anti-mouse for CCRC-M1 and anti-callose) conjugated to 10-nm gold particles (British Biocell International). Finally, grids were washed (six times for 5 min each) in a TBS + BSA 0.2% buffer, 1 min in TBS, 10 min in TBS + glutaraldehyde 2%, 5 min in TBS, and then two times for 5 min each in double deionized water. The sections were stained with 0.5% (w/v) UA in methanol for 10 min in the dark, rapidly rinsed 10 times with water and stained with lead citrate for 10 min, and briefly rinsed 10 times with water. In this experiment, epoxy resin was used as embedding medium. It is generally well admitted that Epoxy resin, which allow good cellular structure preservation, is not recommended for immunocytochemical studies. In the case of XyG and callose epitopes studied in Arabidopsis pollen grain and pollen tube, the detection was done with success (Fig. 5). Subcellular observation of Arabidopsis pollen grains showed that the intine cell wall contains the hemicellulose epitopes associated with fucogalactoXyG (Fig. 5A) and XyG (Fig. 5B). On the exine wall, pollen coat material is also visible. In the pollen tube, a double cell wall can be detected back from the tip region with the inner cell wall containing callose (Fig. 5C).

4.2.1.2 Immunogold labeling of polysaccharides and synthesizing-enzymes in Golgi stacks on acrylic sections

Ultrathin sections of tobacco cells or Arabidopsis root tips were blocked in TBS (Tris-buffered saline: Tris–HCl 20 mM pH 7.2, 200 mM NaCl) supplemented with 0.2% of BSA and milk 3% for 30 min. Sections were then incubated with monoclonal antibodies CCRC-M86 at a 1/5 dilution in TBS + BSA and normal goat serum (NGS; British Biocell International) for 3 h at room temperature. After washing in TBS + BSA 0.2% buffer, grids were incubated for 1 h at room temperature in the rat secondary antibody conjugated to 10-nm gold particles (Tebu-British Biocell International, http://www.tebu-bio.com). After
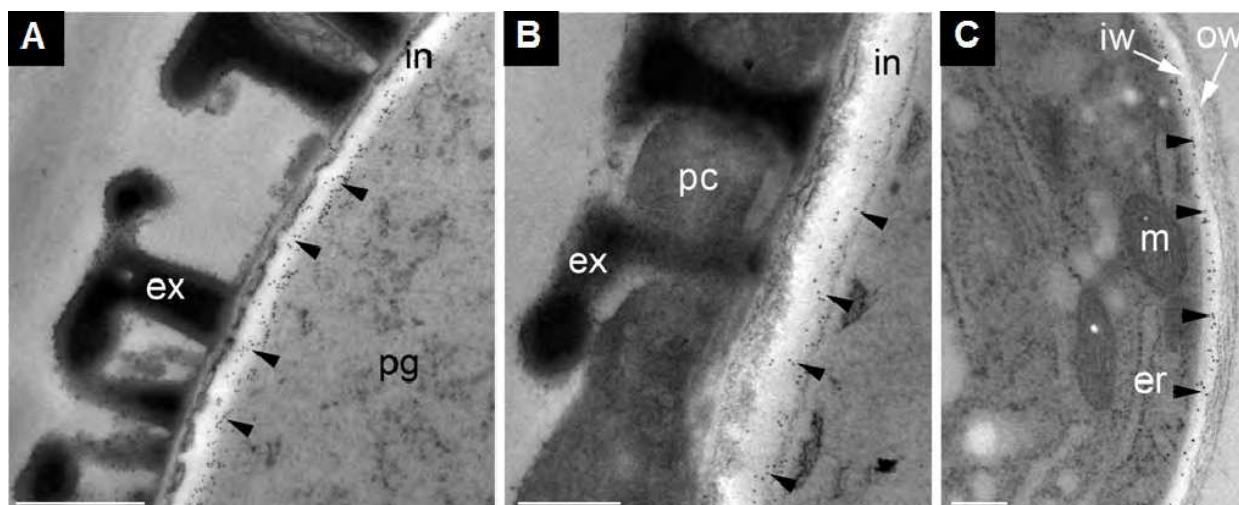
Fig. 5. A. Localization of fucosylated XyG in the intine wall of the pollen grain using the CCRC-M1 MAb. B. Localization of the motif XXXG found in XyG in the intine wall of the pollen grain using the LM15 MAb. C. Localization of callose in the inner cell wall of the pollen tube using the anti-callose antibody. Arrowhead, gold particles; er, endoplasmic reticulum; ex, exine; in, intine; iw, inner cell wall; m, mitochondria; ow, outer cell wall; pc, pollen coat; pg, pollen grain. Bars = 0.2 µm.

washing and fixation in TBS + 2% glutaraldehyde, sections were first stained with 4% vapour osmium for 3 h on a hotplate warmed to 40°C placed under a fume hood. For this step, the grids were put closed to 4% osmium droplets on a parafilm paper. This system was put in a small glass Petri dish who was kept hermetically closed until the total osmium evaporation. Then sections were classically stained 10 min with 0.5% UA diluted in methanol and 10 min in lead citrate.

Ultrathin sections of frozen cells expressing AtXT1–Green Fluorescent GFP (GFP) or AtMURUS 3 (MUR3)–GFP fusion proteins (Chevalier et al., 2010) were blocked in TBS (Tris–HCl 20 mM pH 7.2, 200 mM NaCl) supplemented with 0.2% of BSA and Tween 20 0.05% for 15 min. Sections were then incubated with the polyclonal anti-GFP antibody (BD living colors A.v peptide antibody – Clontech) at a 1/5 dilution in TBS + BSA and NGS for 2 h at room temperature. After washing in TBS + BSA 0.2% buffer, grids were incubated for 1 h at room temperature in the rabbit secondary antibody conjugated to 10 nm gold particles (Tebu-British Biocell International). After washing and fixation in TBS + 2% GA, sections were stained as described above with osmium vapours, uranyl acetate and lead citrate.

The combined use of HPF-FS and cryo-embedding in acrylic resin is particularly adapted to immunodetection of either membrane proteins or cell wall polysaccharides (Fig. 6 & Fig. 7). The cryo-immobilisation by HPF ensures the sample vitrification without modification of molecular structures. While chemical fixatives (such as aldehydes or osmium tetroxyde) are relatively inert with sugar molecules, proteins are often covalently cross linked with fixatives, which can disturb the epitope structure. During the cryo-dehydration step, we have lightly fixed the cells by adding 0.5% uranyl acetate to avoid the modification of the protein epitope. To enhance the electron micrograph contrast, the contact with osmium vapour prior to classical uranyl acetate and lead citrate staining is efficient (Fig. 6, Fig. 7, Fig.8 & 9) to enhance Golgi stack morphology. The non-fucosylated sugar motifs associated

with XyG, already detected in Golgi units in tobacco protoplasts (Fig. 4B), are mainly localized to the secretory vesicles of *trans* Golgi network (TGN) (Fig. 6A). With the same preparation procedure, it is also possible to immunolocalize XyG-synthesizing enzymes fused to GFP (e.g., AtXT1-GFP), within Golgi cisternae in transformed BY-2 cells (Fig. 6B). AtXT1-GFP was shown to localize mainly to the cis Golgi compartment (Chevalier et al., 2010).
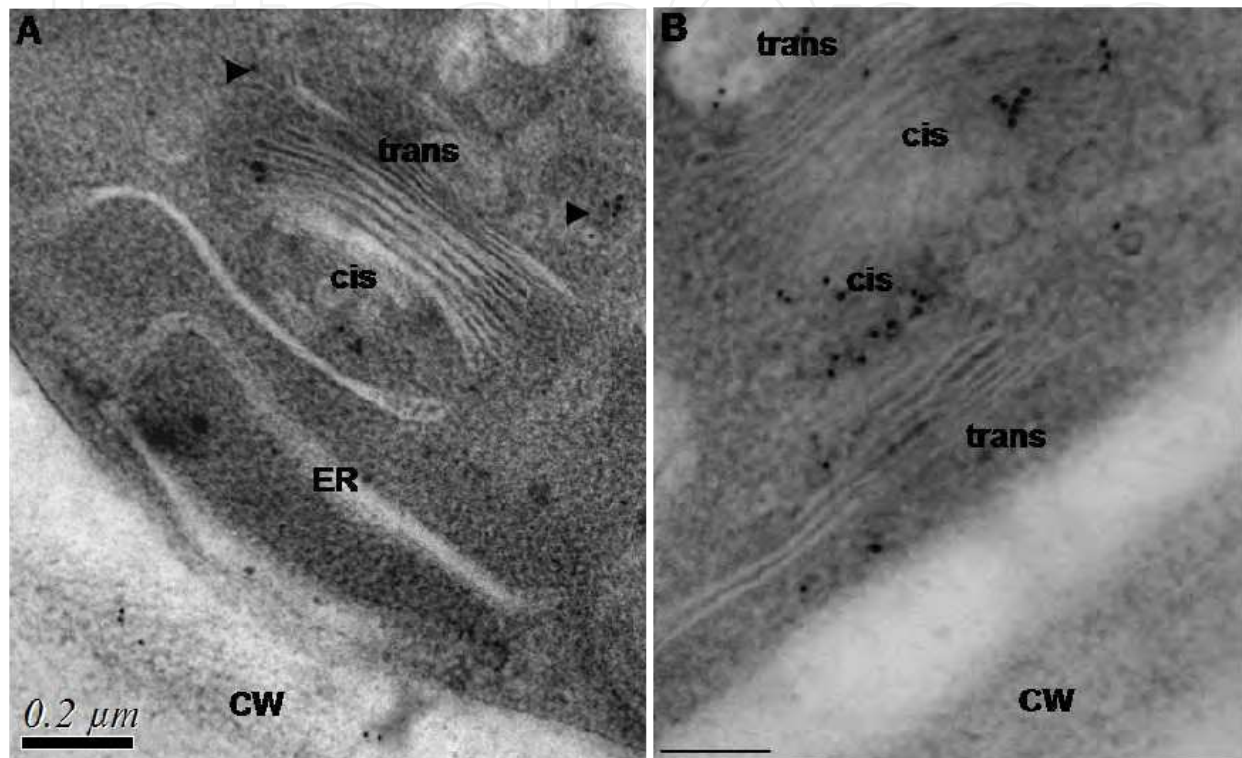

Fig. 6. Electron micrographs of HPF/FS and LR White embedded tobacco cells illustrating immunogold labelling of Golgi stacks and the cell wall. A- Non fucosylated motifs of XyG were immunodetected with the MAb CCRC-M86. B- AtXT1–GFP fusion protein was labelled with anti-GFP Ab, in Golgi stacks of transformed tobacco BY-2 suspension-cultured cells. The black head arrows indicate the secretory vesicles labelled. CW, cell wall; ER, endoplasmic reticulum; bars = 200 nm.

4.2.2 Double immunogold labeling

Information on the distribution of Golgi localized enzymes are currently obtained by using GFP technologies and confocal laser microscopy (Brandizzi et al., 2004). Immuno-electron microscopy is the only approach to study the distribution of GFP-labelling within a given organelle. Therefore, a central issue for immunogold at TEM level is to preserve epitopes of interest while minimizing the loss of ultrastructural details. Here, we study the localization of QUASIMODO2 protein (QUA2) in *qua2* mutant transgenic plants expressing the QUA2-GFP fusion protein (Mouille et al., 2007). QUA2 is a putative methyltransferase involved in the methylesterifcation of HG. It is well admitted that HG are synthesized in their methylesterified form and then possibly de-esterified by pectin methylesterases in the cell wall (Micheli, 2001).

With the sample substituted in 0.5% AU medium and embedded in LR White resin, we have been able to perform a double immunolabeling to localize Golgi enzymes fused to GFP and cell-wall polysaccharides. Ultrathin sections of Arabidopsis root tips of *qua*2-QUA2-GFP (Mouille et al., 2007) were blocked as describe above and incubated 2h at 25°C with the polyclonal anti-GFP antibody (BD living colors A.v peptide antibody – Clontech) at a 1/5 dilution in TBS + BSA and normal goat serum. After washing in TBS + BSA 0.2% buffer, sections were again saturated with 3% milk, 0.2% BSA in TBS buffer for 30 min. Incubation with the MAb JIM7 diluted to 1/5 in TBS + BSA and normal goat serum was done for 2h at 25°C. After six rinsing for 5 min with TBS buffer containing 0.2% BSA, grids were incubated for 1 h at room temperature in the rabbit secondary antibody conjugated to 10 nm gold particles diluted to 1/20 in TBS + BSA 0.2% + NGS 3%. Sections were then washed three times in TBS buffer + BSA 0.2%, and incubated 1 h at 25°C in the goat anti-rat secondary antibodies conjugated to 20-nm gold particles (Tebu-British Biocell International, http://www.tebu-bio.com). Sections were rinsed six times for 5 min with TBS buffer containing BSA 0.2%, and were followed by one washing in TBS buffer. After fixation in TBS + 2% GA, sections were stained as described above with osmium vapours, uranyl acetate and lead citrate.

The fusion protein is detected in the *trans* Golgi cisternae (Fig. 7C) while the methylesterified HG are immunolocalized not only in the *trans* cisternae but also in the TGN as previously described (Zhang & Steahelin, 1992; Toyooka et al., 2009). The lack of strong fixative in the substitution medium like osmium tetroxide or glutaraldehyde, allows the combined detection of pectic epitopes and its Golgi-associated biosynthetic enzyme. The cell wall labelling of the methylesterified HG epitopes (Fig. 7A & 7B) shows that the sample preparation did not alter the polymer localization. Given that the protein epitope is not impaired by strong fixatives, the double labelling of the product (HG) and the Golgi-localized methyltransferase is now possible. Our results suggest that HG methylation is likely to start in trans Golgi cisternae.

4.3 Quantitative mapping of polysaccharide-synthesizing enzymes within Golgi stacks

Statistical analysis of immunogold labelling was used to map XyG-glycosyltransferases within Golgi cisternae (Chevalier et al., 2010). As no antibodies directed against these enzymes are currently available, we have chosen to investigate their localization indirectly by using anti-GFP antibodies in transgenic tobacco cells expressing the glycosyltransferases fused to GFP. Immunogold labelling was quantified over each Golgi cisternae (fifty Golgi stacks with distinguishable cisternal subtypes) and subjected to chi-squared (χ^2) test to check for labelling specificity in transformed BY-2 cells versus wild type. The relative labelling index (RLI) was also determined to evaluate specific gold particles distribution within different subtypes of Golgi cisternae (Mayhew et al., 2002; Mayhew, 2011).

4.3.1 Labelling specificity of signal in transformed BY-2 cells

To evaluate the distribution of gold particles in Golgi stacks of transformed cells as compared with wild type cells, we used a chi-squared test (χ^2) as described by Mayhew et al., (2002). We considered the null hypothesis as 'no difference of gold distribution between wild type and transformed cells'. We analyzed gold labelling in different compartments of Golgi stacks: *cis*, medial, *trans* and the TGN (Fig. 8).
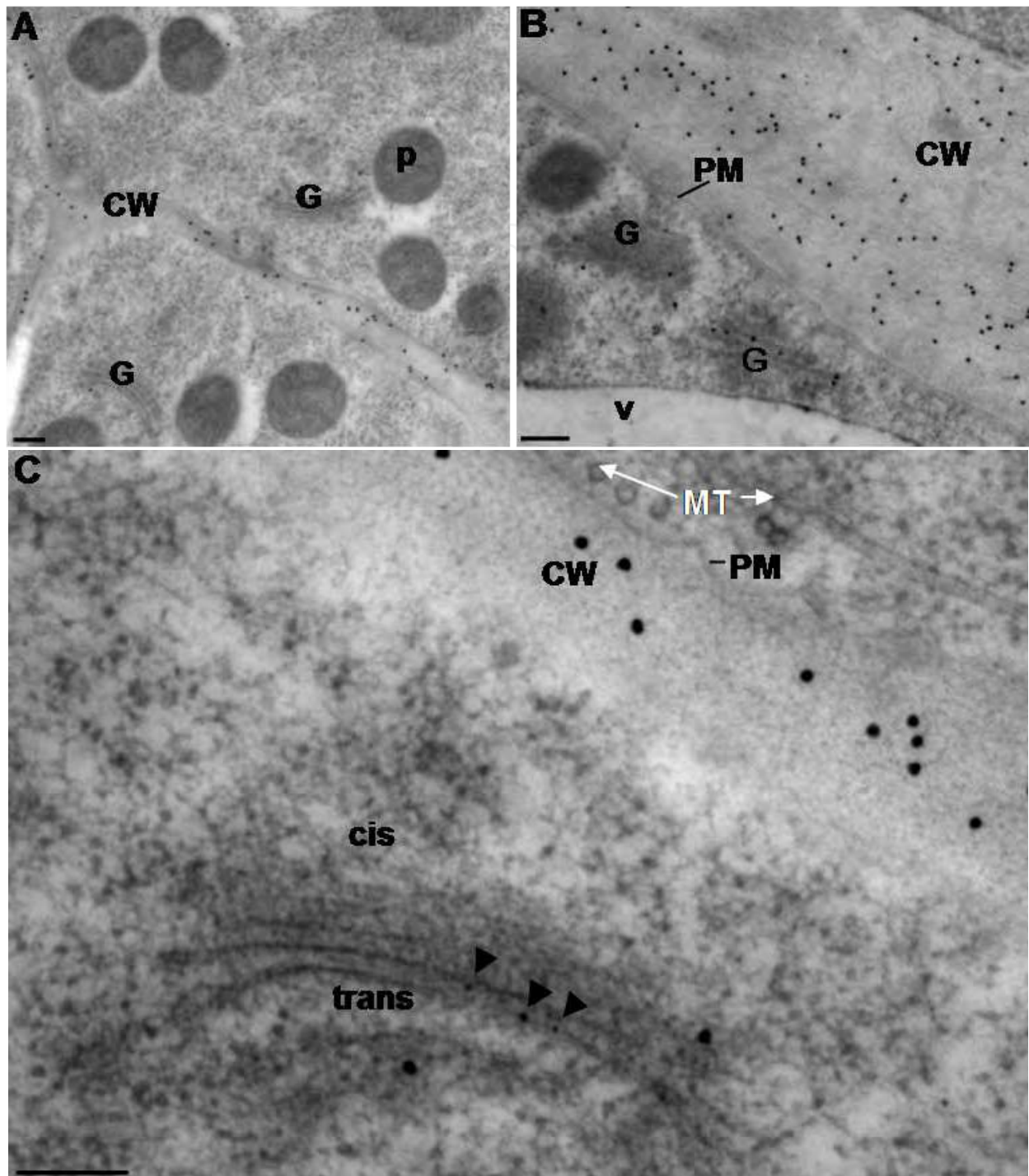
Fig. 7. Electron micrographs of HPF/FS and LR White-embedded ultrathin sections of Arabidopsis root tips of *qua2*-QUA2-GFP. Double localization of AtQUA2-GFP with anti-GFP antibody (10 nm gold particles) and of highly methylesterified HG with MAb JIM7 (20 nm gold particles) in Arabidopsis root meristematic cells. A-B: Note abundant labelling of HG epitopes in the cell wall and in the last Golgi compartments. C, the black arrow heads indicate the 10 nm gold particles corresponding to GFP labelling in the *trans* Golgi cisternae. CW, cell wall; G, Golgi ; MT, cortical microtubules ; P, plastid; PM, plasma membrane ; V, vacuole. Bars = 100 nm.
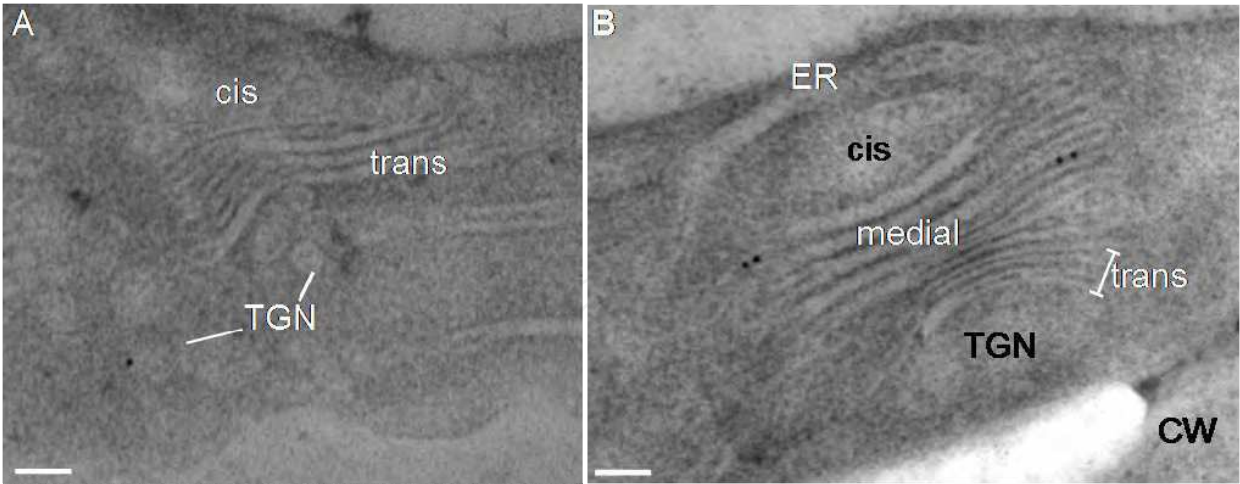
Fig. 8. Electron micrographs of GFP immunodetection on A- 3 day-old wild type BY-2 cells or B- transformed cells expressing the fusion protein AtMUR3-GFP. 50 similar images are used to determine the number of gold particles observed in each Golgi compartment: *cis*, medial, *trans* cisternae and the *trans* Golgi network (TGN). CW, cell wall; ER, Endoplasmic reticulum; At MUR3 is a XyG-galactosyltransferase, bars = 100 nm.

To compare the distribution of gold particles in these compartments between the two tobacco lines, we counted the gold particles observed over 50 Golgi units both in non-transformed cells (Wild type cells, termed WT cells) and transformed tobacco cells (expressing Golgi-enzyme fused to GFP, termed GFP-cells), immunolabelled with anti-GFP antibodies (Fig. 8). We prepared a table of gold particles counted for each tobacco lines and their repartition in different Golgi compartments (Table 2).

For each Golgi compartment, we calculated the expected gold particle number (Eij) according to the null hypothesis as 'no difference of gold distribution between wild type and transformed cells and that the gold particles are randomly distributed across the Golgi units'. The expected gold count (Eij) at two line cells for each selected Golgi compartment are calculated by using the table 2 values1, from the product of column (total gold particles observed in all Golgi stacks for wild type or transformed tobacco lines) and row totals (total gold particles observed in all BY-2 lines for the selected compartment) divided by the big total (total gold particles observed in four Golgi compartments, in wild type and transformed BY-2 cells).

	cis (j_1)	**medial** (j_2)	*trans* (j_3)	**TGN** (j_4)	**Row Total** (j_5)
WT cells (i_1)	O_{11}	O_{12}	O_{13}	O_{14}	O_{15}
GFP cells (i_2)	O_{21}	O_{22}	O_{23}	O_{24}	O_{25}
Column Total (i_3)	O_{31}	O_{32}	O_{33}	O_{34}	**O_{35}**

Table 2. Gold particles counted (Oij) over 50 Golgi stacks from Wild type tobacco cells (WT cells) or transformed BY-2 cells by Golgi localized enzymes fused to GFP (GFP cells). Oij designates the gold particle number indicated in the row (i) and the column (j). O_{15} and O_{25} designate the total gold particle number in WT cells and GFP cells, respectively. O_{31} to O_{34} correspond to the total gold count in the four Golgi compartments of the two tobacco lines. **O_{35}** is the big total (four Golgi compartments, in WT and GFP cells).

Example for the *cis* compartment of WT cells, expected gold number (E_{11}) is calculated as described below[1]:

$$E_{11} = [(O_{31} X\ O_{15})/\mathbf{O_{35}}] \tag{1}$$

To compare the distribution of gold particles between the two tobacco lines in these compartments, we created a contingency table (two columns; four rows) as described in Mayhew et al. (2002) and also in table 3. In this contingency table we report the observed (O_{ij}) and expected (E_{ij}) numbers of gold particles. For each tobacco line, total column should be equal ($O_{15}=E_{15}$; $O_{25}=E_{25}$). If expected number is superior to 5, the χ^2 test can then be applied. The corresponding partial χ^2 value is calculated as the square of the difference between observed and expected gold particle numbers divided by the expected gold number.

Example for the *cis* compartment of WT cells, χ^2 value (χ_{11}^2) is calculated as described below[2]:

$$\chi_{11}^2 = [(O_{11}-E_{11})^2/\ E_{11}] \tag{2}$$

The total χ^2 value ($\chi_{15}^2 + \chi_{25}^2$) is compared with χ^2 value in contingency table analysis with a degree of freedom equal to 3 in our experiment and corresponding to[3]:

$$Degree\ of\ freedom = [(row\ number\ -1)\times(column\ number\ -1)] \tag{3}$$

When the total χ^2 value ($\chi_{15}^2 + \chi_{25}^2$) is found to be superior to that of the contingency table, $\chi_{table}^2 = 12.38$ (with a degree of freedom of 3 and an uncertainty degree $\alpha = 0.005$) indicating that labelling is specific in Golgi stacks of transformed BY-2 cells.

	WT cells			GFP cells			Total observed gold
	(O_{ij})	(E_{ij})	χ_{ij}^2	(O_{ij})	(E_{ij})	χ_{ij}^2	
cis	O_{11}	E_{11}	χ_{11}^2	O_{21}	E_{21}	χ_{21}^2	O_{31}
medial	O_{12}	E_{12}	χ_{12}^2	O_{22}	E_{22}	χ_{22}^2	O_{32}
trans	O_{13}	E_{13}	χ_{13}^2	O_{23}	E_{23}	χ_{23}^2	O_{33}
TGN	O_{14}	E_{14}	χ_{14}^2	O_{24}	E_{24}	χ_{24}^2	O_{34}
Column Total	O_{15}	E_{15}	χ_{15}^2	O_{25}	E_{25}	χ_{25}^2	$\mathbf{O_{35}}$

Table 3. Model of contingency table use for quantitative analysis of anti-GFP labelling over Golgi stacks in tobacco BY-2 cells expressing Golgi enzyme–GFP. This table contains two columns (Oij and Eij) and four rows (related to the four Golgi compartments studied).

4.3.2 Determination of fusion protein distribution in Golgi stacks

Quantification of the labelling was done according to Mayhew et al. (2002), on a total of 50 Golgi stacks with clearly distinguishable subtypes of cisternae (Fig. 9A) from transformed BY-2 cells expressing enzyme-GFP fused construct (noted GFP cells). First, gold particles were counted in each Golgi compartment (*cis*, medial, *trans* and the TGN) in 50 Golgi stacks from WT or GFP cells as described in § 4.3.1. The *'observed gold particles'* (n_0) in a given Golgi compartment in GFP cells is determined by subtracting the number of gold particles found in this compartment (Oij, GFP cells, 50 Golgi stacks) from the one counted in WT cells (Oij,

WT)[4]. Example for the *cis* compartment of WT cells, n_0 value is determined as described below[4]:

$$n_{011} = O_{21} - O_{11} \qquad (4)$$

Second, each electron micrograph is calibrated and a lattice of test points was superimposed on each Golgi stack (so as to be random in position) by the plungins function of Image J computer software (Fig. 9B); hit points were counted ('*Observed points*', P) in each compartment of the Golgi stack.
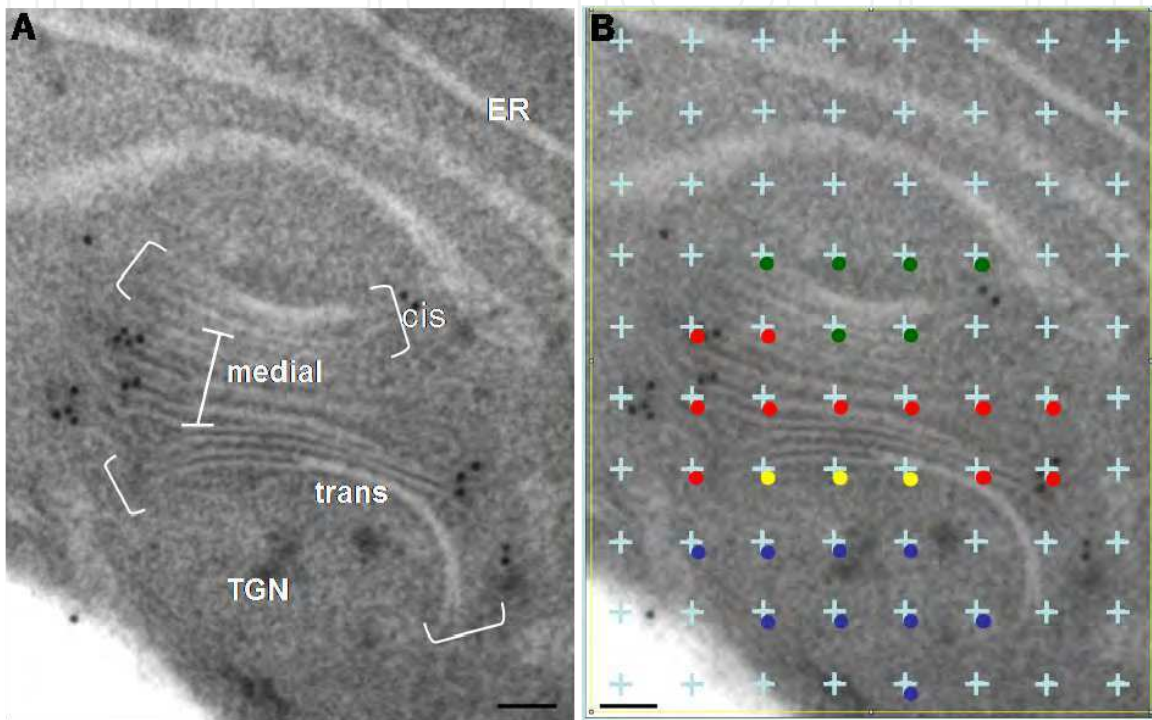

Fig. 9. Electron micrograph of a Golgi unit from At-MUR3-GFP transformed BY-2 cells. (A) *cis*, medial, *trans* and *trans* Golgi network (TGN) are clearly distinguishable. (B) Lattice (1015,43 area per point) was superimposed on the same electron micrograph calibrated (1nm/1.01 pixels) by using Image J computer software. In this example, 6 '*Observed points*, P' are present in the *cis* (green points), 11 in the medial (red points), 3 in the *trans* (yellow points) and 9 in the TGN (blue points) compartments. Scale bars = 100 nm.

"*Observed gold particles*, n_0" and "*observed points*, P" are put in the relative labelling index (RLI) table (Table 4). Then, the '*expected golds particles*' (n_e) (i.e. random distribution) is calculated for each Golgi compartment[5] from the product of the observed points in the Golgi compartments and column total of observed gold particles (total gold particles observed in four Golgi compartments, $n_{0\,51}$), divided by the column total of observed points (total points observed in four Golgi compartments, $P_{\,52}$). Example for the *cis* compartment of WT cells, n_e value is determined as described[5]:

$$n_{e\,13} = (P_{12} \times n_{0\,51})/P_{\,52}. \qquad (5)$$

Observed (n_0) and expected (n_e) gold particles distribution allowed calculation of the relative labelling index[6] (RLI):

$$RLI = (n_0/n_e) \qquad (6)$$

For a random distribution, the relative labelling index (RLI) is inferior or equal to 1, and for a compartment preferentially labelled RLI is superior to 1.

	Observed gold particles (n_0)	Observed points (P)	Expected gold particles (n_e)	RLI (n_0/n_e)
cis	$n_{0\,11}$	P_{12}	$n_{e\,13}$	$n_{0\,11}/\,n_{e\,13}$
medial	$n_{0\,21}$	P_{22}	$n_{e\,23}$	$n_{0\,21}/\,n_{e\,23}$
trans	$n_{0\,31}$	P_{32}	$n_{e\,33}$	$n_{0\,31}/\,n_{e\,33}$
TGN	$n_{0\,41}$	P_{42}	$n_{e\,43}$	$n_{0\,41}/\,n_{e\,43}$
Column total	$n_{0\,51}$	P_{52}	$n_{e\,53}$	

Table 4. Model of relative labelling index (RLI) table comprising 3 columns (n_0, P and n_e) and four rows (related to four Golgi compartments studied). Total observed gold particles and total of expected gold particles must be equal ($n_{0\,51} = n_{e\,53}$).

In a recent published study, we have applied this quantitative analysis to determine the Golgi mapping of three XyG synthesis enzymes in transformed tobacco cell lines (Chevalier et al., 2010). We show that the labelling of AtXT1–GFP (Fig. 6B) was mostly associated with *cis* and medial cisternae, whereas the labelling of AtMUR3–GFP (Fig. 8B & Fig.9) was mainly detected in medial and *trans* cisternae of Golgi stacks. In addition to HPF-FS protocol described here for BY-2 cell preparation, Tokuyasu cryo-sectioning approach is also amenable to this plant cell model (Chevalier et al., 2011). A new approach named the "rehydration method" was proposed by Van Donselaar et al. (2007). It combines HPF/FS procedure followed by a rehydration step, infiltration in sucrose and cryo-sectioning, and is particularly efficient to investigate the localization of epitopes from polysaccharides that are often lost during Tokuyasu process and was recently used to immunolocalize epitopes associated with XyG (Viotti et al., 2010). It could be of interest to adapt the rehydration method to plant suspension cells like tobacco BY-2 cells for quantitative study of cell wall polymers such as hemicelluloses or pectins.

5. Conclusion

In conclusion, many immuno-microscopical methods are available to investigate plant cell walls. A number of antibodies against cell wall carbohydrate epitopes are also available and can be used to examine the distribution of polysaccharides. It is however important to perform experiments with varying parameters to determine the best conditions for a given sample. Optical imaging instrumentation, based on confocal laser scanning microscopy (CLSM), offers the ability to determine the spatial organisation of polymers at the cell surface, across the cell wall compartment as well as within the endomembrane system. The CLSM minimizes interference of innate plant autofluorescence from chlorophylls or vacuole content and provides a quite precise picture of polysaccharides distribution. Moreover, cryo-sample preparation for immunogold EM studies has significantly improved the immunodetection at the subcellular level of the cell wall components and their synthesizing enzymes. Finally, the preservation quality of both the cell ultrastructure and epitopes allows the quantification of the labelling within specific membrane-bound compartments (e.g; Golgi stacks). A careful

combination of microscopical and quantification methodologies is very promising for future studies devoted to imaging of cell wall glycomolecules and synthesizing enzymes.

6. Acknowledgments

7. References

Baskin T.I., Busby C.H., Fowke L.C., Saturant M. & Gubler F. (1992) Improvements in immunostaining samples embedded in methacrylate: localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. *Planta*, Vol. 187, pp. 405-413

Boavida, L.C. & McCormick, S. (2007) Temperature as a determinant factor for increased and reproducible in vitro pollen germination in Arabidopsis thaliana. *The Plant Journal*, Vol.52, pp. 570–582

Brandizzi F., Irons S.L., Johansen J., Kotzer A. & Neumann U. (2004) GFP is the way to glow: Bioimaging of the plantendomembrane system. *Journal of Microscopy*, Vol. 214, pp.: 138-158.

Cheung, A.Y., Wang, H. & Wu, H.M. (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell*, Vol.82, pp. 383-393

Chevalier, L., Bernard, S., Ramdani, Y., Lamour, R., Bardor, M., Lerouge, P., Follet-Gueye, M.L. & Driouich, A. (2010) Subcompartment localization of the side chain xyloglucan-synthesizing enzymes within Golgi stacks of tobacco suspension-cultured cells. *The Plant Journal*, Vol.64, pp. 977-989

Chevalier L., Bernard S., Vicré-Gibouin M., Driouich A. & Follet-Gueye M.L. (2011) Cryopreparation of tobacco Bright Yellow-2 (BY-2) suspensioncultured cells and application to immunogold localization of Green Fluorescent Protein-tagged-membrane-proteins. *Journal of Biological Research*, Vol.16, pp. 202-216

Clausen, M.H., Willats, W.G.T. & Knox, J.P. (2003) Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research*, Vol.338, pp. 1797-1800

Coimbra, S. & Salema, R. (1997). Immunolocalization of arabinogalactan proteins in *Amaranthus hypochondriacus* L. ovules. *Protoplasma,* Vol.199, pp. 75-82.

Coimbra, S. & Duarte, C. (2003). Arabinogalactan proteins may facilitate the movement of pollen tubes from the stigma to the ovules in *Actinidia deliciosa* and *Amaranthus hypochondriacus. Euphytica,* Vol.133, pp. 171-178

Coimbra, S., Almeida, J., Junqueira, V., Costa, M.L. & Pereira, L.G. (2007) Arabinogalactan proteins as molecular markers in Arabidopsis thaliana sexual reproduction. *Journal of Experimental Botany*, Vol.58, pp. 4027-4035

Dardelle, F., Lehner, A., Ramdani, Y., Bardor, M., Lerouge, P., Driouich, A. & Mollet JC (2010) Biochemical and immunocytological characterizations of Arabidopsis thaliana pollen tube cell wall. *Plant Physiology*, Vol.153, pp. 1563-1576

Driouich A., Faye L. & Staehelin L.A. (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *Trends in Biochemistry. Science.*, Vol. 18, pp. 210-214

Driouich A., Durand C. & Vicré-Gibouin M. (2007) Formation and separation of root border cells. *Trends in Plant Science*, Vol. 12, pp.14–19

Driouich A. & Baskin T. (2008). Intercourse between cell wall and cytoplasm exemplified by arabinogalactan-proteins and cortical microtubules in the root of *Arabidopsis thaliana. Am. J. Botany.* 95: 1491-1497.

Durand C., Vicré-Gibouin M., Follet-Gueye M.L., Duponchel L., Moreau M., Lerouge P. & Driouich A. (2009) The organization pattern of root border-like cells of Arabidopsis is dependent on cell wall homogalacturonan. *Plant Physiology*, Vol.3, pp 1411-1421

Ellis, M., Egelund, J., Schultz, C.J. & Bacic, A. (2010) Arabinogalactan-proteins: key regulators at the cell surface? *Plant Physiology*, Vol.153, pp. 403-419

Fitchette A.C., Cabanes-Macheteau M., Marvin L., Martin B., Satiat-Jeunemaitre B., Gomord V., Crooks K., Lerouge P., Faye L. & Hawes C. (1999) Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. *Plant Physiology*, Vol. 121, pp. 333-44

Follet-Gueye, M.L., Pagny, S., Faye, L., Gomord, V. & Driouich, A. (2003) An improved chemical fixation method suitable for immunogold localization of green fluorescent protein in the Golgi apparatus of tobacco bright yellow (BY-2) cells. *Journal of Histochemistry and Cytochemistry*, Vol. 51, pp.931–940

Gibeaut D.M. & Carpita N.C. (1994) Biosynthesis of plant cell wall polysaccharides. *FASEB J.*, Vol. 8, pp. 904-915

Gilbert, H.J. (2010) The biochemistry and structural biology of plant cell wall deconstruction. *Plant Physiology*, Vol.153, pp. 444-455

Harholt J., Jensen J.K., Sørensen S.O., Orfila C., Pauly M. & Scheller H.V. (2006) ARABINAN DEFICIENT 1 is a putative arabinosyltransferase involved in biosynthesis of pectic arabinan in Arabidopsis. *Plant Physiology*, Vol. 140, pp. 49–58

Harris P.J. & Northcote D.H., (1971). Polysaccharide formation in plant Golgi bodies. *Biochemistry and Biophysical Acta.*, Vol. 237, pp. 56-64

Hawes M.C., Bengough G., Cassab G. & Ponce G. (2003) Root caps and rhizosphere. *Journal of Plant Growth Regulation*, Vol. 21, pp.352–367

Hématy, K., Cherk, C. & Somerville, S. (2009) Host-pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology*, Vol.12, pp. 406-413

Jauh, G.Y. & Lord E.M. (1996) Localization of pectins and arabinogalactan-proteins in lily *(Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination *Planta*, Vol.199, pp. 251-261

Johnson-Brousseau, S.A. & McCormick, S. (2004) A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically expressed genes. *The Plant Journal*, Vol. 39, pp. 761–775

Jones, L., Seymour, G.B., & Knox, J.P. (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1->4)-beta-D-galactan. *Plant Physiology*, Vol.113, pp. 1405-1412.

Langhans M., Förster S., Helmchen G. & Robinson D.G. (2011) Differential effects of the brefeldin A analogue (6R)-hydroxy-BFA in tobacco and Arabidopsis. *Journal of Experimental Botany*,. Vol. 62, pp. 2949-2957

Lee, K.J.D., Marcus S.E. & Knox J.P. (2011) Cell wall biology: Perspectives from cell wall imaging. *Molecular Plant*, Vol.4, pp. 212-219

Lehner, A., Dardelle, F., Soret-Morvan, O., Lerouge, P., Driouich, A., & Mollet, J.C. (2010) Pectins in the cell wall of *Arabidopsis thaliana* pollen tube and pistil. *Plant Signaling and Behavior,* Vol.5, pp. 1282-1285

Li, Y.Q., Bruun, L., Pierson, E.S. & Cresti, M. (1992) Periodic deposition of arabinogalactan epitopes in the cell wall of pollen tubes of *Nicotiana tabacum L. Planta*, Vol.188, pp. 532-538

Liepman, A.H., Wightman, R., Geshi, N., Turner, S.R. & Scheller, H.V. (2010) Arabidopsis - a powerful model system for plant cell wall research. *The Plant Journal*, Vol.61, pp. 1107-1121

Marcus, S.E., Verhertbruggen, Y., Herve, C., Ordaz-Ortiz, J.J., Farkas, V., Pedersen, H.L., Willats, W.G.T. & Knox, J.P. (2008) Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BioMedCentral Plant Biology*, Vol.8, pp. 60-71

Mayhew, T.M., Lucocq, J.M. & Griffiths, G. (2002) Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. *Journal of Microscopy*, Vol. 205, pp.153–164

Mayhew T.M. (2011) Quantifying immunogold localization on electron microscopic thin sections: a compendium of new approaches for plant cell biologists. *Journal of Experimental Botany*, Vol. 62, pp. 4101-4113

Micheli F. (2001) Pectin methylesterases: cell wall enzymes with important roles in plant physiology. Trends in Plant Sciences, Vol. 6, pp.414-419

Moller, I., Marcus, S.E., Haeger, A., Verhertbruggen, Y., Verhoef, R., Schols, H., Mikklesen, J.D., Knox, J.P. & Willats, W. (2008) High-throughput screening of monoclonal antibodies against plant cell wall glycans by hierarchial clustering of their carbohydrate microarray binding profiles. *Glycoconjugate Journal* Vol. 25, pp. 37-48.

Mollet, J.C., Park, S.Y., Nothnagel, E.A. & Lord, E.M. (2000) A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. *The Plant Cell*, Vol.12, 1737-1749

Mollet, J.C., Kim, S., Jauh, G.Y. & Lord, E.M. (2002) AGPs, pollen tube growth and the reversible effects of Yariv phenylglycoside. *Protoplasma*, Vol.219, pp. 89-98

Mollet, J.C., Faugeron, C. & Morvan, H. (2007) Cell adhesion, separation and guidance in compatible plant reproduction, In: *Plant Cell separation and adhesion,* Roberts, J. & Gonzalez-Carranza, Z. (Ed.), *Annual Plant Reviews*, Vol.25, pp. 69-90

Mouille, G., Ralet, M.C., Cavelier, C., Eland, C., Effroy, D., Hématy, K., McCartney, L., Truong, H.N., Gaudon, V., Thibault, J.F., Marchant, A. & Höfte, H. (2007) Homogalacturonan synthesis in Arabidopsis thaliana requires a Golgi-localized protein with a putative methyltransferase domain. *The Plant Journal*, Vol.50, pp. 605–614

Pagny S., Bouissonnie F., Sarkar M., Follet-Gueye M.L., Driouich A., Schachter H., Faye L. & Gomord V. (2003) Structural requirements for Arabidopsis beta1,2-xylosyltransferase activity and targeting to the Golgi. *The Plant Journal*, Vol. 33, pp. 189-203

Pattathil, S., Avci, U., Baldwin, D., Swennes, A.G., McGill, J.A., Popper, Z., Bootten, T., Albert, A., Davis, R.H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W.S. & Hahn, M.G. (2010) A comprehensive toolkit of Plant cell wall glycan-directed monoclonal antibodies. *Plant Physiology*, Vol.153, pp. 514-525.

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M. & Roberts, K. (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *The Plant Cell*, Vol.3, pp. 1317-1326.

Puhlmann, J., Bucheli, E., Swain, M.J., Dunning, N., Albersheim, P., Darvill, A.G. & Hahn, M.G. (1994) Generation of monoclonal antibodies against plant cell wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal alpha-(1,2)-linked fucosyl-containing epitope. *Plant Physiology*, Vol.104, pp. 699-710

Qin, Y. & Zhao, J. (2006). Localization of arabinogalactan proteins in egg cells, zygotes, and two-celled proembryos and effects of β-D-glucosyl Yariv reagent on egg cell

fertilization and zygote division in *Nicotiana tabacum* L. *Journal of Experimental Botany*, Vol.57, pp. 2061-2074

Ray P.M. (1980) Cooperative action of beta-glucan synthetase and UDP-xylose xylosyl transferase of Golgi membranes in the synthesis of xyloglucan-like polysaccharide. *Biochimistry and Biophysical Acta*, Vol. 629, pp. 431-444

Sandhu, A.P.S., Randhawa, G.S. & Dhugga, K.S. (2009) Plant cell wall matrix polysaccharide biosynthesis. *Molecular Plant*, Vol. 2, pp. 840-850

Seifert, G.J. & Blaukopf, C. (2010) Irritable walls: the plant extracellular matrix and signaling. *Plant Physiology*, Vol.153, pp. 467-478

Smyth, D.R., Bowman, J.L. & Meyerowitz, E.M. (1990) *Early flower development in* Arabídopsis. The Plant Cell, *Vol.2, pp. 755-767*

Staehelin L.A., Giddings T.H. Jr, Kiss J.Z. & Sack F.D. (1990) Macromolecular differentiation of Golgi stacks in root tips of Arabidopsis and Nicotiana seedlings as visualized in high pressure frozen and freeze-substituted samples. *Protoplasma*, Vol. 157, pp. 75-91

Studer, D., Graber, W., Al-Amoudi, A. & Eggli, P. (2001) A new approach for cryofixation by high-pressure freezing. *Journal of Microscopy*, Vol. 203, pp.285–294

Toyooka K., Goto Y., Asatsuma S., Koizumi M., Mitsui T. & Matsuoka K. (2009) A mobile secretory vesicle cluster involved in mass transport from the Golgi to the plant cell exterior. *The Plant Cell*, Vol. 4, pp. 1212-1229

Van Donselaar E., Posthuma G., Zeuschner D., Humbel B.M. & Slot J.W. (2007). Immunogold labeling of cryosections from high-pressure frozen cells. *Traffic*, 8: 471-485

Vicré, M., Santaella, C., Blanchet, S., Gateau, A. & Driouich, A. (2005) Root border-like cells of Arabidopsis. Microscopical characterization and role in the interaction with rhizobacteria. *Plant Physiology,* Vol.138, pp. 998-1008

Viotti C., Bubeck J., Stierhof Y.D., Krebs M., Langhans M., van den Berg W., van Dongen W., Richter S., Geldner N., Takano J., Jürgens G., de Vries S.C., Robinson D.G. & Schumacher K. (2010) Endocytic and secretory traffic in Arabidopsis merge in the trans-Golgi network/early endosome, an independent and highly dynamic organelle. *The Plant Cell*, Vol. 22, pp. 1344-1357

Willats, W.G.T., Marcus, S.E. & Knox, J.P. (1998) Generation of a monoclonal antibody specific to (1->5)-alpha-L-arabinan. *Carbohydrate Research*, Vol.308, pp. 149-152.

Willats W.G., McCartney L. & Knox J.P. (2001) In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of Arabidopsis thaliana. *Planta*, Vol. 213, pp. 37-44

Willats W.G., McCartney L., Steele-King C.G., Marcus S.E., Mort A., Huisman M., van Alebeek G.J., Schols H.A., Voragen A.G., Le Goff A., Bonnin E., Thibault J.F. & Knox J.P. (2004) A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta*, Vol. 218, pp.673-81

Wu, H., de Graaf, B., Mariani, C. & Cheung, A. Y. (2001). Hydroxyproline rich glycoproteins in plant reproductive tissues: structure, functions and regulation. *Cell and Molecular Life Sciences,* Vol.58, pp. 1418-1429

Yates, E.A., Valdor, J.-F., Haslam, S.M., Morris, H.R., Dell, A., Mackie, W. & Knox, J.P. (1996) Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. *Glycobiology*, Vol.6, pp. 131-139.

Zhang G.F. & Staehelin L.A. (1992) Functional compartmentalization of the Golgi apparatus of plant cells: an immunochemical analysis of high pressure frozen/freeze substituted sycamore suspension-cultured cells. *Plant Physiology*, Vol. 99, pp. 1070-1083
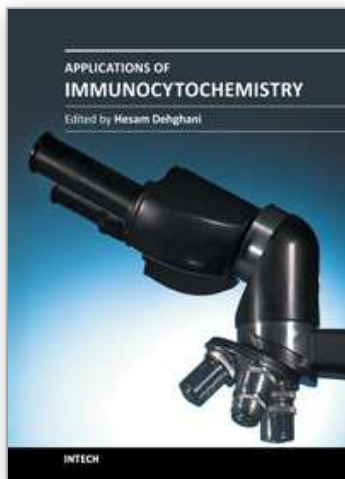
Applications of Immunocytochemistry

Edited by Dr. Hesam Dehghani

Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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