Glycosynthase activity of hybrid aspen xyloglucan *endo*-transglycosylase *Ptt*XET16-34 nucleophile mutants†

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Glycosynthases are active-site mutants of glycoside hydrolases that catalyse glycosyl transfer using suitable activated donor substrates without competing product hydrolysis (S. M. Hancock, M. D. Vaughan and S. G. Withers, *Curr. Opin. Chem. Biol.*, 2006, **10**, 509–519). Site-directed mutagenesis of the catalytic nucleophile, Glu-85, of a *Populus tremula x tremuloides* xyloglucan *endo*-transglycosylase (*Ptt*XET16-34, EC 2.4.1.207) into alanine, glycine, and serine yielded enzymes with glycosynthase activity. Product analysis indicated that *Ptt*XET16-34 E85A in particular was able to catalyse regio- and stereospecific *homo*- and *hetero*-condensations of α-xylogluco-oligosaccharyl fluoride donors XXXGαF and XLLGαF to produce xyloglucans with regular sidechain substitution patterns. This substrate promiscuity contrasts that of the *Humicola insolens* Cel7B E197A glycosynthase, which was not able to polymerise the di-galactosylated substrate XLLGαF. The production of the *Ptt*XET16-34 E85A xyloglucosynthase thus expands the repertoire of glycosynthases to include those capable of synthesising structurally homogenenous xyloglucans for applications.

Introduction

Xyloglucans comprise an important family of plant polysaccharides built upon a β(1→4)-glucan backbone with regular α(1→6)-xylose substitution. Xyloglucans are wide-spread among higher plants, where they act as seed storage polysaccharides[1,2] or vital cellulose cross-linking agents in the cell wall.[3] Although xyloglucans are phylogenetically diverse, with members possessing a variety of backbone branching patterns, those containing the XXXG repeating motif are among the most common[4–6] (where "X" represents α-D-Xyl*p*-(1→6)-β-D-Glc*p*-(1→4) and "G" represents β-D-Glc*p*-(1→4) in the common nomenclature[7]). The xylose residues are regiospecifically extended with β-D-Gal*p*-(1→2) or α-L-Fuc-(1→2)-β-D-Gal*p*-(1→2) units to produce "L" and "F" substructures, respectively (Fig. 1). The widely used xyloglucan from tamarind (*Tamarindus indica*) seeds is comprised of XXXG (**1**), XXLG (**2**), XLXG (**3**), and XLLG (**4**) motifs, while dicot primary wall xyloglucans are distinguished by the presence of fucosylated XXFG (**5**) and XLFG motifs (**6**).[5]

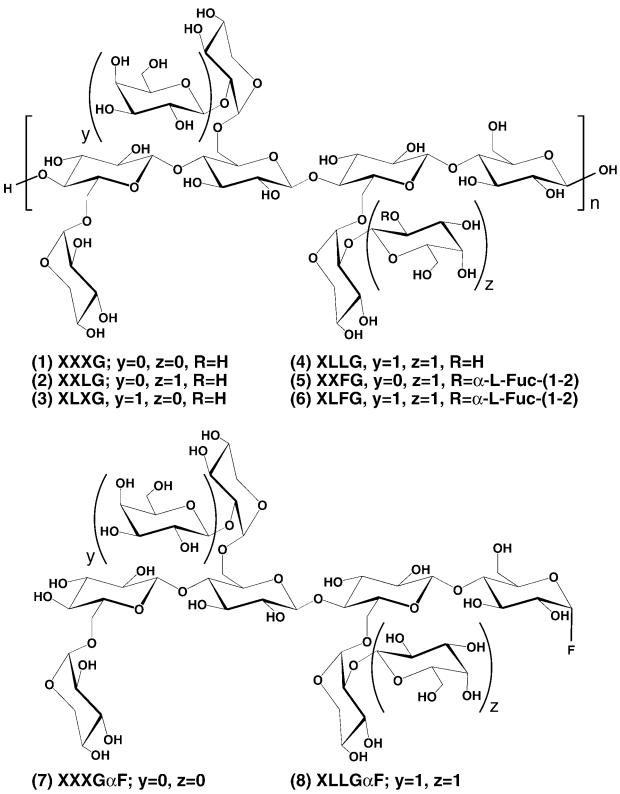

Fig. 1 Structure of XXXG-based xyloglucan, xylogluco-oligosaccharides, and α-xylogluco-oligosaccharyl fluoride substrates.

The microheterogeneity of xyloglucans presents difficulties for the use of the natural polysaccharide in detailed kinetic studies of xyloglucan-active enzymes, *e.g. endo*-xyloglucanses (EC 3.2.1.150

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and EC 3.2.1.151) and xyloglucan *endo*-transglycosylases (XET, EC 2.4.1.207),[8–11] as well as in molecular studies of the structurally important xyloglucan–cellulose interaction.[12–14] Previous work has demonstrated that Withers' glycosynthase technology[15] can be harnessed for the efficient synthesis of a library of differentially xylosylated xylogluco-oligosaccharides for the mechanistic analysis of an XET from hybrid aspen.[9]

In the classic incarnation,[15] a glycosynthase is a retaining β-glycoside hydrolase in which the catalytic nucleophile is converted by site-directed mutagenesis to a non-nucleophilic amino acid. The resulting protein is therefore rendered incapable of performing its normal catalytic function. However, when admixed with a suitable α-glycosyl fluoride, which mimics the covalent glycosyl-enzyme intermediate of the natural reaction, the glycosynthase can catalyse glycosyl transfer to acceptor substrates. Advantages include a reduction in the number of steps required to produce target oligosaccharides (*versus* traditional synthesis), the use of easily manipulated enzymes and comparatively inexpensive glycosyl donor substrates (in contrast to glycosyl transferases and UDP-sugars), and high yields (*versus* glycoside hydrolases employed for kinetically-controlled transglycosylation). Numerous glycosynthases producing a diversity of oligosaccharides have been generated from both *exo*- and *endo*-acting glycoside hydrolases.[16,17]

Glycosynthases derived from the retaining *endo*-β-glucanase from *Humicola insolens*, *Hi*Cel7B, have been extensively used to produce oligosaccharides for enzyme structure–function studies[18–22] and for the synthesis of flavonoid glycosides.[23] Recently, the synthetic capacity of the *Hi*Cel7B E197A glycosynthase variant has been extended to the synthesis of natural non-galactosylated xylogluco-oligosaccharides and analogs.[9,11,24] Although *Hi*Cel7B E197A can use the α-glycosyl fluoride of XXXG (**1**), *viz*. XXXGαF (**7**), as a donor substrate,[11] this glycosynthase is surprisingly not able to catalyse the polymerisation of XLLGαF (**8**) at practically useful rates. To overcome this limitation and further expand the repertoire of glycosynthases to the production of galactosylated xyloglucans and xylogluco-oligosaccharides, we have produced three new glycosynthase variants from a hybrid aspen (*Populus tremula x tremuloides*) xyloglucan *endo*-transglycosylase.[25,26] Thus, mutation of the *Ptt*XET16-34 catalytic nucleophile, Glu-85, to Gly, Ser, and Ala generated glycosynthases with varying abilities to oligomerise both XXXGαF (**7**) and XLLGαF (**8**). We believe this is the first report of a glycosynthase that is capable of producing homogenously galactosylated xyloglucan fragments.

Results and discussion

Wild-type *Ptt*XET16-34 (formerly *Ptt*XET16A[25,26]) is a transglycosylase that cleaves xyloglucan oligo- or polysaccharide donors at internal unbranched Glc residues ("G",[7] Fig. 1) and transfers glycosyl units onto the non-reducing end of xyloglucan oligo- or polysaccharide acceptors.[8,9,11] Despite a high transglycosylation-to-hydrolysis ratio, the wild-type enzyme cannot be harnessed for kinetically controlled glycoside synthesis using aryl β-xylogluco-oligosaccharides[27–30] because such substrates fail to act as donor substrates.[9,25] To explore whether *Ptt*XET16-34 could be converted into a xyloglucan glycosynthase with complimentary activity to that of the well-studied *Hi*Cel7B E197A,[9,11,24] the catalytic nucleophile of *Ptt*XET16-34, Glu-85, was replaced by alanine, glycine and serine.§ In each case, proteins with no detectable wild-type activity were produced. However, kinetic and product analysis indicated that all three mutants were able to catalyse the regio- and stereospecific condensation of the α-xyloglucosyl fluoride donor substrates XXXGαF and XLLGαF to produce homogenous xyloglucans.

Glycosynthase activity on XXXGαF (7)

An initial screen of activity, measuring fluoride ion release from the *homo*-condensation of XXXGαF (**7**), indicated that the *Ptt*XET16-34 E85A, E85G, and E85S glycosynthase mutants exhibited similar pH–rate dependencies which, like that of the wild-type enzyme,[11,26] have maxima at pH 5.0–5.5 and drop sharply below pH 5 (Fig. 2). At pH 5, *Ptt*XET16-34 E85A, E85G, and E85S mutants catalysed the coupling of two molecules of XXXGαF with essentially identical initial rates at substrate concentrations up to 2 mM (Fig. 3A). Activity of wild-type enzyme toward XXXGαF was undetectable. Although it has been shown that glycosynthase efficiency is sometimes increased by the incorporation of glycine or serine instead of the classic alanine,[31–34] this was not the case for the *Ptt*XET16-34 glycosynthases. Due to the catalytic similarity of the three glycosynthase variants, further analysis was primarily focused on *Ptt*XET16-34 E85A.

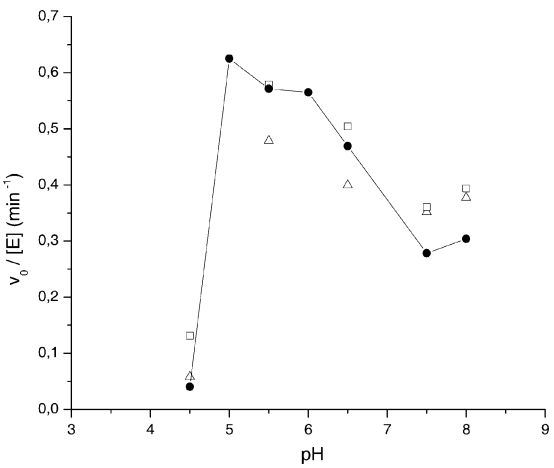

Fig. 2 pH-dependence of the condensation of 2.6 mM XXXGαF (**7**) catalysed by *Ptt*XET16-34 E85A (●), E85G (□), and E85S (△) glycosynthase mutants at 30 °C.

The condensation of XXXGαF by *Ptt*XET16-34 E85A exhibited saturation kinetics at concentrations up to 20 mM (Fig. 3A), yielding apparent k_{cat} and K_m values of 1.06 ± 0.06 min^{-1} and 1.59 ± 0.36 mM (k_{cat}/K_m 0.66 mM^{-1} min^{-1}). The K_m value is comparable to other glycosynthases derived from *endo*-acting glycosidases, which are often in the range 1–10 mM.[31,34–36] The observed k_{cat} value is relatively low (values ranging from 10 to 1100 min^{-1} have been reported for "*endo*"-glycosynthases with certain donor–acceptor pairs[31,35,36]), although it compares

§ These three variants have previously been demonstrated to generate glycosynthases from a number of glycosidases; in several cases the Gly and Ser variants are significantly better catalysts than the Ala variant.[16,31–36]

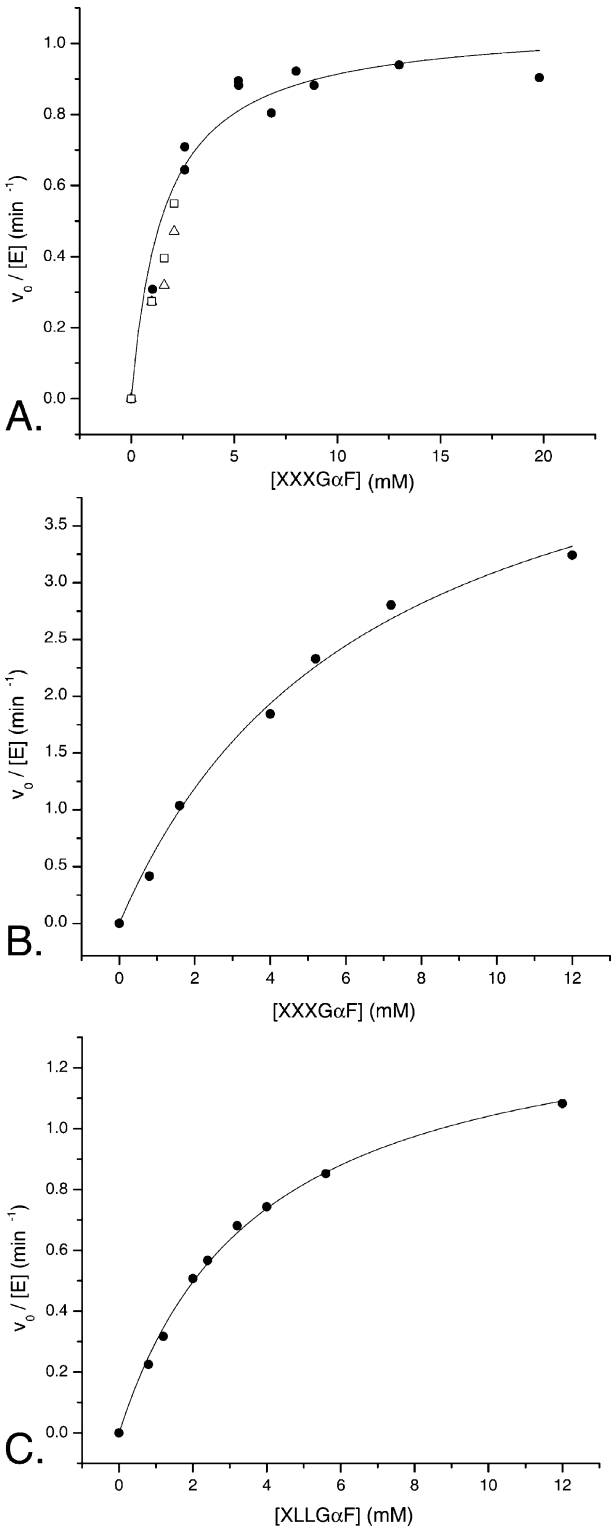

Fig. 3 Initial rate kinetics of *Ptt*XET16-34 and *Hi*Cel7B glycosynthase mutants. (A) XXXGαF (**7**) substrate with *Ptt*XET16-34 E85A (●), *Ptt*XET16-34 E85G (□), and *Ptt*XET16-34 E85S (△). (B) XXXGαF (**7**) substrate with *Hi*Cel7B E197A. (C) XLLGαF (**8**) substrate with *Ptt*XET16-34 E85A.

well with the few published data on the *homo*-condensation of disaccharide donors (0.2–10 min^{-1}).[34,36] Furthermore, the k_{cat} value for the *homo*-condensation of XXXGαF is only *ca.* 5-fold lower than that of *Hi*Cel7B E197A, which has previously been employed for the synthesis of xylogluco-oligosaccharide variants.[9,11,24] *Hi*Cel7B E197A also exhibits Michaelis–Menten kinetics in the condensation of XXXGαF, with k_{cat} 5.17 ± 0.41 min^{-1}, K_m 6.68 ± 1.06 mM, and k_{cat}/K_m 0.77 mM^{-1} min^{-1} (Fig. 3B). When compared to *Hi*Cel7B E197A as a potential catalyst for the synthesis of $(XXXG)_n$, the lower k_{cat} value of *Ptt*XET16-34 E85A is somewhat compensated by a lower K_m value; *Ptt*XET16-34 E85A will be saturated with substrate over a wider concentration range.

To provide a series of reference compounds for glycosynthase product analysis, wild-type *Ptt*XET16-34 was incubated with the tetradecasaccharide XXXGXXXG (Fig. 1; $y = z = 0$, $n = 2$). The initial products of *Ptt*XET16-34 action on XXXGXXXG are XXXG (**1**) and $(XXXG)_3$, where the latter may also act as a glycosyl donor and/or acceptor to yield longer products. As indicated by HPAEC-PAD analysis, a 1 h incubation produced a distribution of $(XXXG)_n$ oligomers, where n ranges from 1 to >10 (Fig. 4A). Supporting ESI-MS data on the reaction mixture is shown in the Supplementary Information (Table S1).

Comparison of products from the wild-type reaction with those formed after incubation of *Ptt*XET16-34 E85A with XXXGαF (**7**) indicated that a series of oligomers of the general structure $(XXXG)_n$αF was formed in a time-dependent manner, along with products resulting from the spontaneous hydrolysis of the C1–F bond (Fig. 4B). The glycosyl fluorides have slightly shorter retention times than the corresponding free sugars (*cf.* XXXGαF and XXXG, Fig. 4), which becomes less pronounced as the length of the oligomer increases. Analogous results were obtained for *Hi*Cel7B E197A, *Ptt*XET16-34 E85G, and *Ptt*XET16-34 E85S (data not shown). Hydrolysis of the glycosynthase products by the highly-specific GH5 *endo*-xyloglucanase from *Paenibacillus pabuli*, which does not cleave β(1→3)-linked polysaccharides,[10] resulted in the production of XXXG and XXXGαF, providing evidence that the XXXG units were joined by β(1→4) linkages (Fig. 4B).

Despite reaching high degrees of polymerisation of $(XXXG)_n$αF ($n \rightarrow 13$, corresponding to M_r 13 600), the spontaneous hydrolysis of the $(XXXG)_n$αF resulted in the unfortunate complication of HPAEC-PAD chromatograms. The addition of an equimolar amount of XXXG (**1**) as an alternate acceptor in the *Ptt*XET16-34 E85A glycosynthase reaction using XXXGαF (**7**) as a donor resulted in the time-dependent conversion of initially formed $(XXXG)_n$αF products into $(XXXG)_n$. As shown in Fig. 4C, a 3.5 h incubation resulted in the production of a mixture of $(XXXG)_n$αF and $(XXXG)_n$, where $n = 2$–4, with a significant amount of the donor remaining in the reaction. However, overnight incubation (24 h) consumed most of the glycosyl fluorides in the reaction, yielding $(XXXG)_{2-10}$ nearly exclusively. Supporting ESI-MS data are shown in the Supplementary Information (Table S2). Compared with reactions where XXXGαF (**7**) is employed as both the donor and the exclusive acceptor (Fig. 4B), the reaction shown in Fig. 4C yielded a product distribution biased toward lower M_r products. This is an effect, in part, of reducing the concentrations of higher mass donors in the $(XXXG)_n$αF series which may condense with other high mass molecules acting as acceptors. The addition of alternate acceptors can therefore be used to both fine-tune the glycosynthase product distribution, as well as control the

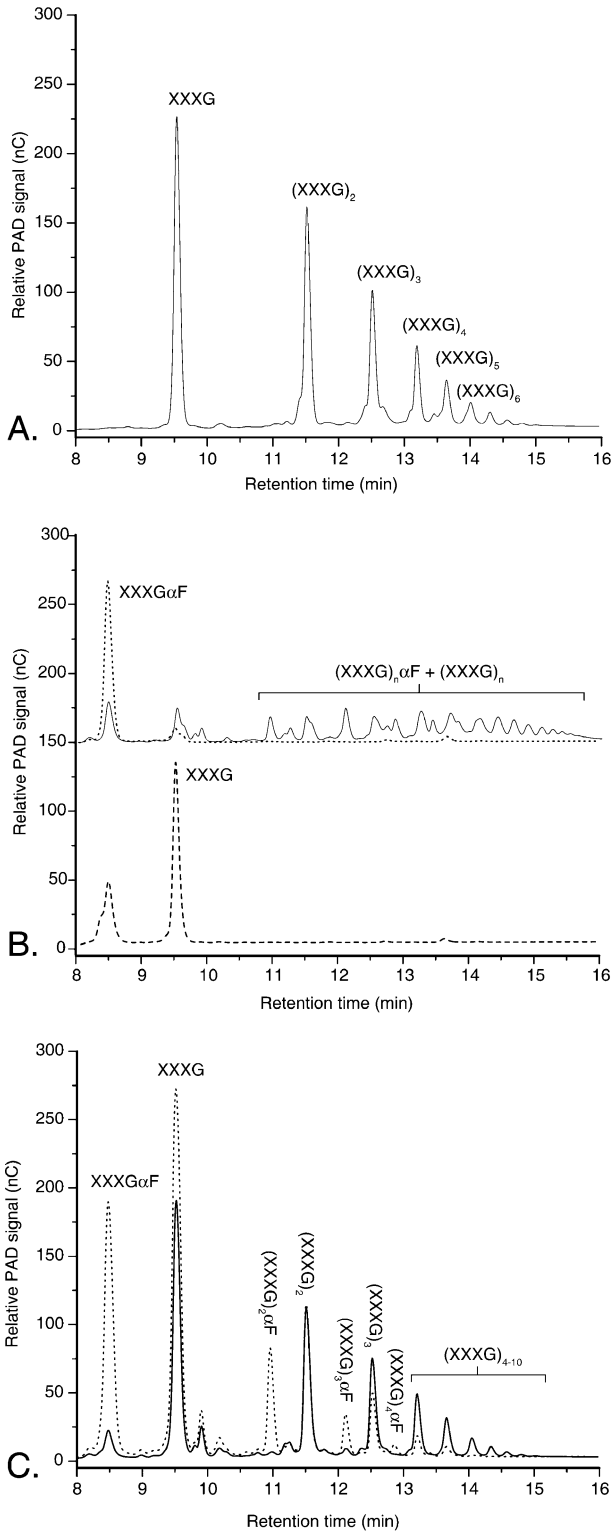

Fig. 4 Product analysis of the reactions catalysed by wild-type *Ptt*XET16-34 and the glycosynthase variant *Ptt*XET16-34 E85A using non-galactosylated donor substrates. (A) Higher-order $(XXXG)_n$ products from the action of wild-type *Ptt*XET16-34 on XXXGXXXG, 1 h incubation. (B) XXXGαF (**7**, dotted line), products after incubation with *Ptt*XET16-34 E85A, 18 h (solid line), and hydrolysis products formed by *endo*-xyloglucanase digestion of the glycosynthase products (dashed line). (C) Products formed after co-incubation of XXXGαF (**7**) and XXXG (**1**) with *Ptt*XET16-34 E85A, 3.5 h (dotted line) and 24 h (solid line).

chemistry at the reducing end of the oligo/polysaccharides for various applications.[37,38]

Glycosynthase activity on XLLGαF

Whereas both *Ptt*XET16-34 E85A and *Hi*Cel7B E197A were able to catalyse the condensation of XXXGαF (**7**) with comparable initial-rate kinetics (Fig. 3A and 3B), analysis of the action of these enzymes on XLLGαF (**8**) revealed a striking difference. Under identical conditions to those used for XXXGαF, addition of *Hi*Cel7B E197A to a solution of XLLGαF did not result in steady-state fluoride ion release nor did it produce condensation products observable by HPAEC-PAD (data not shown). In contrast, *Ptt*XET16-34 E85A catalysed the condensation of XLLGαF (**8**) with apparent saturation kinetics (Fig. 3C). The apparent k_{cat} and K_m values were 1.44 ± 0.04 min^{-1} and 3.78 ± 0.22 mM, respectively (k_{cat}/K_m 0.38 mM^{-1} min^{-1}). The apparent k_{cat} value was slightly favorably increased (1.4-fold), although the apparent K_m value was more than twice that for XXXGαF (**7**). Thus, while the presence of both Gal residues on the xylogluco-oligosaccharide donor effectively precluded condensation by *Hi*Cel7B E197A, galactosylation only slightly affected catalysis by the *Ptt*XET16-34 E85A glycosynthase under initial-rate conditions.

Product analysis indicated that *Ptt*XET16-34 E85A catalysed the *homo*-condensation of XLLGαF (**8**) to produce a series of oligomers $(XLLG)_nαF$ ($n = 2$–6), which spontaneously hydrolysed to $(XLLG)_n$, analogous to the results observed for XXXGαF (**7**) (*cf.* Fig. 5A and Fig. 4B). These products were likewise hydrolysed by the *P. pabuli* GH5 *endo*-xyloglucanase to XLLGαF and XLLG, thus indicating β(1→4) linkages between the XLLG repeats (Fig. 5A). *Ptt*XET16-34 E85G and *Ptt*XET16-34 E85S were similarly able to condense XLLGαF (**8**) to produce multimers, as evidenced by HPAEC-PAD analysis (data not shown). As was observed for the XXXGαF (**7**)/XXXG (**1**) substrate pair (Fig. 4C), incubation of XLLGαF (**8**) with the alternate acceptor substrate XLLG (**4**) reduced the product complexity by the near-exclusive production of $(XLLG)_n$ in overnight reactions (Fig. 5B, supporting ESI-MS data in Supplementary Information, Table S3).

Interestingly, the degrees of polymerisation of products produced by *Ptt*XET16-34 E85A condensation of XLLGαF (**8**) were significantly lower than those from XXXGαF (**7**). The data shown in Fig. 4B and Fig. 5A, in which identical enzyme concentrations and incubation times (18 h) were employed, clearly indicate that the glycosynthase consumed nearly all of the XXXGαF (**7**) in the reaction, whereas a significant amount of XLLGαF (**8**) remained. Similarly, when alternate acceptors were used, the XXXGαF (**7**)/XXXG (**1**) mixture reached the same approximate level of conversion after 3.5 h that the XLLGαF (**8**)/XLLG (**4**) mixture reached after 14 h (*cf.* Fig. 4C and Fig. 5B). Moreover, the XLLGαF (**8**)/XLLG (**4**) reaction essentially stalled at this time point, with very little additional conversion occurring by extending the incubation to 36 h. It is likely that the *Ptt*XET16-34 E85A glycosynthase is inhibited by $(XLLG)_n$ products to a greater extent than $(XXXG)_n$ products, possibly due to additional binding interactions to the pendant Gal residues. Nonetheless, $(XLLG)_n$ oligomers up to $n = 6$ were observed (M_r 8230), indicating that *Ptt*XET16-34 E85A is the first glycosynthase capable of producing homogenously galactosylated xyloglucan fragments.

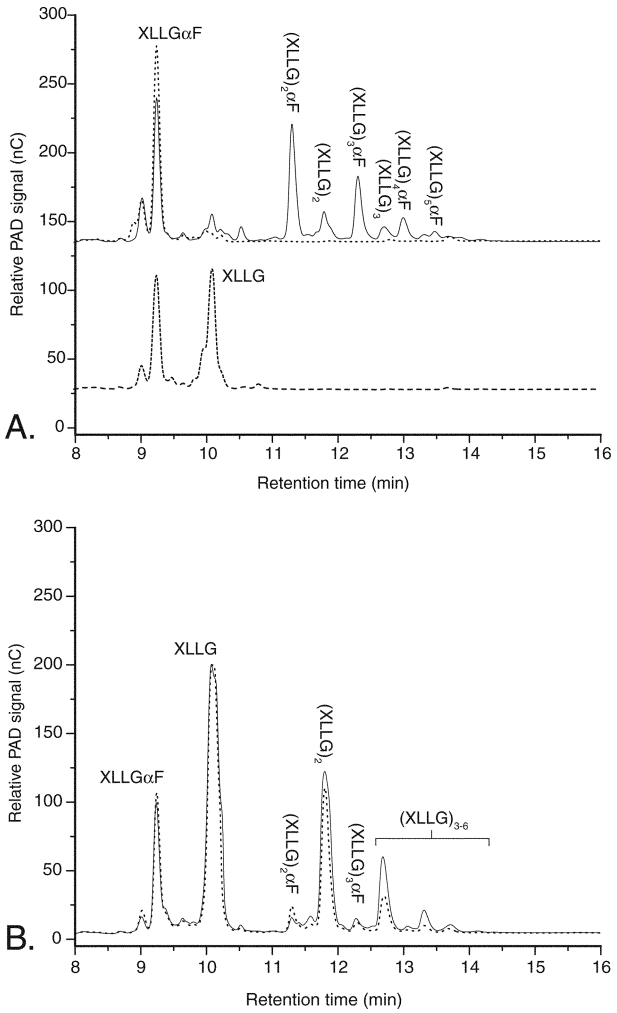

Fig. 5 *Ptt*XET16-34 E85A glycosynthase product analysis using XLLGαF (**8**) as a donor. (A) XLLGαF (**8**, dotted line), products after incubation with *Ptt*XET16-34 E85A, 18 h (solid line), and hydrolysis products formed by *endo*-xyloglucanase digestion of the glycosynthase products (dashed line). (B) Products formed after co-incubation of XLLGαF (**8**) and XLLG (**4**) with *Ptt*XET16-34 E85A, 14 h (dotted line) and 36 h (solid line).

Comparison of transglycosylases capable of synthesising homogenous xyloglucans

The present study has established that *Hi*Cel7B E197A, wild-type *Ptt*XET16-34, and *Ptt*XET16-34 E85A can all be employed for the synthesis of xyloglucans with regular backbone substitution using appropriate donor substrates, as summarised in Table 1.

Whereas *Ptt*XET16-34 E85A was able to oligomerise both XXXGαF (**7**) and XLLGαF (**8**), the *Hi*Cel7B E197A glycosynthase was, intriguingly, not useful for the synthesis of galactosylated xyloglucans. Although directly comparable kinetic data are not available, this is somewhat surprising since the k_{cat} of *homo*-condensation of XXXGαF by *Hi*Cel7B E197A (Fig. 3) is only *ca.* 5-fold lower than that for the coupling of α-lactosyl fluoride to *p*-nitrophenyl β-cellobioside.[31] Thus, while *Hi*Cel7B E197A accommodates extensive xylose branching of the donor and acceptor substrates, it does not tolerate further sidechain extension.

Wild-type *Ptt*XET16-34 is similarly limited to the production of non-galactosylated $(XXXG)_n$ xyloglucans, and suffers from a significant practical drawback when compared to the glycosynthases. *Ptt*XET16-34 requires well-defined Glc_8-based donor substrates, the production of which demands careful kinetic control of the digestion of xyloglucan by an *endo*-(xylo)glucanase, followed by fractionation of the products by size-exclusion chromatography.[8,11] While XXXGXXXG is accessible in reasonable amounts by β-galactosidase treatment of fractionated Glc_8-based oligosaccharides or chemo-enzymatic synthesis,[11] the random distribution of Gal residues in tamarind xyloglucan makes the isolation of alternate donors, such as XLLGXLLG, impractical or impossible.

In contrast, well-defined *Ptt*XET16-34 E85A glycosynthase donor substrates are readily prepared. Glc_4-based XGOs (**1–4**) are the limit digestion products of many *endo*-(xylo)glucanases acting on tamarind xyloglucan and can be produced on multigram scales,[9,11,37,39] while subsequent transformation to the corresponding α-fluorosugars is straightforward (4 steps, including protection/deprotection).

Conclusion

The xyloglucan *endo*-transglycosylase from *Populus tremula x tremuloides* has been converted into glycosynthases capable of producing homogenous xyloglucans from α-xylogluco-oligosaccharyl fluoride donors by mutation of the catalytic nucleophile, a glutamate, to alanine, glycine, or serine. The facile production of XXXGαF (**7**) and XLLGαF (**8**), together with the unique ability of *Ptt*XET16-34 E85A to use both of these substrates as donor substrates, opens the possibility to produce a variety of xyloglucan oligo- and polysaccharides of defined composition for research and practical applications. Optimisation of the reaction conditions to control product distributions in preparative reactions is ongoing.

Table 1 Transglycosylases available for the synthesis of homogenously decorated xyloglucan fragments

	Donor substrate				
	XXXGαF (**7**)	XLLGαF (**8**)	XXXGXXXG	XLLGXLLG	XXXG- or XLLG-β-CNP or β-F
*Ptt*XET16-34	No activity	No activity	$(XXXG)_n$ products	Pure substrate not available	No activity[9,25]
*Ptt*XET16-34 E85A	$(XXXG)_n$ products	$(XLLG)_n$ products	No activity	Pure substrate not available, no activity expected	No activity expected
*Hi*Cel7B E197A	$(XXXG)_n$ products	No activity	No activity	Pure substrate not available, no activity expected	No activity expected

Table 2 PCR primers used for site-directed mutagenesis. The underlined sequences introduced the desired mutations

Primer name	Sequence
F3	5′-TGA CTA CGT AGC TGC CCT GAG GAA GCC AGT-3′
R4	5′-TTA GTA CGT ATT ATA TGT CTC TGG TCT CTC TTG CAT TCT GG-3′
PttXET16-34-E85A-Forw	5′-Biotin-CGG AGC ATG ACG CGA TAG ACT TTG AG-3′
PttXET16-34-E85A-Rev	5′-Biotin-CTC AAA GTC TAT CGC GTC ATG CTC CG-3′
PttXET16-34-E85G-Forw	5′-Biotin-CGG AGC ATG ACG GGA TAG ACT TTG AG-3′
PttXET16-34-E85G-Rev	5′-Biotin-CTC AAA GTC TAT CCC GTC ATG CTC CG-3′
PttXET16-34-E85S-Forw	5′-Biotin-CGG AGC ATG ACT CGA TAG ACT TTG AG-3′
PttXET16-34-E85S-Rev	5′-Biotin-CTC AAA GTC TAT CGA GTC ATG CTC CG-3′

Materials and methods

Protein production

Site-directed mutagenesis was performed using double PCR with biotinylated primers[40] (Table 2) on the wild-type *Ptt*XET16-34 (GenPept AAN87142, formerly *Ptt*XET16A) construct (pAK6) as the template.[26]

Heterologous gene expression in *Pichia pastoris* strain GS115 and protein purification were essentially performed as described by Kallas *et al.*[26] ESI-TOF MS analysis[41] of the purified proteins indicated that the correct variants has been produced: *Ptt*XET16-34 E85A, M_r calc. 33791.7, obs. 33790.9; *Ptt*XET16-34 E85G, M_r calc. 33777.7 obs. 33777.8; *Ptt*XET16-34 E85S, M_r calc. 33807.7, obs. 33807.5. Enzyme concentrations were determined by A_{280} measurements, using $\varepsilon = 74\,490$ M^{-1} cm^{-1}. XET activity (EC 2.4.1.207) was assayed according to the colorimetric method devised by Sulová *et al.*[42] *Hi*Cel7B E197A ($M_r = 44\,519$; 12.6 g L^{-1}) was obtained as previously described.[20]

Substrates

Xyloglucan from *Tamarindus indica* seeds was purchased from Megazyme International Ireland Ltd (Bray, Ireland). XXXG (**1**),[11] XXXGαF (**7**),[11] and XLLG (**4**)[39] were prepared as previously described. XLLGαF (**8**) was synthesised from per-*O*-acetylated XLLG (**9**) by deprotection of the anomeric hydroxyl group and fluorination as follows.

(2,3,4-Tri-*O*-acetyl-α-D-xylopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-1,2,3,6-tetra-*O*-acetyl-α,β-D-glucopyranose (9). This compound was prepared from tamarind xyloglucan (7.5 g) in 37% yield essentially as described.[39] MALDI-MS $m/z = 2502$ $[M + Na]^+$.

^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 6.20 (d, $J_{1,2} = 3.7$ Hz, 0.4H, H-1$^{Glc\,I\alpha}$); 5.64 (d, $J_{1,2} = 8.2$ Hz, 0.6H, H-1$^{Glc\,I\beta}$); 5.44–5.31 (m, 6H, H-3$^{Glc\,I\alpha,\,IV,\,Xyl\,II,\,III,\,IV}$, H-4$^{Gal\,II,\,III}$); 5.24–5.09 (m, 6H, H-2$^{Gal\,II,\,III}$, H-3$^{Glc\,I\beta,\,II,\,III}$, H-4$^{Glc\,IV}$); 5.01–4.88 (m, 11H, H-1$^{Xyl\,II,\,III,\,IV}$, H-2$^{Glc\,I,\,II,\,III}$, H-3$^{Gal\,II,\,III}$, H-4$^{Xyl\,II,\,III,\,IV}$); 4.80–4.75 (m, 3H, H-1$^{Glc\,IV}$, H-2 Glc$^{Glc\,IV,\,Xyl\,IV}$) 4.68–4.51 (m, 5H, H-1$^{Glc\,II,\,III,\,Gal\,II,\,III}$, H-6a$^{Glc\,I}$); 4.15–4.06 (m, 5H, H-6$^{Gal\,II,\,III}$, H-6b$^{Glc\,I}$); 3.96–3.46 (m, 22H, H-2$^{Xyl\,II,\,III}$, H-4$^{Glc\,I,\,II,\,III}$, H-5$^{Glc\,I,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, H-6$^{Glc\,II,\,III,\,IV}$); 3.43 (m, 1H, H-5$^{Glc\,II}$); 2.14–1.95 (m, 78H, C$H_3$CO).

^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 170.7–169.0 (CH_3CO); 101.6, 101.5 (C-1$^{Gal\,II,\,III}$); 101.0, 100.7, 100.6 (C-1$^{Glc\,II,\,III,\,IV}$); 98.8, 98.7, 98.6 (C-1$^{Xyl\,II,\,III}$); 96.5 (C-1$^{Xyl\,IV}$); 91.8 (C-1$^{Glc\,I\beta}$); 89.2 (C-1$^{Glc\,I\alpha}$); 77.4, 76.0, 75.9, 75.4, 75.3, 74.6, 73.9, 73.8, 73.2, 73.1, 72.7, 72.5, 72.4, 72.2 (2C), 71.8 (2C), 71.4, 71.3 (2C), 70.9, 70.8, 70.7, 69.7, 69.6, 69.4 (2C), 69.1 (2C), 68.3 (2C), 67.8, 67.1 (2C), 66.5, 64.5 (C-2$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-3$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-4$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-5$^{Glc\,I,\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-6$^{Glc\,II,\,III,\,IV}$); 61.7 (C-6$^{Glc\,I\alpha}$); 61.5 (C-6$^{Glc\,I\beta}$); 61.4, 61.3 (C-6$^{Gal\,II,\,III}$); 59.4 (2C), 58.9 (C-5$^{Xyl\,II,\,III,\,IV}$); 21.1–20.7 ($CH_3$CO).

(2,3,4-Tri-*O*-acetyl-α-D-xylopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α,β-D-glucopyranose (10). This compound was obtained in 59% yield from compound **9** as described for the synthesis of the corresponding acetylated XXXG.[11] MALDI-MS $m/z = 2460$ $[M + Na]^+$.

^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 5.59–5.27 (m, 7H, H-1$^{Glc\,I}$, H-3$^{Glc\,IV,\,Xyl\,II,\,III,\,IV}$, H-4$^{Gal\,II,\,III}$); 5.22–5.08 (m, 6H, H-2$^{Gal\,II,\,III}$, H-3$^{Glc\,I,\,II,\,III}$, H-4$^{Glc\,IV}$); 5.01–4.84 (m, 11H, H-1$^{Xyl\,II,\,III,\,IV}$, H-2$^{Glc\,I,\,II,\,III}$, H-3$^{Gal\,II,\,III}$, H-4$^{Xyl\,II,\,III,\,IV}$); 4.81–4.48 (m, 7H, H-1$^{Glc\,II,\,III,\,IV,\,Gal\,II,\,III}$, H-2 Glc$^{Glc\,IV,\,Xyl\,IV}$); 4.49 (dd, $J_{5,6a} = 1.5$ Hz and $J_{6a,6b} = 11.0$ Hz, 1H, H-6a$^{Glc\,I}$); 4.27–4.07 (m, 5H, H-6$^{Gal\,II,\,III}$, H-6b$^{Glc\,I}$); 3.89–3.52 (m, 23H, H-2$^{Xyl\,II,\,III}$, H-4$^{Glc\,I,\,II,\,III}$, H-5$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, H-6$^{Glc\,II,\,III,\,IV}$); 2.09–1.95 (m, 78H, C$H_3$CO); 1.79 (br, 1H, O*H*).

^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 170.7–169.0 (CH_3CO); 101.7, 101.6 (C-1$^{Gal\,II,\,III}$); 100.9, 100.6 (2C) (C-1$^{Glc\,II,\,III,\,IV}$); 98.7, 98.6 (C-1$^{Xyl\,II,\,III}$); 96.4 (C-1$^{Xyl\,IV}$); 90.3 (C-1$^{Glc\,I}$); 76.0, 75.9, 75.7, 75.4, 74.9, 74.8, 74.7, 73.7, 73.1, 72.9, 72.6, 72.4, 72.1 (2C), 72.0, 71.9, 71.8, 71.6, 71.4, 71.3, 70.9, 70.8 (2C), 69.4, 69.4 (3C), 69.2, 69.1, 68.6, 68.5, 68.3, 68.2, (C-2$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-3$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-4$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-5$^{Glc\,I,\,II,\,III,\,IV,\,Gal\,II,\,III}$); 67.7 (C-6$^{Glc\,IV}$); 67.2 (C-6$^{Glc\,III}$); 67.1 (C-6$^{Glc\,II}$); 62.1 (C-6$^{Glc\,I\alpha}$); 61.4 (2C) (C-6$^{Gal\,II,\,III}$); 61.3 (C-6$^{Glc\,I\beta}$); 59.4, 58.9 (2C) (C-5$^{Xyl\,II,\,III,\,IV}$); 21.2–20.7 ($CH_3$CO).

(2,3,4-Tri-*O*-acetyl-α-D-xylopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→2)-(3,4-di-*O*-acetyl-α-D-xylopyranosyl)-(1→6)]-(2,3-di-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (11). The hydroxyl compound **10** was fluorinated as described for the synthesis of

heptasaccharidyl fluoride,[11] and the expected mixture of anomeric fluorides was obtained in 97% yield. Anomerisation of fluorides gave after flash chromatography the expected compound **11** in 84% yield. $[\alpha]_D^{25}$ +40 (*c* 1.0 in $CHCl_3$). MALDI-MS $m/z = 2462$ $[M + Na]^+$.

^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 5.62 (dd, $J_{1,2}$ = 2.4 Hz, $J_{H,F}$ = 53.1 Hz; 1H, H-1$^{Glc\,I}$); 5.46–5.33 (m, 6H, H-3$^{Glc\,I\alpha,\,IV,\,Xyl\,II,\,III,\,IV}$, H-4$^{Gal\,II,\,III}$); 5.25–5.09 (m, 6H, H-2$^{Gal\,II,\,III}$, H-3$^{Glc\,I\beta,\,II,\,III}$, H-4$^{Glc\,IV}$); 5.05–4.90 (m, 10H, H-1$^{Xyl\,II,\,III,\,IV}$, H-2$^{Glc\,I,\,III}$, H-3$^{Gal\,II,\,III}$, H-4$^{Xyl\,II,\,III,\,IV}$); 4.86 (m, 1H, H-2$^{Glc\,II}$); 4.80–4.77 (m, 3H, H-1$^{Glc\,IV}$, H-2 Glc$^{Glc\,IV,\,Xyl\,IV}$); 4.71 (d, $J_{1,2}$ = 8,5 Hz, 1H, H-1$^{Glc\,III}$); 4.61–4.55 (m, 4H, H-1$^{Glc\,II,\,Gal\,II,\,III}$, H-6a$^{Glc\,I}$); 4.17–4.03 (m, 5H, H-6$^{Gal\,II,\,III}$, H-6b$^{Glc\,I}$); 3.97–3.62 (m, 21H, H-2$^{Xyl\,II,\,III}$, H-4$^{Glc\,I,\,II,\,III}$, H-5$^{Glc\,I,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, H-6$^{Glc\,II,\,III}$, H-6a$^{Glc\,IV}$); 3.56 (dd, $J_{5,6b}$ = 3.9 Hz and $J_{6a,6b}$ = 12.1 Hz, 1H, H-6b$^{Glc\,IV}$); 3.47 (m, 1H, H-5$^{Glc\,II}$); 2.15–1.95 (m, 75H, CH_3CO).

^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 170.7–169.4 (CH_3CO); 103.0 (d, $J_{C,F}$ = 226.0 Hz, C-1$^{Glc\,I}$); 101.5, 101.4 (C-1$^{Gal\,II,\,III}$); 100.9, 100.7, 100.6 (C-1$^{Glc\,II,\,III,\,IV}$); 98.9, 98.8 (C-1$^{Xyl\,II,\,III}$); 96.4 (C-1$^{Xyl\,IV}$); 77.4, 76.1, 75.8, 75.2, 75.0, 74.5, 74.3, 73.8, 73.2, 73.1, 72.6, 72.4, 72.1 (2C), 71.7 (2C), 71.4, 71.3, 70.9, 70.8 (2C), 70.7, 69.8, 69.4, 69.3 (2C), 69.2 (2C), 68.9, 68.3 (2C), 67.9, 67.1 (2C), 66.6, 66.4 (C-2$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-3$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-4$^{Glc\,I,\,II,\,III,\,IV,\,Xyl\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-5$^{Glc\,I,\,II,\,III,\,IV,\,Gal\,II,\,III}$, C-6$^{Glc\,II,\,III,\,IV}$); 61.4, 61.3 (2C) (C-6$^{Glc\,I,\,Gal\,II,\,III}$); 59.4 (2C), 58.9 (C-5$^{Xyl\,II,\,III,\,IV}$); 21.2–20.3 ($CH_3CO$).

α-D-Xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-[β-D-galactopyranosyl)-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[β-D-galactopyranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl fluoride (XLLGαF, 8). Acetylated compound **11** was deprotected as described for the synthesis of heptasaccharidyl fluoride XXXGαF (**7**).[11] The expected compound **8** was obtained in 93% yield. MALDI-MS $m/z = 1412$ $[M + Na]^+$.

Glycosynthase reactions

The condensation of α-xylogluco-oligosaccharyl fluorides was monitored using an Orion Ionplus 96–09 fluoride ion-selective combination electrode coupled to a Windows-based personal computer *via* a Vernier Electrode Amplifier and LabPro interface; raw data was collected and transformed using the Logger Pro™ software program (Vernier Software & Technology, Beaverton, OR). All glycosynthase assays to determine the rate of fluoride ion release were performed at 30 °C in a jacketed glass vessel in a total volume of 625 μL. The following enzyme concentrations were used: *Ptt*XET16-34 wild-type (2.15 μM), E85A (4.3 μM), E85G (2.8 μM), E85S (2.3 μM) and *Hi*CEL7B E197A (1.13 μM).

pH-dependence

The *Ptt*XET16-34 glycosynthases (enzyme concentrations as stated above) were incubated at substrate concentration 2.6 mM XXXGαF (**7**) in 50 mM sodium acetate for the pH-range 4.5–5.5, or in 50 mM sodium phosphate buffers for the pH-range 6.0–8.0.

Kinetic parameters

*Ptt*XET16-34 E85A, *Ptt*XET16-34 E85G, or *Ptt*XET16-34 E85S were incubated with XXXGαF (**7**) in 50 mM sodium acetate buffer (pH 5.0). *Ptt*XET16-34 E85A was incubated with XLLGαF (**8**) in 50 mM sodium acetate buffer (pH 5.0). *Hi*Cel7B E197A was incubated with XXXGαF (**7**) or XLLGαF (**8**) in 50 mM sodium phosphate buffer (pH 7.5). All enzymatic rates were corrected for spontaneous hydrolysis of the α-xyloglucosyl fluoride substrate and in all cases, substrate conversion was <1%. The apparent kinetic parameters k_{cat} and K_m were calculated by fitting the initial rate data with the Michaelis–Menten model by non-linear regression using Microcal™ Origin® version 6.0.

Product analysis

Wild type *Ptt*XET16-34 (8.1 μM) was incubated 1 h with 2 mM XXXGXXXG and 5 mM sodium acetate buffer (pH 5.0) at 30 °C (total volume 50 μL). *Ptt*XET16-34 E85A (8 μM), *Ptt*XET16-34 E85G (14 μM), or *Ptt*XET16-34 E85S (12 μM) were incubated at 30 °C with 10 mM XXXGαF (**7**) or XLLGαF (**8**) in 5 mM sodium acetate buffer (pH 5.0), total volume 50 μL. Additionally, *Ptt*XET16-34 E85A (8 μM) was incubated at 30 °C with 10 mM XXXGαF (**7**) or XLLGαF (**8**) in the presence of 10 mM XXXG (**1**) or XLLG (**4**) in 5 mM sodium acetate buffer (pH 5.0), total volume 50 μL. *Hi*Cel7B E197A (1.13 μM) was incubated at 30 °C with 2 mM XXXGαF (**7**) or XLLGαF (**8**) in sodium phosphate buffer (pH 7.5). For linkage analysis of the glycosidic bonds formed by the glycosynthases, 10 μL samples were heated to 80 °C (5 min), followed by cooling to room temperature, addition of 1 μL of a 0.14 g L^{-1} stock solution of *Paenibacillus pabuli* GH5 *endo*-xyloglucanase,[10] and further incubation at 37 °C (3 h). Prior to analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), samples (1–2 μL) were diluted 1 : 25 or 1 : 50, as appropriate, with 0.1 M sodium hydroxide and immediately injected; the gradient and equipment were identical to those previously described.[8] Additionally, samples were withdrawn from the reaction, diluted 1 : 5 in 50% aqueous MeOH containing 0.5 mM NaCl, and analysed by mass spectrometry as previously described.[43]

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References

1 R. A. Freitas, S. Martin, G. L. Santos, F. Valenga, M. S. Buckeridge, F. Reicher and M. R. Sierakowski, *Carbohydr. Polym.*, 2005, **60**, 507–514.
2 J. S. G. Reid, *Adv. Bot. Res.*, 1985, **11**, 125–155.
3 M. Pauly, P. Albersheim, A. Darvill and W. S. York, *Plant J.*, 1999, **20**, 629–639.
4 H. Hilz, L. E. de Jong, M. A. Kabel, R. Verhoef, H. A. Schols and A. G. J. Voragen, *Carbohydr. Res.*, 2007, **342**, 170–181.
5 M. Hoffman, Z. H. Jia, M. J. Peña, M. Cash, A. Harper, A. R. Blackburn, A. Darvill and W. S. York, *Carbohydr. Res.*, 2005, **340**, 1826–1840.

6 J. P. Vincken, W. S. York, G. Beldman and A. G. J. Voragen, *Plant Physiol.*, 1997, **114**, 9–13.
7 S. C. Fry, W. S. York, P. Albersheim, A. Darvill, T. Hayashi, J. P. Joseleau, Y. Kato, E. P. Lorences, G. A. Maclachlan, M. McNeil, A. J. Mort, J. S. G. Reid, H. U. Seitz, R. R. Selvendran, A. G. J. Voragen and A. R. White, *Physiol. Plant.*, 1993, **89**, 1–3.
8 M. J. Baumann, J. M. Eklöf, G. Michel, Å. M. Kallas, T. T. Teeri, M. Czjzek and H. Brumer, *Plant Cell*, 2007, **19**, 1947–1963.
9 R. Fauré, M. Saura-Valls, H. Brumer, A. Planas, S. Cottaz and H. Driguez, *J. Org. Chem.*, 2006, **71**, 5151–5161.
10 T. M. Gloster, F. M. Ibatullin, K. Macauley, J. M. Eklöf, S. Roberts, J. P. Turkenburg, M. E. Bjornvad, P. L. Jorgensen, S. Danielsen, K. S. Johansen, T. V. Borchert, K. S. Wilson, H. Brumer and G. J. Davies, *J. Biol. Chem.*, 2007, **282**, 19177–19189.
11 M. Saura-Valls, R. Fauré, S. Ragas, K. Piens, H. Brumer, T. T. Teeri, S. Cottaz, H. Driguez and A. Planas, *Biochem. J.*, 2006, **395**, 99–106.
12 J. Hanus and K. Mazeau, *Biopolymers*, 2006, **82**, 59–73.
13 S. Morris, S. Hanna and M. J. Miles, *Nanotechnology*, 2004, **15**, 1296–1301.
14 M. Madson, C. Dunand, X. M. Li, R. Verma, G. F. Vanzin, J. Calplan, D. A. Shoue, N. C. Carpita and W. D. Reiter, *Plant Cell*, 2003, **15**, 1662–1670.
15 L. F. Mackenzie, Q. P. Wang, R. A. J. Warren and S. G. Withers, *J. Am. Chem. Soc.*, 1998, **120**, 5583–5584.
16 S. M. Hancock, M. D. Vaughan and S. G. Withers, *Curr. Opin. Chem. Biol.*, 2006, **10**, 509–519.
17 G. Perugino, B. Cobucci-Ponzano, M. Rossi and M. Moracci, *Adv. Synth. Catal.*, 2005, **347**, 941–950.
18 S. Blanchard, S. Armand, P. Couthino, S. Patkar, J. Vind, E. Samain, H. Driguez and S. Cottaz, *Carbohydr. Res.*, 2007, **342**, 710–716.
19 S. Blanchard, S. Cottaz, P. M. Coutinho, S. Patkar, J. Vind, H. Boer, A. Koivula, H. Driguez and S. Armand, *J. Mol. Catal. B: Enzym.*, 2007, **44**, 106–116.
20 S. Fort, V. Boyer, L. Greffe, G. Davies, O. Moroz, L. Christiansen, M. Schülein, S. Cottaz and H. Driguez, *J. Am. Chem. Soc.*, 2000, **122**, 5429–5437.
21 V. Boyer, S. Fort, T. P. Frandsen, M. Schülein, S. Cottaz and H. Driguez, *Chem.–Eur. J.*, 2002, **8**, 1389–1394.
22 S. Fort, L. Christiansen, M. Schülein, S. Cottaz and H. Driguez, *Isr. J. Chem.*, 2000, **40**, 217–221.
23 M. Yang, G. J. Davies and B. G. Davis, *Angew. Chem., Int. Ed.*, 2007, **46**, 3885–3888.
24 R. Fauré, D. Cavalier, K. Keegstra, S. Cottaz and H. Driguez, *Eur. J. Org. Chem.*, 2007, **2007**, 4313–4319.
25 P. Johansson, H. Brumer, M. J. Baumann, Å. M. Kallas, H. Henriksson, S. E. Denman, T. T. Teeri and T. A. Jones, *Plant Cell*, 2004, **16**, 874–886.
26 Å. M. Kallas, K. Piens, S. E. Denman, H. Henriksson, J. Fäldt, P. Johansson, H. Brumer and T. T. Teeri, *Biochem. J.*, 2005, **390**, 105–113.
27 M. Faijes and A. Planas, *Carbohydr. Res.*, 2007, **342**, 1581–1594.
28 D. H. G. Crout and G. Vic, *Curr. Opin. Chem. Biol.*, 1998, **2**, 98–111.
29 D. J. Vocadlo and S. G. Withers, in *Carbohydrates in Chemistry and Biology*, ed. B. Ernst, G. W. Hart and P. Sinay, Wiley-VCH, Weinheim, Germany, 2000, pp. 723–844.
30 A. Trincone and A. Giordano, *Curr. Org. Chem.*, 2006, **10**, 1163–1193.
31 V. M. A. Ducros, C. A. Tarling, D. L. Zechel, A. M. Brzozowski, T. P. Frandsen, I. von Ossowski, M. Schülein, S. G. Withers and G. J. Davies, *Chem. Biol.*, 2003, **10**, 619–628.
32 C. Mayer, D. L. Jakeman, M. Mah, G. Karjala, L. Gal, R. A. J. Warren and S. G. Withers, *Chem. Biol.*, 2001, **8**, 437–443.
33 C. Mayer, D. L. Zechel, S. P. Reid, R. A. J. Warren and S. G. Withers, *FEBS Lett.*, 2000, **466**, 40–44.
34 M. Hrmova, T. Imai, S. J. Rutten, J. K. Fairweather, L. Pelosi, V. Bulone, H. Driguez and G. B. Fincher, *J. Biol. Chem.*, 2002, **277**, 30102–30111.
35 M. Jahn, D. Stoll, R. A. J. Warren, L. Szabo, P. Singh, H. J. Gilbert, V. M. A. Ducros, G. J. Davies and S. G. Withers, *Chem. Commun.*, 2003, 1327–1329.
36 M. Sugimura, M. Nishimoto and M. Kitaoka, *Biosci., Biotechnol., Biochem.*, 2006, **70**, 1210–1217.
37 H. Brumer, Q. Zhou, M. J. Baumann, K. Carlsson and T. T. Teeri, *J. Am. Chem. Soc.*, 2004, **126**, 5715–5721.
38 L. C. Gunnarsson, Q. Zhou, C. Montanier, E. N. Karlsson, H. Brumer and M. Ohlin, *Glycobiology*, 2006, **16**, 1171–1180.
39 L. Greffe, L. Bessueille, V. Bulone and H. Brumer, *Glycobiology*, 2005, **15**, 437–445.
40 T. Nordström, K. Nourizad, M. Ronaghi and P. Nyrén, *Anal. Biochem.*, 2000, **282**, 186–193.
41 G. Sundqvist, M. Stenvall, H. Berglund, J. Ottosson and H. Brumer, *J. Chromatogr., B*, 2007, **852**, 188–194.
42 Z. Sulová, M. Lednicka and V. Farkaš, *Anal. Biochem.*, 1995, **229**, 80–85.
43 C. Martinez-Fleites, C. I. P. D. Guerreiro, M. J. Baumann, E. J. Taylor, J. A. M. Prates, L. M. A. Ferreira, C. M. G. A. Fontes, H. Brumer and G. J. Davies, *J. Biol. Chem.*, 2006, **281**, 24922–24933.

