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Localization of methyltransferase activities throughout the endomembrane system of flax (*Linum usitatissimum* L) hypocotyls

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A microsomal fraction from flax hypocotyls (*Linum usitatissimum* L) showed a methylation ability from *S*-adenosylmethionine on to the cell wall polysaccharides. Two kinds of methylation were found: (i) a methyl esterification of uronic acids in the oxalate extracts and (ii) an *O*-methylation of the hydroxyl groups in the NaOH extracts. The methyltransferase study showed a rapid decrease of the methyl esterification abilities, whereas the *O*-methylation on to the hydroxyl groups was maintained throughout the culture duration. The localization of such activities in the flax endomembrane system was performed using isopycnic centrifugation. Enzymic marker tests allowed us to identify the different membrane types. Methyltransferase activities in the different enriched fractions appeared to be associated with the Golgi apparatus for the *O*-methylation, and with the plasma membrane, Golgi apparatus and endoplasmic reticulum compartments for the carboxymethyl esterification.

INTRODUCTION

The development of the primary cell wall during elongation in plants involves displacement and rearrangement of polysaccharides already present in the wall, as well as re-orientation of the syntheses of new material (Northcote, 1982). These changes occur by activation or inactivation of certain key enzymes (Northcote, 1986).

Pectins, important constituents of the primary cell wall in dicotyledonous families, consist of polysaccharide blocks, heavily branched and largely methyl esterified, alternating with unbranched blocks of galacturonic acids with various degrees of esterification (Jarvis, 1984). A high proportion of galacturonic acid in pectins confers a highly acidic character and promotes ionic interactions with calcium (Jarvis, 1984). Plant cells modulate this character by addition or removal of methyl esters from galacturonic acid residues (Maness *et al.*, 1990).

Kauss *et al.* (1969) showed that a microsomal fraction from mung beans contained an enzymic activity which transferred the methyl group from *S*-adenosyl-L-methionine (SAM) to substances already present in the particulate preparation. This methyltransferase activity was shown to occur at the macromolecular level and to be activated by EDTA. Methylations were also observed on to hemicelluloses rich in glucuronic acid residues. Kauss *et al.* (1967) and Kauss & Hassid (1967) with corn cobs and Baydoun *et al.* (1989) with pea microsomal fractions demonstrated that the transfer occurred from SAM to D-glucuronic acid in a preformed polysaccharide.

The biosynthesis of non-cellulosic polysaccharides is known to be localized in the endomembrane system (Northcote & Pickett-Heaps, 1966; Ray, 1977; Moore & Staehelin, 1988) and, more specifically, in the Golgi apparatus from which they are secreted. The new non-cellulosic molecules arrive at the plasmalemma via vesicular transport (Brummel *et al.*, 1990).

Little is known about the intracellular localization of the polysaccharide methyltransferase activities: do they act in the Golgi cisternae or during the vesicular transport to the plasma membrane? Rees (1977) proposed that pectins were synthesized in a completely methyl-esterified form and were de-esterified as required, according to their destination in the wall. Another possibility is the biosynthesis of more or less methyl-esterified pectins, according to elongation or differentiation signals received by cells. The problem of methylation/demethylation is still unclear.

The present work reports a study of methyltransferase activities in flax hypocotyls of *Linum usitatissimum* L. Because of the lack of information about the localization of the methyltransferases we studied these activities in the endomembrane system using isopycnic ultracentrifugation.

MATERIALS AND METHODS

Plant material

Seedlings of flax (*L. usitatissimum* L var. Ariane) were grown, in darkness, on moist paper soaked with milliQ water (Millipore), at 25 °C. Hypocotyls were excised after 4, 7, 9, 11 or 14 days of growth.

Microsomal preparation

Hypocotyl tissues (10–15 g fresh weight) were quickly chopped with scissors in a homogenization medium [50 mM-Tris/HCl, pH 7.5, 12% (w/w) sucrose, 1 mM-EGTA, 1 mM-dithioerythritol (DTE), 0.5% (w/v) BSA, 1.5% (w/v) polyvinylpyrrolidone (PVP), 0.1 mM-$MgCl_2$, and ground in a mortar with a pestle in a minimum amount of the same medium. Homogenization was achieved with a Tenbroeck glass Potter–Elvehjem homegenizer (6 ml for 1 g fresh weight) and the homogenate was strained through a nylon cloth (100 μM mesh). The filtrate was successfully centrifuged at 1000 *g* for 15 min and 10000 *g* for 15 min (rotor JA 18, Beckman J2-21), and finally ultracentrifuged at 180000 *g* for 45 min (rotor 70 Ti, Beckman L8-70). The pellets were resuspended in 2–4 ml of the homegenization medium. They represented the crude microsomal fraction (Baydoun & Northcote, 1980; Chanson *et al.*, 1984).

All these operations were carried out at 0–4 °C in a maximum of 2 h. This type of experiment has been run several times. As the results were similar, they were not all reported.

Subcellular fractionation

The crude microsomal fraction (about 25 mg of protein) was loaded on the top of a 36 ml linear sucrose gradient (15–48% w/w): 10 mM-Tris/HCl, pH 7.5, 1 mM-DTE and 15% or

Abbreviations used: SAM, *S*-adenosyl-L-methionine; DTE, dithioerythritol; PM, plasmalemma; ER, endoplasmic reticulum; β-GS I, β-glucan synthase I; β-GS II, β-glucan synthase II; L2, last washing medium.

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48 % (w/w) sucrose. The gradient was formed with a gradient generator (Büchler, Osi, France) in quick seal polyallomer tubes (Beckman). The tubes were centrifuged at 180000 *g* for 90 min at 4 °C (rotor 70 Ti, Beckman L8-70). The density gradient was separated from the bottom of the tube into 13 fractions of 3 ml volume using a microperspex peristaltic pump. Their absorbance was monitored at 280 nm (Bromma 2138 uvicord; Pharmacia LKB).

The fraction density was determined using a Atago Illuminator refractometer.

Each fraction was assayed for protein content according to the Bradford micro assay (Bio-Rad; Bradford, 1976).

Enzyme assays

Membrane-marker enzyme assays for plasma membrane (PM) [vanadate-sensitive ATPase, β-glucan synthase (β-GS II)], Golgi apparatus [latent IDPase, β-glucan synthase (β-GS I); tonoplast (nitrate-sensitive ATPase), endoplasmic reticulum (ER) (NADH cytochrome *c* reductase; EC 1.6.99.3) and mitochondria (cytochrome *c* oxidase; EC 1.9.3.1) were performed according to standard methods (Hodges & Leonard, 1972; Quail, 1979).

ATPases. ATPase assays were performed in 50 mM-Tris/Mes containing 3 mM-ATP, 3 mM-$MgSO_4$ and 0.15 mM-Na_2MoO_4. The test was carried out with 100 μl of each fraction and in the presence or absence of an activator (25 mM-K_2SO_4), or inhibitors (50 mM-$NaNO_3$ or 0.1 mM-$Na_2V_2O_2$). The required pH was 6.5 and 9 to identify respectively PM and tonoplast fractions.

IDPases. IDPase assays were performed in 50 mM-Tris/Mes, pH 7.5, containing 3 mM-IDP, 3 mM-$MgSO_4$, 0.15 mM-Na_2MoO_4 with and without 0.01 % (w/v) Triton X-100 for latent IDPase and native enzyme respectively.

Substrate (ATP or IDP) and enzyme were incubated for 30 min at 35 °C, then the reaction was stopped and released phosphate was complexed after addition of 2 ml of 1 % (w/v) sodium ascorbate in 0.5 M-H_2SO_4 and 2 ml of 1 % (w/v) ammonium heptamolybdate in 0.5 M-H_2SO_4 for 45 min at room temperature.

Phosphohydrolase activities, in each fraction, were characterized by absorbance at 820 nm (Shimadzu MPS 2000; Kyoto, Japan).

NADH cytochrome *c* reductase. NADH cytochrome *c* reductase was assayed by following the reduction of cytochrome *c* at 550 nm ($\epsilon_{550} = 18.5\ mM^{-1} \cdot cm^{-1}$). Phosphate buffer (0.1 M), pH 7.5, containing 5 mM-KCN, 0.05 mM-oxidized cytochrome *c*, 0.3 mM-NADH and 0.015 % (w/v) Triton X-100, constituted the incubation medium (1 ml). The reaction, with 1 μM-antimycin A, was started by the addition of 100 μl of fraction.

Cytochrome *c* oxidase. Cytochrome *c* oxidase was tested with the same reactives as above, but antimycin A, KCN and NADH were omitted. Cytochrome *c* was previously reduced by sodium dithionite. The decrease in absorbance at 550 nm, corresponding to cytochrome *c* oxidation, was recorded.

β-Glucan synthase assays. The two glucan synthases identified in higher-plant cells were tested at 25 °C with a method adapted from that described by Pierce & Hendrix (1979).

The β-GS I (of high affinity), associated with Golgi apparatus membrane, is activated by Mg^{2+} and synthesizes β1 → 4 glucans. Thus β-GS I was assayed with 1 μM-UDP-glucose spiked with 1.85 kBq of UDP-D-[U-^{14}C]glucose and 20 mM-$MgCl_2$ in a 80 mM-Tris/HCl buffer at pH 8 containing 1 mM-DTE, 5 mM-cellobiose and 0.6 μM-UDP-glucose.

The β-GS II (of low affinity), used as marker enzyme for the PM, synthesizes β1 → 3 glucans *in vitro* (Fèvre, 1983). When β-GS II activity was assayed, $MgCl_2$ was omitted and a higher level of substrate was provided: 1 mM-UDP-glucose with 9.25 kBq of UDP-D-[U-^{14}C]glucose.

Incubation, for 1 h (β-GS I) or 30 min (β-GS II), was started by mixing an aliquot of the fraction (250 μl) and the substrate buffer (250 μl), and the reaction was ended by boiling for 10 min. Glycans, with incorporated radioactive substrate, were precipitated by 70 % (v/v) ethanol with crude mitochondrial fraction as carrier, were washed twice with 70 % (v/v) ethanol containing 10 mM-$MgCl_2$ and collected on to glass fibre filters (GF/F, Whatman). Filters were dried, immersed in scintillation liquid (lipoluma, Lumac, The Netherlands) and radioactivity was measured in a SL Tricarb 2250 CA Packard scintillation counter.

Methyltransferase assay. An aliquot of crude micromosal fraction or of each fraction from the sucrose density gradient (500 μl) was incubated, at 25 °C, in 500 μl of 0.1 M-Tris/HCl, pH 6.8, containing 1 % (w/v) BSA, 12 % (w/v) sucrose and 10 μM-SAM with 18.5 kBq of *S*-adenosyl-[^{14}C]methionine. A pH of 6.8 was chosen to avoid chemical de-esterification which usually occurs above pH 8. Because of the low level of methylation shown in preliminary tests, incubation was performed for 16 h. Contamination was prevented by filtering all the solutions. The enzymes were inactivated by addition of ethanol up to 70 % (v/v) and assay mixtures were brought to 80 °C within 10 min. The reaction mixture was centrifuged (4000 *g*, 15 min; Hettisch Universal, Prolabo, Paris, France). Since SAM, carrying a positive charge, might be linked to polyanions by electrostatic bonds, we modified the procedure used by Kauss *et al.* (1967) as follows. We added 1 M-NaCl to the ethanol solution wash to break non-specific linkages. Then the pellet was washed twice with 70 % (v/v) ethanol containing 1 M-NaCl and centrifuged (4000 *g*, 15 min). The methylated polymers were distinguished by their selective solubility in 0.5 % (w/v) ammonium oxalate or in 6 % (w/v) NaOH. The first substances, usually thought to be pectins, were extracted three times from the pellets by 0.5 % (w/v) ammonium oxalate at 80 °C for 15 min and centrifugation (4000 *g*, 15 min). Oxalate supernatants were collected and their radioactivity measured. The last pellet was treated with 6 % (w/v) NaOH, the extract was expected to be hemicellulose-like polymers. This extraction was carried out at 80 °C for 15 min.

All the extracts were stored at 4 °C in the presence of sodium azide (0.1 %, w/v).

Standards for determination of background levels were prepared in each test from incubations of previously boiled aliquots. They were subjected to all the washing and extraction steps.

The radioactivity of the oxalate and NaOH extracts was measured by liquid scintillation counting and indicated the methyltransferase activity on to pectin and hemicellulose respectively. Supernatants from the washing steps were measured to check non-specific adsorption.

The methyltransferase activities were expressed in c.p.m. per total fraction. Because of the long incubation period, no test for linearity was done.

Size exclusion chromatography

Size exclusion chromatography of substances extracted either with oxalate or with NaOH was run in 1 M-NaCl on a column of Sephacryl S200 (Pharmacia; 600 mm × 25 mm, 100 ml/h). The polysaccharides were detected by measuring absorbance at 214 nm and by counting the radioactivity. The fractions eluted in the exclusion (V_o), elution (V_e) and inclusion (V_i) volumes were further separated on a column of Sephadex G25 (600 mm × 25 mm, 100 ml/h; Pharmacia) to remove NaCl. The oxalate fractions (1–3) and NaOH fractions (4–6) were treated with methanol, trimethylsilylated and then analysed by g.l.c. (Varian instrument capillary column DB 225, 90–180 °C at a rate of 2 °C/min, 180–230 °C at a rate of 5 °C/min) as previously described (Morvan *et al.*, 1991).

Chemicals

UDP-D-[U-^{14}C]glucose (specific radioactivity 10 GBq/mmol) and *S*-adenosyl-[^{14}C]methionine (specific radioactivity 1.8 GBq/mmol) were obtained from C.E.A. (France).

Non-radioactive sugar nucleotides, ATP (sodium salt), IDP (sodium salt), NADH and cytochrome *c* were obtained from Boehringer–Mannheim. Other chemicals were of analytical grade.

RESULTS

Evidence for a methyltransferase activity in a crude microsomal fraction of flax

As described in the Materials and methods section 1 M-NaCl was added to the ethanol solution washing to break non-specific linkages between SAM and anionic polysaccharides. Table 1 shows that radioactivity was removed during the different washing steps with 1 M-NaCl. Radioactivity decreased from the incubation medium to the last washing medium (L2) according to a diffusion film process. When these solutions were separated on a Sephadex G25 column (Pharmacia), radioactivity was found to be associated with a retained volume, eluted beyond V_i, which may correspond to non-incorporated SAM (Fig. 1*a*). The amount of radioactivity was larger in the first extraction by oxalate than in the blank and L2 solutions, and we considered that the radioactivity measured corresponded to the expression of methyltransferase activity. Futhermore, when separated on a Sephadex G25 column (Pharmacia), all the extracts showed radioactivity in volumes eluting between V_o and V_i corresponding to methylated poly- or oligo-saccharides (Fig. 1*b*).

Such a methyltransferase test showed the presence of an activity in a crude microsomal fraction from 4-day-old hypocotyls. Methylation seemed to be slightly more important on to oxalate substances (4066 c.p.m./g of fresh material) than on to NaOH extracts (2711 c.p.m./g of fresh material; Table 2). Studies performed on different days of growth suggested that a change in methylation occurred during the hypocotyl's growth phase (Table 2). Results showed, on the one hand, that activity in oxalate extracts rapidly decreased between the fourth and seventh days, being undetectable at day 9 (Table 2). On the other hand, methylation of alkali extracts decreased by a half at day 9 and then reached a plateau. At day 14, methylation only occurred on to the hemicellulose-like substances.

Methyl linkages and sugar composition

When separated on S200, the oxalate extracts (obtained in large amounts from another experiment) showed a large polydispersity (Fig. 2). The major part of the radioactivity was associated with the high-molecular-mass polymers. After saponification of these polymers (NaOH 1 M, 24 h, 4 °C) and chromatography on S200, all the radioactivity was found to be in V_i and was demonstrated, by distillation, to be methanol. Hence, oxalate polymers were methyl esterified. As shown in Table 3, these polymers were mainly polysaccharides and were composed of 15–50% uronic acids (with a majority of galacturonic acids). The ratio of galacturonic acid to rhamnose (larger than 1) demonstrated the presence of polygalacturonic blocks. Besides this the main neutral sugars were galactose and glucose.

When separated on S200 (Fig. 3), the NaOH polymers appeared homogeneous in size, the mean molecular mass being estimated to be 15000 Da from a calibration curve (Hourdet & Muller, 1991). The radioactivity profile followed quite well the u.v. one and was in agreement with the methylation of the polymers. Due to the alkali treatment, the methylation should be only *O*-methylation on to the hydroxyl groups. As shown in Table 3, these polysaccharides contained 7–16% uronic acids. The main sugars were galactose, glucose and mannose. The ratio of galactose to glucose decreased from the high- to low-molecular-mass polysaccharides.

Table 1. Radioactivity in the incubation medium (M), the ethanol 70% (v/v)/1 M-NaCl washing medium (L1 and L2) and the first oxalate extract (Ox1) of the microsomal fraction from 4-day-old hypocotyls

Radioactivity levels decreased during washing, from the incubation medium (M) to the two washes with 1 M-NaCl (L1 and L2). The first oxalate extract (Ox1) was much more radioactive than the last wash solution L2.

	Radioactivity (c.p.m.)			
	M	L1	L2	Ox1
Assay	86392	10531	1106	7131
Blank	68745	26365	4833	2253

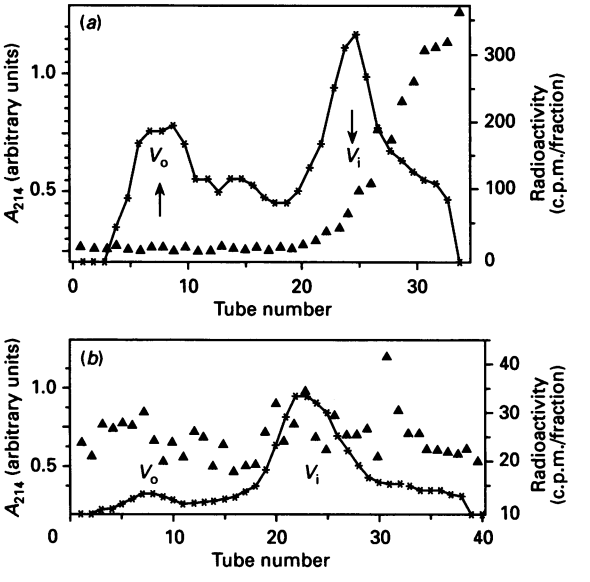

Fig. 1. Chromatograms of washing step solution (*a*) and one oxalate extract (*b*) and radioactivity associated with the eluted fractions

The exclusion and inclusion volumes V_o and V_i were determined with a solution of pectin and galacturonic acid. The compounds recovered in fractions after elution on Sephadex G25 column were followed by absorbance at 214 nm (★) and by counting the radioactivity (▲) present in each fraction.

Table 2. Methyltranferase activities during the etiolation of the flax hypocotyls

Methyltransferase activities were expressed in c.p.m. incorporated per g of fresh material.

Age (days)	Oxalate extracts (c.p.m./g fresh weight)	NaOH extracts (c.p.m./g fresh weight)
4	4066	2711
7	478	2026
9	0	1448
11	0	1123
14	0	1241

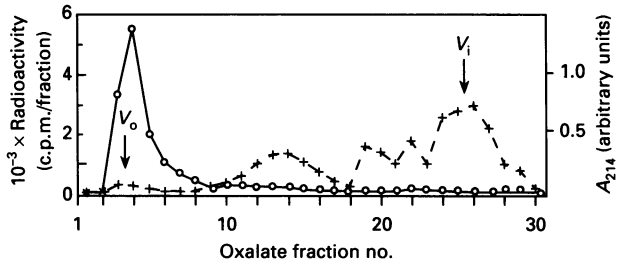

Fig. 2. Size exclusion chromatography on Sephacryl S200 of polymers extracted by oxalate

The exclusion and inclusion volumes V_o and V_i were determined with a solution of Blue Dextran and galacturonic acid. The eluted compounds were followed by absorbance at 214 nm (+) and by counting the radioactivity (○) and profiles of each are illustrated.

Table 3. Molar sugar composition (%) of the polymers extracted from the microsomes and fractionated on Sephacryl S-200

The fractions 1–6 have been defined in the Materials and methods section. The sugar composition was obtained after gas-phase chromatography as described in the Materials and methods section. The percentage of uronic acids was calculated relative to the total amount of sugars.

	Molar sugar composition					
	Oxalate extract			NaOH extract		
Sugars	1	2	3	4	5	6
Uronic Acid	17	17	40	7	11	16
GalA/GlcA	1.3	7.3	1.2	1.1	1.0	1.0
Neutral sugars (100%)						
Rha	1	3	3	1	2	1
Ara	5	13	6	10	9	1
Xyl	2	5	3	2	9	3
Man	4	11	9	16	16	2
Gal	35	35	35	55	30	12
Glc	53	33	44	16	34	81
GalA/Rha	12	6	12	4	3	8
Gal/Glc	0.7	1.1	0.8	3.4	0.9	0.15

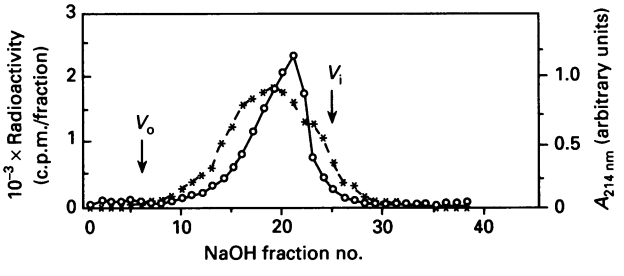

Fig. 3. Size exclusion chromatography on Sephacryl S200 of polymers extracted by NaOH.

The exclusion and inclusion volumes V_o and V_i were determined with a solution of Blue Dextran and galacturonic acid. The eluted compounds were followed by absorbance at 214 nm, (+) and by counting the radioactivity (○) and profiles of each are illustrated.

Distribution of marker activities

According to the observations of Naghashi & Hiraike (1982) and Chanson *et al.* (1984), a large part of the mitochondrial marker activity, cytochrome *c* oxidase, sedimented during the differential centrifugation. One half of the activity sedimented at

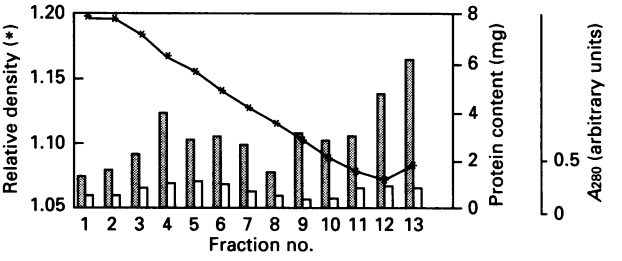

Fig. 4. Relative density and protein profiles in the fractionated gradient

Protein quantity profiles were prepared using absorbance at 280 nm (arbitrary units; open bars) and measurements from the micro-Bradford assay (μg; closed bars). The relative density of each gradient fraction was estimated using an Atago Illuminator refractometer.

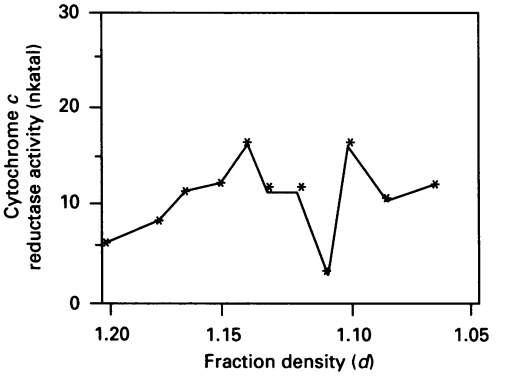

Fig. 5. NADH cytochrome *c* reductase activity: localization of the ER-enriched fractions

10000 *g*, and the other half being found in the 180000 *g* pellet. Furthermore, the 10000 *g* pellet showed the highest specific activity, being considerably enriched in mitochondrial material. Thus we did not consider this activity in the gradient fraction in further isopycnic centrifugation experiments.

When subjected to differential followed by isopycnic centrifugations, membranes of flax hypocotyl cells migrated along the continuous sucrose gradient. The density gradient was linear between fractions 2 and 12, corresponding to a variation in relative densities from 1.196 to 1.072 (Fig. 4). Since they were disturbed under our conditions of gradient fractionation, we did not analyse the fraction at each gradient extremity (fractions 1, 13 and 14). Protein profiles (Fig. 4) showed three peaks corresponding to three zones of *d*: 1.196–1.156, 1.142–1.128 and 1.103–1.072. According to literature data they corresponded respectively to the membrane families: (i) PM, (ii) Golgi apparatus and (iii) tonoplast, with ER spreading over the three zones (Quail, 1979; Baydoun & Northcote, 1980).

Distribution of enzyme activities. Endomembranes were identified with enzymic markers, NADH cytochrome *c* reductase activity identifies the ER. Half of this activity was shown to sediment in the 10000 *g* pellet. Similar observations have been made in other experiments on plant microsomes (Chanson *et al.*, 1984; Naghashi & Hiraike, 1982), in which ER sedimented at around 10000 *g*. However, an ER fraction could be found at relative densities between 1.10 and 1.17, as shown in Fig. 5 where two peaks of NADH cytochrome *c* reductase were detected at $d = 1.10–1.09$ and $d = 1.17–1.14$. They were respectively classed as smooth ER and rough ER.

ATPase activity at pH 6.5, which has been generally used as a PM marker, showed two peaks of high activity around $d = 1.16$

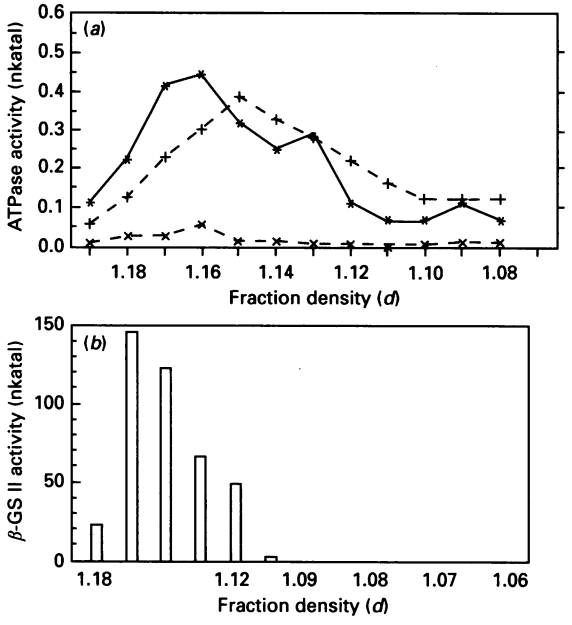

Fig. 6. Phosphohydrolase and β-GS II activities in PM-enriched fractions

ATPase activity (*a*) was measured at pH 6.5 (★), with vanadate (+) and with potassium (×), and expressed in nkatal. β-GS II activity (*b*) expressed in nkatal, was also measured.

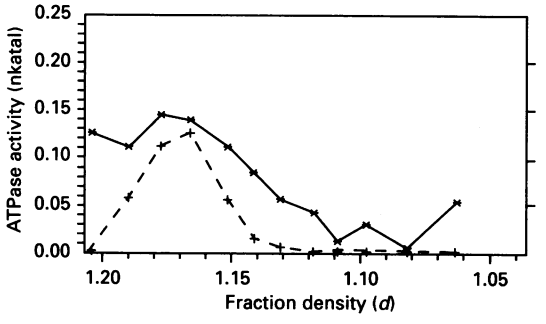

Fig. 7. Phosphohydrolase activities at pH 9 in tonoplast-enriched fractions

ATPase activity at pH 9 with (+) or without nitrate (★) expressed in nkatal.

Table 4. Distribution of the incorporated radioactivity in the gradient fractions

The incubation is expressed as a percentage of the total radioactivity (%RA) selectively extracted either in oxalate (12804 c.p.m.) or in NaOH (1296 c.p.m.). Four-day-old hypocotyls were used since they expressed the greatest activity. The micromosal fraction (10 ml), obtained from 21 g of fresh material, was layered on to two gradients (20 mg of protein each). The fractions (3 ml), except those from either end of the gradient, from only one gradient were tested for methyltransferase activity. Abbreviations: SER, smooth endoplasmic reticulum; GA, Golgi apparatus.

Relative density	Extracts	Radioactivity level (c.p.m./fraction)	% RA	Membrane
1.18–1.15	Oxalate	3204	25	PM
1.14–1.12	Oxalate	6750	53	GA
1.10–1.07	Oxalate	2850	22	SER
1.18–1.15	NaOH	180	14	PM
1.14–1.12	NaOH	936	72	GA
1.10–1.07	NaOH	180	14	SER

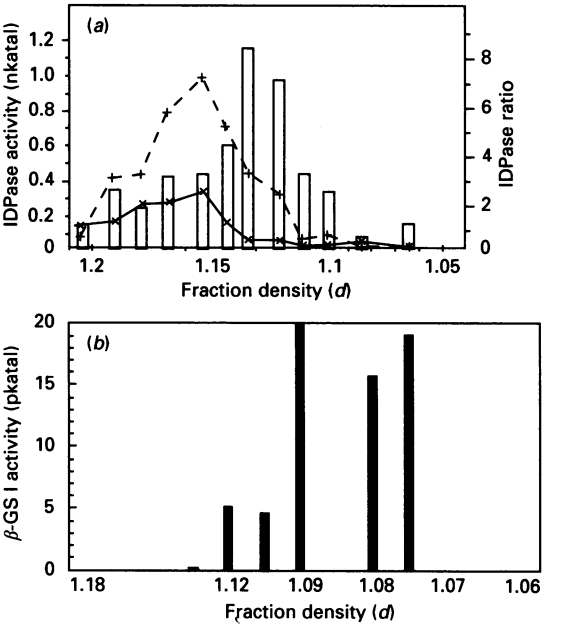

Fig. 8. IDPase (*a*) and β-GS I (*b*) activities

IDPase activities (nkatal) were measured at pH 7.5 in native (★) and latent (+) states. The IDPase ratio (open bars) indicating latent/native IDPase activity values was represented as a histogram. β-GS I activity was expressed in pkatal.

and $d = 1.13$ (Fig. 6*a*). Vanadate inhibition occurred all along the gradient. A small K^+ stimulation of ATPase activity was only observed between $d = 1.15$ and 1.14.

β-GS II activity, a more specific PM marker, reached a maximum at $d = 1.17$ and was present from 1.18 to 1.15 (Fig. 6*b*), reducing the relative density range found for PM-enriched fractions with the ATPase marker. Therefore PM for flax hypocotyls was spread between $d = 1.18$ and 1.15.

Nitrate-sensitive ATPase at pH 9 was observed in the lightest fractions of the gradient around $d = 1.10$ (Fig. 7), and was considered, according to the literature, to correspond to the tonoplast membranes. The high ATPase activity measured at pH 9 and recovered at relative densities from 1.19 to 1.15 was attributed to ATPase activity of PM and/or to contaminating mitochondria.

IDPase marker activities, corresponding to the difference between latent- and native-IDPase activities, should reveal the presence of the Golgi membranes. A large difference between these two activities was detected in the relative density range 1.18–1.13 (Fig. 8*a*). These results suggested a considerable overlap of Golgi membranes with PM in the heaviest fractions. However, when the ratio between latent and native IDPases was calculated, the highest values were found between relative densities of 1.15 and 1.10 (Fig. 8*a*, histogram). Therefore Golgi membranes were thought to be localized in the gradient between $d = 1.15$ and 1.10. Besides, the β-GSI I activities, another specific marker for the Golgi membranes appeared to be associated with membranes in the relative density range 1.12–1.07 (Fig. 8*b*), i.e. membranes of relative lower density than those characterized by IDPase.

Distribution of methyltransferase activity in membrane fractions

To determine the subcellular compartmentation of methyltransferase activities, the microsomal pellet (180000 *g*) was purified on linear sucrose gradients and three main fractions were analysed, either for methyl esterification activity (oxalate supernatants) or for *O*-methylation (NaOH supernatants). The

first fraction ($d = 1.18–1.15$) was recognized above as a PM-enriched fraction. The second one ($d = 1.14–1.12$), corresponded to the fraction identified as Golgi enriched. The third zone ($d = 1.10–1.07$), associated with lighter fractions, might be enriched in ER membranes as reported elsewhere (Baydoun & Northcote, 1980) although they presented a large β-GS I activity.

The oxalate extracted showed high radioactivity in the three relative density ranges (Table 4). The substantial quantity of methyl ester groups incorporated on to uronic acids was mainly associated with the Golgi membranes and accounted for 53% of total incorporated radioactivity. Lower activities were recovered from the lowest (22%) and the highest (25%) density zones. These results suggested either a contamination with Golgi membranes throughout the gradient densities, or/and that methyl esterification of the oxalate polysaccharides could occur throughout the endomembrane system from ER to PM.

Maximum incorporation of *O*-methyl on NaOH polysaccharides (72%) appeared to occur in density interval ($d = 1.14–1.12$), and were characterized as Golgi-enriched fractions. The amount of *O*-methylation was less important than the ester methylation. It might be due to the pH at which the assays were performed (pH 6.8).

DISCUSSION

According to Kauss & Hassid (1967), the treatment of microsomes with hot oxalate would extract methyl-esterified pectins whereas NaOH would solubilize *O*-methylated hemicelluloses. This sequence is generally used to extract polysaccharides from cell walls as a function of their ionic or covalent linkages. Our results indicated, in both fractions, the presence of galacturonic acid (specific to pectins) and glucuronic acid (usually found in hemicellulosic polysaccharides) and showed that the extraction sequence was not highly specific to the polysaccharide nature. However, as suggested by Kauss & Hassid (1967), two kinds of methylation occurred in the endomembrane system of flax cells: (i) an esterification on to the oxalate polysaccharides and (ii) an *O*-methylation on to the NaOH polymers. The methyl esterification was thought to be mainly on to galacturonic acids, but we do not know yet if it is on to some rhamnogalacturonan I or/and on to polygalacturonan blocks. Both kinds of polysaccharides have been found in the cell walls of 3–4-day-old hypocotyls (Moran *et al.*, 1991). Moreover, maximum activity of the methyl esterification was found in early days of hypocotyl development. After day 4, a decrease of the pectic content of the cell wall was reported during elongation of the hypocotyls in darkness (Morvan *et al.*, 1992), and we noted that methyl esterification decreased too. Our results also demonstrated the capacity for methyl esterification in the Golgi compartment. Some radioactivity was also found in fractions enriched in PM- and ER-derived vesicles. Were these data a result of a major contamination with Golgi membranes? As Brummel *et al.* (1990) have suggested the contamination of ER could originate from some Golgi vesicles, since in clover root tips and cultured carrot cells pectic polysaccharides appeared to be formed in the cis and medial Golgi. In the same way, β-GS I activity was found in flax associated with light membrane fractions. Fractionation at higher resolution would answer this question. A second hypothesis would be that some polysaccharides were synthesized in ER-derived vesicles. Bowles & Northcote (1972, 1974) discussed this fact, arguing that in the ER a lipid molecule could play a role in polysaccharide biosynthesis as a first acceptor for a sugar monomer and that polymerization by glycosyl synthases could proceed from this molecule. Otherwise, our results may be attributed to glycosylation occurring in the ER compartment during post-translational modifications of the proteins.

O-methylation of polysaccharides was maintained throughout the 14 days of culture in darkness. These results were in agreement with the corresponding orientation of wall metabolism (Morvan *et al.*, 1992), since in darkness the wall polysaccharide composition changed from a mainly pectic one to a mainly hemicellulosic one. In addition, results showed that *O*-methylation of hemicellulose-like polysaccharides occurred mainly in the Golgi apparatus. Similar results were obtained with xyloglucans by Griffing *et al.* (1986). Brummel *et al.* (1990) suggested that xyloglucans were synthesized in the trans-Golgi compartment.

To better understand how the methyltransferase activities distributed Golgi apparatus through ER and their vesicles were implicated in wall biosynthesis requires the recovery of isolated vesicles with a fine fractionation procedure and assays for polysaccharide-synthesizing enzymes. Pulse–chase experiments with precursors of wall polysaccharides, such as [^{14}C]SAM, from ER or Golgi apparatus through transport vesicles, may lead to further explanations of the way these non-cellulosic polysaccharides are methylated while travelling to the cell wall.

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