Two Xyloglucan Xylosyltransferases Catalyze the Addition of Multiple Xylosyl Residues to Cellohexaose*□

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Xyloglucan (XYG) is the principal hemicellulose found in the primary cell walls of most plants. XYG is composed of a β-(1,4)-glucan backbone that is substituted in a regular pattern with xylosyl residues, which are added by at least one and likely two or three xylosyltransferase (XT) enzymes. Previous work identified seven Arabidopsis thaliana candidate genes, one of which (AtXT1) was shown to encode a protein with XT activity (Faik, A., Price, N. J., Raikhel, N. V., and Keegstra, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7797–7802). We expressed both AtXT1 and a second closely related gene, now called AtXT2, in insect cells and demonstrated that both have XT activity for cellopentaose and cellohexaose acceptor substrates. Moreover, we showed that cellohexaose was a significantly better acceptor substrate than cellopentaose. Product structural characterization showed that AtXT1 and AtXT2 preferentially added the first xylosyl residue to the fourth glucosyl residue from the reducing end of both acceptors. Furthermore, when the ratio of UDP-xylose to cellohexaose and the reaction time were increased, both AtXT1 and AtXT2 added a second xylosyl residue adjacent to the first, which generated dixylosylated cellohexaose. On the basis of these results, we concluded that AtXT1 and AtXT2 have the same acceptor specificities and generate the same products in vitro. The implications of these results for understanding in vivo XYG biosynthesis are considered.

The primary wall surrounding plant cells is composed of cellulose, hemicellulose, pectin, and protein. Xyloglucan (XYG)² is the principal hemicellulose found in the primary cell walls of non-graminaceous plants, where it composes up to 25% of the cell wall. XYG is thought to link via hydrogen bonds to the surfaces of adjacent cellulose microfibrils, thereby forming three-dimensional cellulose-XYG networks that function as the principal load-bearing structure of the primary cell wall (1).

XYG is composed of a backbone of 1,4-linked β-d-Glcp residues that are substituted in a regular pattern at O-6 with an α-d-Xylp residue (where “Xyl” is xylose) to form repeating subunits with specific xylosylation patterns that are highly conserved (2). Most plants have XYG with an XXXG-type repeating subunit (where “G” denotes an unsubstituted glucosyl residue, and “X” denotes a glucosyl residue substituted at O-6 with an α-d-Xylp residue; see Ref. 3 for XYG nomenclature). However, plants in Poaceae and Solanaceae have an XXGG-type repeating subunit and perhaps also an XXGGG-type repeating subunit (for reviews, see Refs. 4–6). Further sugar substitution occurs primarily at specific xylosyl residues within the repeating subunit. These xylosyl residues are substituted at O-2 with a variety of glucosyl moieties, with the most common being β-d-Galp (represented by “L” and found in many plant species), α-L-Araf (where “Ara” is arabinose; represented by “S” and found only in Poaceae and Solanaceae), and the disaccharide α-L-Fucp-(1,2)-β-d-Galp (where “Fuc” is fucose; represented by “F”). Although the structure of the XYG repeating subunit is generally highly conserved within a plant species, the frequency and distribution of further carbohydrate substitutions may vary significantly. For example, endoglucanase digestion of Arabidopsis XYG produces a diverse array of oligosaccharides, including XXXG, XXLG, XXFG, XLFG, XLGG, and XLXG, all containing XXXG as the core XYG repeating subunit (7).

The biosynthesis of XYG requires α-fucosyltransferase, β-galactosyltransferase, α-xylosyltransferase (XT), and β-(1,4)-glucan synthase activities. Recently, much progress has been made in the identification and characterization of the genes and proteins involved in XYG biosynthesis. An XYG α-fucosyltransferase (FUT1) was purified from pea epicotyls using traditional biochemical purification techniques (8). The amino acid sequence was used to identify XYG fucosyltransferase genes in Arabidopsis thaliana (AtFUT1) (8) and Pisum sativum (PsFUT1) (9). Alternatively, a genetic strategy was used to identify one of the XYG galactosyltransferases. A screen of ethyl-methane sulfonate-mutagenized Arabidopsis plants identified a mutant line (mur3) with a reduction in cell wall fucose content (10); further investigation determined that MUR3 encodes an XYG galactosyltransferase (7).

Recently, Faik et al. (11) identified seven putative XYG XT genes that are related to the previously characterized fenugreek galactomannan galactosyltransferase (GMGT) (12) and that belong to CAZy family GT34 (afmb.cnrs-mrs.fr/CAZY/) (13). The results of heterologous expression of the seven candidate

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2 The abbreviations used are: XYG, xyloglucan; XT, xylosyltransferase; GMGT, galactomannan galactosyltransferase; EGI, endoglucanase II; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HPAEC, high performance anion-exchange chromatography.

The implications of these results for understanding in vivo XYG biosynthesis are considered.
Heterologous Expression of AtXT1 and AtXT2

XT genes in *Pichia pastoris* indicate that AtXT1 (At3g62720) encodes a protein with XT activity when cellopentaose is the acceptor substrate (11). However, attempts to express other members of this gene family in *Pichia* cells did not result in detectable XT activity, so the functions of the AtXT2–7 glycosyltransferases remain to be determined. The lack of activity was particularly surprising for At4g02500 because it has high sequence similarity (83% identical and 91% similar at the amino acid level) to AtXT1. Therefore, we revisited the heterologous expression of AtXT1 and At4g02500 in insect cells. In this study, we further characterized AtXT1 activity and present evidence that At4g02500 (henceforth referred to as AtXT2) encodes a protein with XT activity. Furthermore, through product characterization, we show that both proteins can add multiple xylosyl residues to cellohexaose.

EXPERIMENTAL PROCEDURES

**Clones, Chemicals, and Enzymes**—The cDNA clones for At3g62720 (AtXT1; U14458) and At4g02500 (AtXT2; U25215) were from the Arabidopsis Biological Resource Center (www. biosci.ohio-state.edu/pcmb/Facilities/abric/abrichome.htm). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). AccuPrime Pfx DNA polymerase, Gateway cloning and expression vectors, LR Clonase, *Escherichia coli* TOP10 competent cells, and the *Drosophila* expression system, and the Bac-to-Bac baculoviral expression system were from Invitrogen. The GENECLEAN SPIN kit was from Bio-Rad. EDTA were from Roche Applied Science. Micro Bio-Spin chromatography columns and the protein assay kit were from Ireland). Complete mini protease inhibitor tablets lacking 3.2.1.4) were from Megazyme International Ireland Ltd. (Bray, Ireland). UDP-xylose was purchased from CarboSource (www.carbosource/CSS_home.html). The acceptor substrates glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose; Dowex 1-X8 Cl– ion-exchange matrix (200–400 mesh); and Driselase were from Sigma. Cellobiose was from Seikagaku America (East Falmouth, MA). Oligoxyloglucan, Aspergillus niger β-glucosidase (EC 3.2.1.21), and Trichoderma longibrachiatum endoglucanase II (EGLI; EC 3.2.1.4) were from Megazyme International Ireland Ltd. (Bray, Ireland). Complete mini protease inhibitor tablets lacking EDTA were from Roche Applied Science. Micro Bio-Spin chromatography columns and the protein assay kit were from Bio-Rad.

**Cloning of AtXT1 and AtXT2—** AtXT1 (At3g62720) and AtXT2 (At4g02500) were PCR-amplified from full-length cDNA clones with gene-specific primers containing a CACC adapter on the 5′-end of the forward primer, which is required for directional cloning using the pENTR/D-TOPO vector. The primer sequences are as follows: AtXT1, CACCATGATAGAAGTGCTATAGGAGCCGA (forward) and CGGAAAAATTAAAAGTACAAAAACAA (reverse); and AtXT2, CACCATGATTGAGGAGGTGTAGAGCGTTA (forward) and CCTAAAGCAAAAACCGATTT (reverse). PCR products were separated on a 1.5% agarose gel, and the DNA corresponding to the size of the expected PCR product was purified using the GENECLEAN SPIN kit, directionally cloned into the pENTR/D-TOPO donor vector, and transformed into *E. coli* TOP10 competent cells. Proper donor constructs were identified from isolated plasmids that had been sequenced to ensure that no mutations had been introduced.

**Expression of AtXT1 and AtXT2—** The *Drosophila* expression system and the Bac-to-Bac baculoviral expression system were used for heterologous expression of AtXT1 and AtXT2. Constructs of AtXT1 and AtXT2 in pENTR/D-TOPO donor vectors were recombined into pMT-DEST48 and pDEST8 destination vectors via 18-h Clonase reactions.

*Drosophila* S2 (Schneider 2) cells were cotransfected with the pCoBlast vector and pMT-DEST48 containing either the full-length AtXT1 or AtXT2 cDNA sequence. Selection of cell lines stably expressing AtXT1 and AtXT2 was done with blastidicin following the supplier’s instructions (Invitrogen). Expression of AtXT1 and AtXT2 was induced by the addition of copper sulfate (500 μM) to the cell cultures 24 h prior to cell harvest. Control S2 cell lines that were not transfected were cultured following the supplier’s instructions. S2 cell cultures (20–30 ml each) were centrifuged at 500 × g for 10 min; the supernatant was removed; and the cells were solubilized with extraction buffer (100 mM HEPES (pH 7.0) containing 1% Triton X-100, 5 mM MnCl2, and one protease inhibitor tablet/20 ml).

The pDEST8-AtXT1 and pDEST8-AtXT2 constructs were electroporated into *E. coli* DH10Bac cells for transposition of the respective XT gene into *Autographa californica* bacmids, which were subsequently isolated from positive clones, and the inserts were sequenced. Positive recombinant bacmids were transfected into Sf21 (Spodoptera frugiperda 21) cells following the supplier’s instructions (Invitrogen). The Sf21 cell cultures were divided, placed into 50-ml conical tubes, and centrifuged at 5000 × g for 15 min. The supernatant was decanted, and the pelleted Sf21 cells were frozen in liquid nitrogen and stored at –80 °C.

The S2 or Sf21 pellets were suspended in ice-cold extraction buffer. The cells were suspended with a pipette and disrupted by 3 × 15 s sonication with a 45-s pause, with storage on ice between sonication treatments. Cell disruption was verified by microscopy. Protein assays were conducted, and the protein concentration was adjusted to 6.25 mg/ml with extraction buffer. The cell extracts were either placed on ice for immediate use in enzyme assays or frozen with liquid nitrogen and stored at −80 °C.

**Immunoblot Analysis**—Cell extracts (either 40 μg of S2 extract total protein or 20 μg of Sf21 extract total protein) were separated by SDS-PAGE as described previously (14), and the proteins were transferred to polyvinylidene difluoride membranes (15). Membranes were blocked with a 5% solution of milk proteins and probed with a polyclonal antibody (1:1000 dilution) raised in rabbits against a fragment of AtXT1 (amino acids 45–195). This antibody was a gift of Ahmed Falk, and the details of its preparation and characterization will be reported elsewhere. Membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit antibodies and developed using the Super Signal West Pico chemiluminescence system. Membranes were stained with Coomassie Blue to ensure uniform protein loading and transfer.

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3 T. Konishi, A. Falk, and K. Keegstra, manuscript in preparation.
**XT Assay**—The XT assay was based upon conditions described previously (11). Cell extracts from S2 and Sf21 cells expressing AtXT1 and AtXT2 were initially surveyed for XT activity with 0.4 mM UDP-xylose, 44,400 dpm UDP-[14C]xylose, and 4.0 mM acceptor substrate (0.1:1.0 substrate ratio); all other XT reactions were conducted in the presence of either 1.0 mM UDP-xylose, 44,400 dpm UDP-[14C]xylose, and 1.0 mM acceptor substrate (1.0:1.0 substrate ratio) or 2.5 mM UDP-xylose, 111,000 dpm UDP-[14C]xylose, and 1.0 mM acceptor substrate (2.5:1.0 substrate ratio). For each XT assay, 40 μl of cell extract was mixed with 10 μl of substrate solution containing the appropriate amounts of UDP-xylose, UDP-[14C]xylose, and acceptor substrate dissolved in extraction buffer. XT reactions were incubated at 25 °C for either 1 or 18 h and terminated by the addition of 450 μl of Dowex 1-X8 ion-exchange resin suspended in water (1:2 resin/water). Each reaction slurry was loaded onto a Micro Bio-Spin chromatography column and spun in a microcentrifuge at 1300 g for 2 min. The column effluent (either 50 or 300 μl) was mixed with 2.0 ml of scintillation counting fluid; radioactivity was then measured on a Beckman Coulter LS-5000 scintillation counter. XT reactions that were destined to be analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) did not contain UDP-[14C]xylose and were carried out in parallel with XT reactions containing UDP-[14C]xylose.

**XT Product Characterization Strategy**—To structurally characterize XT products, we developed a strategy that used a combination of high performance anion-exchange chromatography (HPAEC) and MALDI-TOF-MS analysis of XT products sequentially digested with β-glucosidase and EGII. The structural characterization strategy consisted of three major steps, each of which involved the parallel analysis of radiolabeled and unlabeled products generated by enzymes with a combination of HPAEC and MALDI-TOF-MS.

Radiolabeled reaction products were partitioned by HPAEC and fractionated into 250-μl aliquots. The radioactivity of each fraction was determined and plotted against time to generate a radioactivity profile that was used to monitor the distribution and amount of radioactivity before and after each enzyme treatment. Unlabeled reaction products were partitioned by HPAEC, desalted, and fractionated into 500-μl
aliquots. Each fraction was analyzed by MALDI-TOF-MS to determine the presence and proportion of each reaction species present at a specific elution time interval.

In the first step, five XT reactions, with or without radiolabel, were pooled (total volume of 1.25 ml), dried down, and suspended in 100 l of water; 25 l of each sample was then analyzed by either HPAEC or HPAEC followed by MALDI-TOF-MS. In the second step, 75-l aliquots of radioactive or nonradioactive XT products were spiked with 10 mol of cellohexaose, dried down, suspended in 75 l of hydrolytic enzyme buffer (25 mM sodium acetate and 0.002% thimerosal (pH 5.0)) containing 50.0 milliunits of -glucosidase, and incubated overnight at 37 °C. Cellohexaose was added as an internal control to verify complete digestion. Reactions were terminated by heating at 100 °C for 10 min, and insoluble material was pelleted by centrifugation at 14,000 × g for 5 min. The supernatant was removed, and a 25-l aliquot was analyzed by HPAEC followed by either liquid scintillation counting or MALDI-TOF-MS.

High pH Anion-exchange Chromatography Instrumentation and Conditions—The XT reaction products were analyzed using a Dionex DX300 carbohydrate analysis system equipped with a CarboPac PA10 column (4 × 250 mm) and an ED50 for pulsed amperometric detection following methods that were adapted from previously published studies (16, 17). Reaction products (25 l/injection) were eluted at a flow rate of 1 ml/min with a gradient of 50 mM sodium acetate and 100 mM sodium hydroxide (0 min) to 100 mM sodium acetate and 100 mM sodium hydroxide (20 min). The column was washed with 300 mM sodium hydroxide (15 min) and re-equilibrated with 50 mM sodium acetate and 100 mM sodium hydroxide (15 min) after each injection. For HPAEC analysis of radiolabeled XT products, each injection was fractionated into 250-l aliquots and mixed with 2.0 ml of scintillation counting fluid, and the radioactivity was determined by scintillation counting. Non-radiolabeled XT reaction products destined for MALDI-TOF-MS analysis were partitioned by HPAEC, desalted with a Dionex in-line desalting module following the manufacturer’s instructions, fractionated into 500-l aliquots, and dried down.

MALDI-TOF-MS—XT reaction products were analyzed by MALDI-TOF-MS based upon previously published methods (18). Briefly, desalted fractions from the HPAEC-partitioned reaction products were dried down, suspended in 2 l of 2,5-dihydroxybenzoic acid dissolved in acetonitrile (10 mg/ml), loaded onto the MALDI-TOF-MS plate, and allowed to crystallize. XT reaction products were analyzed with a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in reflector mode with a 100-ns extraction
time and a 20-kV accelerating voltage. Each spectrum consisted of data accumulated over 50 laser shots.

RESULTS

Heterologous Expression of AtXT1 and AtXT2—AtXT1 and AtXT2 were expressed in two insect cell lines (Drosophila S2 and Sf21) as an alternative to heterologous expression in P. pastoris. The expression of AtXT1 or AtXT2 was verified by immunoblot analyses of S2 and Sf21 cell extracts with an antibody raised against a peptide fragment of AtXT1 (Fig. 1). Because immunoblot analysis verified the presence of AtXT1 and AtXT2 proteins in both expression systems, detergent-solubilized extracts were assayed for XT activity.

In the presence of either cellopentaose or cellohexaose as the acceptor substrate, detergent-solubilized extracts from both S2 and Sf21 cells expressing either AtXT1 or AtXT2 showed significant levels of XT activity (Fig. 2, A and B). Although both enzymes utilized either cellopentaose or cellohexaose as an acceptor, the activities of both enzymes were 4-fold higher with cellohexaose as the acceptor compared with cellopentaose. Therefore, further biochemical studies on AtXT1 and AtXT2 activities were conducted using cellohexaose as the acceptor substrate. Furthermore, because we wanted large quantities of material for biochemical characterization of XT activity, we utilized Sf21 cells expressing either AtXT1 or AtXT2 for the experiments described below.

Preliminary Characterization of AtXT1 and AtXT2 Enzymatic Activities Produced in Sf21 Cells—In initial studies, we used an XT assay with a 1:10 donor/acceptor substrate ratio (0.4 mM UDP-xylose and 4.0 mM cellopentaose or cellohexaose). However, HPAEC analysis of AtXT1 and AtXT2 products conducted at these substrate ratios showed that a significant amount of cellohexaose was not xylosylated (data not shown). Experiments to increase the amount of xylose incorporated into products demonstrated that a 1:1 substrate ratio (1.0 mM UDP-xylose and 1.0 mM cellohexaose) increased the amount of xylose incorporated and decreased the amount of cellohexaose remaining after 1 h compared with the previously published assay conditions (11) (data not shown). Because we were more interested in defining the products of these enzymes than in analyzing the kinetic parameters of each enzyme, the latter reaction conditions were adopted as the standard XT assay conditions and used for further characterization of AtXT1 and AtXT2 substrate specificities and the products generated.

The specificity of AtXT1 and AtXT2 was investigated using various sugar nucleotide donors and oligosaccharide acceptors. AtXT1 and AtXT2 are specific for cellooligosaccharides with a degree of polymerization of >4 (Fig. 3, A and B). Cello-oligosaccharides

| Enzyme | Xyl incorporation (pmol) |
|--------|--------------------------|
| AtXT1  |                          |
| 1.0 mM UDP-Xyl | 20,626 ± 793 |
| 18 h   | 35,525 ± 1165            |
| 2.5 mM UDP-Xyl | 24,656 ± 1097 |
| 1 h    | 50,809 ± 1300            |
| 18 h   |                          |
| AtXT2  |                          |
| 1.0 mM UDP-Xyl | 14,668 ± 598 |
| 1 h    | 33,056 ± 1820            |
| 2.5 mM UDP-Xyl | 18,515 ± 602 |
| 1 h    | 40,243 ± 2928            |

FIGURE 4. HPAEC analyses of products generated by AtXT1. Products were detected via pulsed amperometric detection (PAD; lower panels), and the eluate was fractionated into 250-μl aliquots. The radioactivity in each aliquot was determined by liquid scintillation counting and graphed as a function of time (upper panels). The compound present in each peak was named according to a previously described nomenclature (3). UDP-Xyl, UDP-xylose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose.
longer than cellohexaose were not tested as acceptor substrates because they are only partially soluble in aqueous buffers. Like AtXT1 (11), AtXT2 is specific for UDP-xylose as the donor substrate because it does not catalyze the incorporation of [14C]galactose into products when assayed under XT or GMGT assay conditions (12, 19) with UDP-galactose as the donor and either cellohexaose or mannohexaose as the acceptor substrate (data not shown).

An additional series of experiments was conducted to characterize the enzymatic properties of both AtXT1 and AtXT2. Many glycosyltransferases are stimulated by the presence of divalent cations. Previous work has shown that XyG biosynthesis is stimulated by Mn$^{2+}$ but not Mg$^{2+}$ (20–24). However, the previous studies were done with enzyme preparations in which both XyG-glucan synthase and XT activities were present, so it was unclear whether the XyG-glucan synthase, XT, or both required Mn$^{2+}$. Using AtXT1 and AtXT2 produced in Sf21 cells, we determined that AtXT1 and AtXT2 activities required Mn$^{2+}$ (supplemental Fig. S1A); the maximum amount of xylose incorporation was achieved over a broad concentration range of 3–15 mM Mn$^{2+}$ (supplemental Fig. S1B). Finally, like many \( \beta \)-glucosidase and EGI to determine 1) the structure of the XT products generated when cellohexaose was used as the acceptor and 2) whether the product structure varied when the substrate ratio and reaction time were varied. Although product characterization is shown for only one of the two enzymes (AtXT1 in Figs. 4 and 6–8 and AtXT2 in Fig. 5), all analyses were performed on products generated with both enzymes, and similar results were obtained regardless of which enzyme was used.

The first question we sought to answer was whether AtXT1 and AtXT2 have the ability to add more than one xylosyl residue to cellohexaose. HPAEC analysis of AtXT1 and AtXT2 products generated at the substrate ratios and incubation times presented in Table 1 revealed the presence of radiolabeled peaks that eluted after cellohexaose (Fig. 4). The total amount of radioactivity incorporated and the proportion of radioactivity associated with each peak were dependent upon the time of incubation and substrate ratio. Parallel XT reactions containing only unlabeled UDP-xylose were performed, and the products were separated by HPAEC, fractionated, and analyzed by MALDI-TOF-MS to determine the masses of the oligosaccharides present in each peak. MALDI-TOF-MS analysis of prod-
ucts generated during a 1-h reaction at a 1:1 substrate ratio (the same as the reaction shown Fig. 4A) contained mainly monoxylosylated cellohexaose along with smaller amounts of unreacted cellohexaose and dixylosylated cellohexaose (data not shown).

Analysis of both labeled (Fig. 4B) and unlabeled (Fig. 5A) reactions conducted for 18 h at a 2.5:1 substrate ratio revealed a more complex mixture of products. MALDI-TOF-MS of HPAEC-partitioned unlabeled products demonstrated that the first peak contained mainly monoxylosylated cellohexaose (Fig. 5B); that the second peak contained mainly dixylosylated cellohexaose (Fig. 5C); and that the small shoulder on the second peak contained the same products as the second peak, but with a significantly higher proportion of trixylosylated cellohexaose (Fig. 5D). These results show that AtXT1 and AtXT2 are capable of adding more than one xylosyl residue to cellohexaose and that the three products produced at a 2.5:1 substrate ratio during 18-h reactions (Figs. 4B and 5A) are mono-, di-, and trixylosylated cellohexaose, respectively.

We next sought to determine the xylosylation patterns of XT reaction products. We first investigated the position of the xylosyl residue closest to the nonreducing end by digestion of the reaction products with β-glucosidase. This enzyme will hydrolyze unsubstituted glucosyl residues from the nonreducing end of β-(1,4)-linked oligosaccharides, but cannot remove a substituted glucosyl residue. In reactions containing mainly monoxylosylated products (e.g. as shown in Fig. 4A at a 1:1 substrate ratio and a 1-h incubation), β-glucosidase treatment produced predominantly XGGG (data not shown), thereby leading to the conclusion that the primary product generated under these reaction conditions was GGGXGGG. When a more complex mixture of products generated during an 18-h reaction at a 2.5:1 substrate ratio (as shown in Figs. 4B and 6A) was treated with β-glucosidase and analyzed by HPAEC, a significant shift in the distribution of radioactivity was observed (Fig. 6, compare A and B). MALDI-TOF-MS analyses of a parallel experiment lacking radiolabel indicated that the main peak consisted primarily of XGGG (Fig. 7, A and B), whereas the shoulder contained mainly dixylosylated cellotetraose (Fig. 7, B and C). The second peak of β-glucosidase-digested products (Fig. 6B, peak B-II) contained XGGGGG (Fig. 7, D and E), XGGGG (Fig. 7F), dixylosylated cellopentaose (Fig. 7, D–F), and trixylosylated cellopentaose (Fig. 7, E and F). Although the amount of radioactivity associated with the elution time interval of 7.0 to 9.0 min is very small (Fig. 6B), MALDI-TOF-MS analyses indicated the presence of ions that corresponded to XGG and dixylosylated cellotriose (data not shown).

The presence of ions corresponding to the monoxylosylated products XGG, XGGG, XGGGG, and XGGGGG from β-glucosidase-digested XT products indicated that AtXT1 and AtXT2 were capable of generating GGGXGGG, GXGGGG, GXXGGG, and XGXGGG, respectively. Furthermore, the HPAEC analysis of β-glucosidase-treated XT products presented in Fig. 6B demonstrated that the amount of radioactivity associated with XGG was significantly greater compared with all other species of β-glucosidase products combined, thereby leading to the conclusion that the primary product generated under all reaction conditions was GGGXGGG. Although this scheme identified the first xylose-substituted glucosyl residue from the nonreducing end of the XT products, it had a significant drawback in that it could not resolve the structures of reaction products containing more than one xylose substitution.

To solve this problem, β-glucosidase-digested reaction products were subsequently treated with EGII, an endoglucanase.
that will hydrolyze the glycosidic linkage of unsubstituted \(\beta-(1,4)\)-linked glucosyl residues. The products resulting from sequential digestion with both enzymes were analyzed by HPAEC (Fig. 6C) and MALDI-TOF-MS (Fig. 8). HPAEC analysis revealed three radioactive peaks with \(R_f\) values (Fig. 6C) that corresponded to previously published values for XG, XXG, and XXXG (17). MALDI-TOF-MS analyses of separated unlabeled products showed the presence of ions with \(m/z\) ratios consistent with these peak assignments (Fig. 8).

These results allowed us to deduce the structures of the multixylosylated reaction products identified by MALDI-TOF-MS in Figs. 5 and 6. The structure of the dixylosylated cellotetraose that composed the shoulder of XGGG in Fig. 6B was XXGG; the structures of the di- and trixylosylated cellopentaose that were identified as components of the second peak in Fig. 6B were XXGGG and XXXGG, respectively. On the basis of these deductions, we concluded that the original XT multixylosylated products consisted primarily of GGXXGG, with minor amounts of GXXGGG and GXXGGG. Although we could not completely discount the presence of minor amounts of XT products with alternating xylose substitution patterns (such as GGXGXX and GXGXGG), significant amounts of these reaction products could not have been present given the proportion of radioactivity associated with XG after EGII digestion (Fig. 6C) compared with the proportion of radioactivity associated with either XGGG after \(\beta\)-glucosidase digestion or XT-generated GGXGGG (Fig. 6A). Therefore, these results provided strong evidence that AtXT1 and AtXT2 both added the first xylosyl residue primarily to the fourth glucose from the reducing end to produce GGXGGG and then added the second xylosyl residue to the adjacent glucosyl residue to form predominately GXXGG.

**DISCUSSION**

Early biochemical studies demonstrated that microsomal preparations from peas (20, 23–26) and soybeans (21, 27–29) have the ability to synthesize XyG in vitro when a mixture of UDP-glucose and UDP-xylose are added as a substrate. Under these conditions, the microsomal membrane preparations are capable of producing nascent XyG fragments.
that have the proper substitution patterns (21, 23, 26). However, solubilization with detergents and further biochemical purification of either the XyG β-glucan synthase or XyG XT were not successful, suggesting that XT and β-glucan synthase formed a complex in the Golgi membranes, where their combined activities synthesized the core XyG molecule consisting of repeating subunits.

Recently, Faik et al. (11) demonstrated that XT activity can be detected in detergent-solubilized pea microsomes if a cello-oligosaccharide acceptor is added. Using this in vitro assay, Faik et al. screened a series of P. pastoris lines transformed with one of seven Arabidopsis candidate XT genes and determined that one gene, AtXT1 (At3g62720), encodes a protein with XT activity. In this study, we have shown that another member of the gene family, AtXT2 (At4g02500; formerly AtGT2), encodes a protein with XT activity. We demonstrated that, in vitro, AtXT1 and AtXT2 exhibited similar preferences for the location of xylose addition to cellohexaose. Furthermore, we have shown that AtXT1 and AtXT2 could catalyze the addition of multiple xylosyl residues to cellohexaose.

In the previous study (11), it was unclear whether the inability to detect XT activity in P. pastoris cell lines transformed with the other members of this gene family was due to the lack of protein production, the generation of inactive proteins, or an inadequate enzyme assay. Because we were able to demonstrate that AtXT2 encodes a protein with XT activity and exhibits similar substrate specificity and preferences for the location of xylose addition to cellohexaose as AtXT1, we believe that the most likely explanation for the earlier results (11) is that AtXT2 was produced in P. pastoris.

In vitro XT assays indicated that either AtXT1 or AtXT2 used either cellopentaose or cellohexaose as an acceptor, with cellohexaose acting as the superior substrate. In this study, we could not detect the low level of activity seen previously when cellotetraose was used as the acceptor for AtXT1 (11). However, it is interesting to note that cellotetraose was also not an acceptor for the enzyme solubilized from pea Golgi membranes (11).

Previous work has shown that GXGGG is the predominant reaction product formed by AtXT1 when cellopentaose is used as the acceptor substrate (11). A comparison of the reaction products generated by either AtXT1 or AtXT2 showed that both enzymes preferentially added a xylosyl residue to the fourth glucosyl residue from the reducing end of the acceptor, producing GXGGG from cellopentaose and GGXGGG from cellohexaose.

In contrast to the high specificity of XyG biosynthetic enzymes AtFUT1 (8, 9) and MUR3 (7), which add only a single sugar to their acceptor substrates, both AtXT1 and AtXT2 are capable of adding multiple xylosyl residues to cellohexaose. Further xylosylation of GGXGGG could be achieved when longer reaction times and/or higher substrate ratios were used with either AtXT1 or AtXT2; both enzymes preferentially

![Figure 8](https://example.com/figure8.png)

**FIGURE 8.** MALDI-TOF-MS analysis of reaction products sequentially digested with β-glucosidase and EGII. AtXT1 reaction products synthesized during an 18-h incubation in the presence of 5 mM MnCl₂, 2.5 mM unlabeled UDP-xylose, and 1.0 mM cellohexaose were sequentially digested with β-glucosidase and EGII. Digested reaction products were separated by HPAEC, desalted with an in-line desalting module, and fractionated into 500-μl aliquots. Each aliquot was analyzed by MALDI-TOF-MS.
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added a second xylosyl residue to GGXGG, forming GGXXGG. A higher substrate ratio was needed in vitro to drive the conversion of GGXGG to GGXXGG because the addition of the second xylosyl residue occurred only after a significant amount of cellohexaose had been converted to GGXGG. Although the conversion of GGXGG to GGXXGG appeared to be less efficient than the initial synthesis of GGXGG, equal amounts of GGXGG and GGXXGG could be produced after an 18-h incubation at a 10:1 substrate ratio (1.0 mM UDP-xylose and 0.1 mM cellohexaose were used to minimize UDP inhibition) (data not shown). Indeed, at higher substrate ratios and longer reaction times, either AtXT1 or AtXT2 inefficiently produced GXXGG when cellopentaose was used as the acceptor (data not shown). On the basis of these observations, we concluded that the preferred reaction for each enzyme was to add xylose to an unsubstituted glucan chain. In addition, the observation that cellohexaose was a better acceptor than cellopentaose indicated that the enzymes recognized at least six glucosyl residues.

On the basis of the addition of the first two xylosyl residues to adjacent positions on cellohexaose, we might have expected the third xylosyl residue to be added to the next glucosyl residue toward the reducing end of GGXGG to form GGXXXG; however, this molecule was not detected during product characterization. Rather, a minor amount of GXGGGG was the only trixosylated product that was detected. Given that both enzymes added the second xylosyl residue closer to the reducing end of cellohexaose compared with the first xylosyl residue, it seemed unlikely that the small amount of GXGGGG was produced by adding xylose to GGXGG, which would have required adding a xylosyl residue closer to the nonreducing end of the molecule. Rather, we postulated that, following the formation of GXXGGG, which itself was formed in small quantities, the enzyme continued to add xylose residues to adjacent glucosyl residues closer to the reducing end of cellohexaose to produce GGXXGG and eventually GXGGGG by the addition of the second and third xylosyl residues, respectively.

The substrate specificities of detergent-solubilized AtXT1 and AtXT2 are similar to that of detergent-solubilized GMGT from fenugreek (19). Like GMGT, AtXT1 and AtXT2 utilized acceptor substrates with a degree of polymerization of 5 or more, with GMGT and XT5s having a significant preference for acceptors with a degree of polymerization of 6. GMGT, AtXT1, and AtXT2 preferentially add an α-(1,6)-linked sugar molecule to the fourth glucosyl residue from the reducing end of a β-(1,4)-linked glucan oligosaccharide acceptor with a degree of polymerization of 6. And like GMGT, which can add a second galactosyl residue to a mannosyl residue adjacent to a galactose-substituted mannosyl residue (12), AtXT1 and AtXT2 can add a second xylosyl residue to GGXGGG to form GGXXGG. Edwards et al. (19) proposed that GMGT has a principal substrate recognition sequence of six mannosyl residues. As noted above, we postulated that both AtXT1 and AtXT2 have similar substrate recognition sequences, except that they recognize six glucosyl residues.

Although there are many similarities between GMGT and the XT activities studied here, there are also some important differences. Reid et al. (30) and Edwards et al. (19) have postulated that the addition of galactosyl residues within the GMGT substrate recognition sequence can be described as a second-order Markov chain model. Although irregular galactose substitution patterns of galactomannan (31) can be explained by this stochastic model, we predict that generation of the highly repetitive xylose substitution patterns of XyG will be described by a deterministic model. However, it is currently unknown whether generation of the XXXG xylosylation pattern is due to intrinsic properties of the XT or to a combination of many factors, including associations with the XyG β-glucan synthase or other unknown auxiliary proteins, the structure of the β-glucan chain, and the spatial constraints of being embedded in the Golgi membrane. To explore these issues, more detailed studies aimed at determining whether heterologously expressed AtXT1 and AtXT2 are capable of generating the XXXG xylosylation pattern in vitro will be conducted. We will use custom-synthesized oligosaccharides (such as XXXGGGG and GGGGGGGXXXG) that are soluble in aqueous buffers (32) to further examine the acceptor specificities of AtXT1 and AtXT2.

One of the fundamental questions that remain to be answered is how many XT5s are needed for XyG biosynthesis in vivo. Like the β-glucan chains that compose a cellulose microfibril, the repeating unit of the XyG backbone is a cellubiose, where the adjacent β-(1,4)-linked Glcp residues are rotated 180° relative to each other along the axis of the glycosidic linkage. This structure suggests that at least two and possibly three different XT enzymes are needed to add the three different xylosyl residues found in XXXG-type XyG. Based on the observations reported here that both AtXT1 and AtXT2 can add xylose to an unsubstituted cello-oligosaccharide, it seems that either of them could be involved in adding the first xylosyl residue to the nascent β-glucan chain as it exits XyG glucan synthase. Moreover, both AtXT1 and AtXT2 are capable of adding multiple contiguous xylosyl residues to cellohexaose in vitro, suggesting that either enzyme could be involved in the addition of two of the three xylosyl residues of the XXXG repeating unit of Arabidopsis XyG. Although neither enzyme adds the third xylosyl residue efficiently in vitro, it is possible that the addition of adjacent xylosyl residues could be accomplished more efficiently if the enzymes are in a membrane complex with the β-glucan synthase. Alternatively, it is possible that formation of XyG in vivo requires the action of additional XT5s, possibly some of the other related glycosyltransferases found in the CAZy GT34 family of proteins found in Arabidopsis (11). We are using reverse genetics with Arabidopsis T-DNA insertion mutants to address additional questions regarding the in vivo roles of AtXT1 and AtXT2, as well as other members of the CAZy GT34 family of proteins.

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