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Fucosylation of Xyloglucan: Localization of the Transferase in Dictyosomes of Pea Stem Cells[1]

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ANNE CAMIRAND*[2], DAVID BRUMMELL, AND GORDON MACLACHLAN
Department of Biology, McGill University, 1205 Avenue du Docteur Penfield, Montréal, Québec, Canada H3A 1B1

ABSTRACT

Microsomal membranes from elongating regions of etiolated *Pisum sativum* stems were separated by rate-zonal centrifugation on Renografin gradients. The transfer of labeled fucose and xylose from GDP-[^{14}C] fucose and UDP-[^{14}C]xylose to xyloglucan occurred mainly in dictyosome-enriched fractions. No transferase activity was detected in secretory vesicle fractions. Pulse-chase experiments using pea stem slices incubated with [^{3}H]fucose suggest that xyloglucan chains are fucosylated and their structure completed within the dictyosomes, before being transported to the cell wall by secretory vesicles.

The enzymes responsible for synthesis of the xylosylated (1,4)-β-glucan backbone $(Glc_4Xyl_3)_n$ of xyloglucan chains in the cell walls of higher plants have been localized by density-gradient centrifugation of tissue homogenates and correlated with membrane markers for the Golgi apparatus (13, 21). In order to synthesize the complete xyloglucan structure which in pea stems consists of heptasaccharides (Glc_4Xyl_3) alternating with nonasaccharide subunits (Glc_4Xyl_3Gal Fuc) (9, 11), galactose and fucose residues must be added to the polysaccharide chain. The presence of all of the required glycosyltransferases for nonasaccharide biosynthesis has been demonstrated in pea membrane preparations (3). However, the subcellular location of the final reactions which add galactose and fucose is presently unknown, although the secretion of extracellular fucose-containing polysaccharide slime in maize root cap cells appears to be accomplished within the Golgi apparatus (20). Nevertheless, complete biosynthesis of xyloglucan chains could occur within the Golgi dictyosomes or, alternatively, terminal side-chain residues could be added to xyloglucan molecules elsewhere in the cell, for example in secretory vesicles or at the plasma membrane where cellulose is assembled (6).

Plant noncellulosic polysaccharides are carried from the Golgi apparatus via vesicular transport to the exterior of the cell where they become incorporated into the growing cell wall (22, 23). Electron microscope observations of xyloglucan-synthesizing *Tropaeolum* cotyledon cells revealed that amyloid material was present in both dictyosomes and secretory vesicles, the latter fusing with the plasma membrane (14). In agreement with this secretion model is the report by Chrispeels (5) that monensin, a sodium ionophore interfering with Golgi transport in animal systems, severely inhibited the incorporation of galactose- and fucose-containing macromolecules into the cell wall of bean cotyledons. Similarly, an inhibition of matrix polysaccharide secretion occurred in pea stem cells after treatment of the tissues with monensin (2) or with the potassium ionophore nigericin, in which case it was accompanied by an accumulation of vesicles in the cytoplasm (8). In the present study, we investigate the subcellular localization of xyloglucan fucosyltransferase, and the secretion of fucose-labeled polysaccharide to the cell wall by using a rate-zonal centrifugation technique to separate the main Golgi membrane stacks (dictyosomes) from the lighter secretory vesicles (23).

MATERIALS AND METHODS

Materials. Guanosine diphospho-L-[U-^{14}C]fucose (8.03 GBq/mmol, *i.e.* 217 mCi/mmol) was obtained from Amersham; L-[6-^{3}H]fucose (3.2 TBq/mmol, *i.e.* 86.6 Ci/mmol), uridine diphospho-D-[U-^{14}C]xylose (9.88 GBq/mmol, *i.e.* 267 mCi/mmol), Amplify and Aquasol II were from New England Nuclear. Bio-Gel P-2 (200–400 mesh) and all materials for electrophoresis were from Bio-Rad, Renografin (76%) from Squibb, and α-chymotrypsin (bovine pancreas) from Sigma. *Streptomyces griseus* endocellulase was a gift from Dr. E. T. Reese, U.S. Army Laboratories, Natick, MA, and tamarind seed xyloglucan was obtained from Drs. T. Hayashi and K. Matsuda, Tohoku University, Sendai, Japan.

***In Vitro* Labeling.** Stem segments (1 cm in length) were cut from below the hook of 1-week-old etiolated *Pisum sativum* var. Alaska plants grown in vermiculite. The segments were homogenized in batches of 200 in an ice-cold mortar for 1 min with 15 ml buffer A (0.1 M Hepes/KOH buffer (pH 7.0), 1 mM EDTA, 1 mM DTT, 0.4 M sucrose, and 0.1% BSA). The preparation was filtered through Miracloth and centrifuged at 1,000*g* for 10 min. The supernatant was layered on top of a linear 20 to 35% Renografin gradient containing 10 mM Hepes/KOH (pH 7.0), 1 mM EDTA, and 1 mM DTT. After centrifugation (1 h at 4°C, 100,000*g* on an IEC B-60 ultracentrifuge with SB 283 rotor), 12 1-ml fractions were collected from the top using a 1-ml automatic pipette. The individual fractions were centrifuged in an Eppendorf microfuge at 15,600*g* for 1 h at 4°C. The refractive index of each Renografin supernatant was determined with a Bausch and Lomb refractometer and the membrane pellets resuspended in 250 μl of buffer B (Hepes/KOH 0.1 M (pH 7.0), 10 mM $MnCl_2$, 0.6 mM EDTA, 0.6 mM DTT, 0.25 M sucrose, and 0.06% BSA) (12).

Aliquots (10 μl) of each of the 12 microsomal suspensions were kept for marker enzyme assays (see below), and 200 μl were used for incubation with GDP-[^{14}C]fucose (1.0 μM) or UDP-[^{14}C]

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[2] Present address: Département de Biochimie, Faculté de Médecine, Université de Montréal, C.P. 6128, Succursale A, Montréal, QC, Canada, H3C 3J7.

xylose (0.5 μM), in the presence of the following unlabeled sugar nucleotides: UDP-glucose (2 mM), UDP-xylose (20 μM), and UDP-galactose (20 μM). The reactions were left to proceed at 20°C for 30 min, after which time the tubes were boiled for 5 min and 100 μg of unlabeled tamarind xyloglucan added as a carrier. The mixtures were extracted 4 times with 1 ml 70% ethanol. Finally, the air-dried insoluble residues were resuspended in 0.5 ml of 50 mM K acetate buffer (pH 5.0) and digested with *S. griseus* endocellulase (0.4 mg/tube) for 48 h at 37°C under toluene. After boiling, the digests were separated on a 1 × 95 cm Bio-Gel P-2 column and 1-ml fractions collected. Predigestion aliquots of resuspended pellets were kept and extracted with 0.5 ml 24% KOH containing 0.1% $NaBH_4$ for 30 min at room temperature. After centrifugation, the supernatants were neutralized and counted for radioactivity.

For polyacrylamide electrophoresis of fucose-labeled compounds, reaction mixtures were extracted with 2% SDS according to techniques described by Hayashi and Maclachlan (10). Solubilized fucose-labeled products were precipitated with 80% acetone on ice, the pellets resuspended in 1 ml 0.1 M Tris-HCl (pH 7.4) buffer, and aliquots digested with 0.25 mg α-chymotrypsin for 15 min. Reactions were stopped by acetone precipitation, and pelleted products were solubilized in 4 mM Tris-HCl buffer (pH 6.8) containing 0.1 M DTT, 2.0% SDS, 10% glycerol, and 2 mM bromophenol blue. Samples of chymotrypsin-digested compounds and nondigested controls were run on 1.5-mm thick 10% acrylamide slab gels for 3 h at 30 to 40 mamp (16), after which the gels were stained with 0.1% Coomassie blue R-250 in methanol/water/acetic acid 45:45:10 overnight. The gels were destained in several changes of methanol/water/acetic acid 45:5:1 and photographed. They were then fixed with Amplify for 1 h, dried under vacuum for 1 h at 80°C and exposed to pre-flashed X-AR5 film (Kodak) for 1 week at −70°C. The fluorograms were developed and photographed.

***In Vivo* Labeling.** Stem segments were collected as described above. Each 1-cm segment was cut into six slices, which were combined into batches of 600 slices, washed once with buffer A containing 10 mM $MnCl_2$, and incubated in another 4 ml of the same buffer plus 40 μCi L-[6-^{3}H]fucose. The containers were placed on a rotary plate shaker. After various periods of incubation, slices were rinsed 3 times with ice-cold water, then tissues were homogenized (30 strokes) with mortar and pestle in buffer A at 4°C, and the homogenate filtered through Miracloth. The residue was washed 3 times with water at room temperature, then 2 times with 70% ethanol, and air-dried overnight. This material was resuspended in a mixture of water and Aquasol II (2:5, v/v) and counted as a gel suspension in a Beckman CPM-100 scintillation counter. These counts represented radioactivity incorporated into cell walls. The Miracloth filtrate was centrifuged 5 min at 1000*g*, then the supernatant was layered over a 20 to 35% Renografin gradient and centrifuged as described above. The gradient was fractionated into 12 1-ml fractions which were recentrifuged in an Eppendorf microfuge at 15,600*g* for 1 h. Pelleted membranes were resuspended in 50 mM K-acetate buffer (pH 5.0) and aliquots counted for radioactivity. Other aliquots were used for cellulase digestions, as above.

For pulse-chase experiments, the buffer containing labeled fucose was removed rapidly after the initial 30 min incubation period. Slices were rinsed 3 times with water at room temperature, and incubated in 4 ml buffer containing 50 mM unlabeled fucose. The containers were returned to the shaker plate for various periods of chase. Reactions were stopped by removing the medium and washing the slices 3 times with ice-cold water, after which the slices were homogenized and centrifuged on Renografin as above.

Marker Enzyme Assays. Latent UDPase assays (± Triton X-100) were conducted according to Nagahashi and Kane (19), vanadate-sensitive ATPase assays according to Gallagher and Leonard (7) and inorganic phosphate determinations for these assays according to Ames (1). NADH cytochrome *c* reductase and cytochrome *c* oxidase were according to Lord (17) and Moore and Proudlove (18), respectively.

RESULTS AND DISCUSSION

***In Vitro* Experiments.** Figure 1A shows the distribution of the activity of the Golgi marker enzyme, latent UDPase, in mem-

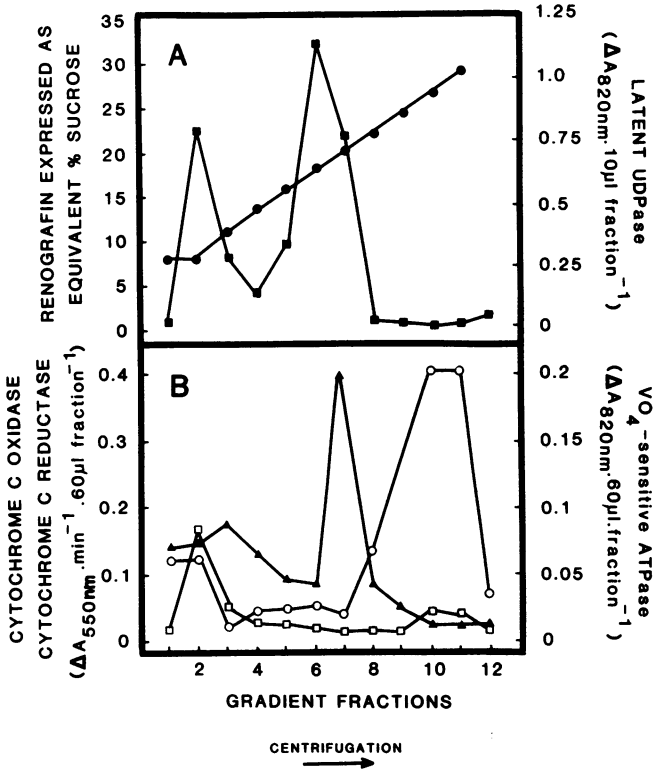

FIG. 1. Distribution of marker enzyme activities on Renografin gradients. (A) Latent UDPase (■) and gradient density (●) expressed as percent equivalent sucrose. (B) Location of other subcellular markers; NADH cytochrome *c* reductase (□), vanadate-sensitive ATPase (▲), and cytochrome *c* oxidase (○).

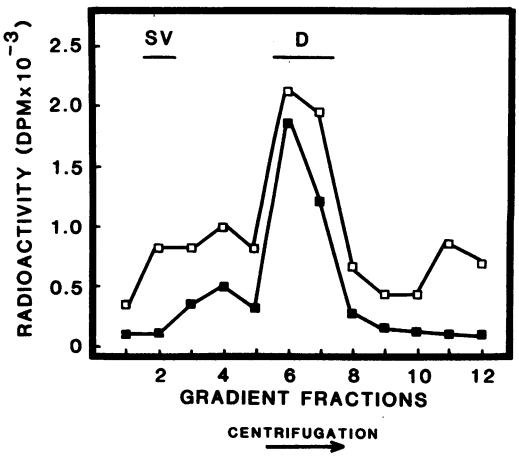

FIG. 2. Distribution of glycosyltransferase activities on Renografin gradients; label incorporated into ethanol-insoluble, alkali-soluble material from UDP-[^{14}C]xylose (□), and GDP-[^{14}C]fucose (■). Bars indicate location of secretory vesicles (SV) and dictyosome-enriched (D) fractions.

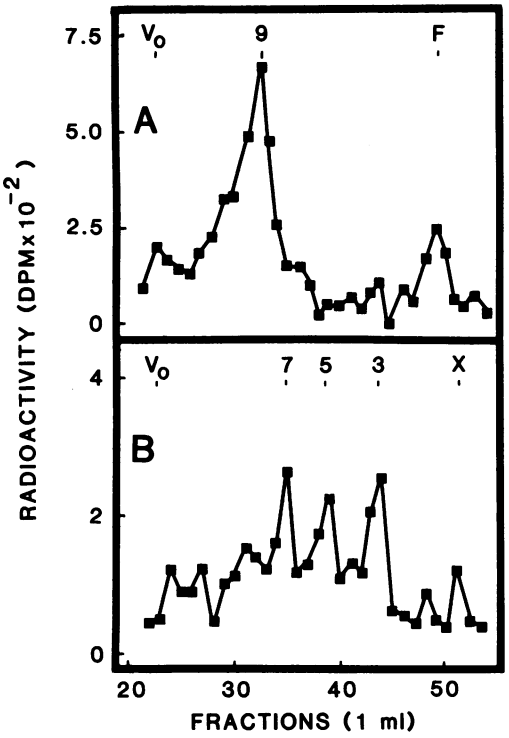

FIG. 3. Fractionation on a Bio-Gel P-2 column of cellulase-digested labeled products synthesized by dictyosome-enriched fractions. Incubations were conducted with GDP-[^{14}C]fucose (A), or UDP-[^{14}C]xylose (B). Size markers: 9, nonasaccharide; 7, heptasaccharide; 5, pentasaccharide; 3, trisaccharide; F, fucose; X, xylose.

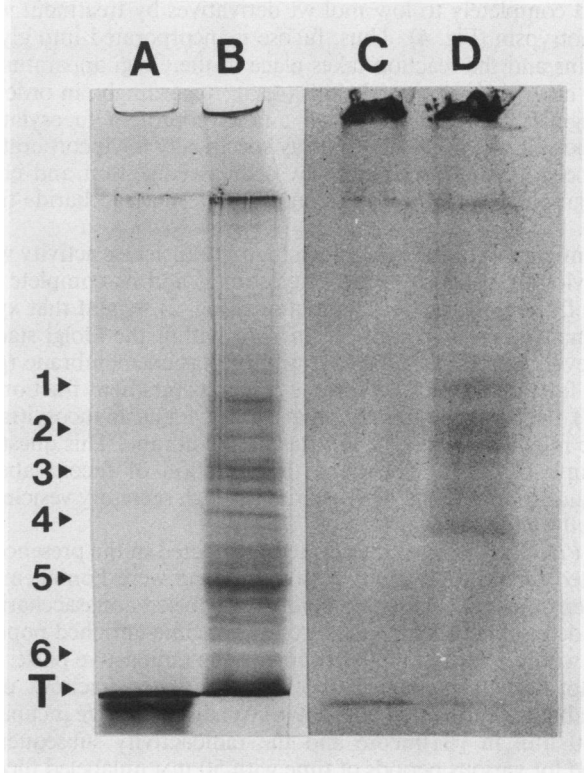

FIG. 4. SDS-polyacrylamide gel migration pattern of components obtained from pea microsomes incubated for 30 min in the presence of GDP-[^{14}C]fucose. (A) SDS-soluble microsomal extract digested with chymotrypsin. (B) Control (no digestion). (C) Fluorograph of (A). (D) Fluorograph of (B). Size markers: (1) 66.0; (2) 45.0; (3) 34.7; (4) 24.0; (5) 18.4; (6) 14.3 kD. (T) Thymol blue.

brane fractions obtained after centrifugation on Renografin gradients. The two peaks of this enzyme activity correspond to pea stem membrane populations separated by rate-zonal centrifugation on Renografin gradients by Taiz *et al.* (23). These workers determined through pulse-chase experiments, electron microscopy of the membranes, as well as by the presence of latent IDPase and the presence or absence of glucan synthase I activity, that these light and heavy membrane peaks were enriched in secretory vesicles and dictyosomes respectively. In our studies, the light peak occurred in gradient fraction 2 and the heavy peak in gradient fractions 6 and 7. The distribution of enzyme markers for endoplasmic reticulum (NADH cytochrome *c* reductase), plasma membrane (vanadate-sensitive ATPase), or mitochondria (cytochrome *c* oxidase) in our gradients was very constant in all experiments and closely resembles the results of Taiz *et al.* (23) (Fig. 1B).

When aliquots of membranes pelleted from individual gradient fractions were resuspended in buffer and incubated in the presence of either UDP-[^{14}C]xylose or GDP-[^{14}C]fucose, the incorporation of both radioactive xylose and fucose into ethanol-insoluble, alkali-soluble products comigrated almost exclusively with the heavier latent UDPase activity, *i.e.* the dictyosome population (Fig. 2). Digestion of fucose-labeled molecules from these fractions with *Streptomyces* cellulase produced low mol wt radioactive fragments. On a Bio-Gel P-2 column, these digestion products coeluted mainly with authentic xyloglucan nonasaccharide (75% of label) found in column fractions 30 to 33, and a small amount (15%) was fucose (Fig. 3A). Xylose-labeled digestion products showed the hepta, penta, and trisaccharide pattern typical of xyloglucan digests (Fig. 3B) (see also Refs. 3, 11, 12). Pea microsomes also incorporated fucose from GDP-[^{14}C]fucose into high mol wt products that were not digestible to smaller fragments by cellulase treatment. These products were mobile during SDS-gel electrophoresis and they could be con-

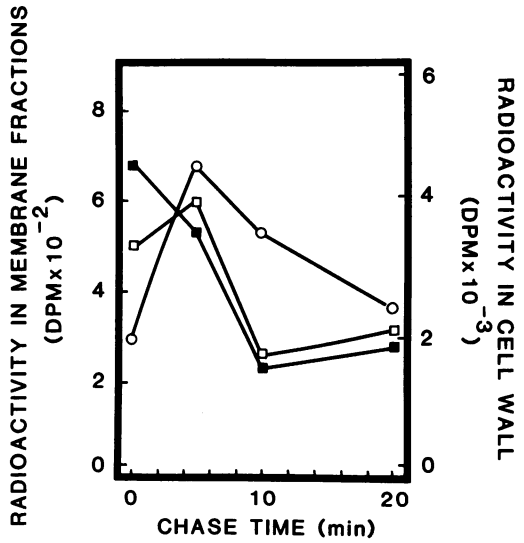

FIG. 5. Kinetics of secretion of fucosylated xyloglucan in pea stem slices. After an initial 30-min pulse with [^{3}H]fucose, the accumulated radioactivity was chased for various periods of time with 50 mM unlabeled fucose. Label was assayed in xyloglucan nonasaccharides isolated from dictyosomes (■), secretory vesicles (□); and in cell wall fractions (○). The data shown are representative of profiles obtained in three separate experiments.

verted completely to low mol wt derivatives by treatment with chymotrypsin (Fig. 4). Thus, fucose is incorporated into glycoproteins and the reaction takes place in the Golgi apparatus, as is the case with bean cotyledons (4, 5). Accordingly, in order to distinguish between xyloglucan and glycoprotein fucosylation products, it was necessary to assay specifically for incorporation of label into nonasaccharides by cellulase digestion and chromatographic separation of the appropriate polysaccharide fragment.

Comigration of the xyloglucan fucosyltransferase activity with the xylosyltransferase in the dictyosomes, and its complete absence from secretory vesicle fractions (Fig. 2) suggest that xyloglucan structure is completed *in vitro* within the Golgi stacks. However, because of the proximity of plasma membrane (gradient fraction 7) and dictyosome fractions (gradient fractions 6 and 7), the possibility remains that some xyloglucan fucosyltransferase might be located at the plasma membrane. This question was investigated by following the secretion of fucose-labeled xyloglucan from Golgi dictyosomes through secretory vesicles to the cell wall *in vivo*.

***In Vivo* Experiments.** Stem slices incubated in the presence of tritiated fucose for various periods of time were homogenized and separated on Renografin gradients. Labeled nonasaccharides were detectable in hydrolysates of dictyosome-enriched populations within 5 min of the beginning of the radioactive pulse, but did not begin to appear in the vesicle-enriched fractions until after about 10 min (data not shown). When slices were incubated for 30 min in [^{3}H]fucose and the radioactivity subsequently chased for various periods of time with 50 mM unlabeled fucose, tritium in nonasaccharides decreased rapidly in the dictyosome-enriched populations from the beginning of the chase period (Fig. 5). The vesicle-enriched population, however, continued to accumulate label in nonasaccharides for approximately 5 min before a rapid reduction in their levels of nonasaccharides was initiated. The observations that nonasaccharides appear in dictyosomes before secretory vesicles upon pulse and can be chased from dictyosomes to secretory vesicles suggest that the major proportion, perhaps all, of the xyloglucan molecule is fucosylated by the time it is transported to the plasma membrane. However, the possibility that plasma membrane possesses some fucosyltransferase activity cannot be entirely discounted. Radioactivity in the cell wall increased immediately upon the start of the chase and reached a maximum after 5 min, after which it decreased slowly with time.

This pattern of chase of fucosylated xyloglucan in pea stem slices is consistent with vesicular transport of this polysaccharide from dictyosomes to the cell wall. A similar exocytosis pathway has been reported for wall polysaccharides containing glucose (22, 23) or arabinose (15). A time lag of about 5 min for chase of the labeled xyloglucan through vesicles to the wall is similar to that found for glucose-labeled matrix polysaccharides in pea cells (22). The discrepancy in the amount of radioactivity chased from the membrane fractions into the cell wall was probably due to differences in recovery of intact dictyosomes and secretory vesicles compared with the greater yield of cell wall material. This observation has also been made in studies using labeled glucose (22). The decrease with time of label in the cell wall fractions indicates that newly secreted xyloglucan is metabolically unstable in the pea stem slice system, possibly due to hydrolytic enzyme activity in the wall.

It is concluded that the complete xyloglucan molecule, including fucose, is formed in Golgi dictyosomes and that the secretory vesicles perform a transport rather than a synthetic function for this polysaccharide.

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