Xyloglucan glucosyltransferase in Golgi membranes from *Pisum sativum* (pea)

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Cell membranes from etiolated *Pisum sativum* (pea) tissues were separated by ultracentrifugation on linear sucrose density gradients and assayed for membrane marker and glycosyltransferase activity. Membrane fractions were shown to incorporate glucose from UDP-D-[^{14}C]glucose into polysaccharides with glycosyl linkages consistent with synthesis of xyloglucan. A combined assay using g.c., radiogas proportional counting and m.s. was employed to determine the identities of ^{14}C-labelled glycosyl residues and the glycosyl linkages between them. In glucan synthase I assays, membrane fractions enriched for Golgi membranes showed ^{14}C incorporation into 4- and 4,6-glucose residues, with minor incorporation into 3-glucose residues. In glucan synthase II assays, all ^{14}C incorporation was into 3- and 3,4-glucose. There was a shift in glycosyl linkage of ^{14}C incorporation from predominantly 4-glucose at low UDP-glucose concentration to predominantly 3- and 3,4-glucose at high UDP-glucose concentrations. Mn^{2+} stimulated incorporation of radioactivity into 4,6-glucose residues characteristic of xyloglucan polysaccharides. Addition of exogenous UDP-xylose to assay mixtures stimulated incorporation into 4,6-glucose, with a maximum at 15 μM UDP-xylose.

INTRODUCTION

The plant Golgi apparatus is responsible for processing of glycoproteins and synthesis of cell-wall-matrix polysaccharides. N-glycosylation and processing of glycoproteins occur in plants in a manner similar to that found in animals (Faye et al., 1989; Griffing, 1991; Levy and Staehelin, 1992), but plant endoplasmic reticulum (ER)–Golgi systems also manufacture large quantities of cell-wall polysaccharides that animals do not make. Thus plant endomembrane systems possess enzymes specific for the biosynthesis of the array of glycosyl linkages found in cell-wall polysaccharides. Hemicellulosic and pectic cell-wall-matrix polysaccharides are synthesized and packaged in the Golgi system and then secreted to the wall (Robinson and Kristen, 1982; Brummell et al., 1990; Levy and Staehelin, 1992).

Xyloglucan is one of the most prominent matrix polysaccharides in non-graminaceous-plant cell walls, making up approx. 20% of total primary cell-wall polysaccharides (McNeil et al., 1984). Xyloglucan is composed of a linear glucan backbone with regular side-chain additions of xylose and galactose and sometimes fucose and arabinose (Hayashi, 1989a). As a polysaccharide that hydrogen bonds to cellulose, it is a major structural component of cell walls and may serve as a linkage between cellulose and other matrix polysaccharides (Talbott and Ray, 1992). Xyloglucan polysaccharides are manufactured in the Golgi apparatus by a combination of enzymes utilizing UDP-glucose for synthesis of the β-1,4-glucan backbone and UDP-xylose for addition of sidechains (Ray, 1980, 1985; Hayashi and Matsuda, 1981; Hayashi et al., 1984; Campbell et al., 1988; Gordon and Maclachlan, 1989).

Several different 1,4-β-glucan 4-β-glucosyltransferases are present in plant cells (Delmer, 1987; Hayashi, 1989a). Glucan synthase I (GS I) may make glucan chains similar to those in cellulose (Ray, 1979, 1980), whereas xyloglucan glucosyltransferase (XGT) may produce the glucan backbone for xyloglucan (Campbell et al., 1988; Gordon and Maclachlan, 1989). GS I and XGT activities may originate from the same enzyme or enzyme complex, although they exhibit differences in K_m and Mg^{2+} or Mn^{2+} stimulation (Delmer, 1987; Hayashi, 1989a). Glucan synthase II (GS II) is a 1,3-β-glucan 3-β-glucosyltransferase that makes glucan chains for the wound polysaccharide callose (Ray, 1979; Delmer, 1987). GS I and XGT are often cited as Golgi-resident enzymes, whereas GS II is usually assigned as a plasma-membrane activity (Quail, 1979; Ray, 1979, 1985; Delmer, 1987), although these two activities are reported as overlapping peaks in linear gradient centrifugation separations (Ray et al., 1976; Dhugga et al., 1991; White et al., 1993). GS I, XGT and GS II have similar requirements for activity. GS I activity is highest with low concentrations (< 50 μM) of UDP-glucose substrate in the presence of Mg^{2+} (Ray et al., 1969; Ray, 1979, 1980); XGT is also most active with low UDP-glucose concentration, but is stimulated more strongly by Mn^{2+} (Ray, 1980; Hayashi and Matsuda, 1981; Hayashi et al., 1984; Gordon and Maclachlan, 1989). GS II activity is highest with high (> 500 μM) UDP-glucose concentrations in the presence of micromolar concentrations of Ca^{2+} and millimolar concentrations of a β-glucoside (Van der Woude et al., 1974; Ray, 1979; Hayashi et al., 1987). Xylosyltransferase (XT) involved in xyloglucan synthesis uses UDP-xylose as a substrate to add xylosyl sidechain residues to the C-6 position of backbone glucosyl residues (Hayashi and Matsuda, 1981; Hayashi et al., 1984; Campbell et al., 1988; Brummell et al., 1990). Other XTs found in plant membranes may be involved with glucuronoxylan synthesis (Baydoun et al., 1989) or xylan synthesis (Rodgers and Bolwell, 1992).

Products from glycosyltransferase assays are usually analysed by degradation with enzymes specific for the β-1,4-glucan backbone and chromatography to separate the hydrolysis products (Ray, 1980; Hayashi, 1989b; Hayashi and Matsuda, 1981; Brummell et al., 1990). Recently, a combination of methylation analysis by g.c.–m.s. and radiogas proportional counting (r.p.c.) have been used to determine amounts of [^{14}C]glucose incor-

Abbreviations used: DTT, dithiothreitol; EMA, eosin-5′-maleimide; f.i.d., flame-ionization detector; r.p.c., radiogas proportional counting; GS I, glucan synthase I; GS II, glucan synthase II; IDPase, inosine diphosphatase; PMAA, partially methylated alditol acetates; UDPase, uridine diphosphatase; XGT, xyloglucan glucosyltransferase; XT, xylosyltransferase; ER, endoplasmic reticulum; TFA, trifluoroacetic acid.

porated into specific glycosyl linkages within reaction products (Hayashi et al., 1987; Shea et al., 1989; Gibeaut and Carpita, 1990, 1991; White et al., 1993). We have used this method to examine [^{14}C]glucose incorporation into glycosyl linkages synthesized by pea membranes separated on linear sucrose gradients. We have also determined the effects of UDP-glucose substrate concentration, cofactors and UDP-xylose concentrations on ^{14}C-labelling of glycosyl linkages.

EXPERIMENTAL

Chemicals and plant material

All sugars and their derivatives were in the D configuration. UDP-[U-^{14}C]glucose (specific radioactivity 11.56 GBq·mmol^{-1}) and UDP-[U-^{14}C]xylose (specific radioactivity 9.57 GBq·mmol^{-1}) were obtained from New England Nuclear (Wilmington, DE, U.S.A.). BSA, UDP-glucose, UDP-xylose, IDP, UDP, NADH, cytochrome *c*, n-butyllithium and methyl iodide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein assay reagent was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Eosin-5′-maleimide (EMA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Peas (*Pisum sativum*, cv. Caprice) were supplied by Asgrow Seed Company (Kalamazoo, MI, U.S.A.) and arrived pretreated with Captan. Tamarind (*Tamarindus indica*) seed kernel powder was supplied by P. L. Thomas and Co. (Bernardsville, NJ, U.S.A.).

Membrane preparation

Pea (*Pisum sativum* L., cv Alaska or Caprice) seedlings were grown 7 days in moist vermiculite in the dark at 25 °C. A stem segment approx. 8 mm long was cut beginning 3 mm below the top of the apical hook (Ray et al., 1976; Ray, 1980). Tissue and all solutions were kept on ice. When tissue was labelled with EMA, segments were chopped into small pieces (less than 1 mm) with a razor blade and rinsed three times with an osmotic solution of 4.5 mM $CaCl_2$, 0.75 M sorbitol, 0.1% BSA and 1 mM KH_2PO_4 to wash membranes away from broken cells. Washed tissue was incubated in 25 μM EMA for 1 h at 4 °C and rinsed with osmotic solution three times to remove unchanged EMA. Tissue was homogenized with mortar and pestle in an equal weight of homogenization medium, which contained 0.4 M sucrose, 10 mM KCl, 0.1 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT and 0.025% (w/v) BSA in 40 mM Tris, pH 8.0. Homogenate was expressed through Miracloth (Calbiochem, La Jolla, CA, U.S.A.) to remove tissue debris.

Fractionation of cellular membranes

Homogenate (14 ml) was loaded on top of a 15–48% (w/w) linear sucrose gradient (24 ml total gradient volume) containing 0.1% (w/v) BSA, 1 mM DTT and 1 mM EDTA in 40 mM Tris, pH 8.0. Gradients were ultracentrifuged at 100000 $g_{av.}$ for 3 h at 4 °C in a Beckman SW-28 rotor. Sucrose gradients were fractionated on a gradient fractionator (Isco, Lincoln, NE, U.S.A.), and the sucrose content of fractions was determined refractometrically.

Standard marker assays

EMA fluorescence

This was used as a PM marker (Spring and Krauss, 1987; White et al., 1993). EMA, a derivative of the fluorescent dye eosin, binds non-specifically to the thiol groups of proteins. If the plasma membrane is intact, it alone will be fluorescently labelled and internal membranes will remain unlabelled. Chopped pea-seedling stem segments were incubated with shaking for 1 h at 4 °C in 25 μM EMA in osmotic solution and then washed three times with osmotic solution. After gradient centrifugation, fluorescence of the EMA marker in sucrose gradient fractions was measured with a fluorimeter using 518 nm excitation and 540 nm emission.

Protein content

This was determined using the Coomassie Brilliant Blue staining method (Bio-Rad Protein Assay) with BSA as a standard.

Antimycin A-insensitive NADH:cytochrome *c* reductase (ER marker)

This was assayed spectrophotometrically by measuring the time-dependent reduction of cytochrome *c* (Hall and Moore, 1988; White et al., 1993). Reaction mixtures contained 0.1 mM cytochrome *c*, 0.1 mM NADH, 0.3 mM KCN and 1 μM antimycin A in 50 mM phosphate buffer, pH 7.5. The reaction was initiated by addition of NADH, and the increase in absorbance at 550 nm was measured over a 1 min interval. An ϵ_{550} for cytochrome *c* of 21.0×10^3 M^{-1}·cm^{-1} (Massey, 1959) was used for calculations of reaction rate.

Inosine diphosphatase (IDPase) and uridine diphosphatase (UDPase)

These activities were used as Golgi membrane markers and were assayed by spectrophotometric determination of P_i released from inosine or uridine diphosphate (Hall and Moore, 1988; White et al., 1993). Both reactions took place in 50 mM Tris, pH 7.5. The UDPase reaction mixture contained 3 mM UDP and 3 mM $MnSO_4$; the IDPase reaction mixture contained 3 mM IDP and 1 mM $MgCl_2$.

[^{14}C]Glycosyl-incorporation assays

Tamarind xyloglucan

Partially purified xyloglucan for incorporation assay product carrier was prepared by dissolving 30 g of tamarind kernel powder in 3 litres of water. The solution was heated, stirred for 24 h at 4 °C and centrifuged at 18000 $g_{av.}$ for 30 min. The pellet was discarded. Soluble polysaccharides were precipitated in 70% ethanol and centrifuged at 18000 $g_{av.}$ for 30 min. The pellet was resuspended in 850 ml of water, re-precipitated, and the pellet was freeze-dried. Carrier xyloglucan (100 μg per assay) was mixed with enzyme assay reaction mixtures by adding 20 μl of a 5 mg/ml stock to each assay mixture.

β-Glucan synthase I (xyloglucan glucosyltransferase)

Reaction mixtures (100 μl final vol.) contained 3 mM $MnCl_2$ and 0.7 μM UDP-[^{14}C]glucose (740 Bq/fraction) in 40 mM Tris, pH 8.0, and 90 μl of fractionated membrane (Ray, 1979, 1980; Hayashi and Matsuda, 1981). Reaction mixtures were incubated for 15 min at room temperature and terminated by adding 100 μg of tamarind xyloglucan carrier and 95% ethanol to make a 70% concentration. Reaction mixtures were chilled to −20 °C. For centrifugation assays, ethanol-insoluble material was extracted three times with 1 ml of 70% ethanol and centrifuged at 3500 $g_{av.}$ for 10 min. Supernatants were discarded. For filtration assays, ethanol-insoluble material was filtered through Whatman GF/A glass-microfibre filters and washed with 70% ethanol.

Incorporation of radioactivity was determined by liquid-scintillation counting of filtration assay mixtures and combined methylation linkage analysis and r.p.c. of centrifugation assay mixtures.

β-Glucan synthase II (callose synthase)

Reaction mixtures contained 80 μl of membranes plus 3 mM $CaCl_2$, 500 μM UDP-glucose and 0.7 μM UDP-[^{14}C]-glucose (740 Bq/fraction) in 100 mM Hepes buffer, pH 6.8 (Ray, 1979; Delmer, 1987). Incubations, centrifugation assays and filtration assays were the same as for GS I above, except that no carrier xyloglucan was added.

Xylosyltransferase

Reaction mixtures contained 80 μl of membranes plus 10 mM $MnCl_2$, 2 mM UDP-glucose and 0.2 mM UDP-[^{14}C]-xylose (740 Bq/fraction) in 10 mM Hepes buffer, pH 7.5 (Hayashi et al., 1984; Brummell et al., 1990). Incubations, centrifugation assays and filtration assays were the same as for GS I above.

Methylation linkage analysis

Membrane fractions containing the Golgi peak, as determined by IDPase, UDPase and GS I markers, were pooled together. Aliquots of membane were assayed for GS I, GS II, and XT. Reaction mixtures were incubated as described above, except that all volumes were increased 5-fold. Reaction products and carrier xyloglucan obtained from GS I and GS II centrifugation assays were derivatized for glycosyl linkage analysis by methylation with n-butyl-lithium (Carpita and Shea, 1989). Reaction products dried over P_2O_5 were dissolved in 0.2–0.5 ml of DMSO and n-butyl-lithium was added slowly, followed by methyl iodide. Methylated samples were extracted with chloroform, washed with water, and the chloroform phase was evaporated to dryness. Samples were fully hydrolysed with 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h and reduced with $NaBH_4$. Per-O-methylated alditols were acetylated with acetic anhydride and 1-methylimidazole as a catalyst (Blakeney et al., 1983).

R.p.c. and g.c.–m.s.

Partially methylated alditol acetates (PMAAs) were dissolved in 100 μl of acetone, and the radioactivity of 1 μl of this sample was measured by liquid-scintillation counting. An aliquot of sample containing approx. 60–300 kBq was evaporated to dryness, redissolved in 5 μl of acetone and introduced by a 3 min splitless injection on a 30 m × 0.75 mm SP-2330 glass capillary column (Supelco, Bellefonte, PA, U.S.A.) at a temperature of 170 °C. After a 3 min delay, column temperature was raised 4 °C/min to 240 °C. G.c. column effluents were split (1:9) between a flame-ionization detector (f.i.d.) and a radiogas proportional counter (r.p. counter). Radioactive PMAAs in the g.c. column effluent were monitored by r.p.c. (Shea et al., 1989; Gibeaut and Carpita, 1990, 1991; White et al., 1993) on a Radiomatic Flo-One/Beta, Model GCR Radiochromatography detector (Radiomatic Instruments and Chemical Co., Meriden, CT, U.S.A.). Radioactive peaks were identified by comparing peaks and retention times with injections of the same samples on a Hewlett–Packard g.c.–m.s. instrument with a 30 m SP-2330 fused silica capillary column and a similar temperature program. Proportions of ^{14}C incorporated into different glycosyl linkages were determined by calculation of area percentages of identified peaks from integrations of r.p.c. detector response.

Characterization of glucan synthase activity

The effect of different concentrations of substrates (UDP-glucose and UDP-xylose) and cofactors ($MgCl_2$ and $MnCl_2$) on enzyme assays were determined by pooling membrane fractions containing the Golgi peak and assaying aliquots. All assays had a total volume of 500 μl and contained 400 μl of membrane fraction and 3.7 kBq of UDP-[^{14}C]glucose substrate in 40 mM Tris, pH 8.0. Concentrations of UDP-glucose, UDP-xylose, $MgCl_2$ and $MnCl_2$ were varied. Reaction mixtures were incubated for 15 min at room temperature and were terminated by addition of carrier xyloglucan (500 μg) and 95% ethanol to make a 70% concentration. Products were isolated and methylated as described above.

RESULTS AND DISCUSSION

Membrane separations

Pea tissue homogenates applied to 15–48% linear sucrose gradients and separated by rate-zonal ultracentrifugation at 100000 $g_{av.}$ for 3 h showed partial separation of ER, Golgi apparatus and PM markers (Figure 1). The ER marker (antimycin A-insensitive NADH:cytochrome *c* reductase) appeared as two peaks, one at the top of the gradient in the 15–21%-sucrose fractions [density (ρ) = 1.057–1.086 g/cm^3] and the other in 38–44%-sucrose fractions (ρ = 1.166–1.195 g/cm^3) towards the bottom of the gradient. The cytochrome *c* reductase activity in the less dense, 15–21%-sucrose, fractions originated from membranes with densities typical for ER membranes. The cytochrome *c* reductase activity found in the more-dense 38–44%

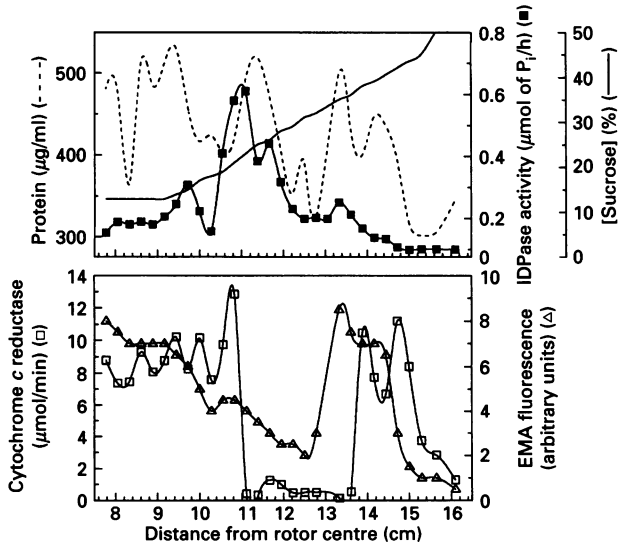

Figure 1 Separation of membrane markers in subcellular fractions

Membranes from pea homogenates were separated on 15–48% linear sucrose gradients by ultracentrifugation at 100000 $g_{av.}$ for 3 h. Experimental protocols for each assay are described in the text.

fractions may have resulted from mitochondrial activity not inhibited by antimycin A.

IDPase and UDPase were used as Golgi markers, and they appeared in two peaks in the sucrose gradients (Figure 1). IDPase and UDPase activities overlapped, and only IDPase activity is presented in Figure 1. The first peak was larger and appeared in the 17–26%-sucrose fractions (ρ = 1.066–1.109 g/cm^3). The second smaller peak was in the 33–35%-sucrose fractions (ρ = 1.142–1.152 g/cm^3). The larger IDPase peak in less-dense fractions has an unknown origin. It does not appear to be a soluble enzyme that would be expected to remain in the 15% sucrose at the top of the gradient, and it was not a form of latent IDPase (Hall and Moore, 1988), as neither time nor inclusion of Triton X-100 changed the pattern of IDPase activity (results not shown). The second IDPase peak was much smaller and corresponded directly with the GS I, GS II, and especially the XT activities located in the sucrose gradients (Figure 2). The fraction density and relative position of these other enzyme markers indicate that the second, smaller, peak marks the presence of Golgi membranes in the gradient.

EMA fluorescence was also observed (Figure 1) at both the top of the gradient in fractions of less than 20% sucrose (ρ < 1.081 g/cm^3) and toward the bottom of the gradient in 35–40%-sucrose fractions (ρ = 1.152–1.176 g/cm^3). The fluorescence in the more dense fractions overlaps with the IDPase Golgi marker and extends down to sucrose densities expected for PM. EMA should bind only to PM if membrane is intact (Spring and Krauss, 1987); however, during tissue preparation and chopping, many cells were certainly broken open. Chopped tissues were washed to remove any internal membranes liberated by the chopping action, but some residual intracellular membranes could have been EMA-labelled, and these membranes may have subsequently migrated into the sucrose gradients during ultracentrifugation. This could explain the EMA fluorescence that overlaps the Golgi fractions. EMA fluorescence in the 15–20%-sucrose fractions may have resulted from EMA bound to soluble proteins that subsequently remained at the top of the gradient. Similar patterns of EMA labelling were observed in rate-zonal separations of membranes from suspension-cultured sycamore (*Acer pseudoplatanus*) cells (White et al., 1993).

Glycosyltransferase assays

Sucrose gradient fractions assayed for standard membrane markers were also assayed for glycosyltransferase activities: GS I, GS II and XT (Figure 2). All three glycosyltransferases overlapped in 31–41%-sucrose fractions. GS I had peak activity in 31–35%-sucrose fractions, whereas XT had a very sharp peak at 35% sucrose. The XT peak corresponded exactly with the smaller IDPase/UDPase peak that represented the Golgi membrane marker (Figure 2). GS II had a small peak of activity that corresponded with the large IDPase/UDPase peak at approx. 20% sucrose. A larger GS II peak was more broad than either of the GS I or XT peaks and it extended into more dense sucrose fractions up to 41%.

GS I activity was found in less dense fractions, beginning in fractions of approx. 31% sucrose. These fractions were above the Golgi marker peak at 35% sucrose, but still in the range of reported densities of Golgi membranes (Ray et al., 1976; Dhugga et al., 1991; Waldron and Brett, 1987). The GS I activity encompassed both the Golgi marker peak and the sharp XT peak and fell off rapidly in more dense fractions above 35% sucrose. GS II increased in the same fractions as GS I, but GS II activity remained high in the more dense fractions from 35 to 41% sucrose, which would be expected to contain PM. There was

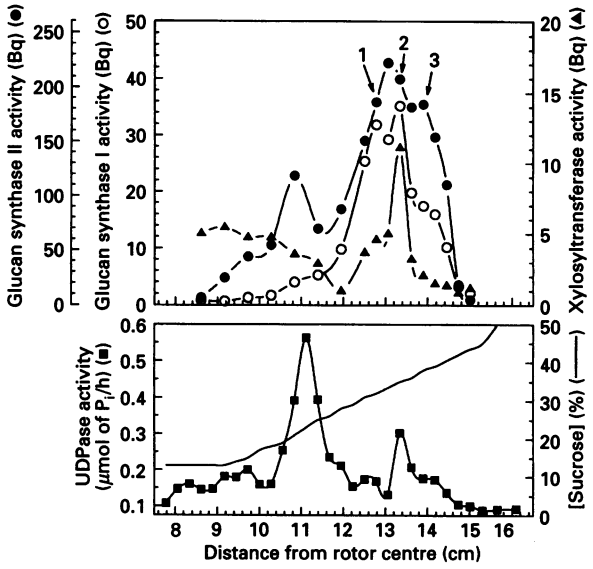

Figure 2 Distribution of UDPase Golgi marker enzyme and glycosyltransferases in subcellular fractions

Membranes from pea homogenates were separated on 15–48% linear sucrose gradients by ultracentrifugation at 100000 $g_{av.}$ for 3 h. Three membrane fractions (1, 2 and 3) were chosen for linkage analysis of UDP-[^{14}C]glucose incorporation into reaction products of GS I and GS II incubations (Figure 4).

some XT activity in the top portions of the gradient and a sharp peak of activity that precisely coincided with the IDPase/UDPase Golgi marker peak and the highest GS I activity at 35% sucrose.

In general, separation of membrane markers on linear sucrose gradients showed that XT was most active in a narrow band of Golgi membranes, whereas both GS I and GS II were active in membranes that spread through a broader range of densities. This indicated that a combination of IDPase/UDPase and XT activities were the best markers for Golgi membranes in this system. GS II activity remained high in membranes migrating to 35–41% sucrose, which had much lower GS I and XT activities. These results are consistent with previously reported distributions of IDPase/UDPase, GS I, and GS II activities (Ray et al., 1976; Ray, 1979; Waldron and Brett, 1987; Brummell et al., 1990; Dhugga et al., 1991). In these membranes, XT appeared in a very narrow band precisely at the density expected for Golgi membranes, whereas the IDPase/UDPase, GS I, and GS II activities were found in much broader density bands.

Three membrane fractions were chosen for linkage analysis of UDP-[^{14}C]glucose into reaction products of GS I and GS II incubations (Figure 2; fractions 1, 2 and 3). Fraction 1 had high activities of both GS I and GS II, but was above the IDPase and XT peaks, fraction 2 corresponded with the highest activity of IDPase and XT and fraction 3 had high GS II activity but lower GS I and XT activities.

Combined g.c.–r.p.c./g.c.–m.s. assay

To determine amounts of radioactivity from UDP-[^{14}C]glucose incorporated into specific glycosyl linkages during enzyme assays, reaction products from GS I and GS II glycosyltransferase assays were analysed with a combination of g.c.–r.p.c. and g.c.–m.s. (Carpita and Shea, 1989; Shea et al., 1989; Gibeaut and Carpita, 1990, 1991; White et al., 1993). Reaction products were

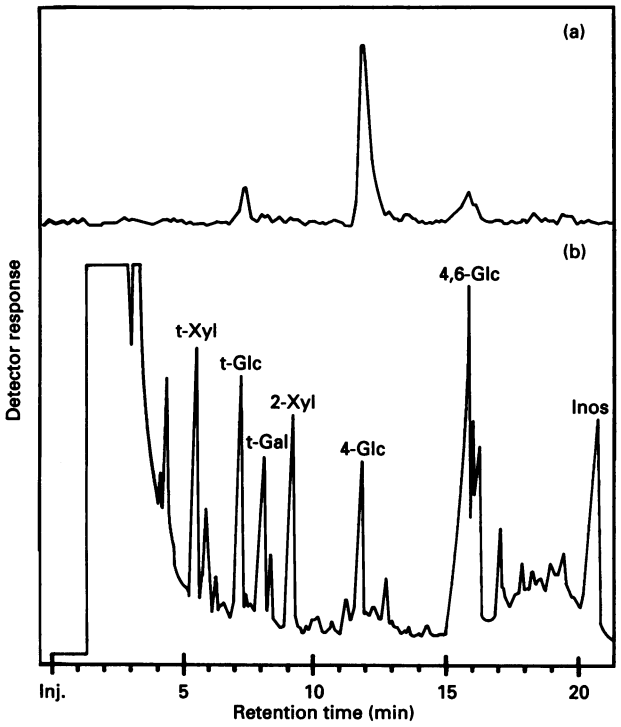

Figure 3 Incorporation of radioactivity from UDP-[U-^{14}C]glucose into specific glycosyl linkages in a GS I assay

Golgi membrane fractions from 15–48% linear sucrose gradients were incubated under GS I assay conditions. Reaction products collected by ethanol precipitation were methylated and injected on a gas chromatograph with effluent split between a flame-ionization detector (**b**) and a r.p. counter (**a**). Major peaks were identified in similar injections by g.c.–m.s. (Carpita and Shea, 1989). Abbreviations: inj, injection points; Inos, inositol.

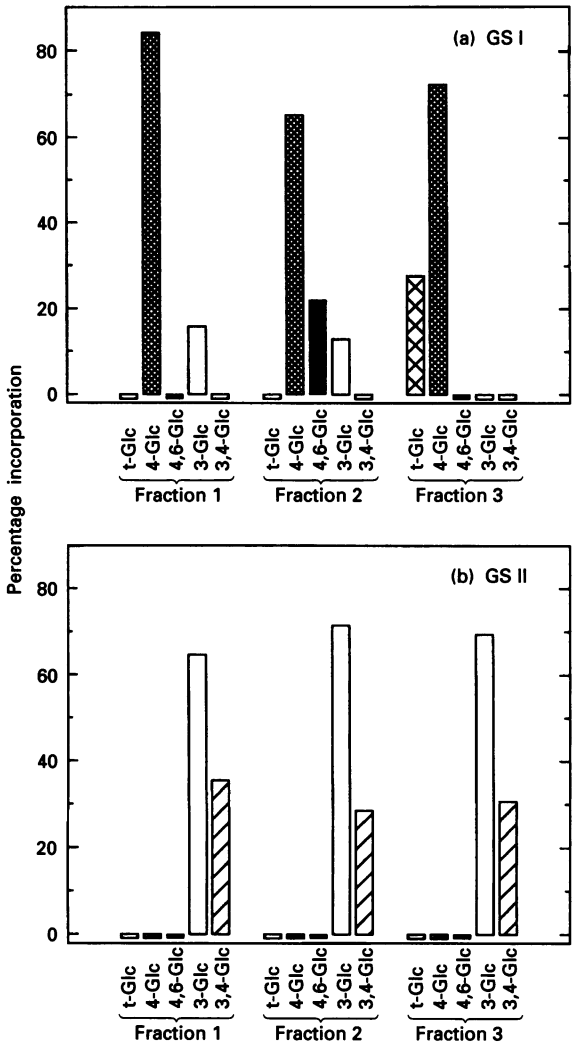

Figure 4 Incorporation of radioactivity from UDP-[U-^{14}C]glucose into specific glycosyl linkages in GS I and GS II assays

Reaction products from GS I (**a**) and GS II (**b**) assays using three different membrane fractions from linear sucrose gradients (Figure 2) were subjected to linkage analysis in a combined g.c.–r.p.c./g.c.–m.s. assay (Figure 3). Data are expressed as percentage incorporation determined by calculation of area percentage from integration of r.p.c. peak areas.

collected by ethanol precipitation, derivatized for linkage analysis, and the resulting PMAAs were separated by g.c. Column effluents were split between two detectors, an r.p. counter (Figure 3a) and f.i.d. (Figure 3b). In addition, the same PMAA samples were analysed by g.c.–m.s. to deduce the glycosyl linkages present in the reaction products. Glycosyl linkages were assigned by comparison of retention times and peak patterns in r.p.c./f.i.d. profiles with those of g.c.–m.s. profiles, where mass spectra were used to identify the PMAA derivatives present (Carpita and Shea, 1989). The f.i.d. profiles (Figure 3b) showed linkages that are characteristic for xyloglucan polysaccharides. These linkages originated mostly from the xyloglucan that was added to each assay as a carrier molecule. Profiles also showed the presence of other glycosyl linkages that may have originated from oligosaccharides, glycoproteins and other glycosylated compounds present in the membranes added to the reaction mixture, or from polysaccharides other than xyloglucan that were synthesized during the assay incubation period. The r.p.c. profiles (Figure 3a) from the split sample effluents showed peaks of radioactivity coinciding with some, but not all, glycosyl linkages in the f.i.d. profile. The data in Figure 3 are from injection of PMAAs from reaction products of a GS I assay (0.7 μM UDP-glucose substrate, 3 mM $MnCl_2$ and no added UDP-xylose) and the r.p.c. in this example showed peaks of radioactivity coinciding with peaks identified by m.s. as t-Glc, 4-Glc, and 4,6-Glc (t in t-Glc stands for terminal glucose; i.e. it is a sugar at the non-reducing chain end and has no other sugars linked to it). Data in Figures 4 and 5 are derived from similar injections and results are expressed as percentage incorporation determined by calculation of area percentage from integration of r.p.c. peak areas in each injection.

Linkage analysis of reaction products

Reaction products from GS I and GS II assays using three different membrane fractions from linear sucrose gradients (Figure 2) were subjected to linkage analysis in the combined g.c.–r.p.c./g.c.–m.s. assay of [^{14}C]glucose incorporation (Figure 4). Pooled Golgi membrane fractions were also used to determine the linkages of glycosyl incorporation resulting from different glucan synthase assay conditions (Figure 5).

GS I assays

Combined g.c.–r.p.c./g.c.–m.s. assay analysis of reaction products of GS I incubations from the same three sucrose gradient

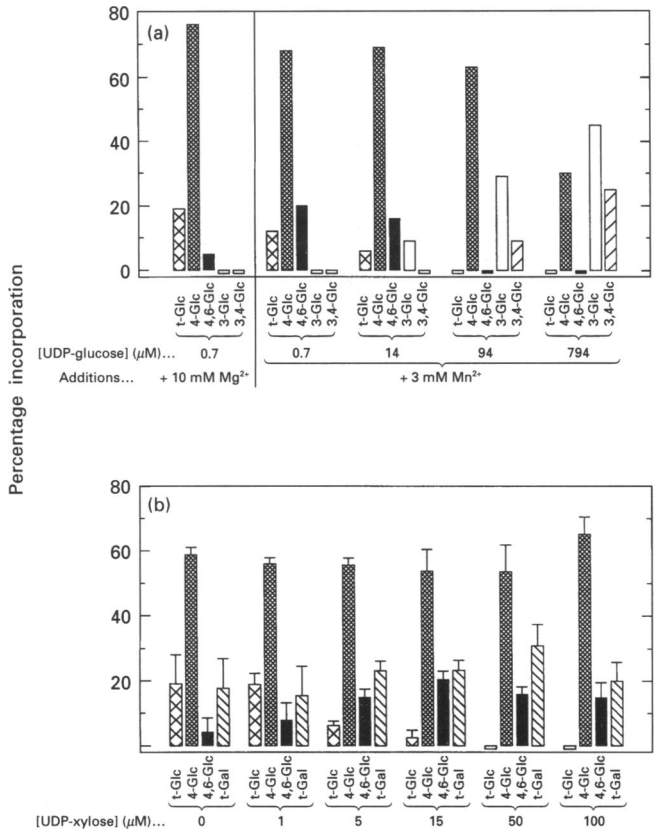

Figure 5 Incorporation of radioactivity from UDP-[U-^{14}C]glucose into specific glycosyl linkages from different GS assay conditions

GS I reaction products from pooled Golgi membrane sucrose-gradient fractions were analysed by combined g.c.–r.p.c./g.c.–m.s. under different assay conditions. Data are expressed as percentage incorporation determined by calculation of area percentage from integration of r.p.c. peak areas. (**a**) Percentage ^{14}C incorporation into specific glycosyl linkages in the presence of Mg^{2+} or Mn^{2+} with different concentrations of UDP-glucose substrate. No exogenous UDP-xylose was added. (**b**) Percentage ^{14}C incorporation into specific glycosyl linkages in the presence of different concentrations of exogenous UDP-xylose. The UDP-glucose concentration was 0.7 μM, and Mn^{2+} was included.

membrane fractions (Figure 2; fractions 1, 2 and 3) showed that the major linkage of ^{14}C incorporation in all three fractions was 4-Glc (Figure 4a). Fractions 1 and 2 produced 10–15% of ^{14}C incorporation into 3-Glc, whereas fraction 3 exhibited no incorporation into 3-Glc. Fraction 2 was the only fraction that had incorporation into 4,6-Glc, and fraction 3 had approx. 25% of its ^{14}C incorporation into t-Glc.

GS II assays

Combined g.c.–r.p.c./g.c.–m.s. assay analysis of reaction products of GS II incubations from the same three sucrose gradient membrane fractions (Figure 2; fractions 1, 2 and 3) showed that 3-Glc was the dominant linkage of ^{14}C incorporation in all three fractions, with approx. 30–35% of total incorporation into 3,4-Glc, but no incorporation into 4-Glc, 4,6-Glc or t-Glc was observed in any GS II assays (Figure 4b).

The differences observed in linkage of incorporation from different sucrose gradient fractions fit with the membrane marker profiles and the distribution of GS I, GS II and XT glycosyltransferase activities as determined by filter assays (Figure 2). The combined g.c.–r.p.c./g.c.–m.s. analysis of GS I assays (Figure 4a) showed incorporation of [^{14}C]glucose into 4-Glc and 3-Glc with membrane fraction 1, into 4-Glc, 4,6-Glc, and 3-Glc with fraction 2 and into t-Glc and 4-Glc with fraction 3. Fraction 1 had high activities of both GS I and GS II (Figure 2), but was above the IDPase and XT peaks. Combined g.c.–r.p.c./g.c.–m.s. assays of fraction 1 showed the expected incorporation into 4-linkages from GS I and 3-linkages from GS II (Figure 4a).

Membrane fraction 2 corresponded with the highest activities of IDPase and XT, and the GS I assays showed incorporation mainly into 4- and 4,6-Glc (totalling 87% of incorporation). The XT activity associated with xyloglucan synthesis would transfer xylosyl residues on to the C-6 position of β-1,4-glucose backbone residues, resulting in 4,6-Glc linkages. Fraction 2 membranes had the highest XT activity, and fraction 2 was also the only one to show [^{14}C]incorporation into 4,6-Glc linkages. GS I assay incubations in these experiments had only UDP-glucose added as substrate, with no exogenous UDP-xylose to act as substrate for XT enzyme in the membranes, yet approx. 20% of [^{14}C]glucose incorporation was observed as 4,6-Glc in membrane fraction 2. Assuming that the 4,6-linkages arose from addition of xylosyl residues to the C-6 position, there was apparently enough endogenous UDP-xylose present in the membrane fractions to allow XT activity.

Glycosyltransferase assays for membrane fraction 3 showed high GS II activity, but lower levels of GS I and XT activities. Combined g.c.–r.p.c./g.c.–m.s. analysis of GS I assays from this fraction showed ^{14}C incorporation only into t-Glc and 4-Glc. The origin of ^{14}C-labelled t-Glc linkages from this fraction is unknown, but t-Glc has been observed as a major incorporation product arising from glycosylation of perhaps phenolic compounds that can be removed by adsorption to charcoal (D. M. Gibeaut and N. C. Carpita, personal communication).

All three membrane fractions showed incorporation of [^{14}C]glucose into 3-Glc in GS II assays (Figure 4b). This result was expected from profiles of glycosyltransferase activities in sucrose gradient fractions (Figure 2), which were characterized by high GS II activity across all three fractions. The appearance of significant ^{14}C incorporation into 3,4-Glc residues in GS II assays was unexpected, and could have originated from dual activity of the GS II enzyme, the presence of a branching enzyme adding glycosyl residues on to C-4 of the 3-linked callose backbone, or selective under-methylation at the C-4 position in glucosyl residues within a 3-linked backbone (Carpita and Shea, 1989). Double methylation of reaction products from GS II assays of pooled Golgi membrane fractions produced essentially 100% incorporation of [^{14}C]glucose into 3-Glc and an absence of 3,4-Glc (results not shown). These results indicate the ^{14}C-labelled 3,4-Glc residues most likely resulted from under-methylation in the C-4 position.

In comparing the data reported in Figures 4(a) and 4(b) it should be noted that the assay conditions for GS I (Ray et al., 1969; Ray, 1979, 1980; Delmer, 1987; Hayashi, 1989a) and GS II (Van der Woude et al., 1974; Ray, 1979; Hayashi et al., 1987; Delmer, 1987; Hayashi, 1989a) incubations were typical of those reported in the literature, with low concentrations of UDP-glucose substrate and Mn^{2+} or Mg^{2+} cofactors for GS I assays and high concentrations of UDP-glucose substrate with Ca^{2+} and a disaccharide as stimulants for GS II. Our results agree with the suggestion by Gibeaut and Carpita (1990) that there is some cross-reaction between the GS I and GS II enzymes under these assay conditions. We observed some incorporation into 3-Glc (GS II activity) under the GS I assay conditions (Figure 4a). In

contrast, there was no 4-Glc incorporation under the GS II assay conditions (Figure 4b).

Effect of Mg^{2+}, Mn^{2+} and UDP-glucose concentration

As noted above, GS I assays are typically performed at low UDP-glucose substrate concentration (< 5 μM), whereas GS II assays are performed at much higher UDP-glucose substrate concentration (> 500 μM). Mg^{2+} is typically added to stimulate GS I and Mn^{2+} to stimulate xyloglucan synthesis. Combined g.c.–r.p.c./g.c.–m.s. analysis of GS I reaction products from pooled Golgi membrane sucrose gradient fractions showed an approximate 3-fold increase in ^{14}C incorporation into 4,6-Glc with the addition of Mn^{2+} to the incubation mixture (Figure 5a). In the presence of Mn^{2+}, a shift of ^{14}C incorporation linkage was observed from predominantly 4-Glc at low UDP-glucose concentration to predominantly 3- and 3,4-Glc at high UDP-glucose concentration (Figure 5a). The 0.7 μM UDP-glucose (3 mM Mn^{2+}) incubations resulted in a total of 88% ^{14}C incorporation into 4- and 4,6-linkages, with minor amounts of additional incorporation into t-Glc. The 14 μM UDP-glucose incubations resulted in a total of 85% ^{14}C-incorporation into 4- and 4,6-linkages, with minor amounts of additional incorporation into t-Glc and 3-Glc. When the UDP-glucose substrate concentration was raised to 94 μM, incorporation into 4,6-Glc disappeared, and incorporation into 3- and 3,4-Glc linkages increased. At 794 μM UDP-glucose substrate concentration, incorporation into 4-Glc decreased to approximately half that observed at 0.7 μM UDP-glucose, and 3- and 3,4-Glc represented approx. 70% of the total ^{14}C incorporation. The observed 3,4-Glc linkages in this experiment are presented to be undermethylation products from a 3-glucan backbone. The 0.7 μM UDP-glucose assay (Figure 5a) was performed under conditions identical with those used for the GS I assays reported above (Figure 4a); however, the membrane fractions reported in Figure 5 were the equivalent of pooling all fractions between membrane fractions 1 and 3 in Figure 2. The 794 μM UDP-glucose incubation resulted in approx. 25% 4-Glc, with the remaining ^{14}C incorporation into 3- and 3,4-Glc. The high UDP-glucose incubations in Figure 5(a) differed from the GS II incubations in Figure 4(b). In Figure 5(a) the high UDP-glucose incubations lacked exogenously added Ca^{2+} and did have exogenously added Mn^{2+} that was not present in the earlier GS II assays. Under these conditions the enzymes present in the pooled Golgi membranes manufactured a mixture of GS I (1,4-glucan) and GS II (1,3-glucan) reaction products. Gibeaut and Carpita (1990) reported the isolation of highest specific GS II activity in membranes that correspond to ER, with lesser GS II activity in plasma-membrane fractions. Our results indicate the presence of membrane fractions enriched for Golgi membranes and possessing GS I activity, but still having significant amounts of GS II activity.

Effect of UDP-xylose substrate concentration

The effect of exogenous UDP-xylose on [^{14}C]glucose incorporation linkage was measured by the combined g.c.–r.p.c./g.c.–m.s. assay (Figure 5b). In these incubations, UDP-glucose concentration was kept at 0.7 μM and Mn^{2+} was included. The major incorporation product at all UDP-xylose concentrations was 4-Glc. All incubations in this experiment showed significant ^{14}C incorporation into a peak with a retention time corresponding to that of t-Gal (Figure 5b). The origin of these residues is unknown; however, similar incorporation from [^{14}C]glucose into t-Gal residues was reported by Gibeaut and Carpita (1990). Incorporation into t-Glc residues decreased to zero as UDP-xylose concentration increased to 50 μM, and incorporation into 4,6-Glc increased to a maximum of approx. 20% at 15 μM. Although the presence of ^{14}C-labelled 4,6-Glc is indicative of xyloglucan synthesis, most xyloglucans have approximately three 4,6-Glc residues for each 4-Glc residue, and none of these membrane fractions was capable of synthesizing polymers with a 4,6-Glc/4-Glc ratio of 3:1. It is clear that these membranes are not efficiently synthesizing glycosyl linkages associated with xyloglucan polysaccharides. Work is currently underway to optimize conditions for these activities.

Enzyme reaction products from glucan and xyloglucan glycosyltransferase assays are usually analysed by degradation with enzymes specific for the β-1,4 glucan backbone and subsequent separation of the hydrolysis products by paper, gel filtration or t.l.c. (Ray, 1980; Hayashi and Matsuda, 1981; Campbell et al., 1988; Hayashi, 1989b; Brummell et al., 1990). Previous results (Hayashi et al., 1987; Shea et al., 1989; Gibeaut and Carpita, 1990, 1991; White et al., 1993) have shown that direct analysis of ^{14}C incorporation into glycosyl linkages can be accomplished by a combination of g.c.–m.s. and r.p.c. The present study extends the use of this method to examine [^{14}C]glucose incorporation into glycosyl linkages by different membranes with a variety of substrate concentrations, cofactor concentrations and incubation conditions.

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REFERENCES

Baydoun, E. A.-H., Waldron, K. W. and Brett, C. T. (1989) Biochem. J. **257**, 853–858
Blakeney, A. B., Harris, P. J., Henry, R. J. and Stone, B. A. (1983) Carbohydr. Res. **113**, 291–299
Brummell, D. A., Camirand, A. and Maclachlan, G. A. (1990) J. Cell Sci. **96**, 705–710
Campbell, R. E., Brett, C. T. and Hillman, J. R. (1988) Biochem. J. **253**, 795–800
Carpita, N. C. and Shea, E. M. (1989) in Analysis of Carbohydrates by GLC and MS (Biermann, C. J. and McGinnis, G. D., eds.), pp. 157–216, CRC Press, Boca Raton, FL
Delmer, D. P. (1987) Annu. Rev. Plant Physiol. **38**, 259–290
Dhugga, K. S., Ulvskov, P., Gallagher, S. R. and Ray, P. M. (1991) J. Biol. Chem. **266**, 21977–21984
Faye, L., Johnson, K. D., Sturm, A. and Chrispeels, M. J. (1989) Physiol. Plant. **75**, 309–314
Gibeaut, D. M. and Carpita, N. C. (1990) Protoplasma **156**, 82–93
Gibeaut, D. M. and Carpita, N. C. (1991) Plant Physiol. **97**, 551–561
Gordon, R. and Maclachlan, G. (1989) Plant Physiol. **91**, 373–378
Griffing, L. R. (1991) J. Electron Microsc. Technique **17**, 179–199
Hall, J. L. and Moore, A. L. (eds.) (1988) Isolation of Membranes and Organelles from Plant Cells, Academic Press, New York
Hayashi, T. (1989a) Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 139–168
Hayashi, T. (1989b) Mod Methods Plant Analysis **10**, 138–160
Hayashi, T. and Matsuda, K. (1981) J. Biol. Chem. **256**, 11117–11122
Hayashi, T., Nakajima, T. and Matsuda, K. (1984) Agric. Biol. Chem. **48**, 1023–1027
Hayashi, T., Read, S. M., Bussell, J., Lin, F. C., Brown, R. M. and Delmer, D. P. (1987) Plant Physiol. **83**, 1054–1062
Levy, S. and Staehelin, L. A. (1992) Curr. Opin. Cell Biol. **4**, 856–862
Massey, V. (1959) Biochim. Biophys. Acta **34**, 255–256
McNeil, M., Darvill, A. G., Fry, S. C. and Albersheim, P. (1984) Annu. Rev. Biochem. **53**, 625–663
Quail, P. K. (1979) Annu. Rev. Plant Physiol. **30**, 425–484
Ray, P. M. (1979) in Plant Organelles (Methodological Surveys, vol. 9) (Reid, E., ed.), pp. 135–146, John Wiley and Sons, New York
Ray, P. M. (1980) Biochim. Biophys. Acta **629**, 431–444
Ray, P. M. (1985) Plant Physiol. **78**, 466–472
Ray, P. M., Shininger, T. L. and Ray, M. M. (1969) Proc. Natl. Acad. Sci. U.S.A. **64**, 605–612
Ray, P. M., Eisinger, W. R. and Robinson, D. G. (1976) Ber. Dtsch. Bot. Ges. **89**, 121–146
Robinson, D. G. and Kristen, U. (1982) Int. Rev. Cytol. **77**, 89–127

Rodgers, M. W. and Bolwell, G. P. (1992) Biochem. J. **288**, 817–822
Shea, E. M., Gibeaut, D. M. and Carpita, N. C. (1989) Planta **179**, 293–308
Spring, O. and Krauss, W. (1987) Plant Sci. **48**, 203–213
Talbott, L. D. and Ray, P. M. (1992) Plant Physiol. **98**, 357–368

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Van der Woude, W. J., Lembi, C. A., Morre, D. J., Kindinger, J. I. and Ordin, L. (1974) Plant Physiol. **54**, 333–340
Waldron, K. W. and Brett, C. T. (1987) Plant Sci. **49**, 1–8
White, A. R., Xin, Y. and Pezeshk, V. (1993) Physiol. Plant. **87**, 31–38