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Xyloglucan Galactosyl- and Fucosyltransferase Activities from Pea Epicotyl Microsomes[1]

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Microsomal membranes from growing tissue of pea (*Pisum sativum* L.) epicotyls were incubated with the substrate UDP-[^{14}C]galactose (Gal) with or without tamarind seed xyloglucan (XG) as a potential galactosyl acceptor. Added tamarind seed XG enhanced incorporation of [^{14}C]Gal into high-molecular-weight products (eluted from columns of Sepharose CL-6B in the void volume) that were trichloroacetic acid-soluble but insoluble in 67% ethanol. These products were hydrolyzed by cellulase to fragments comparable in size to XG subunit oligosaccharides. XG-dependent galactosyltransferase activity could be solubilized, along with XG fucosyltransferase, by the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate. When this enzyme was incubated with tamarind (*Tamarindus indica* L.) seed XG or nasturtium (*Tropaeolum majus* L.) seed XG that had been partially degalactosylated with an XG-specific β-galactosidase, the rates of Gal transfer increased and fucose transfer decreased compared with controls with native XG. The reaction products were hydrolyzed by cellulase to ^{14}C fragments that were analyzed by gel-filtration and high-performance liquid chromatography fractionation with pulsed amperometric detection. The major components were XG subunits, namely one of the two possible monogalactosyl octasaccharides (-XXLG-) and digalactosyl nonasaccharide (-XLLG-), whether the predominant octasaccharide in the acceptor was XXLG (as in tamarind seed XG) or XLXG (as in nasturtium seed XG). It is concluded that the first xylosylglucose from the reducing end of the subunits was the Gal acceptor locus preferred by the solubilized pea transferase. These observations are incorporated into a model for the biosynthesis of cell wall XGs.

All XG chains analyzed to date contain 1,2-β-linked Gal attached to particular Xyl side chains that are linked 1,6-α- to a 1,4-β-glucan (cellulosic) backbone. These Gal units may terminate the side chains as in the storage XG of many seeds, e.g. tamarind (*Tamarindus indica L.*) and nasturtium (*Tropaeolum majus* L.), or they may be fucosylated by 1,2-α-linked Fuc, as in the structural XG of many dicot primary walls, e.g. pea (*Pisum sativum* L.), soybean, and sycamore. In this paper we focus on the detection and solubilization of XG galactosyltransferase activity from microsomes of growing regions of the pea epicotyl. To our knowledge, the only published report of the direct assay of this potentially important enzyme activity is a recent thesis by Chileshe (1995), which provided the starting point for this paper.

XGs are composed of chains of the repeating subunit heptasaccharide Glc_4Xyl_3, more precisely designated -XXXG- (Fry et al., 1993), where X = xylosylglucose (isoprimeverose) and G is Glc at the reducing end. Gal is attached to one or both of the two Xyl units closest to the reducing end to form two possible monogalactosylated octasaccharide subunits (-XLXG- or -XXLG-) and/or a digalactosylated nonasaccharide (-XLLG-). Although elongation of the XG backbone can proceed in vitro without concurrent galactosylation (Gordon and Maclachlan, 1989), the incorporation of Gal in vivo appears to follow soon after chain elongation in the *trans*-Golgi cisternae (Brummell et al., 1990; Zhang and Staehelin, 1992). The addition of Gal interferes with self-association by chains of the XG backbone and prevents its precipitation from aqueous solution (Reid et al., 1988). Galactosylation may be completed before the final decoration with Fuc takes place in the *trans*-Golgi network (Zhang and Staehelin, 1992). The addition of Fuc generates subunits with side chains containing three sugars, a structure that facilitates binding of XG to cellulose microfibrils (Levy et al., 1991, 1996).

PXG was first thought to be composed almost exclusively of alternating heptasaccharide and nonasaccharide in a repeating dimer $(-XXXG.XXFG-)_n$ (Hayashi and Maclachlan, 1984a), since this dimer was the chief intermediate that was formed transiently during cellulase hydrolysis, and equal amounts of the hepta- and nonasaccharides are always major components in complete digestion mixtures. Recent studies (Guillén et al., 1995) have also detected the presence of small amounts of other subunits, including two octasaccharides and the decasaccharide -XLFG-. Thus, pea microsomes must contain XG galactosyltransferase activity that preferentially galactosylates the nascent XG backbone at every second heptasaccharide to form a transient repeating unit (-XXXG.XXLG-) in the backbone, which is then fucosylated.

Studies of pea microsomes (Gordon and Maclachlan, 1989; White et al., 1993) have confirmed results from earlier studies of soybean and French bean microsomes (Hayashi

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PXG, TXG, NXG, xyloglucans derived from growing pea epicotyl, tamarind seed, and nasturtium seed, respectively; TFA, trifluoroacetic acid; V_o, void volume of a chromatography column; V_t, total volume of a chromatography column; XG, xyloglucan. Oligosaccharide subunits of XG are abbreviated according to the nomenclature proposed by Fry et al. (1993).

and Matsuda, 1981; Campbell et al., 1988), which demonstrated that continued elongation of the XG backbone to form a structure with repeating subunits requires cooperativity between XG glucosyl- and xylosyltransferases. Some XG chain elongation may occur in the presence of UDP-Glc alone, with subsequent xylosyl transfer to the β-glucan extension (Ray, 1980), but the fact that Glc incorporation is enhanced by UDP-Xyl and vice versa implies that their transferases act in vitro concurrently and interdependently to generate the observed regularity of the pea XG backbone. It is not known whether XG galactosyl transfer is coupled to the process of chain elongation in vivo.

It is established, however, that PXG fucosyltransferase can act independently of other XG glycosyltransferases to add Fuc to preformed acceptor XG chains in the medium in which microsomes are suspended (Camirand and Maclachlan, 1986). PXG fucosyltransferase has been solubilized from microsomal extracts with the detergent CHAPS, using added TXG (Hanna et al., 1991) or a cellulase-resistant trimer from TXG (Maclachlan et al., 1992) as a fucosyl acceptor. Free XG monomer subunits do not act as fucosyl acceptors, but, on the contrary, as competitive inhibitors of fucosyl transfer to longer chains (Farkas and Maclachlan, 1988). This last study also showed that fucosyltransferase activity in pea microsomes was distinctly enhanced when UDP-Gal was added to the reaction medium, which may mean that galactosylation of nascent PXG could take place in these preparations to form new sites where further fucosylation could then occur.

To demonstrate directly that [^{14}C]Gal is incorporated from UDP-[^{14}C]Gal into XG, a high-M_r product must be identified that is hydrolyzed by purified endo-1,4-β-glucanase (E.C. 3.2.1.4) to authentic XG subunit oligosaccharides. These can be detected tentatively by their elution volumes after gel filtration, e.g. on Bio-Gel columns (Hayashi and Maclachlan, 1984a; Maclachlan et al., 1992; Guillén et al., 1995), and, more precisely, by their elution patterns from HPLC columns (McDougall and Fry, 1990; Buckeridge et al., 1992; Vincken et al., 1995) and their NMR spectra (York et al., 1990, 1993).

In the present study the optimal detergent level that solubilized PXG fucosyltransferase activity (0.3% [w/v] CHAPS) also extracted XG galactosyltransferase activity. However, TXG, although an effective acceptor for fucosyl transfer, was a relatively poor acceptor for galactosyl transfer. This was not surprising, since seed XG is already highly galactosylated (York et al., 1990; Maclachlan et al., 1992). It proved necessary to partially degalactosylate seed XG with an XG-specific β-galactosidase from nasturtium seeds (Edwards et al., 1988) to convert it to an acceptor for PXG galactosyltransferase that was sufficiently effective to identify which Xyl units were galactosylated.

MATERIALS AND METHODS

Chemicals

UDP-D-[U-^{14}C]Gal (11.5 GBq mmol^{-1}) and GDP-L-[U-^{14}C]Fuc (8.3 GBq mmol^{-1}) were purchased from New England Nuclear-Dupont. BSA, CM-Sephadex, CHAPS, DEAE-cellulose, dextrans, β-galactosidase (E.C. 3.2.1.23) from various organisms, leupeptin, PMSF, and pronase were from Sigma. Purified (HPLC grade) NaOH (50% [w/w]) was from Fisher Scientific, and anhydrous sodium acetate was from Fluka. Sephadex G-10, Sepharose CL-6B, and CM-Sephadex column supports were from Pharmacia. CarboPac PA-100 columns for HPLC were supplied by Dionex (Sunnyvale, CA). Partially purified cellulase from *Trichoderma* sp. (1% suspension in dilute $[NH_4]_2SO_4$) and TXG were purchased from Megazyme (Sydney, Australia). NXG was prepared from *Tropaeolum majus* L. var Climbing Giant Tall Forest, as described by Edwards et al. (1985).

Plant Materials

All studies were conducted with microsomes isolated from seedlings of garden pea (*Pisum sativum* L. var Alaska) purchased from Rogers Bros. Seed Co. (Boise, ID). Seeds were washed in 10% (v/v) commercial $NaClO_4$ for 10 min, rinsed in water, and soaked overnight. They were germinated in moist vermiculite in the dark for 7 to 8 d at 22°C until the third internodes were 3 to 5 cm long. The apical 2 to 3 cm of internodes cut just below the hook were used as the source of microsomes.

Membrane Preparation and Solubilization

Pea microsomal membranes were prepared using procedures described previously with minor changes (Camirand and Maclachlan, 1986; Gordon and Maclachlan, 1989). Stem segments were weighed and homogenized with a blender (twice for 30 s) in cold extraction buffer (2 volumes of 0.1 M Pipes-KOH, pH 6.2, 0.4 M Suc, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM DTT, 1 μM leupeptin, 0.1% [w/v] BSA, and 1 mM PMSF dissolved in *n*-propanol). The mixture was filtered through nylon cloth and the filtrate was centrifuged (5000*g* for 10 min) to remove cell wall debris and unbroken cells. The supernatant was then microcentrifuged (model L8–80, Beckman) at high speed (100,000*g*) with angled (80 Ti) or swinging bucket (SW41) rotors for 60 min at 4°C. Membranes in the pellet, referred to here as microsomes, were resuspended in an extraction buffer (1.0 mL per pellet from 10 g fresh weight of segments). Protein concentrations were determined using an assay kit (Bio-Rad) with BSA as the standard. Yields were approximately 5 mg protein mL^{-1}.

Protein was extracted from pea microsomes with CHAPS detergent according to the method of Hanna et al. (1991) for solubilizing PXG fucosyltransferase. Aliquots of microsome suspension prepared as above were mixed with 1 volume of 0.6% (w/v) CHAPS in the extraction buffer and incubated with gentle stirring in the cold for 30 min. The slurry was centrifuged (100,000*g* for 60 min at 4°C), leaving solubilized protein in the supernatant. Approximately one-third of the original microsomal protein was extracted by this procedure at a concentration of about 0.8 mg protein mL^{-1}.

Partial Degalactosylation of XG

The standard reaction mixture for degalactosylating TXG or NXG contained 0.1 g of XG, 20 μg of nasturtium β-galactosidase partially purified by ion-exchange chromatography, as described by Edwards et al. (1988), and 10 mL of 50 mM sodium acetate buffer, pH 5.0. Fractions removed after different incubation times at 45°C were boiled to inactivate β-galactosidase and passed through columns of Sepharose CL-6B using 0.1 N NaOH and 0.01% $NaBH_4$ as the eluant, as described below. High-M_r XG fractions that eluted between the V_o and the elution peak of 500,000-D dextran were combined and neutralized, and XG was precipitated by the addition of 2 volumes of ethanol. Aliquots were dissolved in water and tested for their ability to act as acceptors for XG galactosyltransferase activity. Estimations of free sugars in TFA hydrolysates of the treated XG preparations were made by HPLC as described below. Approximately 7% of total Gal in TXG was lost by 6 h of incubation in the above reaction mixture, and 17% of Gal in NXG was lost by 1 h. Both preparations began to gel when the incubations were prolonged, which confirms the observation of Reid et al. (1988) that galactosylation prevents XG from self-association.

Assays for Transferase Activities

For preliminary tests using native intact XG samples as acceptors for glycosyl transfer, methods were based on those developed by Chileshe (1995) from earlier studies of pea microsomes (Farkas and Maclachlan, 1988) or solubilized microsomal protein (Hanna et al., 1991). Relatively high concentrations of ADP-Rib were included in reaction mixtures to inhibit UDP-Gal:UDP-Glc epimerase, as described previously (Hayashi and Maclachlan, 1984b). Reaction mixtures contained 100 μL of enzyme in the form of either a microsomal suspension (approximately 0.5 mg of protein) or a solubilized protein (approximately 0.1 mg of protein), prepared as described above in extraction buffer with and without CHAPS detergent. This was diluted with 70 μL of extraction buffer supplemented with 0.25 mM ADP-Rib, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ with and without 20 μL of 1% XG (200 μg). Reactions were initiated by the addition of 10 μL of stock UDP-[^{14}C]Gal or GDP-[^{14}C]Fuc, which represented a final concentration of approximately 3 μM sugar nucleotide containing 450,000 dpm, as assayed by liquid-scintillation spectroscopy. Assays were conducted at room temperature until terminated by the addition of 1 volume of 20% TCA (w/v) and chilling the mixture to 4°C. After the sample was centrifuged at low speed to precipitate protein and glycoprotein, the pellet was washed repeatedly with 67% ethanol containing 1 mM EDTA to remove unused substrate or its degradation products that might have precipitated (Saugy et al., 1988). The supernatant was brought to 67% ethanol and chilled to −20°C to precipitate XG, which was washed with 67% ethanol.

In later tests, solubilized microsomal protein was concentrated by centrifugation in a filtration device (Microcon 10, Amicon), and total volumes of reaction mixtures were reduced to 50 μL containing 15 to 30 μg of protein. The buffer was replaced with Hepes-KOH, pH 7.0, which was closer to the optimum pH of solubilized XG galactosyltransferase. Mg^{2+} was omitted from reaction mixtures, since there was no indication that it was required, but Mn^{2+} was included because EDTA inhibited XG galactosyltransferase and Mn^{2+} was the only divalent cation that could overcome this inhibition. Other ingredients were included at the same concentrations as in earlier tests. Reactions were terminated by adding ethanol to a concentration of 67% and chilling to −20°C. Pellets containing XG were resuspended in 50 mM NaOH and reprecipitated four times with 2 volumes of ethanol.

Characterization of Products Synthesized by Pea Enzyme

In tests to examine the size and label distribution of products generated from UDP-[^{14}C]Gal, several standard reaction mixtures were combined to assay ^{14}C and carbohydrates by column chromatography. To ensure that all unused UDP-[^{14}C]Gal had been washed from reaction mixtures before gel chromatography, they were first passed through a column containing 1 mL of DEAE-cellulose. Gel filtration was conducted using columns of Sepharose CL-6B (1.1 × 100 cm), equilibrated, and eluted with degassed 0.1 N NaOH and 0.01% $NaBH_4$ at room temperature. This column gave an estimate of apparent chain length of polysaccharide up to the equivalent of dextran, with a molecular mass of 10^6 D (V_o). Alternatively, fractionation on columns of Bio-Gel P_2 (1.1 × 126 cm) with 0.01% NaN_3 as the eluant separated products in the size range of XG subunit oligosaccharides. The latter were prepared from XG by digestion with freshly prepared *Trichoderma* sp. cellulase (0.5 mg mL^{-1}) in 50 mM sodium acetate buffer, pH 5.8, at 40°C for 16 h. Reactions were terminated by boiling for 15 min.

Fractions containing labeled oligosaccharide peaks that eluted from Bio-Gel P_2 columns were pooled and aliquots were analyzed further by HPLC using a CarboPac PA-100 column. Samples containing up to 10 μg of oligosaccharides were fractionated in 0.1 N NaOH containing 60 mM sodium acetate degassed with helium, and profiles were recorded automatically as they emerged from the column (flow rate 0.8 mL min^{-1}) by a pulsed amperometric detector. When ^{14}C distribution was to be determined, repeated aliquots containing 10 μg of oligosaccharides were fractionated until a total of at least 3000 dpm had been passed through the column. The fractions containing origin material or oligosaccharide peaks were collected manually, combined, neutralized, and counted separately. Free sugars were generated from samples of XG or oligosaccharides by total hydrolysis in 2 M TFA at 110°C in closed tubes for 90 min. Acid was removed by evaporation, and approximately 5 μg of total sugars was fractionated through a CarboPac PA-100 column using 12 mM NaOH for elution. Label distribution was determined as above.

Table I. *Capacities of pea epicotyl microsomes and 0.3% CHAPS extracts of microsomes to incorporate label from UDP-[^{14}C]Gal or GDP-[^{14}C]Fuc into TCA-soluble and -insoluble products*

TXG was added to all reaction mixtures as a potential acceptor for [^{14}C]glycosyl transfer. Reactions were terminated at 30 min by adding TCA to a final concentration of 10% (v/v). This left the polysaccharide soluble (including added TXG), but the protein precipitated (including glycoprotein). The products were washed repeteadly in 67% ethanol to remove unused substrate. Values are the average ± SE of four separate determinations.

Enzyme	Substrate	^{14}C Incorporation	
		TCA-soluble	TCA-insoluble
		dpm min^{-1} mg^{-1} protein	
Microsomes	UPD-[^{14}C]Gal	128 ± 20	475 ± 17
	GDP-[^{14}C]Fuc	271 ± 4	132 ± 1
Solubilized	UDP-[^{14}C]Gal	692 ± 46	133 ± 7
	GDP-[^{14}C]Fuc	1620 ± 13	631 ± 19

RESULTS

Incorporation of Gal and Fuc from Sugar Nucleotides into XG by Pea Microsomes

Microsomal membranes from 1-week-old pea epicotyls, or extracts of microsomes with the detergent CHAPS, were incubated with UDP-[^{14}C]Gal or GDP-[^{14}C]Fuc in the presence of TXG, and the amount of label incorporated into 67% (v/v) ethanol-insoluble products in 30 min was measured. Table I shows results from a typical test in which products were first separated into those that were insoluble in 10% (w/v) TCA (e.g. glycoprotein) and those that were soluble (uncharged polysaccharide, including the added TXG). The microsomes incorporated more ^{14}C from Fuc into TCA-soluble than TCA-insoluble products, which is in keeping with earlier observations that pea microsomes not only fucosylate their own XG more extensively than endogenous glycoprotein (Camirand and Maclachlan, 1986), but also fucosylate exogenously supplied TXG (Farkas and Maclachlan, 1988). Under the same conditions the microsomes were relatively ineffectual at incorporating ^{14}C from Gal into TCA-soluble products. Despite numerous attempts, we have never been able to increase the [^{14}C]Gal incorporation in microsomes by adding UDP-Xyl with or without UDP-Glc under conditions in which endogenous XG backbone was generated (Gordon and Maclachlan, 1989). It appeared that XG chain elongation and galactosylation were not coupled in vitro. However, solubilized microsomal enzyme formed labeled TCA-soluble products from both substrates in the presence of added XG, with specific activities that were 5- to 6-fold greater than activities in intact microsomes.

Progress curves for incorporation of label from UDP-[^{14}C]Gal into TCA-soluble, 67% ethanol-insoluble products by pea microsomes and CHAPS extracts are shown in Figure 1. Membranes continued to incorporate ^{14}C for at least 2 h, and the addition of TXG enhanced this uptake. Detergent extracts incorporated significant amounts of ^{14}C as [^{14}C]Gal only in the presence of added TXG, and the reaction terminated after about 30 min. The level of substrate UDP-[^{14}C]Gal was not significantly depleted in reaction mixtures and was not a limiting factor. However, the addition of fresh enzyme and/or fresh XG did result in renewed ^{14}C incorporation. Evidently, solubilized XG galactosyltransferase activity was less stable under the present conditions than solubilized XG fucosyltransferase activity, which continued at a linear rate for 2 to 3 h (Hanna et al., 1991). The number of acceptor sites in native TXG that were accessible for galactosyl transfer must have also been limited.

The total labeled products that were formed by microsomes from UDP-[^{14}C]Gal plus supplied TXG were fractionated on a column of Sepharose CL-6B, and the distribution of ^{14}C and carbohydrate in the fractions are shown in Figure 2. Almost all of the carbohydrate eluted in the V_o of the column, indicating a size greater than the equivalent of dextran with a molecular mass of 10^6 D (manufacturer's calibration). Native TXG falls in this size range (Maclachlan et al., 1992). Part of the ^{14}C co-eluted with carbohydrate within V_o fractions and part eluted in included fractions with a mass down to the equivalent of 70,000-D dextran. Treatment with *Trichoderma* sp. cellulase under conditions that totally degraded TXG to subunit oligosaccharides re-

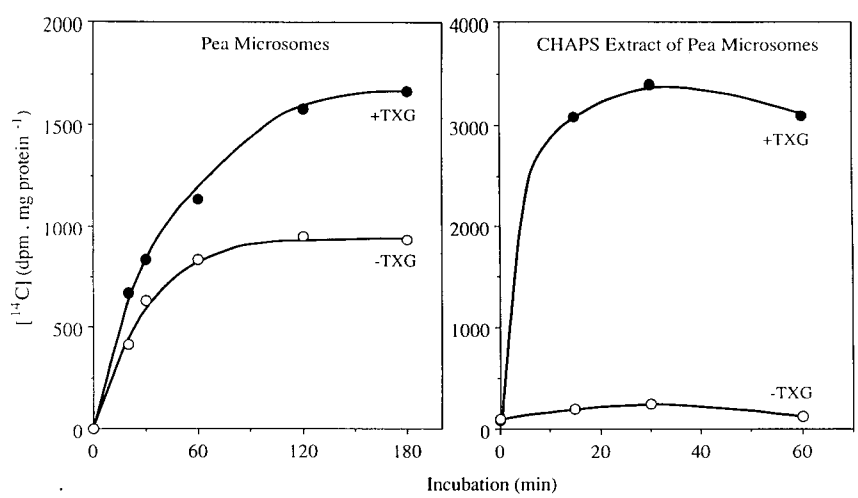

Figure 1. Degree of dependence on added TXG (+TXG) of the incorporation of [^{14}C]Gal from UDP-[^{14}C]Gal into products soluble in TCA but insoluble in 67% ethanol. The enzyme source was either pea microsomes or extracts with the detergent CHAPS, prepared as described in "Materials and Methods." ^{14}C incorporated in the presence of CHAPS extract was in [^{14}C]Gal only, as determined by HPLC of TFA hydrolysates. −TXG, Control.

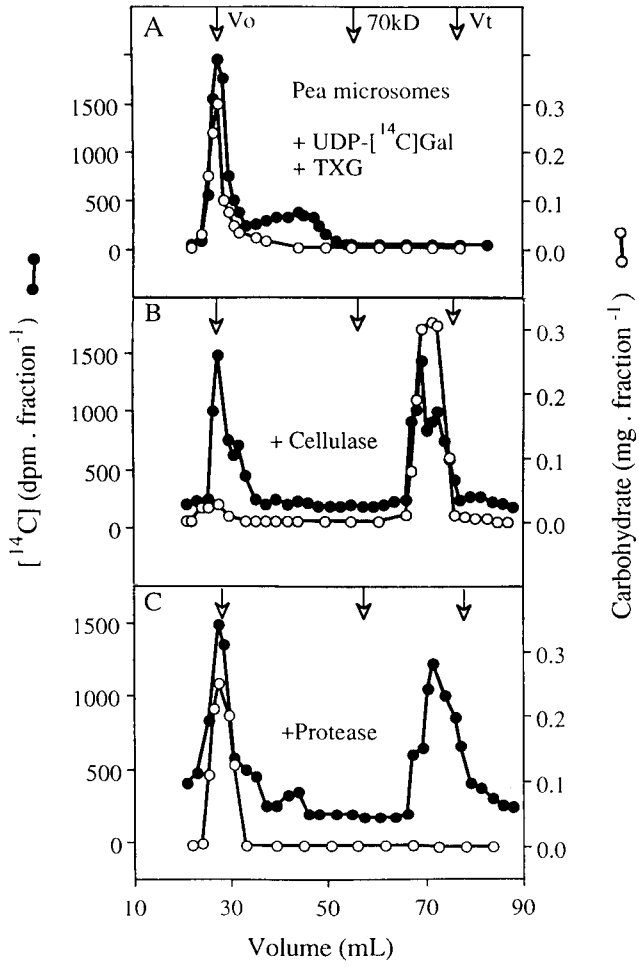

Figure 2. Gel filtration on columns of Sepharose CL-6B of total 67% ethanol-insoluble products formed from UDP-[^{14}C]Gal by pea microsomes in the presence of TXG. A, Radioactivity (●) and carbohydrate (○) profiles eluted with 0.1 M NaOH and 0.01% $NaBH_4$ between the V_o and the V_t. The elution peak of dextran with a mass of 70,000 D is so designated. B, Reaction mixture was digested with *Trichoderma* sp. cellulase. C, Reaction mixture was digested with *Streptomyces* sp. protease (pronase). Details of reaction conditions and gel chromatography are given in "Materials and Methods."

sulted in the complete loss of detectable carbohydrate from the V_o of the column and its elution in fractions near the V_t. At the same time, a major part of the label shifted from V_o fractions to the near V_t, indicating that cellulase digested it to products with the mobility of XG oligosaccharides. The *Streptomyces* sp. protease (pronase) did not degrade any carbohydrate but did shift part of the ^{14}C into products eluting near the V_t, as would be expected if ^{14}C-glycosylated proteins were among the reaction products and converted to [^{14}C]glycopeptides by the protease.

When these tests were repeated with labeled products formed by CHAPS extracts in the presence of TXG, essentially all of the ^{14}C eluted in the V_o fractions, along with carbohydrate. The label and TXG were completely degraded by cellulase to products with low M_r (Chileshe, 1995). Further hydrolysis by heating with TFA followed by chromatography confirmed that all of the ^{14}C remained in Gal. It is concluded that long chains of TXG can act as acceptors for galactosyl transfer from UDP-[^{14}C]Gal when added to pea microsomes or to detergent extracts of microsomes. Despite a lower apparent specific activity for XG galactosyl transfer than fucosyl transfer in CHAPS extracts (Table I), subsequent tests used these extracts in preference to intact microsomes because incorporation by the extracts was confined to and dependent on added XG as acceptor (Fig. 1). Free XG oligosaccharide subunits were not galactosylated by these pea enzyme preparations. The highly galactosylated trimer of subunits found in partial digests of TXG was a weak Gal acceptor compared with intact TXG (data not shown), as was also observed for pea fucosyltransferase (Maclachlan et al., 1992).

Subunit Oligosaccharides of TXG and NXG before and after Partial Degalactosylation

The subunit composition of native TXG and NXG was examined after hydrolysis with *Trichoderma* sp. cellulase, fractionation through a Bio-Gel P_2 column, and HPLC with detection by pulsed amperometry. The resulting oligosaccharide profiles (Fig. 3) were used to calculate the relative yields by weight of the four subunits observed, namely XLLG, XLXG, XXLG, and XXXG, as recorded in Table II. Digalactosylated nonasaccharide was the predominant subunit in both XGs (51% by weight in TXG and 73% in NXG). The nongalactosylated subunit was a minor constit-

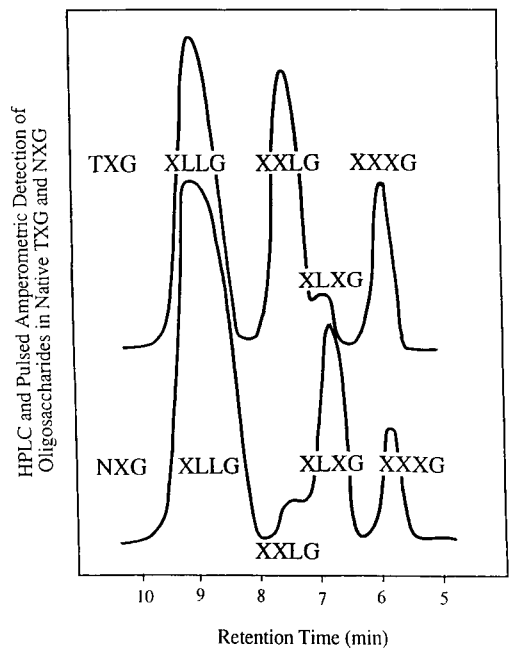

Figure 3. Pulsed amperometric detection profiles of oligosaccharide subunits (5 μg total) in cellulase digests of TXG and NXG following HPLC fractionation. The digestion used *Trichoderma* sp. cellulase, and chromatography was through a CarboPac column, as described in "Materials and Methods." Values for the relative weights of the oligosaccharides as separated in this particular chromatographic run are given in Table II.

Table II. *Relative concentrations of oligosaccharides in TXG and NXG before and after partial de-galactosylation by nasturtium β-galactosidase*

TXG and NXG were incubated with β-galactosidase for 6 and 1 h, respectively. Oligosaccharide subunits were generated by cellulase digestion and after Bio-Gel P_2 chromatography 5 μg was separated by HPLC fractionation through a CarboPac (Dionex) column. Profiles were recorded with a pulsed amperometric detector (Fig. 3) and their relative weights were measured by cutting out peaks from the paper profiles. The molar ratios of Glc:Xyl:Gal in TXG and NXG were calculated from these data at 4:31.25 and 4:3:1.6, respectively. Abbreviations for oligosaccharides are from the nomenclature suggested by Fry et al. (1993) and β-galactosidase is abbreviated to β-gal.

Subunit	Percentage of Yield of Oligosaccharides by Weight					
	TXG			NXG		
	Untreated	+β-gal	Δ[a]	Untreated	+β-gal	Δ
XLLG	50.6	44.1	−6.5	72.5	59.7	−12.8
XLXG	5.1	4.4	−0.7	18.1	19.4	+1.3
XXLG	31.6	36.8	+5.2	2.2	6.0	+3.8
XXXG	12.7	14.7	+2.0	7.2	14.9	+7.7

[a] Δ, Difference between values for untreated and +β-gal.

uent (13% of TXG and 7% of NXG). The most striking difference between the subunits in these seed XGs was in their octasaccharide components. TXG contained far more of the octasaccharide that eluted from the CarboPac column closer to XLLG than the one closest to XXXG, as also observed by Buckeridge et al. (1992) and Vincken et al. (1995). However, in NXG the relative octasaccharide composition was the reverse, as observed also with rapeseed XG (York et al., 1993). The main octasaccharide in TXG was designated as XXLG, and that in NXG was XLXG, in keeping with observations of York et al. (1993), who demonstrated using NMR spectroscopy and fast-atom bombardment MS that XXLG was the structure of the predominant octasaccharide in TXG. In a study published after this paper was submitted, Fanutti et al. (1996) recorded very similar proportions of the four main oligosaccharide subunits and sugars in TXG and NXG to those observed here.

In an effort to increase the capacities of these seed XGs to act as galactosyl acceptors by reducing their Gal content, they were pretreated with several commercial sources of β-galactosidase. None was able to degalactosylate intact XG chains, although most readily attacked XG oligosaccharides. An earlier observation (Maclachlan et al., 1992) that a preparation of *Aspergillus niger* β-galactosidase could generate Gal from XG should not be interpreted as a capacity to degalactosylate long XG chains, because this enzyme was contaminated with a cellulase that rapidly degraded XG to subunits that the enzyme could attack. The only β-galactosidase that appears to hydrolyze XG chains directly is the activity that develops during germination in nasturtium cotyledons, as described by Edwards et al. (1988). The procedure of Edwards and colleagues was followed here for partially purifying this activity using *p*-nitrophenyl-β-galactoside as the substrate and fractionation by $(NH_4)_2SO_4$ precipitation, followed by ion exchange in columns of DEAE-cellulose and CM-Sephadex. The major peak eluting from the final column had a specific activity of 758 nkat mg^{-1} protein, which was identical to that reported by Edwards et al. (1988) at this point in the purification. This preparation was contaminated by traces of XG-cleaving enzyme activity, as assayed viscometrically, but it was only sufficient to depolymerize a minor part of XG during the incubation times used here. The partially purified main activity peak was active against intact XG and nitrophenyl-β-galactoside but showed no capacity to degalactosylate either of the free XG nona- or octasaccharides.

Table II also shows the oligosaccharide composition of high-M_r TXG and NXG after treatment with nasturtium β-galactosidase as described in "Materials and Methods." This treatment evoked a loss of XLLG and increases in the proportions of XXLG and XXXG, along with the generation of free Gal. The levels of XLXG showed little change. This implies that nasturtium β-galactosidase preferentially hydrolyzed the subunit XLLG to Gal plus XXLG, and then this subunit to Gal plus XXXG.

Degalactosylated TXG and NXG as Acceptors for Glycosyl Transfer

The capacities of high-M_r TXG and NXG, before and after β-galactosidase pretreatment, to act as Gal and Fuc acceptors from ^{14}C-sugar nucleotides were assayed with solubilized pea membrane protein. As shown in Figure 4, native (untreated) TXG was a better Fuc acceptor than native NXG, but it rapidly lost this property after a brief preincubation in β-galactosidase. In contrast, the capacities of TXG and especially NXG to act as Gal acceptors increased as degalactosylation proceeded.

Nature of Galactosylated Products

The products formed from UDP-[^{14}C]Gal by solubilized pea enzyme using native or partially degalactosylated TXG and NXG as acceptors were washed in 67% ethanol after incubation and hydrolyzed by extended incubation in high levels of *Trichoderma* sp. cellulase, as described in "Materials and Methods." Carbohydrate was recovered after Bio-Gel P_2 chromatography primarily in a peak corresponding to XG oligosaccharides, which were heavily labeled (Fig. 5). Label eluting in the V_o was probably present in small

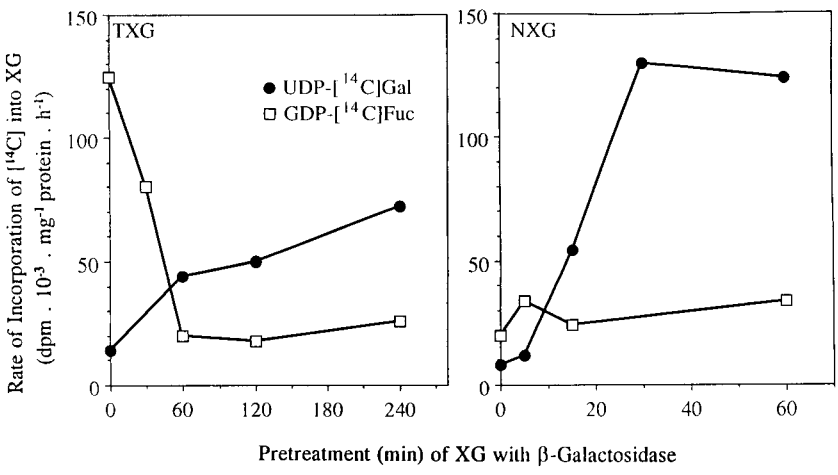

Figure 4. Effects of partial degalactosylation of TXG and NXG on their capacities to accept Gal or Fuc from ^{14}C-sugar nucleotides in the presence of solubilized pea enzyme (CHAPS extract). XG-specific β-galactosidase from nasturtium seed was preincubated with XG for different times, after which the partially degalactosylated XG was fractionated on columns of Sepharose CL-6B. Most of the XG eluted near the V_o as in Figure 2A. These high-M_r fractions were combined and used as acceptor XG in standard reaction mixtures to measure ^{14}C incorporation into 67% ethanol-insoluble products in 20 min. Details of procedures are given in "Materials and Methods."

amounts of oligomers resistant to cellulase (Maclachlan et al., 1992). The peak eluting in the V_t was free [^{14}C]Gal generated as a result of traces of β-galactosidase activity in the cellulase preparation. The peak eluting in a fraction equivalent to a tetrasaccharide has not been identified. Degalactosylation resulted in increased yields of labeled products but no major change in the elution profiles from Bio-Gel columns.

The peaks from Bio-Gel P_2 columns that corresponded to XG oligosaccharides (between 30 and 50 mL, Fig. 5) were divided into samples containing up to 10 μg of carbohydrate (phenol sulfuric assay) and fractionated by HPLC. Pulsed amperometric detection indicated well-separated peaks of the four major seed oligosaccharides, as shown in Figure 3. These peaks were collected as they emerged from the detector, combined with the same peaks from other samples, and evaporated for measurement of ^{14}C content. As shown in Table III most of the ^{14}C (approximately 80%) was recovered in XLLG and XXLG using either TXG or NXG as acceptor. XLLG was more heavily labeled in NXG than in TXG, which probably reflects the preponderance of -XLXG- in NXG over TXG (Fig. 3; Table II) and a preference of the pea enzyme for this octasaccharide as galactosyl acceptor.

The distribution of ^{14}C between the two Gal units in the labeled XLLG has not been measured directly. However, preliminary tests were conducted with β-galactosidase from *A. niger*, which specifically removes Gal from the central Xyl of XLLG (York et al., 1993). The labeled XLLG that was collected in the above experiment was incubated with *A. niger* β-galactosidase, as described previously (Ma-

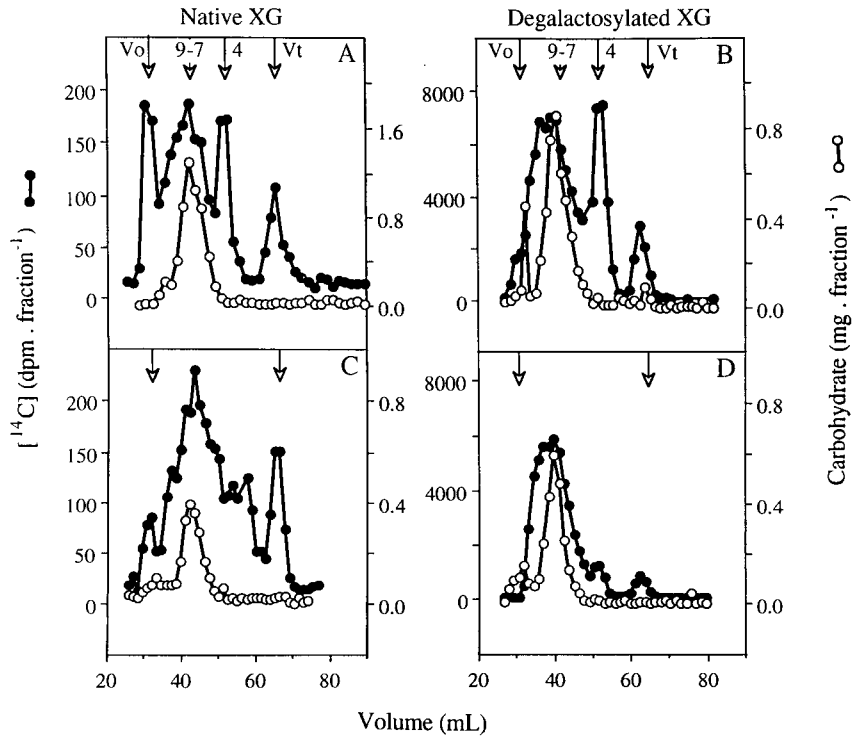

Figure 5. Bio-Gel P_2 profiles of ^{14}C (●) and carbohydrate (○) from cellulase digests of reaction products formed by solubilized pea enzyme from UDP-[^{14}C]Gal with either native or partially degalactosylated TXG or NXG as acceptors.

Table III. *Relative ^{14}C content of oligosaccharide subunits of XG incubated with UDP-[^{14}C]Gal plus solubilized protein from pea microsomes*

The oligosaccharides were recovered from cellulase hydrolysates of seed XG that had been pretreated with nasturtium β-galactosidase and fractionated on Bio-Gel P_2 columns, as in Table II and Figure 5. They were separated by repeated HPLC fractionation as in Figure 3 until sufficient label had been collected (approximately 3000 dpm) for accurate determination of its distribution between peaks.

Subunit	Percentage of Recovered ^{14}C	
	TXG	NXG
XLLG	30	39
XLXG	5	2
XXLG	47	44
XXXG	1	1
Origin	17	14

clachlan et al., 1992), and products were fractionated on a column (0.2 × 7 cm) of Sephadex G-10. Very little label was recovered as free [^{14}C]Gal, which implies that most of the [^{14}C]Gal in XLLG was present next to unsubstituted Glc.

DISCUSSION

These results demonstrate that pea epicotyl microsomes contain galactosyltransferase activity that is capable of transferring label from UDP-[^{14}C]Gal to chains of preformed XG. Sepharose CL-6B profiles (Table I; Fig. 1) showed that added seed XG stimulated incorporation of [^{14}C]Gal by pea microsomes and detergent extracts into high-M_r products. These products were digested by cellulase in part to fragments that had a size equivalent to authentic XG subunit oligosaccharides (Figs. 2, 3, and 5). Partial degalactosylation of TXG or NXG by XG-specific nasturtium β-galactosidase (Table II) increased their capacity to act as Gal acceptors (Figs. 4 and 5). Bio-Gel P_2 profiles of the products after cellulase hydrolysis showed major peaks of ^{14}C that co-eluted with the XG oligosaccharide peaks (Fig. 5). HPLC of these labeled peaks indicated that ^{14}C was incorporated mainly into the subunits -XLLG- and -XXLG- of both TXG and NXG (Table III), even though the predominant octasaccharide in TXG was XXLG and that in NXG was XLXG (Fig. 3; Table II).

It was anticipated that pea XG galactosyltransferase would preferentially galactosylate the Xyl of -XXXG-, which is closest to the unsubstituted Glc, to form -XXLG- and not -XLXG-, because the major fucosylated subunit in PXG is -XXFG- (Hayashi and Maclachlan, 1984a; Guillén et al., 1995), which must have derived from -XXLG-. In the present tests this prediction was confirmed since -XLXG- contained only 2 to 5% of incorporated [^{14}C]Gal versus 44 to 47% in -XXLG- (Table III). This specificity appears to

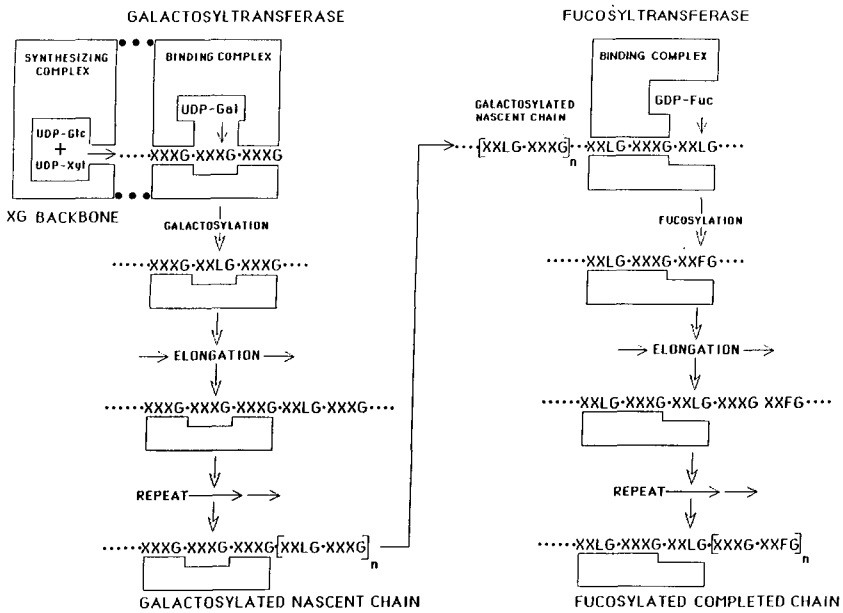

Figure 6. Model for the modes of action of pea galactosyl- and fucosyltransferases during biosynthesis of cell wall XG containing repeating subunits of alternating hepta- and nonasaccharide, i.e. $(\text{-XXXG.XXFG-})_n$ in the nomenclature proposed by Fry et al. (1993). A complex is assumed for generating the XG backbone of $(\text{-XXXG-})_{2n}$ from UDP-Glc and UDP-Xyl. Galactosyltransferase is envisaged as binding to (-XXXG-) subunits on either side of the subunit that it galactosylates. When this action is repeated on subsequent sequences of three contiguous heptasaccharides in nascent XG, a chain is formed with regular repeats of the dimer (-XXXG.XXLG-). Fucosyltransferase binds to a trimer of this galactosylated nascent XG to fucosylate the octasaccharide (-XXLG-) nearest the reducing end of the chain. Repetition of this action by binding and fucosylating the next trimer results in a chain with (heptasaccharide-nonasaccharide) as the repeating unit. Dotted lines connect the complex that forms the XG backbone with galactosyltransferase to indicate the possibility that these events are coupled in vivo. Fucosyltransferase is shown as a separate entity because of evidence that its action does not depend on concurrent or confluent chain elongation.

have been retained in the galactosyl transfer that formed -XLLG-. Whether a separate galactosyltransferase is required in peas to form -XLXG- or the activity detected here is capable of galactosylating either Xyl, albeit at very different rates, can only be determined when the activity is purified. We conclude that solubilized pea galactosyltransferase activity catalyzed the following reactions:

$$\text{-XXXG- + UDP-[}^{14}\text{C]Gal} \longrightarrow \text{-XX[}^{14}\text{C]LG- + UDP}$$

$$\text{-XLXG- + UDP[}^{14}\text{C]Gal} \longrightarrow \text{-XL[}^{14}\text{C]LG- + UDP}$$

The known affinities of pea XG glycosyltransferases for potential substrates, together with our understanding of the structure of wall XG, permit several clear conclusions about the mode of action of these synthases during XG chain elongation. The observation that free XG subunits are unable to act as acceptors for pea galactosyl- or fucosyltransferases indicates that these enzymes must bind to more than a single subunit site within an XG chain before catalysis takes place. In native PXG the major repeating dimer must have derived by fucosylation of repeating subunits of -XXXG.XXLG- in nascent XG. For pea XG galactosyltransferase to generate a structure from nascent backbone XG, which is only galactosylated at every second subunit, it must logically recognize a minimum of three contiguous heptasaccharides and transfer Gal to the central subunit of this trimer. If only a dimer were required for recognition, galactosylation of the Xyl nearest the reducing end would result in long stretches of -XXLG-, which do not exist. A galactosyltransferase requirement for three contiguous heptasaccharides in XG helps to explain why native TXG with 12.7% XXXG is a better Gal acceptor than NXG with only 7.2% XXXG (Table II). It also explains why degalactosylation of NXG, which doubles the XXXG content, results in a marked increase in its capacity to act as a Gal acceptor (Table II; Fig. 4).

These conclusions have been incorporated into a model shown in Figure 6 for the biosynthesis of wall XG with a simple repeating unit of $(\text{-XXXG.XXFG-})_n$ from a nascent XG backbone containing only $(\text{-XXXG-})_{2n}$. Several studies (Hayashi and Matsuda, 1981; Campbell et al., 1988; Gordon and Maclachlan, 1989; White et al., 1993) agree that formation of the XG backbone requires coordinated, interdependant glucosyl and xylosyl transfer. The degree of xylosylation of the β-glucan backbone that is obtained in vitro depends on the UDP-Xyl:UDP-GIc concentration ratio (Gordon and Maclachlan, 1989). If this ratio were limiting on occasion in vivo, it could result in the formation of incompletely xylosylated fragments, which are in fact found as minor constituents of PXG (Guillén et al., 1995).

The observed structure of PXG and substrate affinities of galactosyltransferase appear to require binding to a chain containing $(\text{-XXXG-})_3$ to form (-XXXG.XXLG.XXXG-). If this reaction is repeated when nascent XG elongates by two subunits or when galactosyltransferase shifts to bind to a contiguous heptasaccharide trimer, the result is XG with alternating hepta- and octasaccharides (Fig. 6). Pea fucosyltransferase also logically requires an acceptor chain containing at least three subunits before it acts. Presented with newly formed $(\text{-XXXG.XXLG-})_n$, fucosyltransferase would be expected to bind to one octasaccharide and fucosylate to the next octasaccharide two subunits away, which means that it must recognize at least three subunits. This enzyme is depicted (Fig. 6) as an independent entity from galactosyltransferase because of evidence that the two activities exist in vivo in separate compartments of the Golgi apparatus (Zhang and Staehelin, 1992) and because of the observation that pea enzyme can transfer Fuc to all regions of the TXG chain in the total absence of UDP-Gal (Maclachlan et al., 1992). Minor amounts of unsubstituted octasaccharide occur in PXG (Guillén et al., 1995) and may be expected to persist in stretches of the galactosylated nascent chain that do not reach the site of fucosylation.

This model assumes that newly incorporated [^{14}C]Gal is linked 1,2-β- to Xyl with [^{14}C] Fuc linked 1,2-α- to Gal, as observed in native PXG (Hayashi and Maclachlan, 1984a), but the nature of these linkages has yet to be confirmed. The model will have to be modified to account for other minor subunits and structural deviations that are observed in pea and other primary wall XGs, and it does not apply to the synthesis of periplasmic XG in seeds. Thus, for example, in addition to hepta- and nonasaccharides (Guillén et al., 1995), pea must be able to generate the octasaccharide XLXG and the decasaccharide XLFG, and some irregularity in the sequence of strictly alternating oligosaccharides (Hayashi and Maclachlan, 1984a) must be accommodated. Further refinement in the model may be expected from results of studies with purified XG glycosyltransferases, which are now under way in several laboratories.

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