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Arabinan synthase and xylan synthase activities of *Phaseolus vulgaris*

Subcellular localization and possible mechanism of action

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Membrane fractions from bean hypocotyl or suspension cultures incorporated arabinose from UDP-β-L-arabinose into arabinan and xylose from UDP-α-D-xylose *in vitro*; the level of each activity was dependent on the state of differentiation of the cells. These activities may be due to single transglycosylases, since no lipid or proteinaceous intermediate acceptors were found in either case. Subcellular fractionation studies showed that enzyme activity *in vitro* was localized in both Golgi-derived membranes and endoplasmic reticulum in similar amounts. However, incorporation into the polymers *in vivo* in suspension culture cells incubated with [1-^{3}H]arabinose was considerably greater in the Golgi-derived membranes. Thus, although these enzymes may be translated and inserted at the level of the endoplasmic reticulum, their activities are under other levels of control, so that most of the activity *in vivo* is confined to the Golgi apparatus. Initiation of glycosylation in the endoplasmic activity may, however, occur.

L-Arabinose and D-xylose are closely related epimers that are interconverted in the cell by 4-epimerization of the respective UDP sugar. Arabinan, probably mostly α-[1→3]- and α-[1→5]-linked, is a constituent of neutral pectin, whereas β-[1→4]-xylan forms a main chain for a group of hemicelluloses. The synthesis of these polymers is related to the state of differentiation of the tissue (Northcote, 1972). Preliminary studies using membrane fractions capable of synthesizing polysaccharide from either UDP-sugar show that the peaks of enzyme activity appear at differing times during development (Bolwell & Northcote, 1981; Dalessandro & Northcote, 1981). Therefore, study of the changes in the levels of these enzymes should shed light on the control mechanisms involved in the cessation of pectin synthesis and the increased hemicellulose synthesis during secondary thickening associated with differentiation.

However, any study of the regulation of the synthesis of polysaccharide must require an investigation of the transfer mechanism as additional components to the transglycosylase may be limiting factors during development. Lipid carriers have been shown to be involved in plant glycoprotein synthesis (Bailey *et al.*, 1979, 1980; Staneloni *et al.*, 1980*a*,*b*, 1981; Lehle, 1981; Hori *et al.*, 1982), but these are probably not always necessary for the synthesis of polysaccharides (Northcote, 1979). An involvement for such intermediates in synthesis of cell-wall polysaccharides of higher plants has not yet been demonstrated.

The specific subcellular site within the endomembrane system, i.e. the Golgi apparatus or endoplasmic reticulum, of such glycosylations is also not certain.

In the present study we have further investigated the arabinan synthase and xylan synthase described previously (Bolwell & Northcote, 1981) and have attempted to characterize the transfer mechanism. Furthermore, we have studied the involvement of the membrane system in the export of the respective polymers both by incorporation *in vitro* with the respective UDP-sugar or *in vivo* with L-[1-^{3}H]arabinose. Transglycosylases that are required for polymer synthesis are localized in both endoplasmic reticulum and Golgi apparatus but may be subject to controls that give rise to increased glycosylation in the latter *in vivo*.

Materials and methods

Cultivation of seeds of *Phaseolus vulgaris* and isolation of callus tissue have been previously described (Bolwell & Northcote, 1981). Suspension cultures were derived from callus tissue by vigorously shaking at 26°C in liquid B5 medium of Gamborg *et al.* (1968) supplemented with 2,4-dichlorophenoxyacetic acid (2.0mg/litre), 20% (v/v) deproteinized coconut milk and 2% sucrose (CMD

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medium). Cultures were routinely maintained in this medium. Cultures were induced to differentiate by subculture into liquid induction medium [IM; B5 medium supplemented with 1-naphthylacetic acid (1.0mg/litre) kinetin (0.5mg/litre) and 3% sucrose].

UDP-α-D-[U-^{14}C]xylose (5.7GBq·mmol^{-1}) was purchased from New England Nuclear; UDP-xylose, UDP-glucose and UDP-galacturonic acid were from Sigma. UDP-β-L-[1-^{3}H]arabinopyranose and non-radioactive UDP-β-L-arabinopyranose were synthesized as described previously (Bolwell & Northcote, 1981).

Preparation of membrane fractions

Total membrane preparations were prepared as described by Bolwell & Northcote (1981).

Subcellular fractions were obtained from homogenates of tissue in 50mM-potassium phosphate buffer, pH7.2, containing 1mM-dithiothreitol and 0.4M-sucrose (1g of tissue/ml of buffer) at 4°C either in a pestle and mortar or by using the Polytron PT 20 ST homogenizer (Kinematic, GMbH, Lucerne, Switzerland) at 4000 rev./min for 2min. In experiments designed to maintain the attachment of ribosomes to membranes, 1mM-$MgCl_2$ was added to the homogenization medium and replaced by 1mM-EDTA when detachment was required. The slurry was filtered through muslin and centrifuged at 1000*g* for 15min. The supernatant was transferred to cellulose nitrate tubes, a 60% (w/w) sucrose cushion introduced at the bottom of the tubes and the tubes were centrifuged for 60min at 100000*g* in a Beckman ultracentrifuge at 4°C with an SW 27 rotor. The interface was harvested and diluted with homogenization medium and layered on to a discontinuous step gradient prepared by introducing successively, at the bottom of the tube, layers of 25%, 34%, 39% and 45% (w/w) sucrose containing all the components of the homogenization medium. After centrifugation at 100000*g* for 90min at 4°C, the material at each interface was collected, diluted with homogenization medium containing 10mM-$MgCl_2$ and centrifuged at 100000*g* for 30min at 4°C. Each pellet was re-suspended in homogenization medium containing 10mM-$MgCl_2$.

The probable localizations of the synthases were further investigated by fractionation of microsomes by a modification of the methods of Bloemendal *et al.* (1974) and Bevan & Northcote (1981), the major modification being the use of phosphate buffer and the absence of KCl to preserve enzyme activity. Tissue was homogenized in 50mM-phosphate buffer, pH7.6, containing 1mM-dithiothreitol, 0.4M-sucrose and 10mM-$MgCl_2$ (1g of tissue/ml). The slurry was filtered through muslin and centrifuged at 1000*g* for 25min. The supernatant was then centrifuged at 15000*g* for 10min to sediment most of the larger organelles. The supernatant was transferred in 3ml portions to 5ml centrifuge tubes and 1ml each of 34% (w/w) sucrose and 55% (w/w) sucrose containing all the components of the homogenization medium was introduced successively at the bottom of the tubes. The discontinuous gradients were then centrifuged at 250000*g* for 4h in a Beckman SW50.1 rotor. On completion each fraction was thoroughly washed after harvesting by centrifugation in homogenization medium by pelleting at 100000*g* for 30min. In these experiments designed to isolate relatively pure smooth and rough membranes and free polysomes the sucrose was treated with diethyl pyrocarbonate as described by Martin & Northcote (1981) and all solutions were sterile to minimize the degradation of RNA. Each fraction was resuspended in homogenization buffer, pH7.2, containing 10mM-$MgCl_2$ before enzyme assay.

Characterization of membrane fractions

Protein was determined by a modification of the Coomassie Blue assay (Read & Northcote, 1981). Antimycin-insensitive NADH:cytochrome *c* reductase was assayed by the method of Shore & Maclachlan (1975) modified by Lord *et al.* (1973), latent IDPase was assayed after storing the membrane at 4°C for 48h (Shore & Maclachlan, 1975) and succinate dehydrogenase was estimated by the method of King (1967). Chlorophyll content was estimated by the method of Bruinsma (1961), RNA at 260nm after phenol/chloroform extraction and phospholipid by the method of Ames (1966) after lipid extraction.

Membranes were prepared for thin sectioning, stained with uranyl acetate and alkaline lead citrate and then examined in an AEI EM6B electron microscope at 60kV (Brett & Northcote, 1975).

Polysaccharide synthase activity

Standard assays for the arabinan synthase and xylan synthase activities and the fractionation of the products of the synthase activities into lipid-soluble material and trichloroacetic acid-insoluble products have been previously described (Bolwell & Northcote, 1981). Material insoluble in 10% trichloroacetic acid was prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by resuspension in acetone followed by centrifugation. All the radioactivity was recovered in the pellet, which was then boiled for 20min in 0.1M-Tris/HCl buffer, pH6.8, containing 100mM-dithiothreitol, 10% glycerol, 2% sodium dodecyl sulphate and 0.006% Bromophenol Blue. Samples were then subjected to electrophoresis on 10% slab gels using the system of Laemmli (1970). Radioactive tracks were cut into 10mm × 2mm slices and solubilized by incubating with 100μl of H_2O_2 (100-volume) at 60°C for 24h before counting for radioactivity. Alternatively radioactivity was visually detected by fluorography by the method of Laskey & Mills (1975).

Characterization of the polysaccharide by total acid, partial acid and enzymic hydrolysis has been described (Bolwell & Northcote, 1981). Periodate oxidation was performed by a modification of the procedure of Hay *et al.* (1965) described by Elbein (1969). Radioactive xylan, together with 0.5mg of walnut xylan, was taken up in 1ml of 0.05M-sodium metaperiodate and allowed to react in the dark for 6–12 days. At the end of the incubation period excess periodate was removed by the addition of 100μl of ethylene glycol. Samples were reduced by the addition of two portions of solid $NaBH_4$ at 30min intervals, 72% (w/w) H_2SO_4 was added to a final concentration of 3% and the samples were hydrolysed at 103kPa at 120°C for 1h and neutralized with 15% (v/v) methyl-di-n-octylamine in chloroform (Harris & Northcote, 1970). The borate was removed by repeated addition and evaporation of methanol under reduced pressure at 30°C. Samples were chromatographed on Whatman no. 1 paper in ethyl acetate/pyridine/water (8:2:1, by vol.). Xylose, erythritol and glycerol markers were detected by the alkaline silver nitrate method (Trevelyan *et al.*, 1950) and the identity of the radioactive products established by comparison with markers and the products of walnut xylan oxidation. Radioactivity was measured in 3cm × 1cm strips (Harris & Northcote, 1970) by liquid scintillation counting.

Incorporation of L-[*1*-^{3}*H*]*arabinose in suspension cultures*

Suspension cultures of bean show a peak of arabinan synthase activity approx. 48h after subculture into CMD medium and of xylan synthase activity approx. 90h after subculture in IM (Bolwell & Northcote, 1983). Cells 48h (CMD medium) or 90h (IM) after subculture were incubated with 50μCi of L-[1-^{3}H]arabinose in a total volume of 50ml for 1h. At the end of this period they were harvested by filtration and washed with media. The cells were weighed and then ground with a little coarse sand in a mortar and pestle in 50mM-phosphate buffer, pH7.2, containing 1mM-dithiothreitol, 1mM-$MgCl_2$ and 0.4M-sucrose (1g of tissue/ml). The slurry was filtered through four layers of cheesecloth and centrifuged at 1000***g*** for 15min. The pellet constituted the cell-wall fraction. The supernatant was layered on to a cushion of 60% (w/w) sucrose and centrifuged at 100000***g*** for 1h. The membranes at the interface were then fractioned on the discontinuous step gradient at 25%, 34%, 39% and 45% (w/w) sucrose. Each fraction was thoroughly washed after harvesting by centrifugation in homogenization medium by pelleting at 100000***g*** for 30min. Each pellet was taken up in 50μl of homogenization medium.

Alternatively, microsomes were fractionated on a discontinuous gradient consisting of 34% (w/w) and 55% (w/w) sucrose at 250000***g*** for 4h. In these experiments the buffer was pH7.6 with 10mM-$MgCl_2$. After the low-speed centrifugation the larger organelles were pelleted at 15000***g*** for 10min. This supernatant was subjected to discontinuous gradient centrifugation. Each fraction was thoroughly washed by pelleting in homogenization medium at 100000***g*** for 30min.

Analysis of cell wall

The cell-wall fraction, which contained some other cell debris, was successively washed with 10ml of chloroform/methanol (3:2, v/v) and twice with 2ml each of cold water, hot water, 3% (w/v) NaOH and 17% (w/v) NaOH. The water-soluble fractions were combined and the amount of polymer estimated by precipitation in 85% ethanol and washing the pellet three times with 85% ethanol. The alkali-soluble fractions were treated similarly, but washed with 70% ethanol. The precipitates and the residue were each resuspended in 1ml of distilled water for further determinations. Incorporation into lipid was estimated in the chloroform/methanol-soluble fraction after washing by the method of Folch *et al.* (1957) until the aqueous phase was free of radioactivity. The organic phase was evaporated to dryness under reduced pressure before chromatography or scintillation counting.

Analysis of membrane fractions

Each membrane pellet resuspended in 50μl of homogenization medium was supplemented with 0.5mg of bean callus neutral pectin and 0.5mg of walnut xylan before addition of 1ml of chloroform/methanol (3:2, v/v) for 16h at 4°C. After centrifugation the organic phase was washed by the method of Folch *et al.* (1957) and evaporated to dryness under reduced pressure before chromatography or scintillation counting. The precipitate was then extracted with 500μl of distilled water, which dissolved all the arabinan. The polymer was then recovered by precipitation in 85% ethanol and washed twice in this solvent. The membrane fraction was further extracted with 500μl of chloroform/methanol/water (10:10:3, by vol.) for 30min and then with 500μl of hot 10% trichloroacetic acid, which dissolved the xylan. Polymer was recovered from the 10% trichloroacetic acid-soluble fraction by precipitation in 85% ethanol and washed three times with this solvent. The polysaccharide pellets and residue were resuspended in 250μl of distilled water for liquid-scintillation counting or hydrolysis or dialysis.

Characterization of products

Samples of cell-wall and membrane pellet polysaccharide were hydrolysed in 3% H_2SO_4 at 120°C

and 102kPa for 1h. Hydrolysates were neutralized with methyl-di-n-octylamine (Harris & Northcote, 1970) and evaporated to dryness under reduced pressure. The samples redissolved in water were chromatographed on Whatman no. 1 paper in ethyl acetate/pyridine/water (8:2:1, by vol.). Radioactivity was determined in 3cm × 1cm strips by scintillation counting (Harris & Northcote, 1970).

Lipids soluble in chloroform/methanol (3:2, v/v) were characterized by ascending t.l.c. in chloroform/methanol/water (65:25:4, by vol.) using prewashed Polygram SILG silica-gel plates. Lipid that was soluble in chloroform/methanol/water (10:10:3, by vol.) was applied to strips of Whatman no. 1 paper and eluted with water. Material left at the origin was re-extracted with chloroform/methanol/water (10:10:3, by vol.) and applied together with Thymol Blue marker to a column (5cm × 0.6cm) of DEAE-cellulose prepared and equilibrated as described by Brett (1980). The column was washed with 10ml of chloroform/methanol/water (10:10:3, by vol.), before elution with a linear gradient of 15ml of 0–0.2M-ammonium formate in chloroform/methanol/water (10:10:3, by vol.). Fractions (0.5ml) were collected.

Results

Characterization of products of incorporation of UDP-sugars in vitro by membrane preparations from hypocotyls and suspension cultures

Membranes showing maximum arabinan synthase activity were prepared from 6-day-old hypocotyls or cells 48h after subculture in CMD medium (primary-wall formation); those showing maximum xylan synthase were prepared from 9–10-day-old hypocotyls or cells 96h after subculture in IM (secondary-wall formation) (Bolwell & Northcote, 1981, 1983).

Incorporation of UDP-arabinose or UDP-xylose was not enhanced by the addition of a primer polysaccharide (neutral pectin from bean callus or walnut xylan). Also, neither UDP-galacturonic acid in the presence of UDP-arabinose nor UDP-glucose in the presence of UDP-xylose over a range of concentrations (200μM–2mM) increased incorporation. The xylan synthase is isolated from tissue synthesizing secondary-wall xylan (Bolwell & Northcote, 1981) and not the xyloglucan of the primary wall (Ray, 1980; Hayashi & Matsuda, 1981). Solubilization in 0.1% Triton inhibited activity for arabinan synthase by 80% and xylan synthase by 40%.

The polysaccharide products have been previously partially characterized (Bolwell & Northcote, 1981). The predominant linkage of the xylan was shown then by enzymic hydrolysis to be probably β-[1→4]. That the linkage for the xylan synthesized by membranes from hypocotyls is mainly [1→4] has been confirmed by periodate oxidation (Table 1), which produced an increasing proportion of radioactive glycerol to xylose with increasing time of oxidation, reaching 82% in glycerol after 12 days.

Table 1. *Periodate oxidation of the synthesized [^{14}C]xylan from UDP-[U-^{14}C]xylose incubated with membranes from hypocotyl tissue*

The radioactive samples (approx. 1500c.p.m.) and walnut xylan were each oxidized with sodium metaperiodate for 6 or 12 days, reduced with $NaBH_4$ and hydrolysed with 3% (w/v) H_2SO_4. Samples of walnut xylan after oxidation, reduction and hydrolysis were co-chromatographed with standards of xylose, erythritol and glycerol as controls, at the same time as the radioactive samples. The c.p.m. were corrected for the number of C atoms in the product. Values in parentheses are percentages of total radioactivity recovered.

	Periodate oxidation of synthesized [^{14}C]xylan	
Products	Corrected c.p.m. after 6 days	Corrected c.p.m. after 12 days
Glycerol	1575 (78)	1748 (82)
Erythritol	33 (2)	57 (2)
Xylose	403 (20)	332 (16)

Other products were apparently formed in addition to polysaccharide during the incubations (Table 2); incorporation into these fractions was much less than that found in some other glycosylating plant systems (Green & Northcote, 1979*a*; Mellor & Lord, 1979; Roberts *et al.*, 1980; Barr & Nordin, 1980; Lehle, 1981). Conventional fractionation by t.l.c., ion-exchange chromatography on DEAE-cellulose and investigations by hydrolysis with phosphatase/phosphodiesterase (Bolwell & Northcote, 1981) did not indicate any significant incorporation into material that behaved as a polyprenyl phosphate monosaccharide or oligosaccharide in the material formed either with UDP-xylose or with UDP-arabinose. Furthermore, the presence of tunicamycin (100μg/ml) or bacitracin (2mM), which have been shown to inhibit glycosylations in plants (Ericson *et al.*, 1977, 1978), or the inclusion of dolichyl monophosphate (added as described by Green & Northcote, 1979*a*) in the standard assays failed to affect incorporation into either polymer.

Very little incorporation into material insoluble in trichloroacetic acid occurred. The majority of this material (50–65%) did not enter the gel on electrophoresis and of the material that did 70% had an M_r of greater than 100000. The radioactive insoluble

Table 2. *Distribution of radioactivity from enzyme incubation into aqueous and lipid solvent fractions*
Membranes isolated from hypocotyl tissue were incubated for 10min with UDP-[U-^{14}C]xylose or UDP-[1-^{3}H]-arabinose and analysed. The source of the membranes was 6-day-old hypocotyls in the case of arabinan synthase and 9-day-old hypocotyls for xylan synthase. Mean values ± S.D. for six incorporations are shown in each case.

	Radioactivity incorporated			
	Arabinan synthase		Xylan synthase	
Fractions	(c.p.m.)	(% of mean total incorporation)	(c.p.m.)	(% of mean total incorporation)
Material soluble in chloroform/methanol (3:2, v/v)	288 ± 118	0.5	115 ± 49	0.9
Pooled aqueous washings	838 608 ± 23 978	—	67 806 ± 1134	—
Material soluble in chloroform/methanol/water (10:10:3, by vol.)	22 170 ± 6387	40.8	580 ± 265	4.6
Material insoluble in 10% trichloroacetic acid	4385 ± 1577	8.1	651 ± 416	5.2
Material soluble in 10% trichloroacetic acid	27 457 ± 8589	50.6	11 168 ± 4698	89.2

material was not rendered soluble by β-elimination at pH 9 (Green & Northcote, 1978).

It seemed therefore that lipid and protein carriers were not involved in the synthesis of arabinan or of xylan.

Characterization of membranes derived from hypocotyl or suspension cultures of bean

Distribution of marker activities in membrane fractions. The membrane fractionation procedure was adapted from Green & Northcote (1979*b*). Membranes were prepared in the presence of Mg^{2+} to maintain attachment of ribosomes to endoplasmic reticulum or in the presence of EDTA to affect their removal from the membrane.

The membranes were separated into five fractions according to their positions on a discontinuous sucrose gradient; fraction 1, 12–25%; fraction 2, 25–34%; fraction 3, 34–39%; fraction 4, 39–45%; fraction 5, >45% sucrose. The results of the assays of the marker enzymes are summarized in Table 3.

Antimycin-insensitive NADH:cytochrome *c* reductase has been used as a marker for the endoplasmic reticulum in plants (Lord *et al.*, 1973; Bowles & Kauss, 1976; Nagahashi & Beevers, 1978). In the presence of EDTA the highest activity of this enzyme occurred at the top of the gradient. In the presence of Mg^{2+} the peak of the activity shifted to the bottom of the gradient.

Succinate dehydrogenase, localized in mitochondrial membranes, was found mainly in fractions 3 and 4 and there was no activity at the top of the gradient (Table 3). Latent IDPase has been used as a marker for plant Golgi apparatus (Ray *et al.*, 1969, Morré & Buckhout, 1979). The highest latent IDPase activity in the membrane fractionation of the bean tissue was associated with fraction 2 in the presence or absence of Mg^{2+}. In hypocotyl preparations chlorophyll was associated with fractions 3 and 4.

The fractionation procedure produced a partial separation of Golgi apparatus (fraction 2) from mitochondrial membranes (fractions 3 and 4), chloroplast membranes (fractions 3 and 4) and, in the presence of Mg^{2+}, endoplasmic reticulum (fractions 3–5). Tonoplast, chloroplast envelope and plasmalemma were not tested for, but, in the presence of EDTA, they might be expected to be present in fraction 1, fraction 1 and 2 and fraction 3 respectively (Leigh *et al.*, 1979; Douce & Joyard, 1979; Baydoun & Northcote, 1980).

Electron microscopy of the membrane fractions. Since the membranes were not prepared in the presence of glutaraldehyde, the mode of extraction used caused considerable damage to the organelles. Nevertheless, the presence of mitochondria in fraction 4 and of chloroplast lamellae and mitochondria in fraction 3 was clearly shown. These fractions also contained vesicles of various sizes. Fractions 1 and 2 were made up of small and large vesicles with smooth membranes. Fraction 5 contained mainly ribosomes with membrane vesicles and occasionally large chloroplast fragments, when fractionated in the presence of Mg^{2+}. This was also true for fractions in the presence of EDTA, although the recovery of ribosomes in this case was very poor.

Distribution of synthase activities

Table 4 shows the distribution of arabinan synthase and xylan synthase activities in membrane fractions separated on sucrose gradients and derived from intact hypocotyls. The distribution of synthases derived from suspension culture cells show similar distributions as do the characteristic marker enzymes.

Membranes fractionated in the presence of EDTA

Table 3. *Distribution of marker enzymes in membrane fractions prepared in the presence of either EDTA or Mg^{2+}*

Tissue was homogenized at 4°C either in the presence of EDTA to effect the removal of ribosomes from endoplasmic reticulum or in the presence of Mg^{2+} to maintain their attachment to the endoplasmic reticulum. The distribution of enzyme activities in fractions prepared from 6-day-old hypocotyls is shown; similar distributions were found for membranes from older hypocotyls and tissue cultures. Recoveries of enzyme activity were 75–85%. The values given are the relative specific activity (RSA), calculated as the ratio of the specific activity in the subcellular fraction to that in the homogenate after centrifugation at 1000 *g* for 15 min. The values also show the percentage in each membrane fraction of the total activity recovered on the gradient (%TA).

	NADH : cytochrome *c* reductase				Succinate dehydrogenase				IDPase				Chlorophyll			
	EDTA		Mg^{2+}		EDTA		Mg^{2+}		EDTA		Mg^{2+}		EDTA		Mg^{2+}	
Fraction	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)	(RSA)	(%TA)
1 (12%/25% interface)	6.71	17	1.57	3	0	0	0	0	1.60	12	0.78	9	0	0	0	0
2 (25%/34% interface)	6.86	57	2.33	13	0	0	0	0	4.32	61	4.48	59	0	0	0	0
3 (33%/39% interface)	0.59	11	1.22	18	6.33	64	5.75	52	0.68	19	1.38	17	12.6	81	14.22	76
4 (39%/45% interface)	1.80	6	2.51	23	16.33	27	6.38	39	0.29	2	0.75	9	7.48	8	3.00	19
5 (45% pellet)	1.82	6	15.61	42	5.33	9	4.75	9	0.23	1	0.08	1	2.52	3	1.88	5
Supernatant	0.38	3	1.052	7	0	0	0	0	0.35	3	0.54	5	0.42	2	0.07	1

gave the bulk of the activity for arabinan synthase in fractions 1 and 2 and these had the highest specific activity. In the presence of Mg^{2+} about 25% of the total activity moves from fractions 1 and 2 to fractions further down the gradient.

For the distribution of xylan synthase activity in homogenates in EDTA, the bulk of the activity was found in fraction 2, the high specific activities at the bottom of the gradient being due to low protein recovery in these fractions when derived from differentiating cells. There was, however, a characteristic shift of some 11% of the total activity down the gradient in the presence of Mg^{2+}, although there was little change in specific activity of the bottom fractions.

These results indicated that some activity for both synthases was probably associated with the endoplasmic reticulum as well as the Golgi apparatus.

Incorporation in vivo of [1-^{3}H]arabinose into cell tissue cultures

Incorporation of [-^{3}H]arabinose into cell-wall fractions. The 1000 *g* pellet contained the bulk of the cell wall and cell debris. There was a 10-fold difference in incorporation of [1-^{3}H]arabinose on a per gram fresh weight basis into cell-wall products between the culture forming primary wall (CMD medium, cultured for 48 h) and secondary wall (IM, cultured for 96 h) (Table 5). There was also a considerable difference in the type of polysaccharide being formed. The bulk of the labelled polysaccharide products were water-soluble from CMD-medium-cultured cells, whereas those from the IM-cultured cells have solubilities characteristic of hemicellulose (Table 6). The molecular weight of most of these products, since they did not pass through a dialysis sac, was greater than 10000, but they differed in their sugar contents. Total acid hydrolysis of each fraction gave radioactive arabinose or xylose as the only products and there were significant differences in the relative proportions between cells from the two culture media. The radioactive composition of the polymers was dependent on the rate of incorporation into the combined monomers of the polysaccharides from the supplied radioactive arabinose. The radioactive composition did not reflect the chemical composition of the material but rather indicated shifts in the flow of carbon into the different polysaccharides of the cells grown in the different media and undergoing either primary-wall growth or differentiation to give secondary walls. The water-soluble products had ratios characteristic of neutral pectin, although there was a 20-fold higher incorporation into the primary wall. In contrast, there was a 34-fold increase in incorporation into polysaccharides, with increasingly high proportions of xylose, soluble in NaOH

Table 4. *Distribution of synthetase activity in subcellular fractions*

Tissue was homogenized and membrane fractions prepared as described in the legend to Table 3. Each fraction was assayed for arabinan synthetase or xylan synthetase and the distribution of either enzyme is shown. The fractions assayed for arabinan synthetase were derived from 6-day-old hypocotyls and for xylan synthetase from 9-day-old hypocotyls. Enzyme activities are given as the ratio of the specific activity in the subcellular fraction to that in the homogenate (relative specific activity, RSA) or as the percentage of the total activity on the gradient recovered in each fraction (% TA).

	Arabinan synthase				Xylan synthase			
	EDTA		Mg^{2+}		EDTA		Mg^{2+}	
Fraction	(RSA)	(% TA)	(RSA)	(% TA)	(RSA)	(% TA)	(RSA)	(% TA)
1 (12%/25% interface)	3.45	28	3.58	20	1.77	6	2.29	14
2 (25%/34% interface)	4.27	42	3.47	23	7.74	48	4.52	29
3 (33%/39% interface)	0.38	7	1.77	19	0.60	9	0.14	2
4 (39%/45% interface)	2.79	16	1.38	13	3.30	22	3.34	34
5 (>45% pellet)	1.36	8	4.20	24	4.37	15	3.27	21
Supernatant	0		0		0		0	

Table 5. *Incorporation of [1-^{3}H]arabinose into subcellular fractions of suspension cultured cells*

Suspension culture (40ml) either 48h after subculture into CMD medium or 96h after subculture into IM was incubated for 1h with 50μCi of [1-^{3}H]arabinose. Cells were harvested by filtration and homogenized in 5ml of 0.1M-phosphate buffer, pH 7.2, containing 1mM-dithiothreitol, 1mM-$MgCl_2$ and 0.4M-sucrose. Total radioactivity incorporated into each fraction is shown. Fractions were prepared from 6g of cells from cultures in CMD medium or 4g of cells in IM medium. (Radioactive material in the 100000*g* supernatant and the combined final supernatants was not insoluble in 85% ethanol and was not investigated further.)

	Total c.p.m. recovered in each fraction	
Fraction	CMD cultured	IM cultured
1000*g* pellet (cell wall)	0.44×10^6	2.3×10^6
1000*g* supernatant	4.2×10^6	9.4×10^6
100000*g* supernatant	2.81×10^6	6.7×10^6
100000*g* pellet (>60% sucrose)	9288	164916
Fractionation of interface material at 60% sucrose		
1 (12%/25% sucrose)	12998	135168
2 (25%/34% sucrose)	23230	255408
3 (34%/39% sucrose)	12850	74484
4 (39%/45% sucrose)	5480	38880
5 (>45% sucrose)	4550	14148
Combined final supernatants from fractions 1–5	0.43×10^6	1.7×10^6

from cells forming secondary walls. The fractions soluble in NaOH contained a high proportion of arabinose. However, it is possible that this may be due to inefficient washing of the material precipitated by ethanol. The final residues had similar but high proportions of incorporated arabinose.

The lipid material was soluble in chloroform/methanol (3:2, v/v) (as shown in Table 6) and was characterized by t.l.c. as non-polar. It was probably steryl glycoside.

Incorporation into membrane fractions in vivo. In cells grown in either media the bulk of the incorporation from [1-^{3}H]arabinose into polysaccharide was found in fraction 2 (the Golgi-apparatus-enriched fraction) in the presence of Mg^{2+}, with relatively little at the bottom of the gradient (Table 5). This result of the incorporation *in vivo* contrasts with the finding that about 50% of the synthase activities were found in fractions 3, 4 and 5 with the *'in vitro'* system.

The proportion of polysaccharide material retained in the sac on dialysis and therefore with a molecular weight greater than 10000 was the same as that found in the cell-wall extracts (75%) and suggests that the polymers are transported ready-made. Most of the polysaccharide in the cells grown in CMD medium was water-soluble containing 90% arabinose, whereas that in the membranes derived from cells grown in IM was approx. 50% soluble in water and 50% soluble in hot 10% trichloroacetic acid. Xylans are soluble in the latter solvent and the proportion of xylose to arabinose is higher in this fraction than in the water-soluble material. No other radioactive sugars were detected in any membrane-derived polysaccharide.

Very little incorporation of arabinose into polymer insoluble in 10% trichloroacetic acid, such as glycoproteins, was found (Table 7).

Material extracted in chloroform/methanol (3:2, v/v) and chloroform/methanol/water (10:10:3, by vol.) was radioactive. The radioactive products in the

Table 6. *Fractionation and characterization of cell-wall preparations of suspension cultured cells*
The 1000*g* pellets (see Table 5) were resuspended in 10ml of chloroform/methanol (3:2, v/v) and extracted for 16h at 4°C before centrifugation. The chloroform/methanol-soluble material was then washed by the method of Folch *et al.* (1957). The pellet was then sequentially extracted. Incorporation (c.p.m.) into polymer in each fraction was measured. The percentage of radioactivity recovered in each sugar after hydrolysis is shown. No other sugars were significantly labelled. The percentage of radioactivity retained in the sac after dialysis against distilled water is also shown. Abbreviation: n.d., not determined.

	Analysis of fractions				
	Chloroform/methanol (3:2, v/v)	Water-soluble material	3% NaOH-soluble material	17% NaOH-soluble material	Residue
Cell wall, CMD-medium cultured cells (c.p.m. incorporated)	10150	355660	33012	13050	9126
Arabinose (%)	n.d.	91	86	85	67
Xylose (%)	n.d.	9	14	15	33
Retained in dialysis sac (%)	—	79	64	100	76
Cell wall, IM-cultured cells (c.p.m. incorporated)	8985	16056	298284	1283820	410660
Arabinose (%)	n.d.	86	70	21	64
Xylose (%)	n.d.	14	30	79	36
Retained in dialysis sac (%)	—	56	76	78	100

Table 7. *Fractionation and characterization of radioactivity incorporated from [1-^{3}H]arabinose into membrane fractions prepared by gradient centrifugation*
Membrane fractions 1–5 (see Table 5) prepared from cells cultured either in CMD medium or in IM were prepared and fractionated. Material soluble in chloroform/methanol (3:2, v/v) and material soluble in chloroform/methanol/water (10:10:3, by vol.) was washed before estimation by the method of Folch *et al.* (1957) and/or was washed by elution with water on Whatman no. 1 chromatography paper respectively. The aqueous washes of the chloroform extractions were pooled with the ethanolic (85%) washes of the polymers precipitated by 85% ethanol and represent radioactivity present in low-molecular-weight compounds (results not shown). Polysaccharide was extracted by water or by hot 10% trichloroacetic acid. The percentage radioactivity recovered in either arabinose or xylose after hydrolysis is shown. No other sugar was significantly labelled. Abbreviation: n.d., not determined.

	Analysis of radioactivity incorporated into polymers						
	Water-soluble material			Hot 10% trichloroacetic acid-soluble material			10% trichloroacetic acid-insoluble material (c.p.m.)
	(c.p.m.)	Arabinose (%)	Xylose (%)	(c.p.m.)	Arabinose (%)	Xylose (%)	
CMD-medium cultured cells							
Fraction 1	4119	92	8	489	n.d.	n.d.	501
Fraction 2	12756	91	9	1198	n.d.	n.d.	895
Fraction 3	4926	89	11	446	n.d.	n.d.	439
Fraction 4	1919	n.d.	n.d.	197	n.d.	n.d.	238
Fraction 5	834	n.d.	n.d.	158	n.d.	n.d.	164
IM-cultured cells							
Fraction 1	27648	76	24	21265	55	45	450
Fraction 2	53988	75	25	60910	56	44	665
Fraction 3	13512	77	23	6875	n.d.	n.d.	600
Fraction 4	7980	n.d.	n.d.	4645	n.d.	n.d.	65
Fraction 5	2553	n.d.	n.d.	3700	n.d.	n.d.	120

chloroform/methanol extract had an R_F value of approx. 0.9 on t.l.c. and were probably steryl glycosides. The chloroform/methanol/water extracts were different for the cells grown in CMD medium from those grown in IM. Material from cells grown in CMD medium was soluble in water and was not lipid; that from cells grown in IM ran as neutral material during ion-exchange chromatography.

Table 8. *Characterization of microsomal fractions*

Tissue from either hypocotyls or suspension cultured cells was homogenized and microsomes fractionated and characterized. The recoveries shown are from 5g of tissue in each case. Incorporation of [1-^{3}H]arabinose was carried out as described in the legend to Table 5. Incorporation into total polysaccharide (85% ethanol-insoluble fraction) is shown. Experiments shown in Table 5 were carried out with cultures grown after approx. 30–40 subcultures (in CMD medium) after initial derivation; the experiments shown here were carried out with cultures grown after 65–70 subcultures (in CMD medium) when the morphogenic response was probably reduced (Bevan & Northcote, 1979).

Fraction and origin	Protein (mg)	RNA (μg)	Phospholipid (μg)	Enzyme activity (nmol · min^{-1})	Enzyme activity [nmol · min^{-1} · (mg of protein)$^{-1}$]	[1-^{3}H]Arabinose incorporated (c.p.m.)
1 (suspension cultures 48h after subculture into CMD medium)						
Arabinan synthase						
Smooth membrane	0.23	1	46	0.819	3.56	57190
Rough membrane	0.45	63	46	0.695	1.54	26413
Free polysomes	0.22	32	2	0.030	0.14	4170
2 (suspension cultures 96h after subculture into IM)						
Xylan synthase						
Smooth membrane	0.31	5	36	0.127	0.406	26530
Rough membrane	0.45	30	22	0.110	0.244	7460
Free polysomes	0.22	21	0	0	0	4270
3 (6-day-old hypocotyls)						
Arabinan synthase						
Smooth membrane	0.28	9	32	0.892	3.18	—
Rough membrane	0.61	123	33	0.909	1.50	—
Free polysomes	0.46	92	1	0	0	—
4 (10-day-old hypocotyls)						
Xylan synthase						
Smooth membrane	0.45	2	42	0.316	0.70	—
Rough membrane	0.59	46	37	0.385	0.65	—
Free polysomes	0.25	23	2	0	0	—

Glycosylation in microsomal fractions

The probable localization of the synthases in both endoplasmic reticulum and Golgi apparatus was also investigated by fractionation of microsomes. The method of separation with *Phaseolus vulgaris* gives free polysomes in the pellet (Bevan & Northcote, 1981) and it separates smooth and rough membranes (Bloemendal *et al.*, 1974). Table 8 shows that in these experiments the relative amounts of protein, RNA and phospholipid are in accordance with assigning the fractions isolated here as smooth membranes, rough membrane and free polysomes. This conclusion was reinforced by electron-microscopy examination of the three fractions. The areas of representative fields of each fraction were measured and the relative amount of smooth and rough membranes estimated. This showed the absence of any rough membrane in the smooth-membrane fraction, less than 20% smooth-membrane vesicles in the rough-membrane fraction and only ribosomes in the pellet. Treatment of the membranes with Triton or EGTA and analysis of the RNA profiles on sucrose gradients (Bevan & Northcote, 1981) have shown the presence of polysomes attached only to the rough-membrane and not the smooth-membrane fractions. The results of incorporation of radioactive arabinose both *in vivo* and *in vitro* indicated significant roles in glycosylation only for the membrane fractions. Bearing in mind that the method of isolation could always result in an artificial redistribution of polysomes, the fractionation procedure has also shown that considerable activity for both synthases was associated with the rough endoplasmic reticulum as well as the smooth membrane. However, the incorporation of arabinose *in vivo* into polysaccharides in tissue culture cells indicated a much greater involvement for the smooth membrane.

Discussion

Two distinguishable mechanisms for polysaccharide synthesis within the endomembrane system have previously been demonstrated. The synthesis of slime polysaccharide by differentiated root-cap cells

of maize is initiated in the endoplasmic reticulum and involved intermediate transfers from nucleotide sugars on to lipid before addition to protein carriers. Subsequent polymerization takes place within the Golgi apparatus before the slime is secreted as polysaccharide (Northcote, 1982). With the synthesis of wall matrix polysaccharides, the mechanism and site of synthesis may be different. It has been clearly shown that the bulk of the synthesis of these polysaccharides occurred in the Golgi apparatus and transport to the cell wall was via smooth-membrane vesicles (Ray *et al.*, 1976; Robinson *et al.*, 1976; Kawasaki, 1981). Some of the enzymes involved have been shown to be localized in the Golgi (Ray, 1973; van der Woude *et al.*, 1974; Powell & Brew, 1974; Ray, 1980).

The synthesis of some cell-wall polysaccharides may therefore contrast with other secreted products of glycosylation, such as the maize-root slime and animal glycoproteins, in that neither protein carriers nor the endoplasmic reticulum are involved. We have investigated the transfer from UDP-sugar to the growing polysaccharide for the synthesis of arabinan and xylan by a search for possible lipid or protein carriers. None were found. It is therefore unlikely that such intermediates are involved in the majority of the transglycosylations that bring about the synthesis of these particular high-molecular-weight cell-wall polysaccharides. However, the possibility that lipid or protein intermediates could be necessary for the initiation of the polymerization, occurring in small amounts, remains. Nevertheless, these intermediates were not detected and the glycosylations were neither stimulated by added dolichyl phosphate nor inhibited by compounds that act at the level of transfers involving polyprenyl phosphate intermediates.

The presence of the transglycosylase within an intracellular compartment does not necessarily indicate that this is the site of polymerization *in vivo*. For example, Golgi membranes contain a $\beta(1\rightarrow4)$-glucan synthase (Northcote, 1974; van der Woude *et al.*, 1974; Ray *et al.*, 1976), but *in vivo* these membranes produce no significant amounts of cellulose (Shore & Maclachlan, 1975; Ray *et al.*, 1976), which is produced at the cell surface. Furthermore, glycosyltransferases may well be translated on membrane-bound polysomes and inserted at the level of the endoplasmic reticulum. Restraints on enzyme activity *in vivo* therefore probably exist and could include transport of nucleotide diphosphate sugars into the lumen of the particular membrane compartment and the availability of dolichyl intermediates, if these are involved in transport or transfer of sugar residues (Haselbeck & Tanner, 1982) or activation of the enzyme only at a particular site during the membrane flow.

Arabinan synthase and xylan synthase activities were found in both endoplasmic reticulum and Golgi vesicles in similar amounts. However, evidence from labelling *in vivo* of polymers containing arabinose during primary-wall synthesis and polymers containing xylose during secondary-wall synthesis indicated polymerization in the endoplasmic reticulum was low and the bulk of the incorporation occurred in Golgi vesicles. Even taking into account possible membrane contamination there did, however, seem to be some incorporation in the endoplasmic reticulum. It follows therefore that although these synthases may well be translated and inserted at the level of the endoplasmic reticulum and are potentially active there, their activity is probably limited to the initiation of glycosylation.

These deductions depend essentially on the quality of the membrane fractionations. We have identified the endoplasmic reticulum by the characteristic shift in position on sucrose gradients induced by attachment of ribosomes in the presence of Mg^{2+} (Lehle *et al.*, 1978; Hopp *et al.*, 1979; Bollini & Chrispeels, 1979; Green & Northcote, 1979*b*; Owens & Northcote, 1981) as well as by the isolation of relatively pure rough endoplasmic reticulum. However, this assumption is dependent on the cation not causing non-specific membrane aggregation. This probably does not occur, since enzyme markers, such as IDPase, localized on other membranes do not shift significantly under the same conditions.

In suspension cultured cells, radioactive arabinose was incorporated into high-molecular-weight polysaccharide within the Golgi, the proportion of label appearing in xylose compared with arabinose being much higher in differentiated cells, and could be related to the type of polymer accumulated in the wall. During secondary-wall formation pectin synthesis is therefore suppressed and hemicellulose synthesis enhanced. Although the rate-limiting steps remain unidentified, the qualitative regulation of the type of polysaccharide accumulated appears to reside in the complement of synthases present within the endomembrane system (Dalessandro & Northcote, 1981; Bolwell & Northcote, 1981) and not the availability of primary substrate, since the enzymes involved in the interconversion of the nucleotide sugars change little during differentiation (Dalessandro & Northcote, 1977*a*,*b*). Similar types of control exist during the glycosylation of animal glycoproteins, where synthesis is directed in part by the presence or absence of the enzymes of oligosaccharide modification (Schachter, 1982).

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