DOI: 10.1002/ejoc.200700395

Glycosynthase-Assisted Synthesis of Xylo-Gluco-Oligosaccharide Probes for α-Xylosyltransferases

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Keywords: Carbohydrate / Enzyme catalysis / Glycosyltransferase / Xyloglucan / Oligosaccharide

An efficient chemo-enzymatic synthesis involving glycosylation catalyzed by E197A nucleophile mutant of the retaining *endo*-cellulase Cel7B from *Humicola insolens* afforded well defined xylo-gluco-oligosaccharides. The synthesis of these complex oligosaccharides requires the use of an enzymatic protection/deprotection concept to allow a single-step condensation of donors onto acceptors. These molecules were tested as potential acceptor substrates for two *Arabidopsis thaliana* putative xyloglucan α-xylosyltransferases expressed in insect cells. Both AtXT1 and AtXT2 catalyzed the incorporation of xylosyl unit(s) onto all of these substrates, but with various efficiencies.

Introduction

Xyloglucan (XyG) is the main hemicellulose of the plant primary cell wall of dicots and non-graminaceous monocots where it is thought to hydrogen-bond to the surfaces of adjacent cellulose microfibrils to form a three-dimensional cellulose-XyG network that functions as the primary load-bearing structure of the plant primary cell wall.[1–3] Xyloglucan is a complex heteropolysaccharide that is composed of a cellulose-like backbone of β(1→4)-linked D-Glc*p* residues that are regularly substituted at O-6 position with an α-D-Xyl*p* residue. An unambiguous XyG nomenclature was developed where the repeating units of XyG are described from the non-reducing end to the reducing end with a single letter that denotes a specific glucosyl residue substitution pattern. An unsubstituted D-Glc*p* is assigned "G", and an α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment is assigned "X".[4] Xyloglucan from most plants is composed of XXXG repeating subunits, while XyG from plants in *Poaceae* and *Solanaceae* is composed of XXGG motifs.[5] Further substitution occurs primarily on the O-2 position at specific xylosyl residues with a variety of sugar moieties; the most common being β-D-Gal*p*, α-L-Ara*f* (only in *Poaceae* and *Solanaceae*) or the disaccharide α-L-Fuc*p*-(1→2)-β-D-Gal*p*.[2,6] The β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* unit is assigned "L", while α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* is assigned "F".[4]

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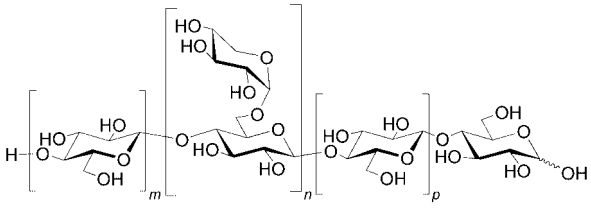
Xyloglucan biosynthesis and its chemical and structural modifications during cell-wall biosynthesis are still poorly understood. The biosynthesis of XyG requires an array of glycosyltransferases and at least one glucan synthase.[7,8] Recently, significant progress in understanding XyG biosynthesis has been made with the identification and characterization of the genes that encode the fucosyltransferase (*AtFUT1*, At2g03220 and *PsFUT1*),[9,10] one of the galactosyltransferases (*AtMUR3*, At2g20370),[11] and two xylosyltransferases (*AtXT1*, At3g62720 and *AtXT2*, *At4g*02500)[12,13] involved in XyG biosynthesis. The latter genes are members of the CAZy[14] family GT34;[15] this family is known to encode seven genes in *Arabidopsis thaliana*.[12] However, only one of these genes (*AtXT1*) was shown to encode a protein with α-xylosyltransferase activity that could catalyze the transfer of an α-D-Xyl*p* from UDP-Xyl to the O-6 position of the penultimate glucosyl residue (i.e., the fourth glucosyl unit from the reducing terminus) of cellopentaose to generate GXGGG.[12] More recent work has shown that *AtXT2* encodes xylosyltransferase with the same substrate specificity and generates the same products as AtXT1.[13] This study demonstrated that AtXT1 and AtXT2 have a strong preference for cellohexaose over cellopentaose, with an approximately 8-fold increase in xylose incorporation when cellohexaose is used as an acceptor with respect to cellopentaose. As with cellopentaose, AtXT1 or AtXT2 added the first xylosyl residue to the antepenultimate glucosyl residue (fourth glycosyl residue from the reducing end of cellohexaose) to generate GGXGGG. Finally, it was also demonstrated that both enzymes can add multiple xylosyl residues onto cellohexaose to generate GGXXGG and limited amounts of GXXXGG if higher concentrations of UDP-Xyl and longer reaction times are used.[13]

While these studies demonstrate that *AtXT1* and *AtXT2* encode XyG xylosyltransferases with very similar activities towards cellopentaose and cellohexaose, it remains to be shown if the other putative glycosyltransferase genes (*AtGT3–7*) of this gene family also encode XyG xylosyltransferases. One of the limiting factors in determining if AtGT3–7 are XyG xylosyltransferases may be the lack of a specific acceptor substrate. For example, a T7-tagged version AtGT5 (At1g74380) has been successfully expressed in *Pichia pastoris* and *Drosophila* Schneider 2 cells; however, this protein does not have xylosyltransferase activity towards cellohexaose (Cavalier and Keegstra, unpublished data). Thus, it becomes important to have a library of pure XyG oligosaccharides (XGOs) with specific xylosylation patterns to be used in the further characterization of substrate specificities AtXT1 and AtXT2 and to determine if other members of this gene family encode XyG xylosyltransferases with unique substrate specificities.

We have recently developed an efficient method for the construction of a library of XGOs,[16,17] with the aim to study the substrate specificity of XyG *endo*-transglycosylases (XETs for a review see ref.[18]) that are involved in catalyzing the molecular grafting of XyG chains during cell wall growth and differentiation. In this paper we have extended this methodology to the preparation of potential XGO acceptors that can be used to further investigate the substrate specificities of AtXT1 and AtXT2, and used as acceptor substrates to determine if other members of this gene family encode XyG xylosyltransferases.

Results and Discussion

Potential XGO substrates **1**–**5** having one to three xylosyl residues on the cello-oligosaccharide backbone were selected as target molecules (Figure 1).



1	$m = 2, n = 1, p = 2$	**GGXGGG**
2	$m = 1, n = 2, p = 2$	**GXXGGG**
3	$m = 4, n = 3, p = 0$	**GGGGXXXG**
4	$m = 0, n = 3, p = 4$	**XXXGGGGG**
5	$m = 6, n = 3, p = 0$	**GGGGGGXXXG**

Figure 1. Structures of the target molecules prepared and tested in this work.

The choice of compounds **1** and **2** is obvious; they may mimic reaction products obtained by the action of AtXT1 and AtXT2 onto cellohexaose. The xyloglucan biosynthesis was thought to require the simultaneous incorporation of glucose and xylose to a nascent xyloglucan acceptor and not by transferring xylose to a preformed β(1→4)-glucan.[1] Thus, compounds **3**–**5** were synthesized in order to study the effect of the position and number of unsubstituted consecutive glucosyl residues for xylosylation on nascent xyloglucan catalyzed by AtXT1–2 and the putative xylosyltransferases AtGT3–7.

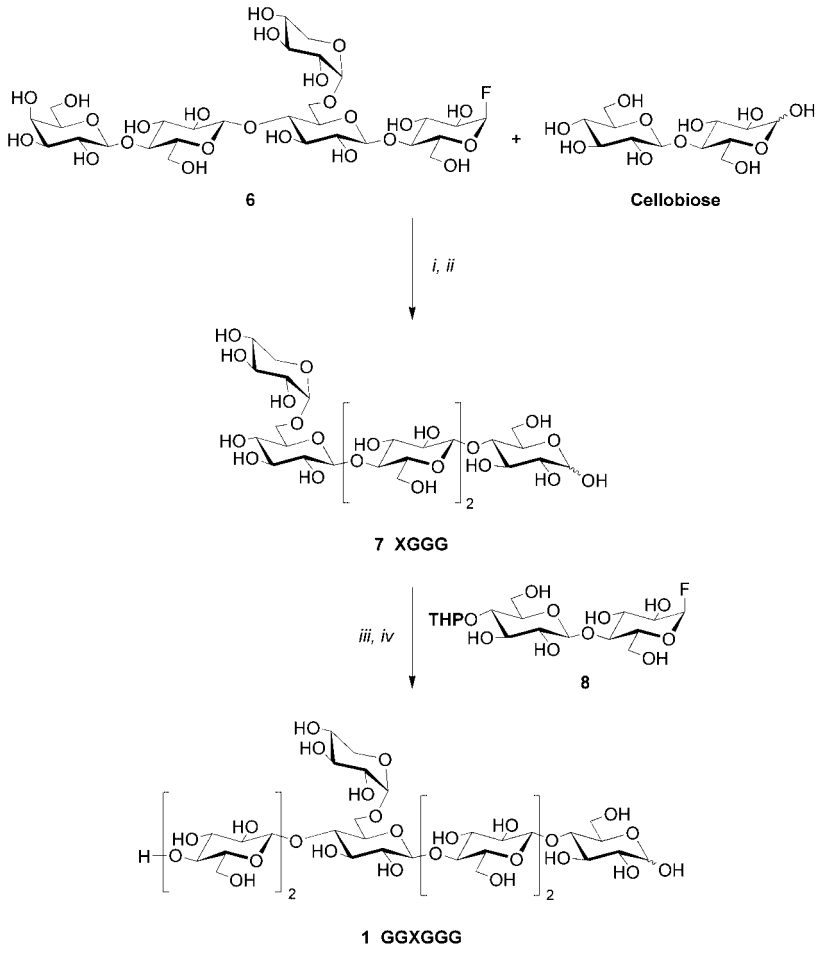
Xylo-gluco-oligosaccharides **1**, **2** and **5** were prepared with the help of our previous results on the syntheses of natural and unnatural β(1→4)-linked D-glucosyl oligosaccharides;[17,19–21] XGOs **3** and **4** were obtained as recently described.[17] The methodology was based on protein engineering of retaining β-glucosidase, as described by Withers and co-workers.[22] Glycoside-hydrolase in which the active-site carboxylate nucleophile was replaced by a non-nucleophilic amino acid was unable to form the glycosyl-enzyme intermediate and had no hydrolytic activity. However, because the rest of the active site is unchanged, these mutated glycosidases might be expected to catalyze the transglycosylation of an activated α-glycosyl fluoride, used in place of the glycosyl-enzyme intermediate, to a suitable acceptor sugar, with the advantage that the reaction products cannot be hydrolyzed and were accumulated almost quantitatively.[23]

The key pentasaccharide **7** was prepared by employing the recently developed concept of lactosyl protection/deprotection at the 4^{ω}-OH group of donors (Scheme 1).[17] The fluoride **6**, obtained from its corresponding acetylated derivative,[17] was condensed onto cellobiose. This reaction was catalyzed by the Cel7B E197A glycosynthase from *Humicola insolens* as already reported.[19] The pentasaccharide **7** was isolated in 76% yield after lactosyl removal by treatment with β-glucosidase/galactosidase from *Agrobacterium* sp.[24] At the opposite, the enzymatic condensation with a non-blocked fluoride afforded the pentasaccharide **7** in 26% yield only (unpublished data). This result shows the impact to use the lactosyl protective group during the synthesis of complex branched oligosaccharides. In glycosidase-assisted syntheses for β(1→4)-D-gluco-oligosaccharides, the common glycosidic donors are lactosyl or D-galactosyl fluorides because the axial orientation of the hydroxy group at C-4 prevents these substrates from being acceptors and leads to a single-step condensation with an appropriate acceptor.[25]

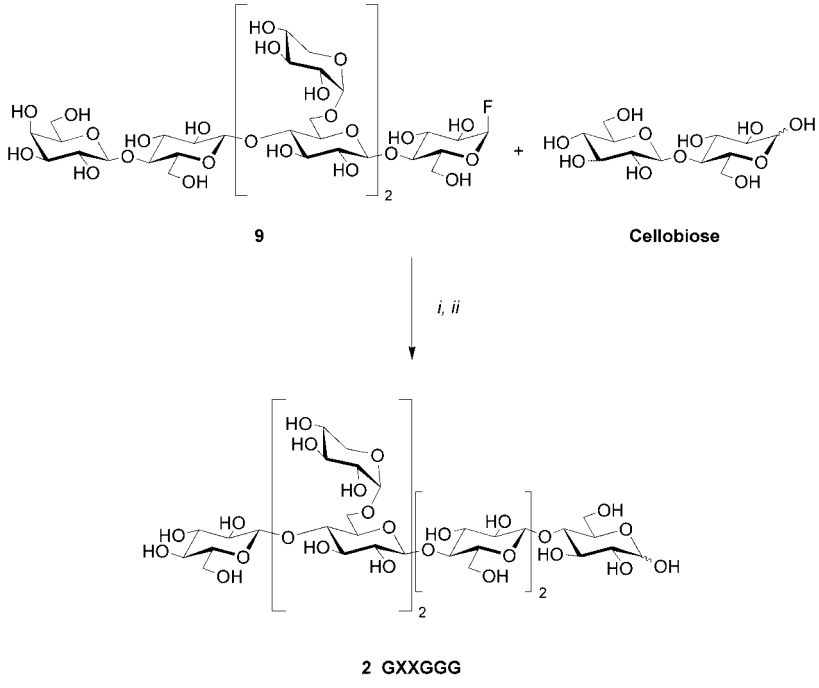
The glycosynthase also catalyzed the coupling of 4^{II}-*O*-THP-cellobiosyl fluoride **8**,[20] and XGGG acceptor **7**, to give the heptasaccharide GGXGGG **1** in 57% yield after acidic hydrolysis of the THP group. This compound GGXGGG **1**, obtained in 33 steps, from D-xylose and cellobiose with an overall yield of 3%, is the initial compound formed by AtXT1 or AtXT2 when cellohexaose was used as an acceptor.[13]

Because GXXGGG **2** was thought to be also an intermediate characterized during the addition of multiple xylosyl residues onto cellohexaose,[13] it was of interest to obtain this XGO in large amounts.

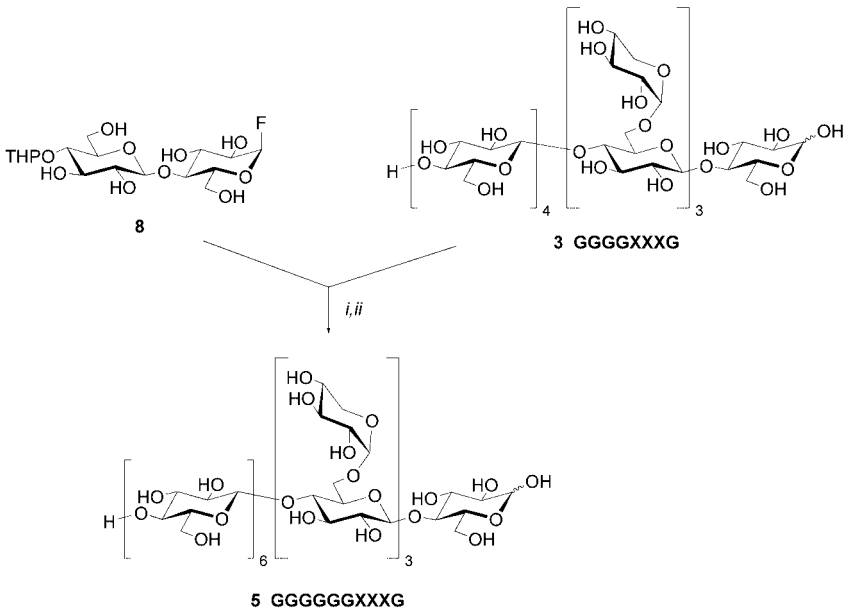
The fluoride **9** was obtained from its corresponding acetylated derivative as recently described.[17] Enzymatic condensation of **9**, catalyzed by the Cel7B E197A glycosynthase from *H. insolens*, onto cellobiose gave the expected nonasaccharide that was submitted to controlled enzymatic degalactosylation using the commercially available β-galac-



Scheme 1. Synthesis of xylo-gluco-oligosaccharide GGXGGG **1**. Reagents and conditions: (i) Cel7B E197A glycosynthase, phosphate buffer (0.2 M, pH = 7); (ii) *Agrobacterium* sp. β-glucosidase/galactosidase, phosphate buffer (0.2 M, pH = 7); (iii) Cel7B E197A glycosynthase, carbonate/hydrogen carbonate buffer (0.1 M, pH = 10); (iv) 1 M HCl.



Scheme 2. Synthesis of xylo-gluco-oligosaccharide GXXGGG **2**. Reagents and conditions: (i) Cel7B E197A glycosynthase, carbonate/hydrogen carbonate buffer (0.1 M, pH = 10); (ii) *A. oryzae* β-galactosidase, acetate buffer (0.1 M, pH = 4.6).



Scheme 3. Synthesis of xylo-gluco-oligosaccharide GGGGGGXXXG **5**. Reagents and conditions: (i) Cel7B E197A glycosynthase, carbonate/hydrogen carbonate buffer (0.1 M, pH = 10); (ii) 1 M HCl.

tosidase from *Aspergillus oryzae* giving **2** in 29% over the two steps (Scheme 2).

Elongation of GGGGXXXG **3**[17] with 4^{II}-*O*-THP-cellobiosyl fluoride **8** and workup as described above afforded GGGGGGXXXG **5** in 87% yield (Scheme 3).

Structure and purity of the compounds were confirmed using ^{1}H NMR spectroscopy[26] and HR-ES mass spectrometry. The anomeric region of xylo-gluco-oligosaccharides **1**, **2** and **5** encompassed seven, eight, and thirteen low-field signals, respectively, including the resonance of 1-H of Glc$^{I\alpha}$ (δ = 5.21 ppm, $^3J_{1,2} \approx$ 3.8 Hz), overlapping doublets for the xylose residues (δ = 4.95 ppm, $^3J_{1,2} \approx$ 3.5 Hz), the 1-H of Glc$^{I\beta}$ signal (δ = 4.65 ppm, $^3J_{1,2}$ = 8.0 Hz), and overlapping doublets for the nonreducing glucose residues ($\delta \approx$ 4.57–4.49 ppm). The high-field region included an extended overlapping region integrating for 41, 46 and 75 H, respectively (Figure 2). The regiochemistry of the new glycosidic linkages formed during the enzymatic condensations was also confirmed by enzymatic hydrolysis using wild-type Cel7B from *H. insolens*.

Previous work showed that reaction time and the UDP-Xyl/acceptor substrate ratio had a significant effect on the amount of xylose incorporated and the structure of the reaction products generated.[13] In this study, the ability of XGOs **1**–**5** to act as acceptors for either AtXT1 or AtXT2 was assessed using a high UDP-Xyl/acceptor (10:1) and a reaction time of 1 h. After 1 h, both AtXT1 and AtXT2 catalyzed the incorporation of xylosyl unit(s) onto these substrates, but with efficiencies less than those observed with cellohexaose (Figure 3). The acceptor substrate specificities of AtXT1 and AtXT2 for XGOs **1**–**5** are similar to each other, which is in agreement with earlier observations using unsubstituted cello-oligosaccharides as acceptors.[13]

Previously, it was proposed that AtXT1 and AtXT2 have a substrate recognition sequence similarities with a fenu-

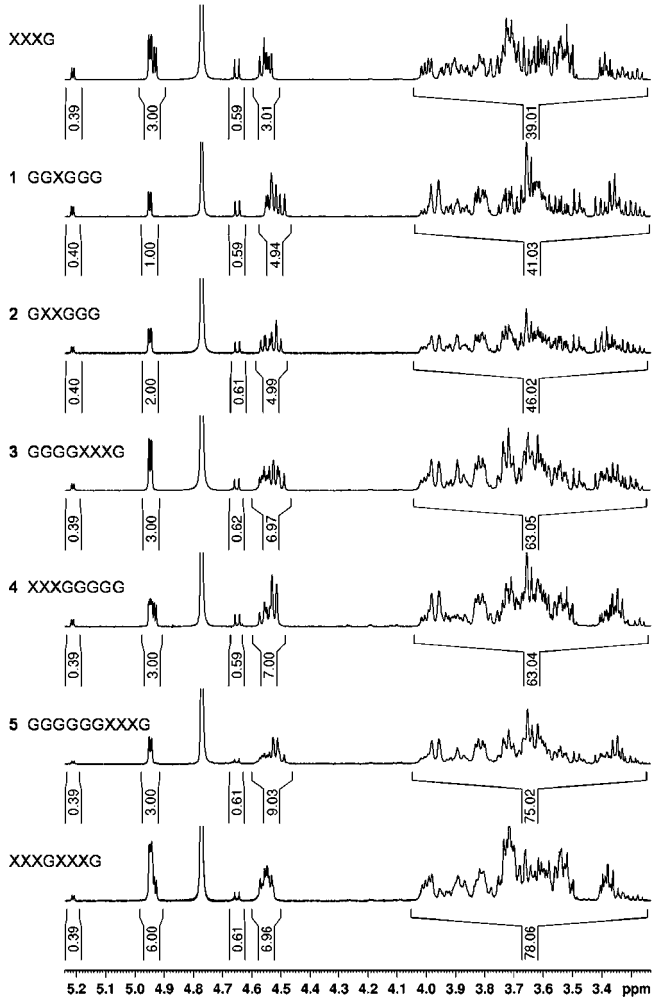


Figure 2. ^{1}H NMR spectra of target xylo-gluco-oligosaccharides **1**–**5**, and xyloglucan oligosaccharides repeating subunits XXXG and XXXGXXXG.

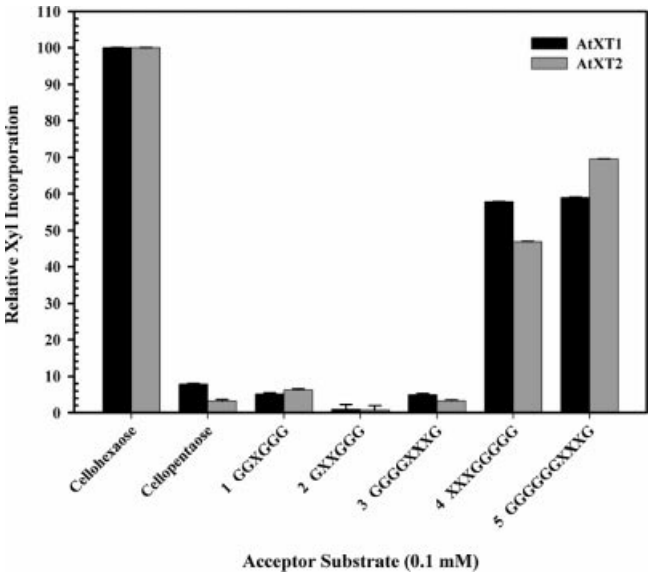


Figure 3. AtXT1 and AtXT2 substrate specificity. Triton-solubilized extracts from Sf21 cells expressing either *AtXT1* or *AtXT2* were assayed using cellopentaose, cellohexaose, and XGOs **1**–**5** as acceptors. All XT reactions were incubated in the presence of 5 mM $MnCl_2$, UDP-[^{14}C]xylose (44400 dpm), 1.0 mM UDP-Xyl, and 0.1 mM acceptor substrate for 1 h. The values are the average ±S.D.s from three assays minus control reactions that lacked acceptor substrate.

greek galactomannan galactosyltransferase (GMGT).[27,28] These similarities include (1) the utilization of acceptor substrates with a degree of polymerization (DP) of 5 or more, with the GMGT, AtXT1, and AtXT2 having a significant preference for acceptors with a DP of 6; (2) the preferential addition of an α(1→6)-linked sugar molecule to the fourth glycosyl residue from the reducing end of a β(1→4)-linked gluco-oligosaccharide acceptor with a DP of 6; and (3) the ability to add a second glycosyl residue, albeit at a reduced efficiency, to the fifth glycosyl residue from the reducing end of an oligosaccharide acceptor with a DP of 6.[13,28] Based upon the similarities between GMGT and the XTs it was proposed that AtXT1 and AtXT2 have a principal substrate recognition sequence of six glucosyl residues. However, further investigation of the substrate recognition sequences of AtXT1 and AtXT2 has been hampered by the limited solubility of β-glucans with a DP greater than 6. Because the xylo-gluco-oligosaccharides **1**–**5** are soluble in an aqueous solution, they have the potential to be useful in the further exploration of AtXT1 and AtXT2 substrate recognition sequence and substrate specificity.

In this study, AtXT1 and AtXT2 had low activity using XGOs **1** and **2** as acceptors. Earlier observations using cellohexaose as an acceptor found that both AtXT1 and AtXT2 add the first xylosyl residue to the fourth glucosyl residue from the reducing end of cellohexaose to produce GGXGGG as the major reaction product. In addition, AtXT1 and AtXT2 inefficiently produce small amounts of GXGGGG, which is subsequently xylosylated to produce GXXGGG.[13] Further xylosylation of these reaction products to form significant amounts of GGXXGG and limited amounts GXXXGG occur, but only when long incubations times (18 h) were used.[13] Because the XGO compounds **1** and **2** are already xylosylated on their fourth glucosyl residue from the reducing end, it is not surprising that they were poor acceptor substrates. Indeed, the results obtained using the XGO compounds **1** and **2** reinforce the conclusions from the previous study that AtXT1 and AtXT2 are much less efficient at adding second and third xylosyl residues to cellohexaose.[13]

The xyloglucan oligosaccharides **3**–**5** have glucosyl backbones that satisfy the predicted substrate recognition sequence length of DP = 6, but differ in either the location of the three contiguous xylosyl residue substitutions or the length of contiguous unsubstituted glucosyl residues. While both AtXT1 and AtXT2 will use XGOs **3**–**5** as acceptor substrates, there were significant differences in the amount of xylose incorporation between XGO **3** and XGOs **4**, **5** (Figure 3). These results show intriguing trends in AtXT1 and AtXT2 specificity with respect to the predicted substrate recognition sequence and the length of contiguous unsubstituted glucosyl residues required for XT activity. The undecasaccharide GGGGXXXG **3** is similar to cellopentaose in acceptor activity and considerably better than cellotetraose (data not shown; cellotetraose is a very poor acceptor with approximately 15–20% of the incorporation of cellopentaose).[12,13] Given that XGO **3** has four unsubstituted glucosyl units and should behave more like cellotetraose, it seems likely that the xylosyl residue on the fifth glucose from the non-reducing end does not interfere with recognition by XT1 and XT2. On the other hand, the amount of xylose incorporated when undecasaccharide XXXGGGGG **4** and the tridecasaccharide GGGGGGXXXG **5** are used as an acceptor is considerably higher than when cellopentaose is used as acceptor and nearly as high as with cellohexaose. The high activity with XGO **5** is not surprising because it has 6 unsubstituted glucosyl residues, but the high activity with XGO **4** again argues that the xylosyl residue on the 6th glucosyl residue from the reducing end does not interefere extensively with recognition by XT1 and XT2.

However, a question remains to be addressed. Why is the addition of the second and third xylosyl residue so inefficient? One possibility is that the low efficiency of adding the second and third xylosyl residue is caused by the artificial in vitro assay using solubilized enzymes and acceptor substrates that may have xylosyl residues that cause steric hindrance of either AtXT1 or AtXT2 activity. Another possibility is that in vivo, the efficient addition of xylosyl residues may require an enzymatic complex consisting of XyG glucan synthase and XyG xylosyltransferase(s). Still a third possibility is that the subsequent xylosyl residues are added by other members of the CAZy family GT34 so that in vivo AtXT1 and AtXT2 add only one or two of the xylosyl residues found in the XXXG repeating subunit of XyG. In this case, XGOs **1**–**5** will be useful as acceptor substrates for exploring the possibility that other members of the CAZy family GT34 are XyG xylosyltransferases.

Conclusion

These preliminary biochemical results that show the interest of using well-defined XGOs with different chemical structures will be extended and the characterization of the products reported in due time.

Experimental Section

General Methods: β-Galactosidase from *Aspergillus niger* was purchased from Megazyme (Bray, Ireland). Evolution of reactions was monitored by analytical thin-layer chromatography using silica gel 60 F254 precoated plates (E. Merck, Darmstadt). Compounds were purified by column chromatography using silica gel Si 60 (63–200 μm, E. Merck, Darmstadt) and subsequently filtered with a syringe-driven filter unit (0.45 μm; Millex®-HV) before lyophilisation. Roman numerals in ascending order are given to the residues from the reducing end. The same Roman numeral is given to a glucosyl residue and its C-6 substituted xylosyl residue. NMR spectra were recorded at 298 K with a Bruker Avance 500 MHz spectrometer. Proton chemical shifts (δ) are reported in ppm with the residual HDO peak as an internal reference (δ_{HOD} = 4.77 ppm).[29] Coupling constants (J) are in Hertz (Hz); multiplicities are singlet (s), doublet (d), and multiplet (m). Low-resolution mass spectra (MS) were recorded with Bruker Autoflex or Waters Micromass ZQ spectrometers and high-resolution mass spectra (HRMS) were recorded with a Micromass ZABSpec-TOF at the CRMPO (Rennes University, France).

α-D-Xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (XGGG 7): Cellobiose (14 mg, 41 μmol, 1.3 equiv.) in phosphate buffer (0.2 M, pH = 7, 1.5 mL) and then *H. insolens* Cel7B E197A glycosynthase (2 mg) were added to the fluoride **6** prepared as already described[17] (25 mg, 31 μmol). The solution was placed in a rotative shaker at 37 °C for 12 h, then Cel7B E197A glycosynthase (0.5 mg) was added again. After 24 h at 37 °C, recombinant β-glucosidase/galactosidase (*Agrobacterium* sp., 0.2 mg)[24] was added. The reaction mixture was kept at 37 °C for 8 h, then another quantity of β-glucosidase/galactosidase (0.1 mg) was added. After 12 h at 37 °C, the solution was diluted with a mixture of acetonitrile/water (1:1 v/v, 10 mL), silica gel was added and the suspension was concentrated under reduced pressure. The powder was deposited at the top of a silica gel column and a gradient (acetonitrile then acetonitrile/water, 95:5, 9:1, 85:15, 8:2 and 75:25 v/v) was used to elute the pentasaccharide **7** (19 mg, 24 μmol) in 76% yield. ES-MS: m/z = 821 $[M + Na]^+$. ES-HRMS: m/z calcd. for $C_{29}H_{50}O_{25}Na$ $[M + Na]^+$ 821.2539; found 821.2544.

β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (GGXGGG 1): THP fluoride **8**[20] (13.3 mg, 31.0 μmol, 1.3 equiv.) and the pentasaccharide **7** (19.0 mg, 23.8 μmol) in carbonate/hydrogen carbonate buffer (0.1 M, pH = 10, 1.5 mL) were incubated with Cel7B E197A glycosynthase (2 mg) at 37 °C for 12 h. The solution was diluted with water (3.5 mL) and HCl (1 M) was added to lower the pH to 1–2. After 5 min, the solution was neutralized with Et_3N. The process was repeated twice until complete hydrolysis of the tetrahydropyranosyl group was achieved. Silica gel was added and the suspension was concentrated under reduced pressure. The resulting solid was deposited at the top of a silica gel column and a gradient (acetonitrile then acetonitrile/water, 95:5, 9:1, 85:15, 8:2, 75:25 and 7:3 v/v) was used to elute the heptasaccharide **1** (15.2 mg, 13.5 μmol) in 57% yield. ^1H NMR (D_2O, 500 MHz): δ = 5.21 (d, $J_{1,2}$ = 3.8 Hz, 0.4 H, 1-H of $Glc^{I\alpha}$), 4.95 (d, $J_{1,2}$ = 3.7 Hz, 1 H, 1-H of Xyl^{IV}), 4.65 (d, $J_{1,2}$ = 8.0 Hz, 0.6 H, 1-H of $Glc^{I\beta}$), 4.55–4.49 (m, 5 H, 1-H of $Glc^{II\ to\ VI}$), 4.02–3.25 (m, 41 H, 2-H to 6-H of Glc and 2-H to 5-H of Xyl) ppm. MALDI-TOF-MS: m/z = 1145 $[M + Na]^+$. ES-HRMS: m/z calcd. for $C_{41}H_{70}O_{35}Na$ $[M + Na]^+$ 1145.3595; found 1145.3590.

β-D-Glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (GXXGGG 2): The fluoride **9**[17] (30.0 mg, 27.4 μmol) and cellobiose (12.2 mg, 35.6 μmol, 1.3 equiv.) in carbonate/hydrogen carbonate buffer (0.1 M, pH = 10, 1.5 mL) were incubated with Cel7B E197A glycosynthase (4 mg). The solution was heated at 37 °C for 12 h, then the solution was diluted with a mixture of acetonitrile/water (1:1 v/v, 10 mL), silica gel was added and the suspension was concentrated under reduced pressure. The resulting solid was deposited at the top of a silica gel column and a gradient (acetonitrile then acetonitrile/water, 95:5, 9:1, 85:15, 8:2 and 75:25 v/v) was used to elute the expected lactosyl compound (33.0 mg, 23.3 μmol) in 85% yield, characterized only by MALDI-TOF-MS: m/z = 1439 $[M + Na]^+$. A solution of this nonasaccharide (30.0 mg, 21.2 μmol) in acetate buffer (0.1 M, pH = 4.6, 2 mL) was treated with commercial β-galactosidase (*Aspergillus oryzae*, 5 mg) at 30 °C for 2.5 h. The reaction mixture was diluted in acetonitrile/water (1:1 v/v, 10 mL), silica gel was added and the suspension was concentrated under reduced pressure. The resulting solid was deposited at the top of a silica gel column and a gradient (acetonitrile then acetonitrile/water, 95:5, 9:1, 85:15, 8:2 and 75:25 v/v) was used to elute the expected octasaccharide **2** (9.1 mg, 7.3 μmol) in 34% yield. ^1H NMR (D_2O, 500 MHz): δ = 5.21 (d, $J_{1,2}$ = 3.9 Hz, 0.4 H, 1-H of $Glc^{I\alpha}$), 4.95 (d, $J_{1,2}$ = 3.4 Hz, 2 H, 1-H of $Xyl^{IV\ and\ V}$), 4.65 (d, $J_{1,2}$ = 8.0 Hz, 0.6 H, 1-H of $Glc^{I\beta}$), 4.57–4.50 (m, 5 H, 1-H of $Glc^{II\ to\ VI}$), 4.01–3.25 (m, 46 H, 2-H to 6-H of Glc and 2-H to 5-H of Xyl) ppm. MALDI-TOF-MS: m/z = 1277 $[M + Na]^+$. ES-HRMS: m/z calcd. for $C_{46}H_{78}O_{39}Na$ $[M + Na]^+$ 1277.4018; found 1277.4020.

β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-D-glucopyranose (GGGGGGXXXG 5): The THP fluoride **8** (12.7 mg, 29.6 μmol, 1.3 equiv.) and undecasaccharide **3** (39.0 mg, 22.8 μmol) in carbonate/hydrogen carbonate buffer (0.1 M, pH = 10, 5 mL) were incubated on a rotative shaker in an oven with Cel7B E197A glycosynthase (3 mg) at 37 °C for 20 h. The solution was diluted with water (5 mL) and HCl (1 M) was added to lower the pH to 1–2. After 5 min, the solution was neutralized with Et_3N. The process was repeated twice until complete hydrolysis of the tetrahydropyranosyl group was achieved. Silica gel was added and the suspension was concentrated. The powder was deposited at the top of a silica gel column and a gradient (acetonitrile then acetonitrile/water, 95:5, 9:1, 85:15, 8:2, 75:25 and 7:3 v/v) was used to elute the tridecasaccharide **5** (40.2 mg, 19.8 μmol) in 87% yield. ^1H NMR (D_2O, 500 MHz): δ = 5.21 (d, $J_{1,2}$ = 3.8 Hz, 0.4 H, 1-H of $Glc^{I\alpha}$), 4.95 (d, $J_{1,2}$ = 3.5 Hz, 3 H, 1-H of $Xyl^{II,\ III\ and\ V}$), 4.65 (d, $J_{1,2}$ = 8.0 Hz, 0.6 H, 1-H of $Glc^{I\beta}$), 4.57–4.49 (m, 9 H, 1-H of $Glc^{II\ to\ X}$), 4.02–3.26 (m, 75 H, 2-H to 6-H of Glc and 2-H to 5-H of Xyl) ppm. MALDI-TOF-MS: m/z = 2057 $[M + Na]^+$. ES-HRMS: m/z calcd. for $C_{75}H_{126}O_{63}Na_2$ $[M + 2\ Na]^{2+}$ 1040.3226; found 1040.3217.

AtXT1 and AtXT2 Assays Utilizing Cellopentaose, Cellohexaose, and XGOs 1–5: AtXT1 and AtXT2 were expressed in *Spodoptera frugiperda* 21 (Sf21) cells and solubilized with extraction buffer consisting of 100 mM HEPES buffer (pH = 7.0) containing 1% Triton X-100, 5 mM $MnCl_2$, and one Complete-mini protease inhibitor tablet lacking EDTA (Roche Diagnostics, Alameda, CA) per 20 mL of extraction buffer as described previously.[13] Xylosyltransferase assays were conducted in the presence of cellopentaose, cellohexaose, and XGOs **1**–**5** according to previously published conditions with some modifications.[13] Briefly, cell extracts of Triton-solubilized Sf21 cells expressing AtXT1 and AtXT2 surveyed for XT activity with 1.0 mM UDP-Xyl, 44400 dpm UDP-[^{14}C]xylose, and 0.1 mM acceptor substrate. For each XT assay, 40 μL of cell extract (6.25 mg mL^{-1}) was mixed with 10 μL of substrate solution containing the appropriate amounts of UDP-Xyl, UDP-[^{14}C]xylose, and acceptor substrate dissolved in extraction buffer. XT reactions were incubated at 25 °C for 1 h and terminated with the addition of 450 μL of Dowex 1X8 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 microfuge at 1300 *g* for 2 min. The column effluent was mixed with 2.0 mL of scintillation counting fluid; radioactivity was then measured with an LS-5000 scintillation counter (Beckman Coulter, Fullerton, CA).

Acknowledgments

We would like to thank late Dr. M. Schülein from Novozymes A/S (Denmark) and Professor S. Withers from University of British Columbia (Canada) for a generous gift of Cel7B E197A and β-glucosidase/galactosidase, respectively. This work at Centre National de Recherche sur les Macromolécules végétales (CERMAV) was supported in part by the Centre de la Recherche Scientifique (CNRS) and the European Union (EU contract QLK5-CT-2001-00443). Work at Michigan State University (MSU) was supported in part by a grant from the Plant Genome Research Program at the National Science Foundation and in part by a grant from the Biosciences Program at the Department of Energy of the United States of America.

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Received: May 2, 2007
Published Online: July 3, 2007

