

Biochemical Characterization and Molecular Cloning of an α -1,2-Fucosyltransferase That Catalyzes the Last Step of Cell Wall Xyloglucan Biosynthesis in Pea*

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Pea microsomes contain an α -fucosyltransferase that incorporates fucose from GDP-fucose into xyloglucan, adding it preferentially to the 2-*O*-position of the galactosyl residue closest to the reducing end of the repeating subunit. This enzyme was solubilized with detergent and purified by affinity chromatography on GDP-hexanolamine-agarose followed by gel filtration. By utilizing peptide sequences obtained from the purified enzyme, a cDNA clone was isolated that encodes a 565-amino acid protein with a predicted molecular mass of 64 kDa and shows 62.3% identity to its *Arabidopsis* homolog. The purified transferase migrates at ~63 kDa by SDS-polyacrylamide gel electrophoresis but elutes from the gel filtration column as an active protein of higher molecular weight (~250 kDa), indicating that the active form is an oligomer. The enzyme is specific for xyloglucan and is inhibited by xyloglucan oligosaccharides and by the by-product GDP. The enzyme has a neutral pH optimum and does not require divalent ions. Kinetic analysis indicates that GDP-fucose and xyloglucan associate with the enzyme in a random order. *N*-Ethylmaleimide, a cysteine-specific modifying reagent, had little effect on activity, although several other amino acid-modifying reagents strongly inhibited activity.

of dicotyledonous and non-graminaceous monocotyledonous cell walls. Within the wall it is firmly bound to cellulose microfibrils (2) via hydrogen bonds and thus may have a stabilizing function. The structure of XG differs among species, although all have a β -1,4-glucan backbone with xylosyl side chains attached to the 6-*O*-position of the glucosyl residues in a regular pattern of three substituted glucosyl residues followed by one unsubstituted glucose at the reducing end. L-Fucose commonly terminates these side chains and is usually present in a nonasaccharide subunit (Glc₄Xyl₃GalFuc), designated by agreement (3) as XXFG, where X is isoprimeverose (xylosyl- α -1,2-glucose) and F is the tetrasaccharide GlcXylGalFuc. Pea cell wall XG is thought to be constituted almost completely of alternating -XXXG- (Glc₄Xyl₃) and -XXFG- subunits (4), but recent study (5) has also detected the presence of small amounts of other subunits including two octasaccharides and the decasaccharide -XLFG- (Glc₄Xyl₃Gal₂Fuc). This subunit (-XLFG-) has also been detected in sycamore cell XG (6), *Arabidopsis* and rapeseed (7), apple fruit (8), and developing nasturtium fruits (9). An unadecasaccharide, -XFFG- (Glc₄Xyl₃Gal₂Fuc₂), has additionally been observed in sycamore cell XG (6).

Although extensive progress has been made in understanding the structure of cell wall components such as XG, the processes of their biosynthesis and assembly are still poorly understood. Therefore, to understand the process of cell wall biosynthesis, isolation of glycosyltransferases and polysaccharide synthases becomes critical. With the exception of cellulose and callose, which are synthesized at the plasma membrane, wall polysaccharides are made in the Golgi (10), and many glycosyltransferase activities associated with their biosynthesis have been identified in plant microsomal fractions (11–14). However, only two genes have been cloned at this time (15, 16).

In previous work from our cell wall group (15), procedures were developed to purify the XG-fucosyltransferase (XG-FTase) from pea microsomes. Two polypeptides (~63 and ~68 kDa) co-purified with the activity, and both of the bands were subjected to tryptic digestion and sequencing. Peptides from the ~68-kDa band showed high sequence similarity to binding protein (BiP), a molecular chaperone. However, amino acid

Plant cells are surrounded by a primary cell wall that is ~10–100 times thicker than the plasma membrane. The cell wall has several important physiological roles including control of morphogenesis, intercellular signaling, and pathogen interactions (1). Plant primary walls are composed mainly of a polysaccharide framework that is deposited during growth, thus determining the cell shape and size. The major components of the primary cell wall are cellulosic microfibrils, hemicellulosic polysaccharides, pectins, and glycoproteins (2).

Xyloglucan (XG)¹ is the major hemicellulosic polysaccharide

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF223643.

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¹ The abbreviations used are: XG, xyloglucan; XG-FTase, xyloglucan

fucosyltransferase; PsFT1, *Pisum sativum* fucosyltransferase 1; AtFT1, *Arabidopsis thaliana* fucosyltransferase 1; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; HPAEC, high pH anion exchange chromatography; PAD, pulsed amperometric detection; GDP-hexanolamine, *P'*-(6-amino-1-hexyl)-*P*²-(5'-guanosine)pyrophosphate; DEPC, diethylpyrocarbonate; NEM, *N*-ethylmaleimide; PLP, pyridoxal 5'-phosphate; NBS, *N*-bromosuccinimide; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Pipes, 1,4-piperazine-diethanesulfonic acid; TMD, transmembrane domain.

sequences from the ~63-kDa polypeptide identified one clone of undetermined function in the *Arabidopsis* expressed sequence tag data base. The full-length *Arabidopsis* cDNA clone was isolated and shown to encode an ~63-kDa polypeptide with XG-FTase activity (15). The goal of the present study is to complement and extend our earlier work by isolating a pea cDNA clone for XG-FTase (PsFT1), analyzing the biochemical characteristics of the purified pea enzyme, determining the substrate acceptor specificity, and characterizing the product of this enzyme in more detail. We show that the purified transferase is indeed XG-specific. When tamarind XG was used as an acceptor, the fucosylated subunits were the nona- and decasaccharides, -XXFG- and -XLFG-, respectively. These two subunits are found in native pea cell wall XG and also in many other plant XGs. The characteristics of PsFT1 are compared with other described fucosyltransferases.

EXPERIMENTAL PROCEDURES

Chemicals and Plant Material

GDP-[³H]Fuc (299.7 GBq/mmol) and GDP-[¹⁴C]Fuc (10.1 GBq/mmol) were purchased from NEN Life Science Products. GDP-hexanolamine-agarose was a gift from Prof. Gerald Hart (The Johns Hopkins University, Baltimore). BioSep-SEC-S4000 was purchased from Phenomenex (Torrance, CA). Tamarind seed xyloglucan and *Trichoderma* sp. cellulase were from Megazyme International (Ireland). For earlier studies, tamarind seed XG was isolated as described previously (17). Nucleotides, nucleotide sugars, all the protease inhibitors, detergents, and the amino acid-modifying reagents were from Sigma. Nasturtium seed XG and pea XG were a gift from Prof. Gordon MacLachlan (McGill University, Montreal, Canada). Rhamnogalacturonan-I and -II were a gift from Dr. Alan Darvill (Complex Carbohydrate Research Center, University of Georgia, Athens, GA).

Garden pea seeds (*Pisum sativum* L. var. Alaska) purchased from Olds Seed Company (Madison, WI) were washed in 10% bleach for 10 min, rinsed thoroughly, soaked 6–8 h with aeration, and grown in moist vermiculate in the dark for 7 days until third internodes reached 3–5 cm in length. Growing regions (2 cm just below the hook) were excised and used as the source of enzyme.

Purification Procedures

The purification was performed as described in the previous work (15) except that in this study we did not include the anion exchange column in purification steps.

PCR Amplification and Molecular Cloning

The cloning of *PsFT1* was accomplished in several steps. The first round PCR was performed using a plasmid cDNA library as template and degenerate primers designed from peptides P5 and P2 (Fig. 1A) (5'-GCIGAYGGITTYGAYGAAA-3' and 5'-ICCCAIACRTRTRTIGTIGGRTG-3'), where I indicates inosine; R indicates purines (G + A), and Y indicates pyrimidines (T + C). The cDNA library was derived from apical hooks of auxin-treated etiolated pea seedlings in vector pBlue-script SK(+) and was kindly provided by Dr. Hans Kende (Department of Energy Plant Research Laboratory, Michigan State University).

A second round PCR was performed to amplify fragments covering the 5'- and 3'-ends. Primers were designed from the first round PCR products (5'-TTCTACTAGTTGACCCGGGAGT-3' and 5'-ACTCCCGGGTCAACTAGTAGAA-3') and the cDNA library vector (5'-TCAGGAAACAGCTATGACCATG-3' and 5'-GTAATACGACTCACTATAGGGC-3').

To identify the 5'- and 3'-ends of the *PsFT1* transcript, 5'- and 3'-RACE were carried out using the 5'-RACE System, version 2.0 (Life Technologies, Inc.), according to the manufacturer's instructions. Poly(A)⁺ RNA (gift from Dr. Hans Kende, Department of Energy Plant Research Laboratory, Michigan State University) was isolated from apical hooks of etiolated pea seedlings 4 h after auxin treatment (indoleacetic acid, 100 μM), using the poly(A)/TRACT mRNA isolation system (Promega). For 5'-RACE, 5'-ACTCCCGGGTCAACTAGTAGAA-3' was the primer for cDNA synthesis, and 5'-GGCTGACTGATACCTGCTGAGACAAGATTTT-3' was the nested primer. For 3'-RACE, poly(dT) primer was used for cDNA synthesis, and 5'-CCAAAAGCC-TGGGCAGAAAT-3' was the nested primer.

Full-length cDNA was amplified using Pwo polymerase (Roche Molecular Biochemicals), end-specific primers (5'-AATGAATATGCTGATAAAGAGAGTC-3' and 5'-CTAATGTCTACGAGCTTAAGGC-3'), and the same cDNA library as template. The same reaction was also

performed using 300 ng genomic DNA as template to confirm the obtained sequence.

All PCR products were recovered from low melting point agarose gel after electrophoresis and cloned onto the pGEM-T Easy vector (Promega) for sequencing. All alignments were carried out using the software package DNASTAR version 4.0 (Madison, WI).

Enzyme Assays

High Molecular Weight Polysaccharide Acceptors—The standard fucosyltransferase assay was performed essentially as developed (14, 18) with minor modifications. The reaction mixture (50 μl) contained enzyme (up to 10 μg of protein), polysaccharides (100 μg) as acceptor, GDP-[³H]Fuc (~3.3 nM, ~30,000 dpm) with 50 μM cold GDP-Fuc as donor, dissolved in the assay medium (40 mM Pipes-KOH, pH 6.8, 0.4 mM DTT, 0.16 M sucrose), and supplemented with 5 mM MgCl₂. In some experiments GDP-[¹⁴C]Fuc (~2 μM, ~60,000 dpm) was used without adding cold GDP-Fuc. Reactions were conducted for 20–30 min at room temperature and halted with 1 ml of 67% ethanol. Washed ethanol-insoluble products were dissolved in 200 μl of water, and the incorporated label (dpm) was measured in a Beckman LS 5000 TD Counter, after adding at least 2 volumes of liquid scintillation mixture (ICN). Control reactions without XG, without protein, or with boiled protein typically resulted in 50–100 dpm/reaction.

Low Molecular Weight Acceptors—When lactose (5 mM), *N*-acetylglucosamine (5 mM), or tamarind XG oligosaccharides (0.2% w/v) were used as acceptors, the reactions were conducted as above and terminated by adding 300 μl of water and then 500 μl of Dowex 1-X80 resin (Cl⁻ form) as described earlier (19). After centrifugation, the supernatants were saved, and the resin was washed with 2 × 1 ml of water. All the supernatants were combined, and the incorporated label was assessed as above.

Glycoprotein α₁-Acid as Acceptor—When glycoprotein α₁-acid (200 μg per reaction) was used as acceptor, the reactions were halted by addition of 1 ml of 0.1% bovine serum albumin and 1 ml of an acid mixture of 1 M HCl containing 2% (w/v) phosphotungstic acid as described earlier (19). After centrifugation, the pellets were washed three times with 1 ml of acid mixture and 1 ml of methanol and then resuspended in 400 μl of water. The labeled product was counted as above.

Biochemical Characterization of the Purified XG-FTase

Size Estimation of the Purified Enzyme—The apparent *M_r* of the enzyme subunit was determined by SDS-PAGE under reducing conditions (20) and by gel filtration using a BioCAD Perfusion Chromatography system (Perspective Biosystems) and a BioSep-SEC-S4000 column (Phenomenex) as described earlier (15).

pH Variation and Effectors—pH stability of the enzyme was investigated by incubating the enzyme (1 μl, ~0.3 μg) with 3 μl of 66 mM phosphate buffer at pH 3.5, 4.2, 5.2, 6.3, 6.8, 7.8, 8.4, or 9.2 for 30 min on ice. The fucosyltransferase reaction was then performed for 20 min in the standard assay using a higher concentration of Pipes, pH 6.2 (46 μl of 0.1 M), to equilibrate the pH in the reaction medium. Reactions were terminated as above. The pH optimum was determined by conducting the assay in 50 mM solution of the following buffers: phosphate buffer, pH 5.2, 6.3, 6.8, 7.8, or 8.4; acetate buffer, pH 5.2; Bis-Tris-HCl, pH 6.8; Hepes-NaOH, pH 6.9; Tris-HCl, pH 7.5 or 8.0, containing all the ingredients of the standard assay medium.

To assess whether divalent cations are required for activity, MgCl₂, MnCl₂, or CaCl₂ were included at concentrations up to 10 mM in fucosyltransferase assays lacking metal ions using ~2 μM GDP-[¹⁴C]Fuc (~60,000 dpm) as described above. The effects of a chelator (EDTA) and DTT were similarly tested.

Kinetic Studies—Determination of kinetic parameters was carried out using a 4 × 3 matrix of GDP-Fuc and tamarind XG in a standard assay conditions (in absence of MgCl₂). XG-FTase velocity was measured as a function of tamarind XG (0.5, 1, or 4 mg/ml) at fixed GDP-Fuc concentrations (12.5, 25, 50, or 100 μM). Reaction mixtures contained a fixed amount of GDP-[³H]Fuc (~3.3 nM, ~30,000 dpm) and enough purified enzyme to have an accurate initial reaction rate in 20 min incubation time. Data were evaluated by double-reciprocal plots according to the Lineweaver-Burk method, and *K_m* and *V_{max}* values were calculated from duplicate data points (Fig. 3).

Amino Acid Modification—Purified enzyme was subjected to amino acid group-specific modification. The following reagents were used: *N*-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC), pyridoxal 5'-phosphate (PLP), and *N*-bromosuccinimide (NBS). Modifying reagents were used according to the methods described by Britten and Bird (21). Standard assays were conducted as described above in the presence of

up to 5 mM of each modifying reagent using GDP- $^{[3]H}$ Fuc. The reactions were halted by adding 67% ethanol and washed as described above before assessing label incorporation. Controls were conducted in the absence of these modifying reagents.

Substrate Specificity—The substrate acceptor specificity was investigated by including alternative acceptors only or by conducting competitive inhibition experiments. The reactions used GDP- $^{[14]C}$ Fuc (~2 μ M). The polysaccharides used were tamarind XG, nasturtium XG, pea cell wall XG, and rhamnolacturonan-I and -II. Tamarind XG oligosaccharide subunits monomers (7–9 degrees of polymerization) and trimers (21–27 degrees of polymerization) were prepared by partial or complete digestion with *Trichoderma* cellulase and purification on Bio-Gel columns. Known acceptors for glycoprotein fucosyltransferases (lactose, *N*-acetylglucosamine, and glycoprotein α -acid) were also tested.

Donor substrate specificity was investigated in a competition assay using tamarind XG as acceptor. Nucleotides, nucleotide sugars, and monosaccharides were tested by inclusion in the standard fucosyltransferase assay at various concentrations (up to 10 mM).

Identification of the Fucosylated Subunits of the XG

Fucosylated subunits in $^{[14]C}$ tamarind XG were determined as described earlier (9, 14), with minor modifications. After digestion by *Trichoderma* cellulase (0.05%, w/v) and fractionation by gel filtration on a Bio-Gel P2 (Bio-Rad) column, the samples (~10 μ g of oligosaccharides, ~100–200 dpm/ μ g) were further separated by HPAEC using a CarboPac PA-100 column (Dionex Corp.) with pulsed amperometric detection (PAD). Fractions of 0.65 ml were collected, neutralized, and analyzed for ^{14}C distribution. The positions of labeled oligosaccharides were compared with those of authentic XG subunits as described (9, 14) and identified according to the suggested nomenclature (3). Labeled products formed by pea microsomes were also analyzed.

RESULTS

Molecular Cloning of *PsFT1* and Sequence Analysis

Following purification, tryptic peptides from each purified protein were subjected to limited amino acid sequencing. Peptide sequence information derived from this process has been published previously (15), and Fig. 1A depicts the location of the six peptides derived from the 63-kDa polypeptide (P1 to P6).

Molecular Cloning of *PsFT1*—As a first step, a 726-base pair internal fragment of the pea XG-FTase was PCR-amplified. The nature of this product was confirmed by its 61.6% identity with *AtFT1* and the presence of a region encoding previously sequenced peptide P1 (Fig. 1A). Primers designed from the middle of this fragment, together with vector-specific primers were used to amplify the cDNA fragments toward the 5'- and 3'-ends of this gene. This generated a combined cDNA sequence encompassing the entire first round PCR product. It encoded all six peptide sequences previously obtained from the purified enzyme (15) with one amino acid change in peptide P1 (Fig. 1A), which may be a sequencing error. To ensure that we had the entire cDNA sequence of this gene, we further performed 5'- and 3'-RACE experiments. The full-length *PsFT1* cDNA was then obtained by PCR from the cDNA library using primers specific to the 5'- and 3'-ends of the open reading frame and was consistent with the corresponding genomic clone of *PsFT1* (data not shown). The deduced amino acid sequence of *PsFT1* is shown in Fig. 1A.

Characterization of the Deduced Amino Acid Sequence—The deduced translation product is 565 amino acid residues long, has a predicted molecular mass of 64 kDa, and has a theoretical pI of 5.91 as determined by DNASTAR (Madison, WI). A transmembrane domain was predicted by TMHMM from residues 39 to 61 (Fig. 1), consistent with the observation that Golgi-localized glycosyltransferases are type II integral membrane proteins. An overall 62.3% identity was found between *PsFT1* and *AtFT1*. The highest similarity was observed at the COOH terminus, postulated in other characterized fucosyltransferases to contain the catalytic domain (22).

Analysis of Conserved Motifs—Although the two XG-FTase

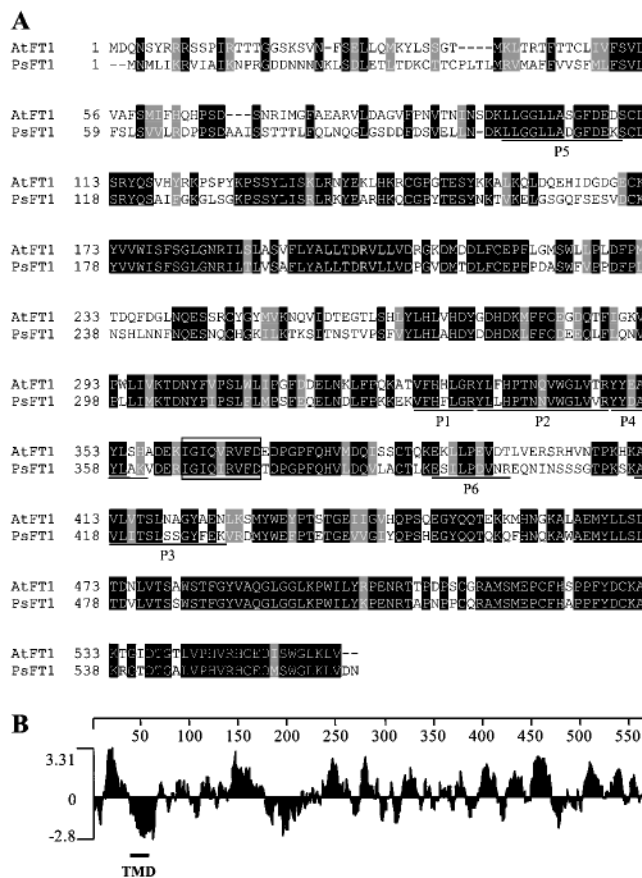


Fig. 1. Predicted amino acid sequences of XG-FTase from pea (*PsFT1*) and *Arabidopsis* (*AtFT1*). A, sequence alignment using the ClustalW algorithm. The six tryptic peptides obtained from microsequencing of purified pea enzyme (P1 to P6) are underlined. Black shading represents conserved amino acids found in *AtFT1* and *PsFT1*, and gray shading represents similar amino acids. The conserved motif found in all known α -1,2- and α -1,6-fucosyltransferases is boxed. B, hydropathic plot of *PsFT1* according to the method of Kyte and Doolittle (31). The predicted transmembrane domain is indicated in B by TMD.

proteins have very low overall sequence similarity with the known fucosyltransferases (less than 20% identity), three conserved motifs were found. By using the PATTERNFIND program, we searched for motifs (α -2/6-motif I, α -2-motif II, α -2-motif III, α -6-motif II, and α -6-motif III) previously identified by Oriol *et al.* (23) that are present in all known α -1,2- and α -1,6-fucosyltransferases. The α -2/6-motif I, present in all these fucosyltransferases, was found in both *PsFT1* and *AtFT1*. An additional motif was also found in *PsFT1* and *AtFT1* at the proper spacing for motifs III observed in all known fucosyltransferases. Both the α -2-motif III and α -6-motif III are present in *PsFT1* and *AtFT1* (Fig. 2). Interestingly, the α -2-motif III and α -6-motif III were previously thought to be specific to known α -1,2- and α -1,6-fucosyltransferases, respectively (23). The α -2-motif II and α -6-motif II were not found in *PsFT1* and *AtFT1*. Fig. 2 summarizes the relative locations of the conserved motifs, their spacing, and their comparison to the consensus motifs of α -1,2- and α -1,6-fucosyltransferases originating from other species.

Biochemical Characterization of Purified XG-FTase

The enzyme recovered from the gel filtration column was used for subsequent biochemical characterization studies.

pH Stability and Optimum—We investigated the effect of pH on the stability and activity of the purified enzyme, which was outside of a membrane environment and surrounded only by

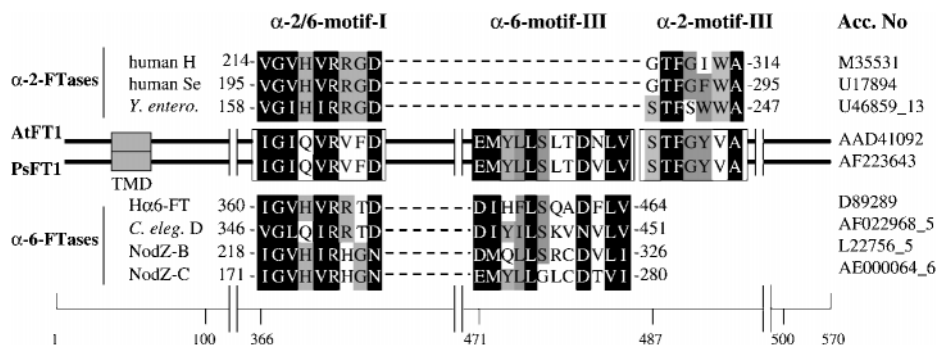


FIG. 2. Schematic illustration of peptide motifs shared by XG-FTases and known α -1,2- and α -1,6-fucosyltransferases. Conserved motifs in AtFT1 and PsFT1 (boxed) are aligned (ClustalW) with the corresponding motifs from some α -1,2-fucosyltransferases from human (*H* and *Se*) and bacteria (*Yersinia enterocolitica*), from some α -1,6-fucosyltransferases from human (*ha6-FT*), *Caenorhabditis elegans*-D (*C. eleg. D*), and two bacterial proteins (*NodZ-B* and *NodZ-C*) from *Rhizobium* species (*Bradyrhizobium japonicum* B and *Rhizobium* sp. C, respectively). The invariant or highly related residues are shown in white letters on black shading and other conserved amino acids are in black letters on gray shading. The positions of the first residues of each motif found in the PsFT1 sequence are indicated on the bottom. The GenBankTM accession numbers of the fucosyltransferases are indicated on the right.

detergent. Purified pea XG-FTase was remarkably stable at extreme pH levels. Even after 30 min treatment with acidic (pH 3.5) or alkaline (pH 9.2) conditions, the enzyme retained over 80% of its activity (data not shown). This is consistent with stability during carbonate wash at pH 10.5. The fucosyltransferase was active between pH 5 and 8 with an optimum activity between pH 6 and 7 (data not shown).

Complete inhibition of the pea XG-FTase activity was observed in acetate buffer at pH 5.2, whereas a decrease of ~40% of the activity was observed with phosphate buffer at pH 5.2 (data not shown). Hepes, Pipes, and Bis-Tris at pH 6.8 did not interfere with the activity.

Effects of Divalent Cations and DTT—Divalent metal ions are required for many proteins that bind nucleotide sugars. To investigate the metal ion dependence of XG-FTase, activity was measured in the presence and absence of various divalent cations. Activity was detected in the absence of cations or in the presence of EDTA (up to 10 mM) (data not shown), indicating that divalent cations are not required for its activity. However, the presence of 2 mM $MgCl_2$ or $CaCl_2$ in the assay medium enhanced the activity up to 30%. In contrast, $MnCl_2$ had no effect at concentrations lower than 2 mM but at higher concentrations inhibited the enzyme (up to 60%) (data not shown). Adding DTT (up to 0.5 mM) enhanced the activity up to 2-fold and did not prevent the inhibitory effect of $MnCl_2$ (data not shown).

Kinetic Analysis of XG-FTase—The rate of XG-FTase activity was determined at various concentrations of XG or GDP-Fuc, respectively, and assessed by Lineweaver-Burk plots. A reciprocal plot of $1/v$ against $1/S$ at a range of GDP-Fuc and tamarind XG concentrations gave a series of straight lines that intersect on the $-1/S$ axis (Fig. 3). This pattern indicates that GDP-Fuc and tamarind XG have separate binding sites and that the binding of one substrate has no consequence for binding of the other. Thus, we concluded that XG-FTase interacted with the substrates in a random order.

The K_m for GDP-Fuc was ~30 μM and V_{max} was ~150 mm/h/mg protein. Earlier data (24) for the unpurified XG-FTase activity indicated a K_m of 72 μM , with a V_{max} of ~0.8 mm/h/mg protein for GDP-Fuc. These results showed that the purified XG-FTase was more active with higher affinity. For tamarind XG, the K_m was ~0.46 mg/ml (~0.40 μM) and V_{max} ~200 mm/h/mg protein. The very low K_m for XG may be because of a higher number of potential acceptor sites throughout the tamarind XG polymer, which increases its interaction with the enzyme.

Chemical Modification of Amino Acids—To gain some in-

sight about the structure-function relationship in XG-FTase, catalytically essential amino acid residue types were identified. The sulfhydryl reagent NEM, a cysteine-specific modifying agent, has been widely used in discriminating between glycoprotein fucosyltransferase isotypes (25). Our results indicated that NEM had little effect on the XG-FTase activity, with 5 mM giving ~40% inhibition of activity (Table I). However, *N*-bromosuccinimide (NBS), DEPC, and PLP reagents (which react with tryptophan, histidine and lysine residues, respectively) decreased the activity about 90–99% when applied at levels up to 5 mM (Table I). Preincubation of the XG-FTase with GDP-Fuc did not prevent inactivation by NBS treatment (data not shown). More studies using site-directed mutagenesis are needed to determine the locations and the roles of these essential residues.

Substrate Specificity—To study the acceptor specificity of the purified XG-FTase, various substrates (polysaccharides, oligosaccharides, and glycoprotein) were first tested as potential acceptors (Table II). In a second experiment, some of these potential acceptors were tested for their ability to inhibit competitively the transfer of fucose onto tamarind XG (Fig. 4).

Table II and Fig. 4 show that rhamnogalacturonan-I and -II, both known to contain terminal α -fucosyl residues (26), were not substrates for the purified pea XG-FTase. In addition, no incorporation was obtained with α_1 -acid glycoprotein, despite the fact that it is a good acceptor for animal glycoprotein α -1,2- and α -1,3-fucosyltransferases. Tamarind XG is the best acceptor for the fucosyltransfer reaction, and although nasturtium XG is also an acceptor, it shows ~50% less incorporation than tamarind XG. One likely explanation is that nasturtium XG has less (~2 mol %) octasaccharide XXLG than tamarind XG (~31 mol %), and the position of galactose in XXLG is preferentially fucosylated by the enzyme (14) (see below). When pea XG was used, very little [¹⁴C]Fuc incorporation (13% of the control) was obtained, probably because pea XG is already almost completely fucosylated.

Tamarind XG monomers and trimers are comparatively poor acceptors (Table II, low M_r acceptors) but are able to compete with tamarind XG polymer. Strong inhibition was exhibited by the trimers (Fig. 4). Rhamnogalacturonan-II, lactose, and *N*-acetylglucosamine could not inhibit the fucosyltransferase reaction (Fig. 4). All these data indicate that the purified enzyme is indeed a xyloglucan-specific fucosyltransferase without any contaminant fucosyltransferase activities for glycoproteins or pectins.

To examine the specificity of the GDP-fucose-binding site, nucleotides and nucleotide sugars were used as inhibitors of

FIG. 3. Double-reciprocal plots of the initial rate data with GDP-fucose and tamarind XG (TXG) as the varied substrates. GDP-Fuc and tamarind XG were used at various concentrations in a standard assay medium as described under "Experimental Procedures." A, kinetic values for XG-FTase at three concentrations of tamarind XG are as follows: 0.5 mg/ml (●), 1 mg/ml (□), or 4 mg/ml (▲). B, kinetic values for XG-FTase at four concentrations of GDP-Fuc as follows: 12.5 μ M (◆), 25 μ M (○), 50 μ M (▼), or 100 μ M (■). GDP-[3 H]Fuc was used, and data are expressed as picomoles of fucose incorporated per min.

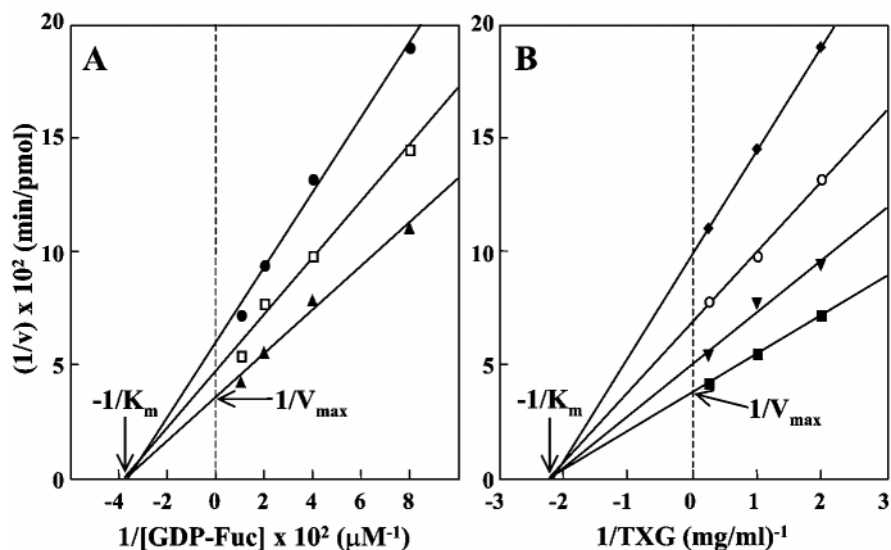


TABLE I

Effect of group-specific reagents on the purified pea XG-FTase activity

The purified enzyme ($\sim 0.2 \mu$ g) was preincubated with the indicated modifying reagent for 20 min and assayed for [3 H]fucose transfer to tamarind XG under standard assay conditions as described under "Experimental Procedures." Values are expressed as % inactivation. Shown are results from one experiment that was repeated three times.

Reagent	Acts on	Inactivation (%)		
		0.5 mM	2.5 mM	5 mM
NEM	Cysteine	5.9	34.4	41.5
PLP	Lysine	51.4	81.0	87.9
DEPC	Histidine	63.5	88.5	95.1
NBS	Tryptophan	96.0	96.3	96.4

TABLE II

Substrate specificity of purified pea fucosyltransferase

Purified enzyme ($\sim 0.2 \mu$ g) was incubated (30 min) with GDP-[14 C]Fuc ($\sim 2 \mu$ M) plus or minus various polysaccharides, glycoprotein, or oligosaccharides as acceptors. Reactions were terminated as described under "Experimental Procedures." The higher background in assays with low M_r acceptors is a result of free [14 C]Fuc released from GDP-[14 C]Fuc.

Additive to reaction mix	[14 C]Fuc Incorporation	
	(+) Enzyme	(-) Enzyme
	<i>dpm/h/reaction mixture</i>	
High M_r acceptors (0.2% w/v)		
None	42.45	41.51
Tamarind XG (TXG)	4391.12	154.32
Nasturtium XG (NXG)	2306.15	185.55
Pea XG	659.17	120.35
Rhamnogalacturonan I	201.00	300.89
Rhamnogalacturonan II	383.29	475.89
Glycoprotein α_1 -acid	149.75	153.94
Low M_r acceptors		
None	574.97	— ^a
TXG monomers (0.2% w/v)	601.81	—
TXG trimers (0.2% w/v)	800.00	—
Lactose (5 mM)	620.42	—
N-Acetylactosamine (5 mM)	603.11	—

^a —, values were not determined.

the fucosyltransferase reaction. The result indicated that guanosine nucleotides provided strong inhibition of XG-FTase in the following order: GMP \cong GDP > GTP (data not shown). This can be explained as end product competition. GDP-fucose showed the same inhibitory effect as GDP or GMP, but no inhibition was obtained with fucose alone, suggesting that the enzyme principally recognizes the nucleotide portion of the

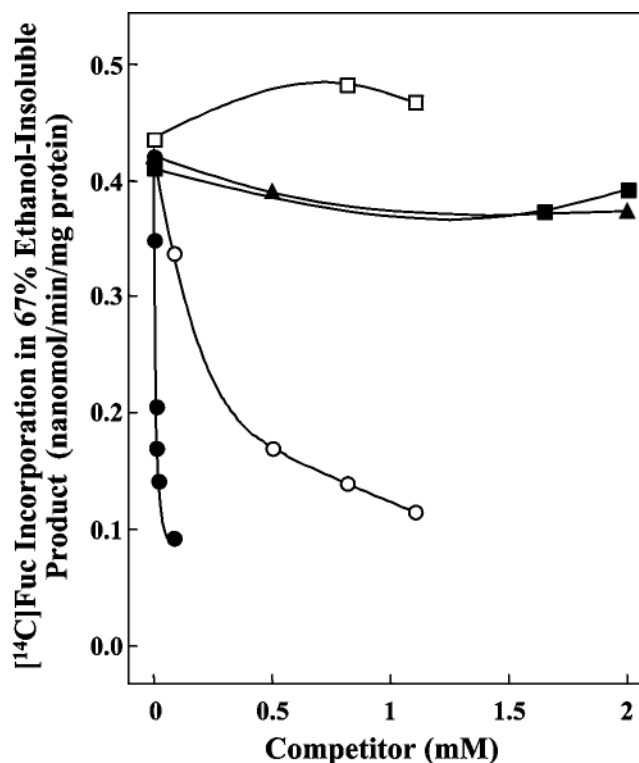


FIG. 4. Competitive inhibition of fucose transfer to tamarind XG by potential acceptors. Purified pea enzyme ($\sim 0.2 \mu$ g) was incubated (30 min) with GDP-[14 C]Fuc ($\sim 2 \mu$ M) and tamarind XG (0.2% w/v) in the absence or presence of N-acetylactosamine (■), lactose (▲), tamarind XG monomers (○), trimers (●), or rhamnogalacturonan-II (□) at final concentrations indicated. Reactions were halted by precipitation with 67% ethanol, and pellets were washed before incorporation of [14 C]Fuc was measured. Results are expressed as nanomoles of fucose incorporated per min per mg of proteins.

GDP-fucose. However, the enzyme is specific for GDP-Fuc because GDP-Man and GDP-Glc did not inhibit the reaction as efficiently as GDP-Fuc (data not shown). Adenosine nucleotides did not provide significant inhibition.

Product Analysis, Identification of Fucosylated Subunits of the XG

Our previous work (15) demonstrated that the purified pea enzyme transfers fucose to position 2 of tamarind XG side chain galactosyl residues. However, we wanted to determine which of

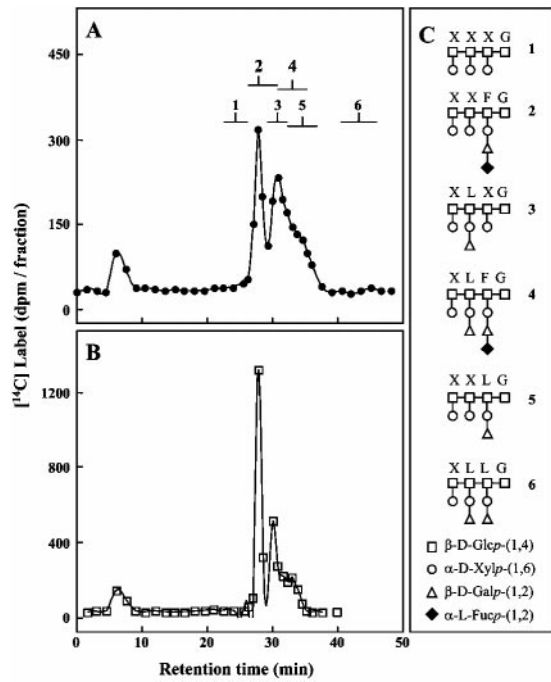


FIG. 5. Distribution of [¹⁴C]Fuc incorporated into tamarind XG subunits in relation to the elution positions of known XG oligosaccharides by HPAEC-PAD through a CarboPac column (Dionex). Separation by the HPAEC-PAD system of cellulase-generated oligosaccharides (~10 μg) from products obtained by action of purified pea XG-FTase (●) (A) or by total microsomal preparation (□) (B), as described under "Experimental Procedures." Known XG oligosaccharides were obtained by cellulase digestion of pea cell wall and tamarind seed XGs, and their structures are presented (C). The locations of XXFG and XLFG are taken from their relative elution positions (numbered 2 and 4, A) from this column as described (8, 9).

the three different subunits found in tamarind XG, namely -XXLG-, -XLXG-, and -XLLG- (14), was the preferred site of fucosylation for pea enzyme. Moreover, we sought to determine which of the two galactosyl residues was fucosylated when -XLLG- was used. To address these questions, tamarind XG was fucosylated by the purified pea enzyme and was digested with *Trichoderma* cellulase before fractionation on Bio-Gel P2 columns. The radiolabeled fragments eluted in a peak that overlapped with the higher M_r side of the XG oligosaccharides peak as expected (data not shown). The products were further fractionated by HPAEC on a CarboPac PA-100 column (Fig. 5), and positions of labeled fragments were compared with those of authentic XG oligosaccharides as described previously (9, 14). Double peaks (one large and one very small) were observed, both with the radiolabeled products and with the marker oligosaccharides. The cause of the double peaks is not certain, but a possible explanation could be that the minor peak represents the oxidized form of the oligosaccharides at the reducing carbon at position 1 of glucose to form gluconic acid residue. These charged forms would elute slightly later than the parent oligosaccharides. Since all the authentic oligosaccharides behave in the same way, our assignments regarding their structures are correct.

Radiolabel was recovered only in fractions where XXFG and XLFG eluted, at a dpm ratio of approximately 1.5:1, respectively (Fig. 5). There was no sign of products fucosylated closer to the non-reducing end of the subunits, e.g. XFXG or XFLG. We would expect these subunits to elute differently from one another, just as the octasaccharides do (Fig. 5). There was no indication of any labeled XFFG in these products, which would have been expected to elute from this column at a different locus from nona- and decasaccharide, as it does after high

pressure liquid chromatography on a Dynamax-60A NH₂ column (6). We conclude that the purified pea XG fucosyltransferase is specific for fucosylation of galactosyl residues nearest the reducing end of XG subunits.

This experiment was repeated using a crude enzyme preparation from microsomal membranes in an effort to identify any isozymes present that could generate different fucosylated subunits, namely XFXG, XFLG, or XFFG. The data in Fig. 5 show that the crude enzyme exhibits the same profile as the purified enzyme, but the dpm ratio between XXFG and XLFG has changed to 3:1. This suggests that, in tamarind XG, the membrane-bound form of the enzyme specifically fucosylates the -XXLG- subunit over -XLLG-. This could be due to a regulatory factor present in the microsomes or a modification of -XLFG- by either α-fucosidase or β-galactosidase, which could increase the amount of XXFG over XLFG.

DISCUSSION

The XG-specific fucosyltransferase was first detected *in vitro* in microsomal membrane preparations from pea epicotyl tissue (27). Fucosylation did not require concurrent addition of UDP-Glc, UDP-Xyl, or UDP-Gal and was evidently due to transfer of fucose to endogenous nascent XG. Microsomal membranes incorporated several times more [¹⁴C]Fuc from GDP-[¹⁴C]Fuc into added tamarind XG than into endogenous XG (18). Isopycnic centrifugation provided evidence that the XG-FTase is present in both a dense Golgi fraction and in vesicles from the *trans*-Golgi network (28). Later studies using antibodies to the fucosylated region of XG determined that the fucose-containing product is observed in the *trans*-Golgi cisternae and in the *trans*-Golgi network (10). Taken together, these studies provide evidence that fucosylation occurs after XG backbone biosynthesis has been completed.

This XG-specific fucosyltransferase from pea epicotyl microsomes was purified as described earlier (15). Analysis of the purification data indicates that XG-FTase is present at very low levels in the cells (less than 0.01% of total protein).

Gel filtration and SDS-PAGE data indicated that the native form of XG-FTase exists as an oligomer (~250 kDa), possibly including the BiP chaperone protein (~68 kDa polypeptide) as part of the complex. The biological relationship between the two polypeptides is still unknown. One possibility is that the chaperone protein BiP may be involved directly or indirectly in XG biosynthesis. A similar dependence has been recently shown for the cell wall β-1,6-glucan synthesis in *Saccharomyces cerevisiae* (29), where β-1,6-glucan biosynthesis depends partly on the molecular chaperone BiP/Kar2p. The other possibility is that the interaction between XG-FTase and BiP is simply an artifact resulting from the carbonate treatment of the microsomes. Nonetheless, many α-1,2-fucosyltransferases for glycoprotein studied to date have an oligomeric form with a molecular mass of ~150 kDa, but SDS-PAGE estimations of molecular mass are ~50 kDa (22). It has been postulated that oligomerization might function as a signal for retention in the Golgi, avoiding transport to the plasma membrane (30).

As expected, PsFT1 shared a high degree of amino acid sequence similarity with its *Arabidopsis* homolog (AtFT1). Comparison of both sequences provided further support for the conclusion that the cloned pea cDNA encoded a XG-FTase enzyme. The Kyte-Doolittle hydrophilicity plot (31) suggested the presence of a 23-amino acid transmembrane domain (TMD) at the NH₂ terminus, indicating that PsFT1 is a type II membrane-bound protein with the active site located in the lumen, like all other fucosyltransferases described to date (with the exception of the bacterial fucosyltransferases that lack the TMD). The conclusion that PsFT1 is a type II membrane protein is also supported by the observation that activity was

greatly stimulated upon the addition of detergents (*i.e.* BRIJ 58) to sealed microsomal membrane vesicles without protein solubilization.² Computer analysis indicated that PsFT1 and AtFT1 shared three conserved motifs with known α -1,2- and α -1,6-fucosyltransferases from vertebrate, invertebrate, and bacteria. One very recent study (23) using Fasta search and hydrophobic cluster analysis showed that α -1,2- and α -1,6-fucosyltransferases have two common motifs (motif I and motif II) in addition to one motif (motif III) specific to each group. Interestingly, for the *Arabidopsis* and pea XG-FTases the motif I is present, but the motif III in these XG-FTases contains elements from both known α -2-motif III and α -6-motif III. This favors a common genetic origin for these three groups of transferases.

Biochemical characterization revealed that PsFT1 enzyme was stable over a wide pH range (3–9), required no divalent cations for full activity, and had a random order interaction with its substrates as has been observed for other fucosyltransferases (32–35).

Several amino acid modification effects were observed. A characteristic that clearly distinguishes different subtypes of fucosyltransferases is the inactivation by a low amount of NEM due to an essential cysteine residue involved in GDP-Fuc binding (35). NEM had little or no effect on the pea XG-FTase activity. However, reagents that modify tryptophan, histidine, and lysine residues (NBS, DEPC, and PLP, respectively) significantly reduced the activity. Histidine is often found at active sites of enzymes where its imidazole group charge can change depending on local environment and thus catalyze the cleavage of bonds. PLP is known to inhibit effectively enzymes that bind phosphorylated substrates via a Schiff base with an amino group. For example, a GDP-fucose-protectable pyridoxal-5'-P-modifiable Lys residue has been shown to be present in the α -1,3-fucosyltransferase from human small cell lung carcinoma NCI-H69 cells (36). A more recent study showed that a Lys residue located within an α -1,3-fucosyltransferases motif is required for activity but not substrate binding (37). It has been shown also that this basic residue is involved in a hydrogen bond between donor and acceptor hydroxyl groups during the catalysis (38). Interestingly, two conserved Lys residues are found located within this segment in PsFT1 and AtFT1 enzymes. Since divalent cations were not required for the activity of PsFT1, it is possible that the Lys residues may serve the same function as the cations by neutralizing the negative charge of the phosphate on GDP-Fuc to enable it to associate with the enzyme. The purified pea XG-FTase was extremely sensitive to NBS, demonstrating an essential tryptophan residue that could not be protected by GDP-Fuc. This suggests that the tryptophan residue is located at a site distinct from the sugar nucleotide-binding site. PsFT1 has 32 lysine, 12 histidine, and 8 tryptophan residues in its amino acid sequence. Most of them are conserved with its *Arabidopsis* homolog AtFT1 and are located at the COOH terminus.

The purified XG-FTase was indeed specific for XG, being unable to utilize acceptors derived from either plant cell wall polysaccharides or glycoprotein carbohydrate moieties. It was also unable to fucosylate free tamarind XG oligosaccharides. However, competitive inhibition experiments indicate a higher affinity of the enzyme for the trimers than for the monomers. This result supports the recently proposed model for the mode of action of pea XG-FTase activity (14) wherein the enzyme binds to a trimer in galactosylated nascent chains and fucosylates the octasaccharide (-XXLG-) nearest the reducing end of the chain. Repetition of this action results in a chain with

repeating hepta:nonasaccharide subunits (-XXXG-XXFG-). It will be of interest to investigate the minimal structure recognition of the acceptor with this enzyme in the future. Also, it is of interest to know the structural features that dictate acceptor substrate specificity. It has been shown that only a few amino acids found within a "hypervariable" region at the NH₂ terminus of human α -1,3- and α -1,4-fucosyltransferases determine the substrate specificity for these enzymes (39).

The products formed by the purified enzyme were identified as XXFG and XLFG following cellulase digestion of the ¹⁴C-fucosylated tamarind XG. This result was predicted because both of these subunits are found in XG (4–9). In pea XG, however, the predominant natural fucosylated subunit is XXFG; XLFG is a minor component (5). Thus, pea XG-FTase generated the same two fucosylated subunits *in vitro* as *in vivo*, but the ratio of their yields is different from that found in natural pea cell wall XG. This difference occurs because the products recovered in this experiment must have arisen by ¹⁴C-fucosyl transfer to the subunits -XXLG- and -XLLG-, the major subunits in tamarind XG, with a ratio of 3:5 (8, 14). It is interesting to point out that no products such as XFXG or XFLG were observed. Moreover, the existence of these oligosaccharides has not yet been demonstrated to date in any plant cell wall XG. The fact that the fucosylation occurs only at the galactosyl residues closest to the unbranched glucose in the XG indicates the existence of a recognition system that can discriminate between the two galactosyl residues in the same subunit (*e.g.* -XLLG-). This discrimination prevents the fucosylation of the second galactose in pea. A crude enzyme preparation from microsomal membranes produced mostly XXFG, showing that the pea tissues from growing regions probably have only one XG-FTase that makes this linkage. However, sycamore cells have fucose on both galactosyl residues in the same subunit (-XFFG-) (6). In this species it will be interesting to determine whether a second FTase exists or whether they have a single enzyme that is able to add fucose to both galactosyl residues.

The availability of the cDNA clones and information about the biochemical properties of XG-FTase will allow future studies aimed at understanding how this enzyme activity is regulated as cell wall synthesis progresses during growth. It will also be important to identify other enzymes involved in XG biosynthesis so that the entire process can be analyzed.

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