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Influence of xyloglucan oligosaccharides on the micromorphology of the walls of suspension-cultured *Rubus fruticosus* cells

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SUMMARY

The deposition of xyloglucans in the incipient wall of *Rubus fruticosus* cells grown in suspension was examined by transmission electron microscopy. The general distribution of polysaccharides and the formation of a middle lamella was followed along the cell growth period with cytochemical reagents. A predominant morphological change of the cell walls, which are in direct contact with the culture medium, is the reorientation of the superficial cellulose microfibrils which form a loose layer oriented towards the medium. Changes of xyloglucan localization during growth were observed via a polyclonal antibody. When the cells age the loosened superficial microfibrils are progressively depleted in the associated xyloglucans. This phenomenon was accelerated when the nonasaccharide XG_9 (XXFG) was added to the culture medium. This is the first observation of an effect of oligosaccharides on the morphology of the plant cell wall at the ultrastructural level.

Key-words: growing cell walls, immunocytochemistry, oligosaccharides, suspension culture, xyloglucans.

INTRODUCTION

During a growth period, cell walls of suspension cultured cells, undergo morphological and biochemical changes which reflect the changes in the physiological state of the cells. From a morphological point of view, the most evident change lies in the observation that the cells, which aggregate during the first phases of growth, tend to separate during the exponential phase of the cycle (Street *et al.* 1972). However, this study was focused essentially on cytoplasmic modifications and gave little information about the changes that may occur in the ultrastructural organization of the walls during the growth cycle. From a biochemical point of view, variations in the non-cellulosic components of the cell walls of *Vinca rosea* and *Catharanthus* suspension cells, during growth, have been noted (Takeuchi & Komamine 1978; Amino *et al.* 1984). These changes are related to the physiological state of the cells, in particular whether the cells are actively dividing or not.

Xyloglucans (XGs), the typical primary wall hemicelluloses of dicots, have been suggested to be implicated in cell wall loosening during cell growth and enlargement (Keegstra *et al.* 1973; Hayashi 1989). They are multi-form molecules which consist of a

β-1,4-linked glucan main chain highly substituted with xylosyl, galactosyl and fucosyl residues, and which are basically made up of nonasaccharide and heptasaccharide units. Suspension cells of cotton contain three XGs having different ratios of nona- to heptasaccharides and having different molecular weights (Hayashi & Delmer 1988). In pea epicotyl, XG apparent molecular mass has been shown to be reduced after IAA treatment, and this reduction is supposed to cause wall loosening that leads to cell enlargement (Talbott & Ray 1992). In suspension cultured cells, xyloglucans are released extracellularly during growth, forming a part of the extracellular polysaccharides (ECP) (Cartier *et al.* 1987). The quantities of ECP released in the culture medium increase with the age of the cells (Paull & Jones 1978). Recently, McDougall & Fry (1991) showed that in cell suspension cultures of spinach, the ECP was the source of biologically active oligosaccharides of the XG series, generated *in vivo* by hydrolysis of the ECP.

From all this biochemical evidence of xyloglucan rearrangement during growth, it is to be expected that modifications in the ultrastructural distribution of these hemicelluloses must occur during the growth period of cells cultured in suspension.

Concerning the ultrastuctural distribution of XGs within the walls, only a few papers have been published, due to the difficulty of obtaining good specific markers of these molecules. A β-galactosidase–gold complex was used by Reis *et al.* (1987) to label XG *Tamarindus* seedlings. However, because of the ambiguity of the labelling, due to the presence of terminal galactose-containing polymers in the cell walls, other markers, using other enzymes with specificity for more precise parts of the XG molecule, were assessed (Ruel & Joseleau 1990).

Immunocytochemical localization of wall xyloglucans was first introduced in 1986 by Moore *et al.* who showed that XGs were distributed throughout the entire cell walls of sycamore suspension cells, including the middle lamella. Later, using the same antibody, Moore & Staehelin (1988) localized XGs within the Golgi cisterna and vesicles in root-tip cells of red-clover (*Trifolium pratense*). In *Tropaeolum majus* seeds, the transformations undergone by the primary walls of cotyledon cells during the deposition of storage xyloglucans were followed using a polyclonal anti-XG (Ruel *et al.* 1990). More recently, Vian *et al.* (1992) used a polyclonal anti-XG to show the close interaction existing between cellulose and XG in wall ghosts coming from mung bean hypocotyl.

In the authors' studies of the cell-wall polysaccharides from *Rubus fruticosus* cells in suspension, the polyclonal antibody raised against an XG purified from the ECP (Ruel *et al.* 1990) was used in order to follow the changes in the distribution of this polysaccharide at different stages of the growth period. This immunolabelling was also used to study the effect of the XG nonasaccharide on XG distribution, when this oligosaccharide was added to the culture of *Rubus* cells. This oligosaccharide is able to regulate plant growth by inhibiting auxin action (York *et al.* 1984; McDougall & Fry 1989). The ultrastructural modifications of the cell walls following the addition of the oligosaccharide to the culture were studied.

MATERIAL AND METHODS

Plant material

R. fruticosus cells were derived from callus of *R. fruticosus* and subcultured in Heller's medium supplemented with vitamin B_1 (1 mg litre^{-1}) and glucose (20 g litre^{-1}). The

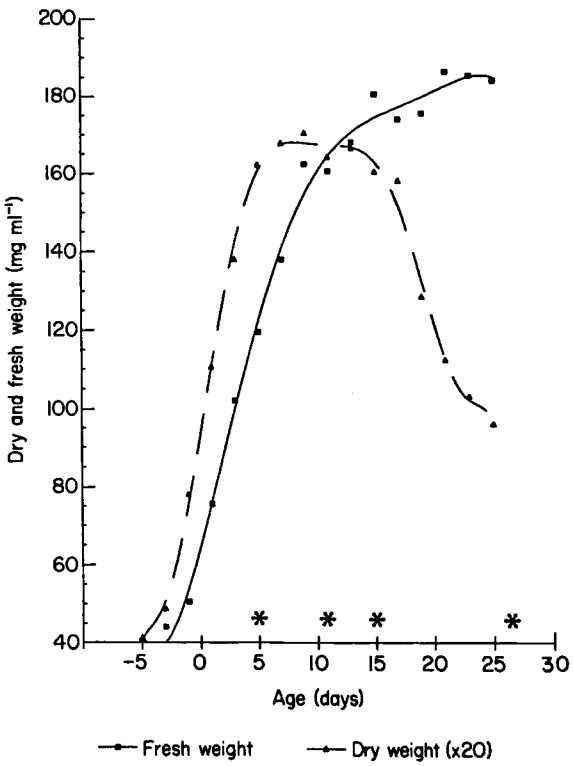


Fig. 1. Growth curve of *Rubus fruticosus* cells cultivated in liquid medium. Asterisks indicate the stages studied by electron microscopy.

cells were harvested after transfer by filtration of the culture medium through a sintered glass funnel and washed with fresh culture medium at intervals between 5 and 30 days. The growth curve of the cells is given in Fig. 1.

Preparation of antibodies

Polyclonal anti-xyloglucan (anti-XG) was raised against a purified xyloglucan extracted from the polysaccharides released into the culture medium of suspension cultures of 18-day-old *Rubus* cells (Joseleau *et al.* 1992). Three rabbits were immunized by subcutaneous injections, using respectively 1 mg ml^{-1}, 2 mg ml^{-1} and 3 mg ml^{-1} of XG. The first injection was in complete Freund's adjuvant and the following injections in incomplete Freund's adjuvant. Rabbits were boosted with the same concentration of XG every 3 or 4 weeks over 3–4 months. The best results were obtained 8 weeks after the first injection.

Antisera were tested, using both enzyme-linked immunoadsorbent assay (ELISA) and electron microscopy affinity assays. ELISA tests were carried out by coating each well of the microtitre plate (NUNC maxisorb 96 wells) with 100 µl of XG (starting concentration: 50 µg ml^{-1}) in 0·05 M sodium carbonate–bicarbonate buffer, pH 9·6. The plates were left overnight at 4°C and then washed with PBS-Tween (0·02% Tween 20 in 10 mM phosphate buffer). The plates were then incubated for 2 h at 37°C with a blocking buffer (3% BSA/PBS with 0·02% sodium azide). Antibody dilutions were carried out in 3% BSA/PBS with 0·02% sodium azide; 100 µl of the antibody test solution were then

added to each well and the plates were incubated for 2 h at 37°C. The plates were washed with PBS (without sodium azide). The antigen–antibody complex was revealed with horse-radish peroxidase labelled with protein A (Immunopure Recomb Protein A Horse-radish Peroxidase conjugate–PIERCE) using *o*-phenylenediamine hydrochloride (OPD) as substrate. The colour was measured at 492 nm with a microtitre reader (UNISCAN II LABSYSTEM).

For electron microscopy affinity assays, the antigen and related polysaccharides, i.e. *Rubus* XG, arabinogalactan, polygalacturonan and galactoglucomannan all purified from the extracellular culture medium (ECP), β-1,3-glucan and XG purified from isolated walls of *Rubus* cells, plus bacterial cellulose and an arabinoxylan extracted and purified from the reed *Arundo donax*, were enrobed in LR White (London Resin Co. Ltd.). The antisera were tested on thin sections using the immunocytochemical process described in a later section for *Rubus* cell sections (see Immunocytochemistry). Two types of control were performed: pre-absorption of the antiserum with 1 mg ml^{-1} of purified xyloglucan and omission of the anti-XG.

Elicitation experiments

Fifteen-day-old culture cells were elicited by adding to the culture medium purified XG_9 (nonasaccharide) at a $1{\cdot}40 \times 10^{-8}$ M final concentration. After 1 h, cells were filtered through nylon cloth and immediately fixed by high pressure freezing (53 s) or with a mixture of glutaraldehyde/paraformaldehyde in phosphate buffer.

Electron microscopy

Normal cells or elicited ones were embedded in LR White after chemical or high pressure freezing (HPF) fixation.

Chemical fixation. Cells were fixed in a mixture of 2% glutaraldehyde, 2% paraformaldehyde in 0·05 M phosphate buffer, pH 7·0 for 3 h at room temperature. They were then washed in phosphate buffer before being dehydrated through 25, 50, 70% ethanol. The cells were then progressively infiltrated and embedded in LR White resin. Polymerization was carried out in an oven at 50°C for 24 h.

High pressure freezing–freeze substitution (HPF–FS). This part of the preparation of the samples was performed in the Laboratory for Electron Microscopy at the Federal Institute of Technology in Zurich (Switzerland). *Rubus* cells were filtered through nylon cloth (mesh 85 μm), placed in the cavity (diameter 2 mm; depth 0·2 mm) of a cylindrical aluminium platelet and sandwiched with a second platelet as described in Studer *et al.* (1989). The space between the sample and the platelets was filled with 1-hexadecene (Fluka, Buchs, Switzerland). The high pressure freezer was an HPM 010 (Balzers Union, Balzers, FL). Cryofixation was performed in 53 s. The sandwiches were immediately transferred to liquid nitrogen and opened. Freeze substitution was performed by transferring the samples which were stored in liquid nitrogen to substituting medium precooled to −92°C, composed of 0·5% glutaraldehyde in absolute ethanol. The apparatus was a Balzers FSU 010 freeze-substitution unit. The samples were kept at −92°C, −62°C and −32°C for 8 h at each step, and finally brought to 0°C for 1 h as described by Studer *et al.* (1989). They were washed three times in absolute ethanol and

embedded stepwise in LR White (30%, 50%, 75%, 100% resin). Polymerization was performed for 24 h at 50°C.

Chemical staining of thin section

Staining with the PATAg (periodic acid, thiocarbohydrazide, silver proteinate) technique was carried out, using a protocol (Ruel 1976) which was a modification of Thiéry's method (1967), adapted to plant cell wall polysaccharides. Periodic oxidation was conducted for 30 min–1 h 30 min at a concentration of 1 or 5% depending on the polysaccharides (cellulose microfibrils are less reactive than pectic or hemicellulosic polysaccharides). Thiocarbohydrazide (TCH) was used at a concentration of 0·02% in 20% acetic acid for 48 h and silver proteinate treatment (1% in water) was conducted for 30 min in the dark.

Immunocytochemistry

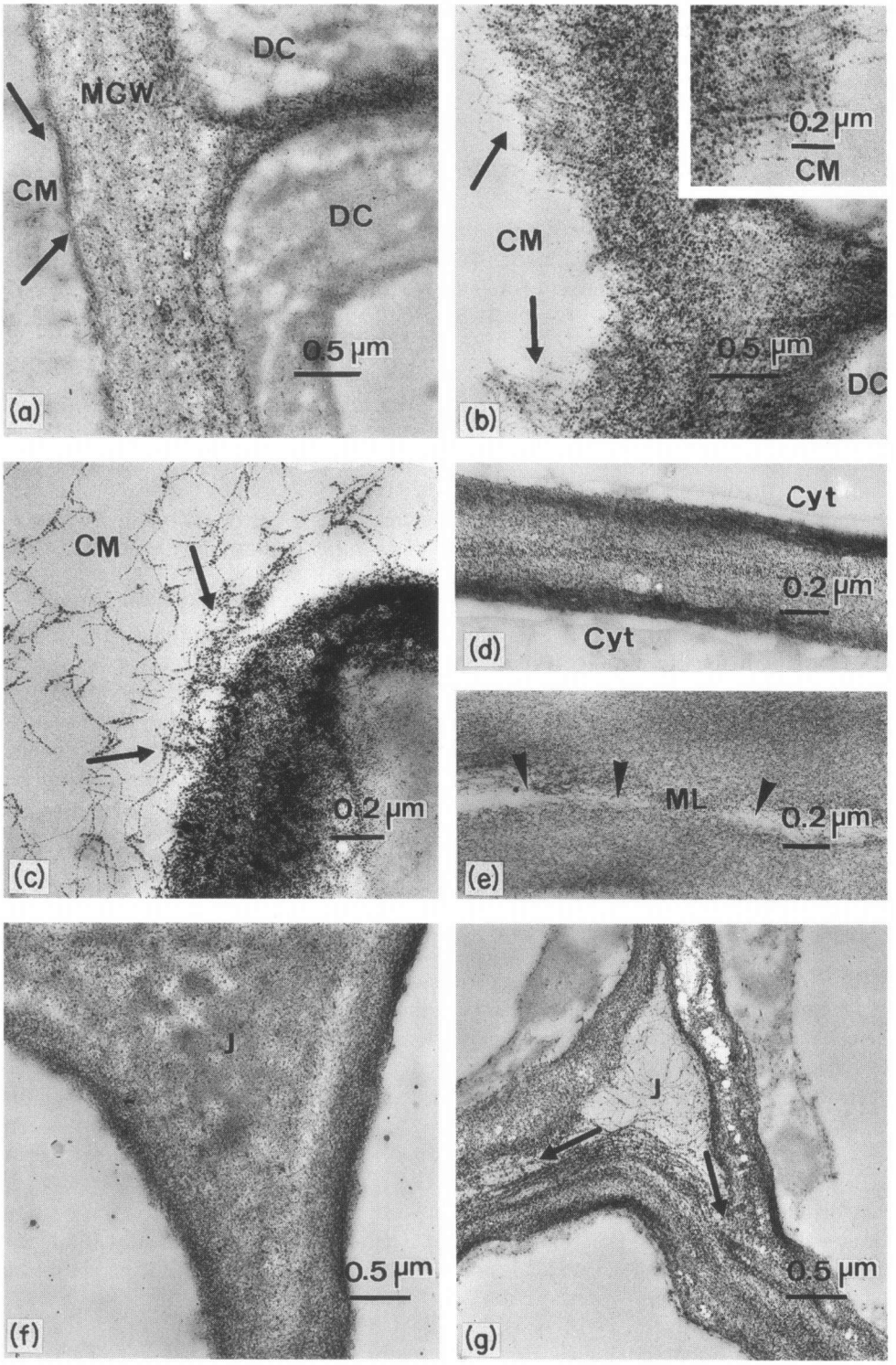
Antibodies were used as post-embedding markers. Sections were floated in plastic rings and labelling was carried out on 50 µl drops placed on clean parafilm. The following procedure was adopted: (1) treatment for 15 min on 0·15 M glycine in 10 mM Tris buffer (2-amino-2-(hydroxymethyl)-1,3-propanediol), pH 7·4, followed by Tris-buffer rinse; (2) floating for 30 min on a blocking solution consisting of 0·1% (w/v) bovine serum albumin (BSA), 0·5% gelatin fish in TBS 500 (Tris buffer containing 500 mM NaCl); (3) incubation (15–16 h) at room temperature on anti-XG (diluted 1/20 in TBS 500 containing BSA 0·1% gelatin fish 0·5% diluted in TBS 500); (4) washing in TBS 500 followed by Tris-HCl rinsing; (5) 2 h incubation on 10 nm gold-conjugated protein A (PA10, JANSSEN Life Science Products) diluted 1:25 in Tris/HCl, 0·1% BSA, 0·5% gelatin fish, pH 7·4; (6) Tris-buffer rinsing; (7) 5 min fixation by floating on 2% (v/v) glutaraldehyde in Tris buffer; (8) final rinsing with water before transfer of the sections on carbon-coated copper grids; (9) grids were post-stained for 2 min on a 2% aqueous solution of uranyl acetate before examination in a Philips 400 T electron microscope.

Controls. The specificity of the labelling was tested on thin section incubated with antisera pre-absorbed with their respective antigens or with pre-immune sera.

RESULTS

In this work, developing cell walls of *R. fruticosus* cells were observed from the cell plate formation to the mature stages at the end of the culture period. Depending on whether the cells were forming aggregates or single elements in the liquid culture, the morphological evolution of the wall was considered according to two different situations, whether the walls were adjacent to each other or in direct contact with the liquid culture medium.

Two techniques were used for the fixation of the samples: high pressure freezing (HPF), followed by freeze substitution (FS) and the classical glutaraldehyde/paraformaldehyde mixture. Comparison between the images obtained from sections coming from the HPF–FS treatment and chemical fixation, indicates that wall preservation was the same. The weak point of the HPF–FS method, in the conditions that were used, was a poor preservation of the cytoplasm. Its strong point was a better retention of the antigenicity of XGs as shown by the optimal dilution of the anti-XG which was 5–10-fold higher than that required in chemical fixation. Therefore,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considering that this study was focused on cell wall organization, samples fixed by either of the two techniques were used, adapting the anti-XG dilution to the type of fixation used.

In this study, the general distribution of all the polysaccharides as the wall develops will be described first, then the XG distribution will be followed via the anti-XG antibody labelling. The action of the XG_9 oligosaccharide will be essentially envisaged from a micromorphological point of view.

General aspects of cell-wall polysaccharide distribution during the growth period

Four steps on the general growth curve were studied in electron microscopy: 5, 11, 15 and 27 days of culture (see Fig. 1). The polysaccharide distribution was investigated using the general PATAg staining (Thiéry 1967; Ruel 1976). This staining provides an overview of almost all the acid and neutral cell wall polysaccharides. As it was previously demonstrated in the kinetic study of PATAg reaction on purified isolated wall polysaccharides (Ruel 1976; Sugiyama & Harada 1986), that the non-cellulosic polysaccharides reacted much more rapidly and strongly to periodate oxidation than cellulose did, controlled conditions of oxidation were applied to *R. fruticosus* cells at different stages of their growth.

From these observations, several statements may be made. At the EM level the general aspect of *Rubus* cell walls varies both with age and situation in the culture medium. In the young stages of growth (5-day old culture), the walls which are in direct contact with the culture medium exhibit a smooth surface limited by a thin outer layer of PATAg reactive material (Fig. 2a). As the cell matures, (15-day old) the outer layer thickens and takes on a fibrillar aspect (Fig. 2b). The microfibrils, weakly reactive to PATAg but underlined by strongly PATAg positive components, undergo a drastic change in the direction of their orientation (Fig. 2b and insert). In 27-day old culture (Fig. 2c) fibrillar elements of the outer layer are detached in the culture medium and leave a loose but conspicuous network attached to the wall.

When cell walls are adjacent (i.e. forming aggregates), no distinct border can be observed in the young stages of growth (5-day old cells) (Fig. 2d). A separation zone progressively individualizes as the cells age (Fig. 2e) and the tricellular junctions which were first filled with polysaccharide material (Fig. 2f) become progressively empty (Fig. 2g). This phenomenon starts from the intercellular spaces and then extends along the middle lamella.

Fig. 2. TEM observation of *Rubus* cell walls at different stages of maturity, glutaraldehyde/paraformaldehyde fixation, PATAg staining. (a,b,c) Cell walls facing the culture medium (CM). (a) 5-day old culture: the outer part of the mother cell wall (MCW) surrounding two daughter cells (DC) still attached to the mother cell, has a smooth surface (arrows). The distribution of PATAg positive polysaccharides is regular but denser inside the associated daughter cell walls (DC). (b) 11-day old culture: daughter cells are still associated with mother cell's wall. The outer part facing the culture medium becomes hairy and takes a fibrillar aspect (arrows). Enlargement shows the cellulose microfibrils (less reactive), with strongly PATAg reactive associated polysaccharides. (c) 27-day old culture: the outer face loses its coherence, microfibrillar elements without any orientation detached from the walls (arrows). (d,e) Adjacent cell walls. (d) 5-day old culture: polysaccharide distribution is the same within the two adjacent walls. No clear border can be observed. (e) 11-day old culture: cells begin to separate along the middle lamella (arrowheads). (f,g) Tricellular junctions. (f) 5-day old culture: the tricellular junction (J) is filled with polysaccharides. (g) 11-day old culture: the junction is depleted of a part of its polysaccharide content. This lysis progresses towards the middle lamella (arrows).

Table 1. Immunoaffinity of anti-XG for thin sections of purified polysaccharides from *Rubus*

	Extracellular XG	Wall XG	Extracellular arabino-galactan	Extracellular galactogluco-mannan	Wall β-1,3-glucan	Wall galacturonan
Observed labelling	(+++)	(++)	(−)	(−)	(−)	(−)

Purified polysaccharides were embedded in LR White and submitted to the anti-XG at the same dilution. (+++): Very strong labelling; (++): strong labelling; (−): no labelling.

Xyloglucan distribution during growth

Antiserum specificity. The polyclonal antibody used in this study was raised against a pure xyloglucan extracted from the ECP of 18-day old cultured cells. The purity of the polysaccharide was evaluated as described in Joseleau *et al.* (1992). It is interesting that, contrary to that used by Moore *et al.* (1986), the XG used in this study gave antibodies without necessitating attachment to a carrier protein. The antisera, coming from three rabbits were tested by two different methods. The first was the classical indirect ELISA and the second was an EM technique used previously by the author (see Ruel & Joseleau 1984) to test the ability of enzyme–gold complexes to recognize their substrates.

Indirect ELISA gave clear positive responses both with ECP and cell-wall XGs. However, the adsorption capacity of XGs inside the wells of NUNC plates was poor and the minimal detectable quantity of XGs was 62 ng ml^{-1}. No interaction with arabinogalactan, galactoglucomannan, galacturonan or β-1,3-glucan antigens coming from *Rubus* ECP was observed. A positive but much weaker response than the XGs was obtained with a xylan from hardwood and with bacterial cellulose.

In EM, affinity of the anti-XG was tested against XGs and the various aforementioned polysaccharides. The enrobed polysaccharides were sectioned and submitted to immunolabelling under the same conditions as those used on thin sections of *Rubus* cells. The immunoaffinity was estimated on micrographs of the thin sections of these preparations, from the intensity of the observed labelling (Table 1). The anti-XGs showed specific recognition only for xyloglucans. Controls showed that the labelling on XGs was eliminated by preincubation with the proper antigen and not with other polysaccharides extracted from *Rubus* cell walls (Figs 3a and b). No labelling was observed using pre-immune serum as primary antibody and protein A complexed with gold, and PA10 used alone had no affinity for the *Rubus* cell walls.

Immunogold labelling of xyloglucans at various stages of the growth period. The anti-XG was applied to label thin sections of *Rubus* cells embedded at three stages of growth: 5, 15 and 27 days of culture. In dividing cells, at the very beginning stage of the cell-plate formation, vesicles containing XG aligned in the equatorial plane of the cell, can be seen (Fig. 4a). The enlargement in the insert shows that XG molecules are delivered in the forming cell plate by Golgi vesicles (Fig. 4, upper part).

In young cells (5-day old culture) XGs are evenly distributed both in mother and daughter cell walls (Fig. 4b). There is no apparent organization or zonation and no true middle lamella can be distinguished between contiguous cells (Fig. 4c). As the cells age and as the middle lamella differentiates (15-day old culture), XGs seem to be

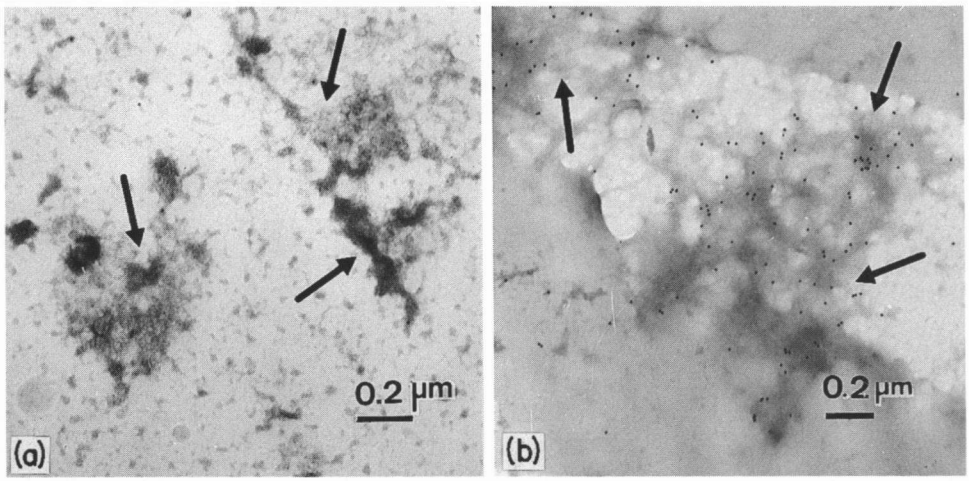


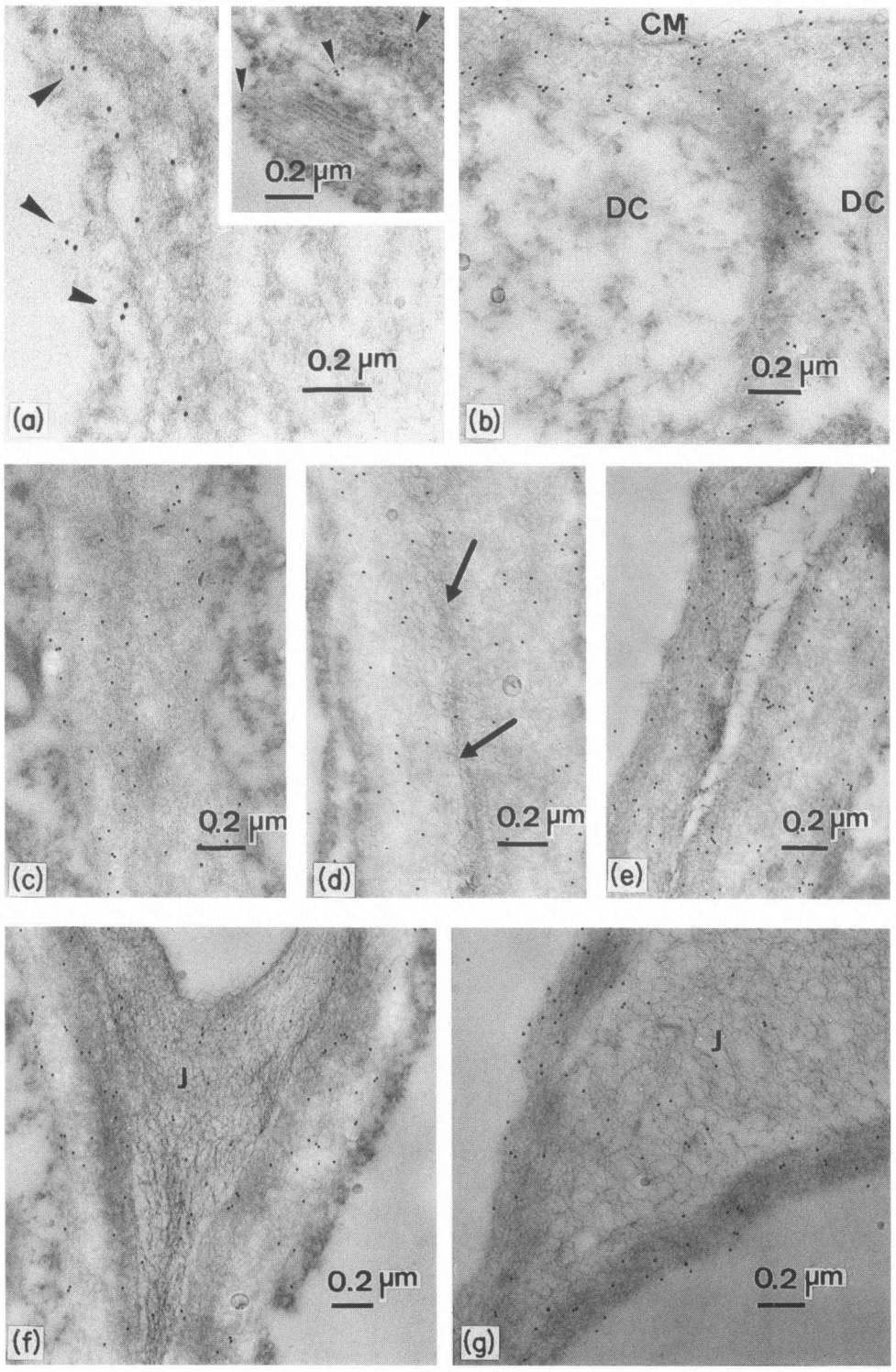
Fig. 3. Anti-XG labelling of a thin section of a purified XG isolated from *Rubus* cell wall. (a) Control, anti-XG was preincubated with XG. (b) Labelling of the XG polysaccharide (arrows indicate the polysaccharide).

progressively eliminated from the junction zone (Fig. 4d). Finally, when the cells separate, some fibrillar material remains attached to the outer part of the wall, but no XG is seen in association with this fibrillar material (Fig. 4e). It can also be seen that at 5 days, tricellular junctions are filled with electron dense material in which some XGs are present (see Fig. 4b and f). As the cells age (15-day old), this junction area becomes progressively depleted of material, leaving a reticulated substrate underlined by uranyl acetate post-staining and devoid of XG (Fig. 4f and g).

In the walls of those cells which are in direct contact with the culture medium, an abundant labelling of XG can be seen both on the wall itself and on the hairy face in contact with the liquid culture (Fig. 5a). The enlargement seen in Fig. 5a shows that the reoriented microfibrils pointing outwards, are lined up with the gold grains, thus demonstrating that the cellulose microfibrils and XG are closely associated. When these cells reach a mature state (27-day old), the most conspicuous transformation is the presence of an abundance of extracellular material which has been released into the culture medium leaving a fibrillar material still attached to the cell. It has to be noted that this fibrillar material is devoid of gold deposits (Fig. 5b).

Effect of XG_9 nonasaccharide on wall morphology and XG distribution

The XG_9 oligosaccharide was applied at a concentration of *c.* 10^{-8} M to the growing cells at the exponential phase of growth (11 or 15-day old) and its effects were followed after 10, 30 and 60 min of action. After 10 min, the first signs of a modification could be seen on the outer part of the walls situated in direct contact with the culture medium (Fig. 6a and b). This modification is characterized by a loosening accompanied by a reorientation of the outermost microfibrils of the wall which produces an abundant extracellular fibrillar material as underlined by uranyl acetate staining. After 30 min of elicitation, adjacent cells also undergo a modification of their architecture. Again, it is the outer part of the walls which responds and swells individualizing a middle lamella junction (Fig. 6c and d). For the walls in contact with the culture medium, in addition to their reorientation, the outer microfibrils were peeled from the rest of the wall, giving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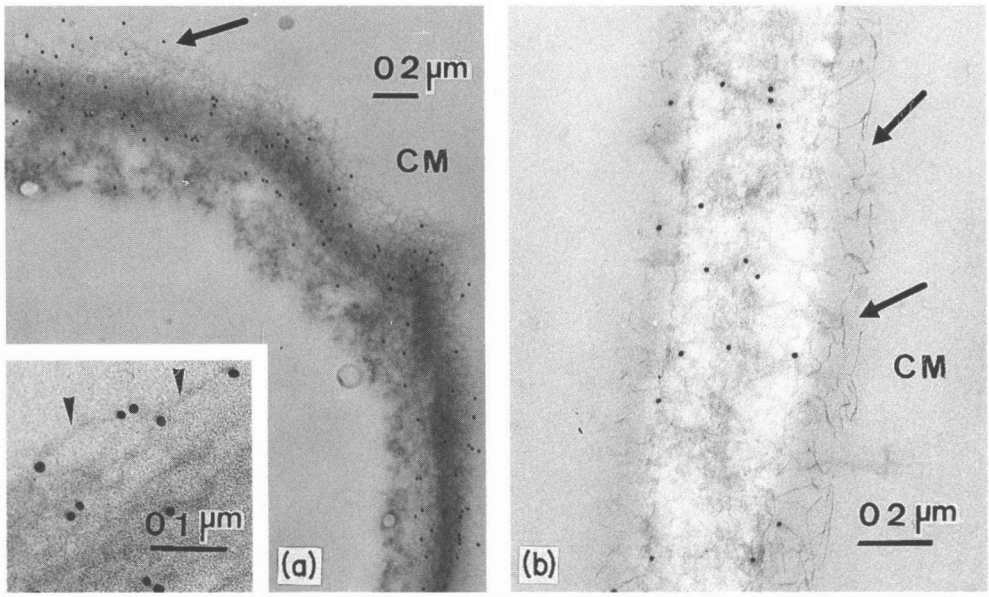


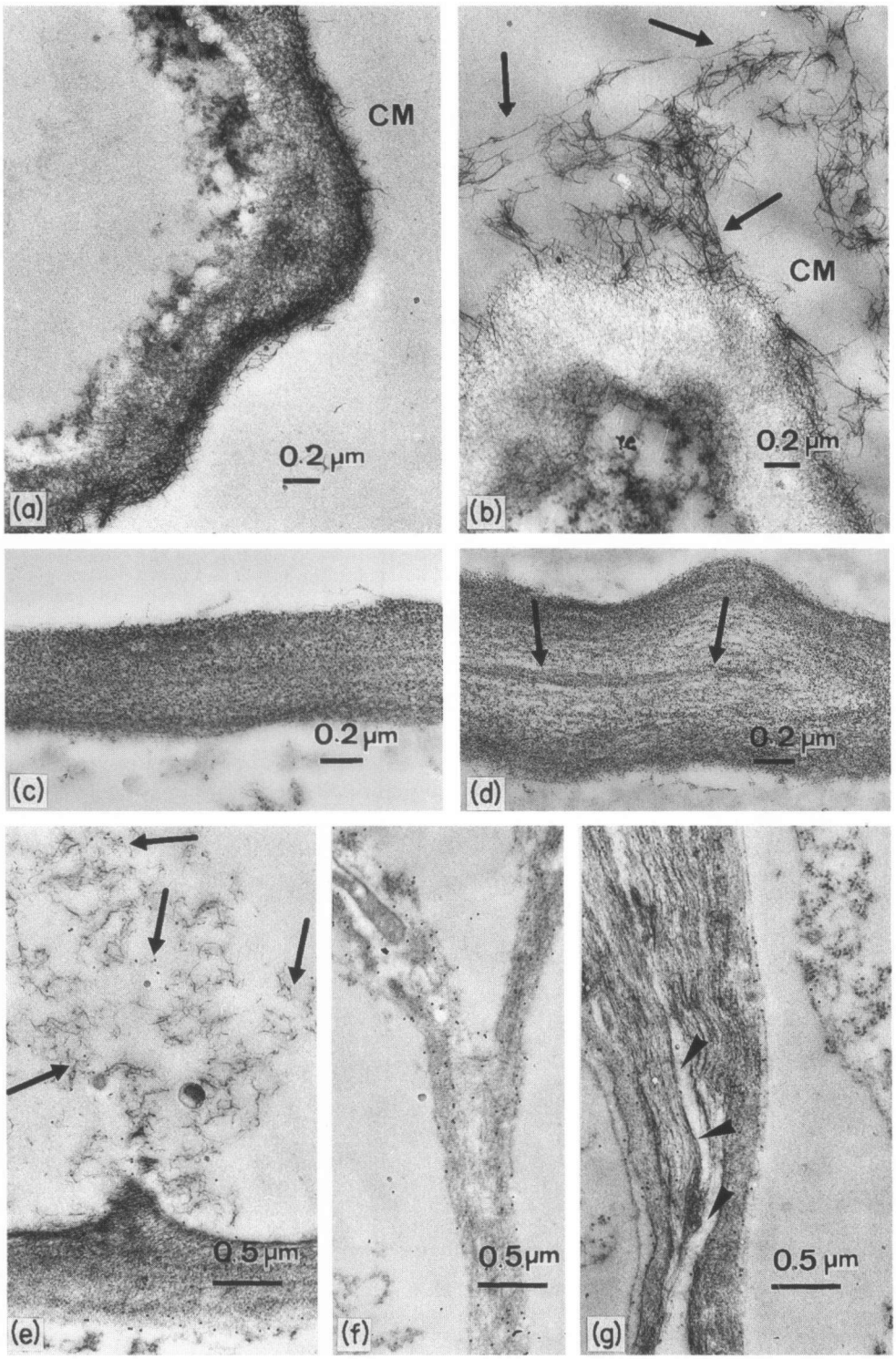
Fig. 5. Evolution of XG distribution in the walls which are in direct contact with the culture medium (glutaraldehyde, paraformaldehyde fixation). (a) 11-day old culture. The hairy layer (arrow) characterized by a reorientation of the microfibrils in the outer layer of the wall, contains XGs. The detail in the upper part shows that XGs are associated with the reoriented microfibrils (arrowheads). (b) Final stage of the growth period (27-day old culture). The outer part of the wall is now organized in a reticulated form and no XGs are seen associated with this network (arrows).

rise to a loose chopped fibrillar material which can extend far from the wall. The anti-XG antibody gave partial labelling of this material with some gold particles aligned along the fibrillar material (Fig. 6e). After 60 min of XG_9 elicitation, most of the cells separate and a clear delamination appears inside the wall as compared to the non-elicited control (Fig. 6f, g). The xyloglucan immunolabelling follows the cellulose microfibrils more clearly than in the control. No modification in the density of the distribution of the gold grains can be detected.

DISCUSSION

As underlined in the results section, the morphological aspect of the walls of controls being the same after chemical or HPF–FS treatment, images from samples fixed by either technique were used.

Fig. 4. Anti-XG labelling of *Rubus* cells during growth. (a,b) Glutaraldehyde/paraformaldehyde fixation. (C–G) HPF–FS technique. (a) Very young stage of the formation of the cell-plate. Golgi vesicles containing XG are associating (arrowheads). Insert: Golgi stack delivering small vesicles filled with XGs towards the newly formed primary wall. (b) Two daughter cells (DC) still associated with the mother cell wall (MCW). Homogeneous distribution of XGs inside walls and the tricellular junction. (c,d,e) Evolution of the median layer between two adjacent cells. (c) Young cell walls. XGs are homogeneously distributed, no distinct area separates the cells. (d) 15-day old culture. Differentiation of a criss-crossed material with very little XGs associated (arrows). (e) Older stage, the adjacent cells separate and XGs disappear. (f, g) Evolution of cell corner area (J). (f) 5-day old culture. A few gold grains corresponding to XGs are present inside the junction. (g) A reticulate material differentiates inside the intercellular space (J) and XGs disappear.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Changes in the morphological distribution of non-cellulosic polysaccharides during growth

The variations observed in the mode of association of the cells aggregated in the early stage of growth, and later dissociated, are in agreement with previous other studies on suspension cultures (Henshaw *et al.* 1966; Street *et al.* 1974; Hayashi & Yoshida 1988). The variation in reactivity of the different polysaccharide components as a function of oxidation conditions (Ruel 1976; Sugiyama & Harada 1986) gave information about the relative distribution of the more reactive pectic and hemicellulosic polysaccharides vs. the less reactive cellulose microfibrils. During the modification of the outer microfibrils (visible in 15-day old culture, Fig. 2b and insert) the images obtained illustrate a stage at which hemicellulosic and pectic polysaccharides are still sticking to cellulose microfibrils (McCann *et al.* 1990). This observation seemed to be of the same type as those by Mora *et al.* (1986) and Vian & Roland (1991) who showed the intimate association of cellulose microfibrils with xylans and with polygalacturonans, respectively.

In adjacent walls, the 'middle lamella' differentiation corresponds to the formation of a more PATAg reactive zone. This suggests a modification in the polysaccharides distribution as proposed by Matar and Catesson (1988). For those cells which are in direct contact with the culture medium, the PATAg reaction permits one to follow the evolution of the smooth outermost layer into a hairy and loose surface with reoriented cellulose microfibrils. This indicates both structural and chemical rearrangements with ageing. In the oldest cells this outer layer extends far from the wall and the overall phenomenon may be related to the sloughing of the ECP described by Fry (1986) in suspension cultured cells.

Such a modification in the microfibril arrangement during growth has been described in the epidermal cell walls of the epicotyl of *Vigna angularis* by Takeda & Shibaoka (1981) and more recently by Wolters-Arts & Sassen (1991). This had not been observed in the walls of cultured cells.

Validity of the anti-XG antibody

To control the specificity of the antibody, two different techniques were used. Effectively, checking the specificity of the antibodies for polysaccharides by ELISA derived methods is sometimes impaired by the poor fixation of the antigen inside the wells. This probably explains the low titre observed for the anti-xyloglucan serum. On the other hand the electron microscopy assays checking the affinity of the antibody for different polysaccharides obviates this problem and clear recognition responses could thus be obtained.

Fig. 6. Addition of XG_9 to the culture medium. (a,b) Glutaraldehyde/paraformaldehyde fixation. 11-day old culture. Uranyl acetate staining—10 min of elicitation. (a) Control without addition of XG_9 to the culture medium (CM). (b) 10 min after the addition of XG_9. Note the strong modification of the outer part of the wall: a fibrillar criss-crossed material replaces the smooth border of the control and extends outwards (arrows). (c,d) Glutaraldehyde/paraformaldehyde fixation. 11-day old culture. PATAg staining. 30 min after XG_9 addition. (c) Control without XG_9 added. Adjacent cell walls are joined. (d) 30 min after XG_9 addition, the outer part of the adjacent walls becomes loose and a middle lamella (arrows) individualizes. (e,f,g) HPF–FS technique—15-day old culture—anti-XG labelling. (e) 30 min after addition of XG_9. XGs are still present inside the wall and a part of them remains associated with the extracellular fibrillar material (arrows). (f,g) 1 h after XG_9 addition. (f) Control without XG_9 added—XG labelling is evenly distributed across the adjacent walls. (g) After 1 h of XG_9 contact, the cell walls separate (arrowheads) and take a lamellar organization. XGs are still present inside the walls.

Changes in xyloglucan localization during growth

Along a cell growth period, antibody labelling showed that XGs appear at the earliest stages of the formation of the cell plate via Golgi vesicles as described by Moore & Staehelin (1988). The absence of a distinct middle lamella differentiation at young stages also corresponds to observations by Moore *et al.* (1986). The other ultrastructural aspects observed during growth have not been described in the literature. However, studies on wall regeneration around carrot protoplasts (Shea *et al.* 1989) showed that hemicelluloses are actively synthesized at the beginning of new wall elaboration. This agrees with the observation of the accumulation of XGs in the inner part of the wall during maturation. It seems that the most important reorganizations take place in the outer part of the wall. This is true not only in adjacent cells, but also in walls which are in contact with the culture medium. The reorganization mainly consists in the reorientation of cellulose microfibril and in the loosening of their association. The observed dissociation can be correlated with the removal of XGs as indicated by the decrease of the intensity of the xyloglucan labelling. This is in agreement with the proposed role of XGs in keeping cellulose microfibrils together by hydrogen bonding (Hayashi *et al.* 1987; Hayashi & Delmer 1988; McCann *et al.* 1990). The observed loosening of cellulose microfibril associations also agrees with the suggestion by Paull & Jones (1978) working on sycamore cells, of a hydrolysis process which could accompany growth and whereby XGs became solubilized in the culture medium.

The presence of wall-bound enzymes (Keegstra & Albersheim 1970) which could participate in cellulose microfibril dissociation also goes along with the authors' observations. A consequence of these wall rearrangements is the release in the culture medium of soluble extracellular fractions. Recently McDougall & Fry (1991) demonstrated that extracellular XGs arise *in vivo* from the wall of cultured spinach cells. This agrees with the labelling of extracellular material by anti-XG antibodies seen in Fig. 6e. It thus appears that outer surface wall loosening is the sign of an ageing cell.

Changes in the morphological aspect and XG distribution after nonasaccharide elicitation

Interestingly, when the biologically active XG nonasaccharide (XG_9) (Joseleau *et al.* 1992) was added to the culture, the cells collected at 10 min, 30 min or 1 h after the addition of the oligosaccharide, showed a rapid response as indicated by the reorientation of cellulose microfibrils and the loosening of the wall surface observed, compared to the controls in which no oligosaccharide had been added. The fact that cell walls which are in direct contact with the culture medium were the first to undergo modifications can be interpreted as being due to the best accessibility to the XG_9 receptors. As in the case of pathogen elicitation, the response is very rapid.

An enhancement of the modifications undergone by the walls can be seen as the time of contact with XG_9 grows. The modification of the external cellulose microfibrils orientation is accompanied by the release of XGs into the extracellular medium, thus a sloughing of XGs into the culture medium may be visualized (McDougall & Fry 1991).

This is the first report visualizing ultrastructural modifications brought about by XG. This result suggests that a possible role of XG fragments could be to activate the hydrolytic enzymes involved in cellulose–xyloglucan dissociation, thus mimicking in a short period, the modifications observed during a growth period going from 5 to 27 days.

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