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Rice BGlu1 glycosynthase and wild type transglycosylation activities distinguished by cyclophellitol inhibition

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ABSTRACT

The rice BGlu1 β-D-glucosidase nucleophile mutant E386G is a glycosynthase that catalyzes the synthesis of cellooligosaccharides from α-D-glucopyranosyl fluoride (GlcF) donor and *p*-nitrophenyl (*p*NP) cellobioside (Glc2-*p*NP) or cello-oligosaccharide acceptors. When activity with other donors and acceptors was tested, the initial enzyme preparation cleaved *p*NP-β-D-glucopyranoside (Glc-*p*NP) and *p*NP-β-D-fucopyranoside (Fuc-*p*NP) to *p*NP and glucose and fucose, suggesting contamination with wild type BGlu1 β-glucosidase. The products from reaction of GlcF and Fuc-*p*NP included Fuc-β-(1→3)-Fuc-*p*NP, Glc-β-(1→3)-Fuc-*p*NP, and Fuc-β-(1→4)-Glc-β-(1→3)-Fuc-*p*NP, suggesting the presence of both wild type BGlu1 and its glycosynthase. Inhibition of the BGlu1 β-glucosidase activity within this preparation by cyclophellitol confirmed that the E386G glycosynthase preparation was contaminated with wild type BGlu1. Rice BGlu1 E386G-2, generated from a new construct designed to minimize back-mutation, showed glycosynthase activity without wild type hydrolytic or transglycosylation activity. E386G-2 catalyzed transfer of glycosyl residues from GlcF, α-L-arabinosyl fluoride, α-D-fucosyl fluoride, α-D-galactosyl fluoride, α-D-mannosyl fluoride, and α-D-xylosyl fluoride donors to Glc2-*p*NP acceptor. The synthetic products from the reactions of α-fucosyl fluoride and α-mannosyl fluoride donors were confirmed to result from addition of a β-(1→4)-linked glycosyl residue. Moreover, the E386G glycosynthase transferred glucose from GlcF donor to glucose, cellobiose, Glc-*p*NP, Fuc-*p*NP, *p*NP-β-D-galactopyranoside, and *p*NP-β-D-xylopyranoside acceptors, but little to *p*NP-β-D-mannopyranoside. Production of longer oligosaccharides occurred most readily on acceptors with an equatorial 4-OH. Elimination of wild type contamination thereby allowed a clear assessment of BGlu1 E386G glycosynthase catalytic abilities.

1. Introduction

Glycosynthases are nucleophile mutants of glycosidases that effect synthesis of oligosaccharides and glycoconjugates from glycosyl fluoride donors and suitable acceptors without hydrolysis of the products (Fig. 1).[1–3] Glycosynthases derived from endoglycosidases use oligosaccharyl fluorides as donor substrates and have high regioselectivity.[3–8] Glycosynthases derived from exoglycosidases are typically capable of efficient glycoside assembly with monosaccharyl fluoride donors, but sometimes with lower substrate specificity and regioselectivity.[3,9,10]

A glycoside hydrolase family 1 β-D-glucosidase from rice (BGlu1, systematically called Os3BGlu7) has a broad substrate specificity, with particularly high hydrolytic activity toward β-(1→3)- and β-(1→4)-linked oligosaccharides and pyridoxine 5′-O-β-D-glucoside.[11–13] It also has high transglucosylation activity using 4-nitrophenyl β-D-glucoside (Glc-*p*NP) as donor for glucosyl transfer to pyridoxine or Glc-*p*NP acceptors, resulting in the synthesis of pyridoxine 5′-O-β-D-glucoside and *p*NP gluco-oligosaccharides. Rice BGlu1 has previously been mutated to form a glycosynthase (Fig. 1C) that can synthesize *p*NP-cellooligosaccharides of at least 11 β-(1→4)-linked glucosyl residues.[14] In that study, it was found that the E386G mutation (designated E414G in the previous study, based on the nucleophile position in the BGlu1 precursor) resulted in much higher glycosynthase activity than the E386A or E386S mutations. The ability to synthesize long cellooligosaccharides was attributed to their binding in a long oligosaccharide binding cleft, which has recently been verified in the X-ray crystal structures of BGlu1 acid-base and glycosynthase mutants.[15,16]

In this paper we expand our exploration of the glycosynthase activity of the BGlu1 E386G enzyme, in particular, we explore the ability of the enzyme to utilize alternate glycosyl fluoride donors and acceptors, thereby broadening the spectrum of di- and trisaccharides produced by this glycosynthase. Wild type

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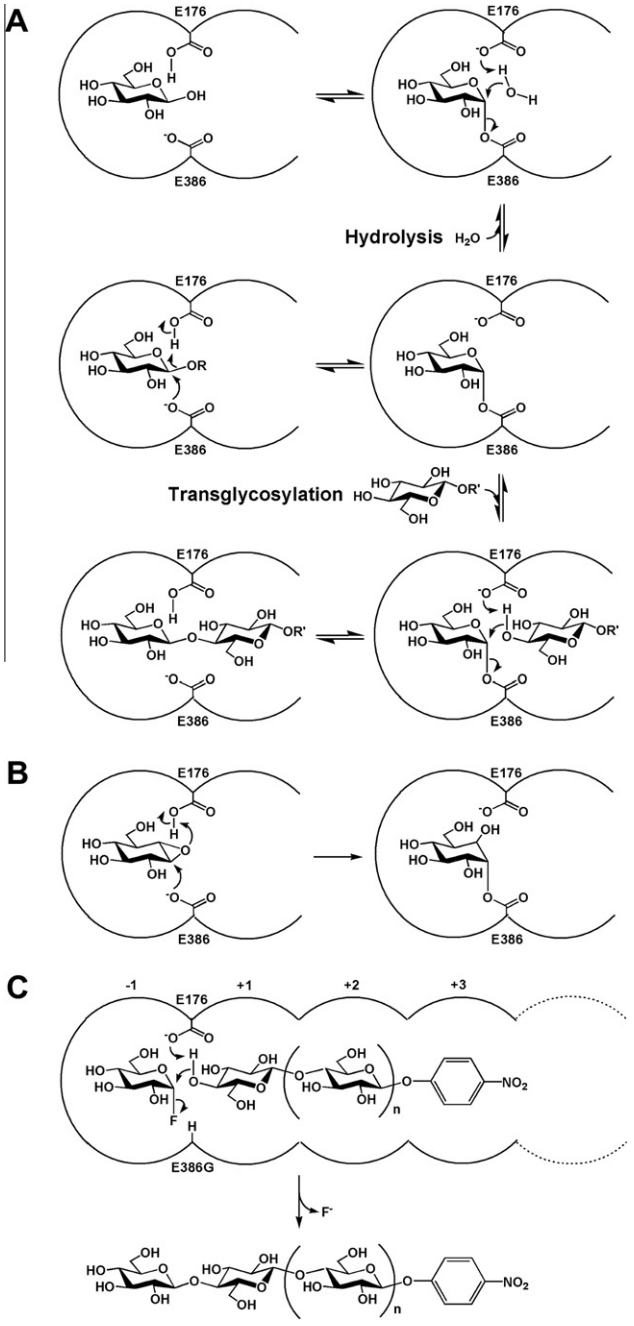


Figure 1. Reaction mechanisms of hydrolysis and transglycosylation catalyzed by rice BGlu1 β-glucosidase (A); covalent intermediate trapping of rice BGlu1 by cyclophellitol (B), and rice BGlu1 E386G glycosynthase (C). Note that cyclophellitol cannot react with BGlu1 E386G due to its lack of a catalytic nucleophile.

BGlu1 contamination of BGlu1 E386G was confirmed and eliminated by inhibition with cyclophellitol, thereby allowing the actual glycosynthase products to be identified.

2. Results and discussion

2.1. Evidence of wild type BGlu1 contamination in the BGlu1 E386G preparation

As is the case with many GH1 glycosidases,[17] the wild type BGlu1 β-D-glucosidase has relatively broad substrate specificity, since it can hydrolyze 4-nitrophenyl β-D-glucopyranoside (Glc-*p*NP), *p*NP β-D-fucopyranoside (Fuc-*p*NP), *p*NP-β-D-galactopyranoside (Gal-*p*NP), *p*NP-β-D-mannopyranoside (Man-*p*NP), *p*NP β-D-xylopyranoside (Xyl-*p*NP), and *p*NP α-L-arabinopyranoside, as well as β-(1→2), β-(1→3), β-(1→4), and β-(1→6) glycosidic linkages.[11,12] This suggests that BGlu1 glycosynthase might also utilize other glycosyl fluoride donors and thus make mixed products.

When the acceptor specificity of the BGlu1 E386G glycosynthase preparation was investigated, both transglycosylation and hydrolysis products were detected. The Glc-*p*NP and Fuc-*p*NP acceptors were cleaved to *p*NP, glucose and fucose, while Glc-*p*NP was produced from Glc2-*p*NP, which suggested wild type contamination of the BGlu1 E386G glycosynthase preparation. Since new column packings were used for all glycosynthase preparations, cross-contamination during purification was unlikely. It thus appeared that a mutation in the Origami(DE3) *Escherichia coli* strain being used for expression may have led to a higher than normal reversion rate, possibly by misreading on the ribosome or reversion mutations in a few cells in the expression culture.[18,19] This suggested that some of the products formed may have been generated by a combination of glycosynthase and wild type transglycosylation activities. Characterization of purified products provided further evidence for the combined action of BGlu1 wild type and E386G glycosynthase, as described below.

Mass spectrometric and NMR characterizations identified the purified products of reaction of α-glucosyl fluoride (GlcF) and Fuc-*p*NP catalyzed by the BGlu1 E386G preparation as Fuc-β-(1→3)-Fuc-*p*NP, Glc-β-(1→3)-Fuc-*p*NP, and Fuc-β-(1→4)-Glc-β-(1→3)-Fuc-*p*NP (Table 1, products 1, 2, and 3, respectively). Since glycosynthase mutants of BGlu1 show very little *p*NP-glycoside hydrolysis,[14] the nonreducing end fucosyl moieties in products of Fuc-β-(1→3)-Fuc-*p*NP and Fuc-β-(1→4)-Glc-β-(1→3)-Fuc-*p*NP from reaction of GlcF and Fuc-*p*NP may have come from transfucosylation from Fuc-*p*NP by wild type BGlu1 contamination, as diagrammed in Figure 1A.

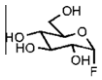
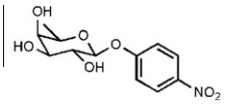
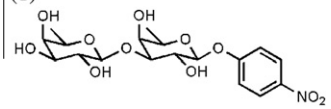
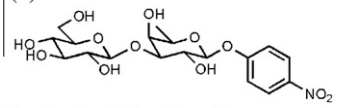
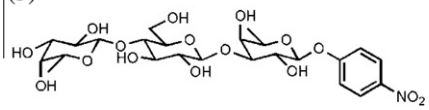
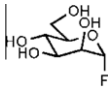
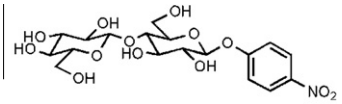
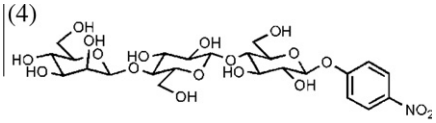
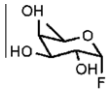
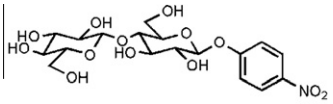
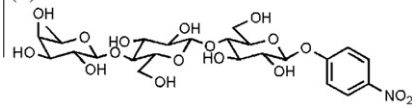
2.2. Cyclophellitol inhibition

The presence of wild type BGlu1 contamination in the E386G preparation was confirmed by inhibition of the wild type enzyme with the covalent inhibitor cyclophellitol. As shown in Figure 1B, cyclophellitol inactivates retaining β-D-glucosidases by forming a covalent adduct with the catalytic nucleophile. This is a stable adduct that does not undergo reactivation by transglycosylation.[20] Figure 2 shows that inactivation of wild type rice BGlu1 by cyclophellitol follows pseudo-first order kinetics of inactivation, similar to previous reports for other β-glucosidases.[20,21] Cyclophellitol acts as an irreversible inactivator of the rice BGlu1 β-glucosidase with a K_i of 5.7 μM and a k_i of 0.35 min^{-1} (Fig. 2B and C).

The E386G preparation was thus pre-incubated with cyclophellitol for 2 h, which resulted in complete inhibition of wild type BGlu1 activity (Fig. 2A), before testing the transglycosylation of GlcF onto Fuc-*p*NP. The E386G glycosynthase preparation synthesized two products in the range of *p*NP-disaccharides and a few trisaccharides in the reaction without cyclophellitol pre-incubation (Fig. 3 lane EG −) and a single product, corresponding to Glc-Fuc-*p*NP, after pre-incubation with 2 mM cyclophellitol (Fig. 3 lane EG +).

As expected, untreated wild type BGlu1 β-glucosidase synthesized transglycosylation products, while that pre-incubated with cyclophellitol did not (Fig. 3, lanes BG). Moreover, uninhibited wild type BGlu1 synthesized only one product in the *p*NP-disaccharide range: Fuc-Fuc-*p*NP, which was also seen with the untreated E386G preparation, but not when it was preincubated with cyclophellitol. This result confirms that the original E386G glycosynthase was contaminated with wild type BGlu1, and that, as would be expected, the wild type BGlu1 does not transfer from GlcF, suggesting

Table 1
Structures of products of glycosynthase reactions of BGlu1 E386G

#	Donor	Acceptor	Product
1	 GlcF	 β-D-Fuc-*p*NP	(1)*  β-D-Fuc-(1→3)-β-D-Fuc-*p*NP (2)  β-D-Glc-(1→3)-β-D-Fuc-*p*NP (3)**  β-D-Fuc-(1→4)-β-D-Glc-(1→3)-β-D-Fuc-*p*NP
2	 ManF	 Glc2-*p*NP (β-D-Glc-(1→4)-β-D-Glc-*p*NP)	(4)  β-D-Man-(1→4)-β-D-Glc-(1→4)-β-D-Glc-*p*NP
3	 FucF	 Glc2-*p*NP (β-D-Glc-(1→4)-β-D-Glc-*p*NP)	(5)  β-D-Fuc-(1→4)-β-D-Glc-(1→4)-β-D-Glc-pNP

* Formed by wild type BGlu1 β-glucosidase contaminant.
** Formed by successive action of glycosynthase and wild type BGlu1 contaminant.

it is unlikely to have contributed to the production of hetero-disaccharide products where the added glycosyl residues were derived from an α-glycosyl fluoride donor.

The cyclophellitol inhibition results show that the combination of wild type BGlu1 and E386G glycosynthase seen in the contaminated prep was likely responsible for synthesis of the novel *p*NP-oligosaccharide Fuc-β-(1→4)-Glc-β-(1→3)-Fuc-*p*NP, which could not be produced by either enzyme alone. This points to the importance of testing for wild type hydrolase activity when assessing glycosynthase products, since the transglycosylation products arising from small amounts of contaminating hydrolase can be significant, given the large amount of glycosynthase needed for the transfer reactions.

2.3. Verification of linkages of glycosynthase products

As noted above, the wild type BGlu1 β-glucosidase could not transfer glycosyl residues from α-glycosyl fluoride donors, so any products generated from such transfers reflect the glycosynthase regiospecificity, even when derived from the mixture with wild type in the E386G preparation. It was previously demonstrated that reaction of BGlu1E386G with GlcF and Glc2-pNP specifically yields β-(1→4)-linked oligosaccharides.[14] The MS and NMR analyses described above identified a product generated from transfer of a single glucosyl residue onto Fuc-*p*NP as β-D-Glc-(1→3)-β-D-Fuc-*p*NP (Table 1, product 2). Similarly, in reaction products with Glc2-*p*NP as acceptor, a single transfer product from α-D-mannosyl fluoride (ManF) was identified as Man-β-(1→4)-Glc-β-(1→4)-Glc-*p*NP (Table 1, product 4) and the corresponding product with α-D-fucosyl fluoride (FucF) donor was Fuc-β-(1→4)-Glc-β-(1→4)-Glc-*p*NP (Table 1, product 5). Thus, in each case, β-(1→4)-linkages were preferred on Glc residues with equatorial 4-OH groups, but the glycosyl residue was added onto the equatorial 3-OH of Fuc residues, where the 4-OH is axial.

Streptomyces β-glucosidase glycosynthase is regiospecific for production of β-(1→3)-linkages to *p*NP-monosaccharide acceptors and can use Fuc-*p*NP and Gal-*p*NP acceptors,[9] while *Agrobacterium* β-glucosidase has a strong preference for formation of β-(1→4)-linkages.[22–25] As such, rice BGlu1 appears to be more similar to *Streptomyces* β-glucosidase glycosynthase in its activity toward these acceptors.

2.4. Transglycosylation activity of E386G-2

A new construct for the production of BGlu1 E386G was made by changing the codon used from GAA (Glu) to GGC instead of GGG to give Gly at the nucleophile position. Use of this double-changed codon should decrease the probability of misreading as Glu (GAG) by the ribosome or of a reversion mutation in the expression culture. The protein expressed from this mutation (E386G-2) indeed exhibited 33-fold lower Glc-*p*NP hydrolysis activity compared to the original E386G glycosynthase (the Glc-*p*NP hydrolysis activities of BGlu1, BGlu1 E386G, and BGlu1 E386G-2 were 1900, 1.1, and 0.033 nmol min^{-1} mg^{-1} protein, respectively). When the E386G-2 glycosynthase preparation was used to catalyze the reaction between Fuc-*p*NP and GlcF, only a single product, corresponding to Glc-Fuc-*p*NP, was detected after pre-incubation either without or with 2 mM cyclophellitol (Fig. 3, lanes EG2 − and +).

Since no wild type hydrolysis and transglycosylation activities were detected in the E386G-2 preparation under the conditions of the standard reaction, this sample was used to test the BGlu1

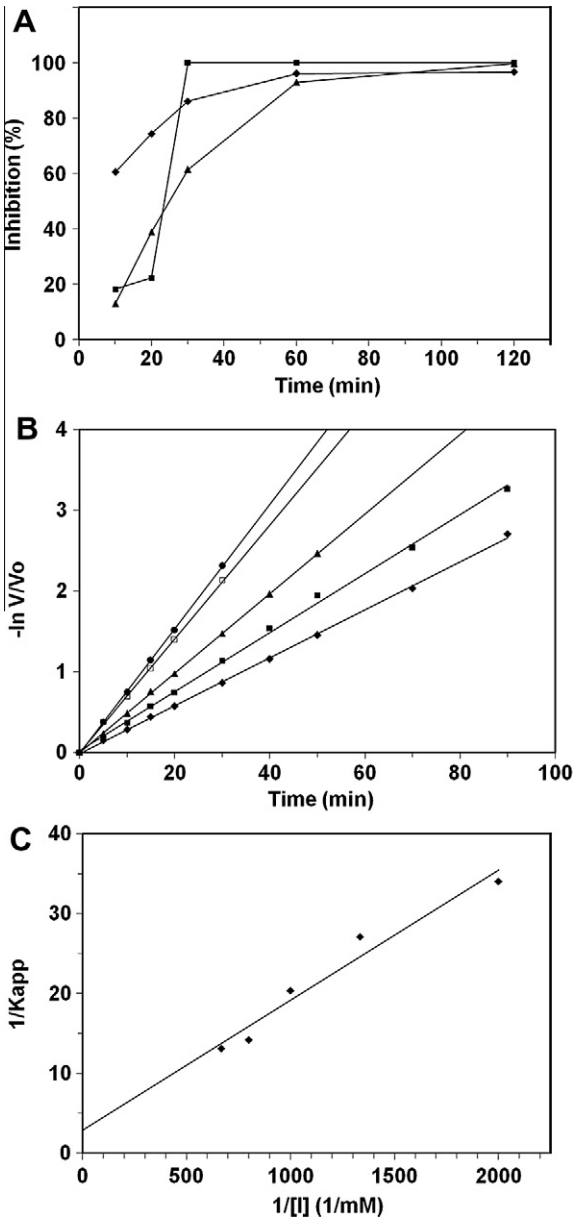


Figure 2. Cyclophellitol inactivation of rice BGlu1. (A) Time course for incubation of the enzymes with cyclophellitol. The enzyme solutions of 0.2 μM BGlu1 (▲), 40 μM E386G (♦), and 40 μM E386G-2 (■) were pre-incubated with 1 μM cyclophellitol for different times and then assayed for release of *p*NP from Glc-*p*NP. (B) Semi-logarithmic plot of wild type BGlu1 β-glucosidase activity versus time at various concentrations of cyclophellitol, (●), 0.5 μM; (□), 0.75 μM; (▲), 1 μM; (■), 1.25 μM; (♦), 1.5 μM. (C) Replot of the inverse of the apparent first order rate constants (k_{app}) from B versus the inverse of the inhibitor concentration, 1/[I].

E386G glycosynthase activity with various donors and acceptors. The E386G-2 glycosynthase successfully transferred a range of donors (α-L-arabinosyl fluoride (AraF), FucF, α-D-galactosyl fluoride (GalF), GlcF, ManF, and α-D-xylosyl fluoride (XylF)) onto *p*NP-cellobiose to produce trisaccharides, and a faint spot of tetrasaccharide was seen with FucF donor (Fig. 4).

The E386G-2 glycosynthase could also use glucose, cellobiose, Fuc-*p*NP, Gal-*p*NP, Glc-*p*NP, Xyl-*p*NP, and Glc2-*p*NP as acceptors (Fig. 4). *p*NP-Oligosaccharides resulting from addition of more than one Glc could only be detected by TLC with Glc2-*p*NP as acceptor. Longer oligosaccharides were, however, synthesized by E386G-2 using GlcF donor when Glc-*p*NP, Man-*p*NP, Xyl-*p*NP, or Glc2-*p*NP acceptors were employed, similar to what had been seen with glycosynthases from *Agrobacterium* β-glucosidase.[22,23] By contrast, the *Streptomyces* E383A glycosynthase could only synthesize disaccharides when using GlcF and *p*NP-monosaccharide acceptors.[9] When using Fuc-*p*NP as acceptor E386G-2 synthesized only Glc-Fuc-*p*NP disaccharide and a tiny amount of Glc-Glc-Fuc-*p*NP trisaccharide, and showed no hydrolytic activity toward acceptors, verifying that the longer Fuc-*p*NP oligosaccharides seen with the original E386G prep resulted from the wild type BGlu1 transglycosylation activity. The *p*NP-disaccharide product spot seen when Glc-*p*NP was used as an acceptor was very faint (Fig. 4B, lane pG), explaining why we failed to detect it in our previous work.[14] This spot had a mobility slightly lower than Glc2-*p*NP, suggesting it might be *p*NP-gentiobioside, but this identity was not confirmed due to the small amount produced. Similarly, the product spots obtained with cellobiose as acceptor were very faint, though at least two products could be detected by sulfuric acid charring (Fig. 4D, lane C2).

Surprisingly, a clear spot of laminaribiose was observed when glucose was used as an acceptor, and this spot was also seen in the other reactions, likely due to the presence of glucose from hydrolysis of GlcF. The production of laminaribiose is in line with the fact that laminaribiose is a much better hydrolytic substrate for the wild-type BGlu1 β-glucosidase than are cellobiose or other disaccharides[12] and consistent with structural studies in complexes with laminaribiose.[15] The enzyme also catalyzed autocondensation of GlcF, as seen by the smear above glucose in the TLC and LC–MS peaks with masses of 379 *m/z* ($[M+^{35}Cl]^-$).

Transglycosylation yields for reaction of BGlu1 E386G-2 with GlcF and a variety of acceptors are shown in Table 2, and those with a variety of donors and Glc2-*p*NP acceptor are shown in Table 3. Relatively high yields were obtained with FucF and ManF donors, but only a low yield with XylF. The absence of a substituent at C-5 apparently affects binding at the −1 subsite, although the activity was higher than that of non-evolved glycosynthases derived from *Agrobacterium* β-glucosidase[22–24] and *Streptomyces* E383A,[9] for which XylF was not a donor substrate. Moreover, E386G-2 uses AraF as a donor, something which has not previously been reported for a GH1 glycosynthase. The E386G-2 glycosynthase gave low yields with Fuc-*p*NP, Gal-*p*NP, Glc-*p*NP, and Xyl-*p*NP acceptors, and GlcF donor, compared to the *Streptomyces* E383A.[9]

Although Gal-*p*NP and Fuc-*p*NP acceptors gave the highest yields for disaccharide production, only very small amounts of trisaccharides and no tetrasaccharides were detected on LC–MS (Table 2). In contrast, Glc-*p*NP gave higher production of trisaccharide and tetrasaccharide products were also seen for Glc-*p*NP, Xyl-*p*NP, and Man-*p*NP, which all have equatorial 4-OH groups. This agrees with the previous report that the BGlu1 E386G glycosynthase appears to require a β-(1→4)-linked acceptor, such as Glc2-*p*NP or cellotriose, before it can efficiently transfer successive glucosyl residues onto a β-(1→4)-linked chain with high regioselectivity.[14]

With Glc2-*p*NP as acceptor, the BGlu1 E386G-2 glycosynthase gave lower yields of trisaccharide products (26% with GlcF), but higher yields of tetrasaccharide (16%) and longer products, compared to *Agrobacterium* E358A (79% trisaccharide and 13% tetrasaccharide under comparable conditions) and E358S (88% trisaccharide, 7% tetrasaccharide) and *Streptomyces* E383A glycosynthase (80% trisaccharide and 3% tetrasaccharide). As noted previously, the unique feature of rice BGlu1 glycosynthases is their ability to produce long oligosaccharides,[14] so that BGlu1 E386G also produced cellopentaose in 11% yield. At donor to acceptor ratios above 1:1, the yields could not be assessed by HPLC with GlcF donor and Glc2-*p*NP acceptor, since they resulted in precipitated products. Thus, BGlu1 E386G is more useful for synthesis of longer oligosaccharides, while the bacterial enzymes are better for synthesis of di- and tri-saccharides.

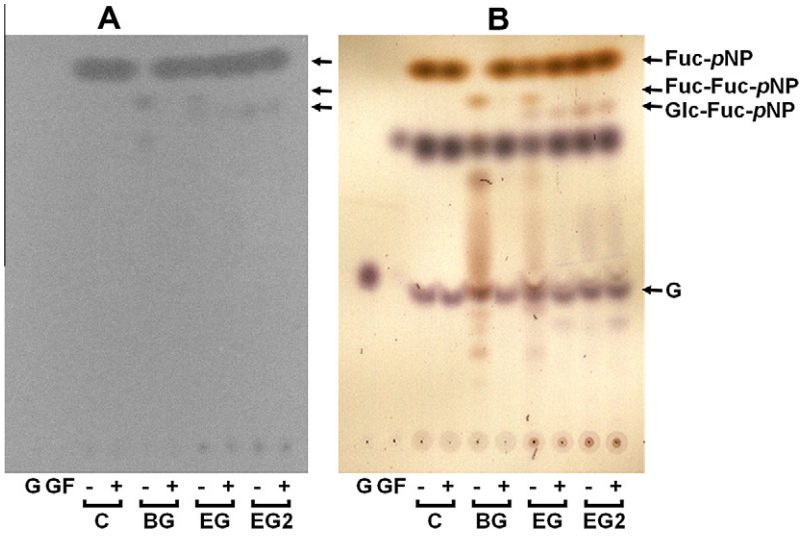


Figure 3. Effect of cyclophellitol inhibition on transglycosylation products of rice BGlu1, BGlu1 E386G, and BGlu1 E386G-2 with Fuc-*p*NP and GlcF. The 0.2 μM BGlu1, 40 μM E386G, and 40 μM E386G-2 were pre-incubated without (−) or with (+) 2 mM cyclophellitol for 2 h, then transglycosylation reactions with 10 mM Fuc-*p*NP and 10 mM α-GlcF in 150 mM NH_4HCO_3, pH 7.0, were incubated at 30°C for 16 h. Silica gel TLC analysis was performed with EtOAc–MeOH–water (7:2.5:1) as a developing solvent, and products detected under UV light (A) or by dabbing with 10% sulfuric acid in ethanol followed by charring (B). Lane G, glucose standard; GF, GlcF; C, control of 10 mM Fuc-*p*NP and 10 mM GlcF without enzyme; BG, BGlu1; EG, BGlu1 E386G; EG2, BGlu1 E386G-2.

2.5. Conclusion

Pure rice BGlu1 E386G glycosynthase transfers glycosyl residues from a variety of α-glycosyl fluoride donors, in the reactivity order Glc > Fuc > Ara > Man > Gal > Xyl. Likewise it transfers glucosyl residues from GlcF to several different acceptors, with a preference for formation of 1–4 linkages. Only equatorial hydroxyls on the ring appear to be glycosylated, so if the C-4 hydroxyl is axial, the monosaccharide is added to the C-3 hydroxyl. However the products formed in this way do not appear to serve as acceptors for further transfer reactions. Care must be taken to avoid contamination by wild type enzyme arising either from laboratory procedures or translational misincorporation, since otherwise, initially formed glycosynthase products can be hydrolyzed, and unexpected transglycosylation products formed.

3. Experimental

3.1. Materials

Cello-oligosaccharides (DP 3–6) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Glucose, cellobiose, Glc2-*p*NP, Glc-*p*NP, Fuc-*p*NP, Gal-*p*NP, Man-*p*NP, and Xyl-*p*NP were purchased from Sigma Chemical Co. (St. Louis, MO). GlcF, AraF, FucF, GalF, ManF, and XylF were synthesized by previously described methods.[26] Cyclophellitol was a generous gift from Kah-Yee Li and Professor Herman Overkleeft, University of Leiden.

3.2. Protein expression and purification

The recombinant BGlu1 and BGlu1 E386G proteins were expressed in *E. coli* strain Origami(DE3) and purified by immobilized metal affinity chromatography (IMAC), enterokinase digestion, and subtractive IMAC, as previously described.[11,14,27] The buffer was exchanged with 50 mM phosphate buffer, pH 6.0, and concentrated in an Amicon ultra-centrifugal filter (Ultracel-10K, Millipore, USA). The purified protein concentration was determined by measuring its absorbance at 280 nm. An extinction coefficient ε_{280} of 113560 M^{-1} cm^{-1}, calculated from the amino acid composition by the method of Gill and von Hippel,[28] was used to calculate the BGlu1 enzyme concentrations.

3.3. Transglycosylation activity of E386G with various donors

The E386G glycosynthase was incubated at 40 μM with 10 mM of each of the donors: AraF, FucF, GalF, ManF, or XylF and 10 mM Glc2-*p*NP acceptor in 150 mM ammonium bicarbonate buffer, pH 7.0, at 30 °C for 16 h.[14] Products were analyzed by TLC (Silica Gel 60 F_{254}, aluminum-backed, Merck) with ethyl acetate (EtOAc)–methanol (MeOH)–water (7:2.5:1) as solvent. Plates were visualized under ultraviolet (UV) light and by exposure to 10% sulfuric acid in ethanol followed by charring. Samples (5 μL) were diluted in 200 μl of 20% MeOH, 0.1% formic acid, and 5 μL of the solution was injected onto an ESI-MS (Waters, Alliance HPLC, Micromass ZQ ESCi, USA). The quadrupole mass analyzer was scanned over a range of 100–1000 *m/z*.

3.4. Transglycosylation by BGlu1 E386G to various acceptors

The BGlu1 E386G glycosynthase was incubated at a concentration of 40 μM with 10 mM GlcF donor and 10 mM acceptor: glucose, cellobiose, Glc-*p*NP, Fuc-*p*NP, Gal-*p*NP, Man-*p*NP, or Xyl-*p*NP, in 150 mM ammonium bicarbonate, pH 7.0, at 30 °C for 16 h. The products were analyzed by TLC with EtOAc–MeOH–water (7:2.5:1 as solvent for glucose and *p*NP-oligosaccharides, and 2:1:1 for cellobiose). The products were further verified by ESI-MS, as described above. Samples (3 μL) of the products from the reactions with 1:1 molar ratios of GlcF: Glc-*p*NP or GlcF: Fuc-*p*NP were loaded onto a ZORBAX carbohydrate column (4.6 mm × 250 mm, Agilent, USA) connected to an Agilent 1100 series LC–MSD. The column was eluted with a linear gradient from 90% to 60% acetonitrile in water over 30 min at a flow rate of 0.8 mL/min. The eluted peaks were detected by UV absorbance at 300 nm. Reaction yields were determined by integration of the product peaks within the HPLC profile and the molecular masses of the eluted products were determined by mass spectrometry.

3.5. Determination of linkages within glycosynthase products

Oligosaccharide products from reactions of FucF and Glc2-*p*NP, ManF and Glc2-*p*NP, and GlcF and Fuc-*p*NP were synthesized with E386G. Reaction mixtures containing FucF or ManF (14.6 mg, 4 equiv) and Glc2-*p*NP (9.2 mg, 1 equiv); GlcF (48 mg, 1 equiv)

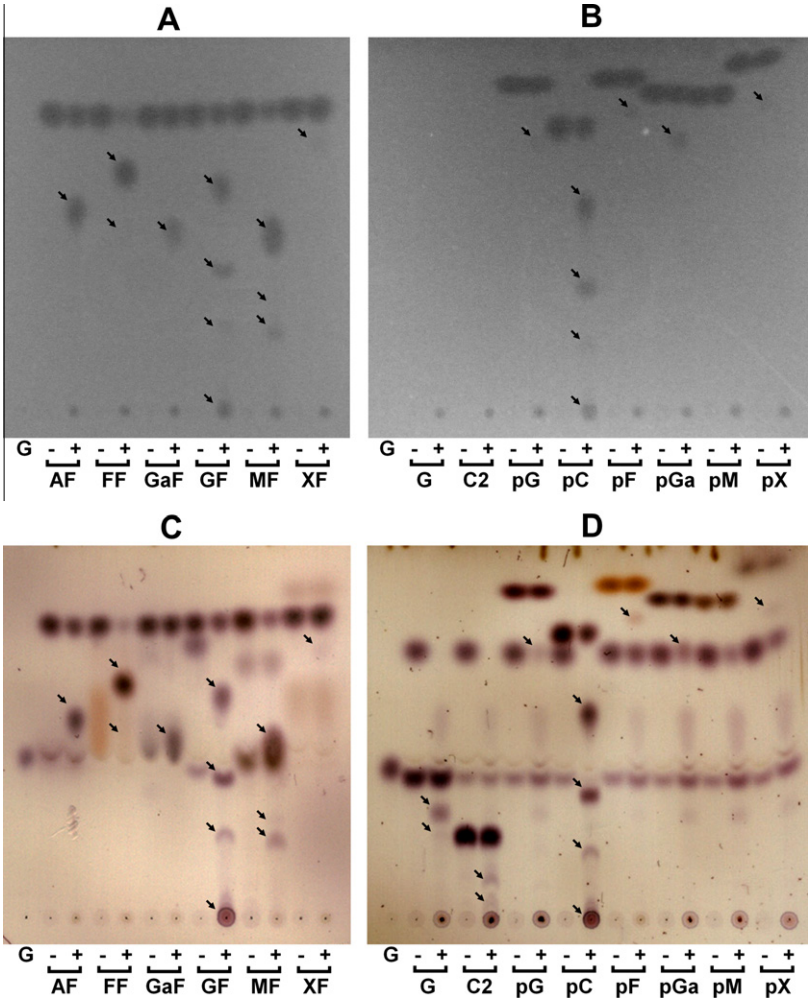


Figure 4. TLC detection of transglycosylation catalyzed by BGlu1 E386G-2 with various donors or acceptors. The E386G-2 was incubated with 10 mM Glc2-*p*NP and 10 mM donors (A and C) or 10 mM GlcF and 10 mM acceptors (B and D) in 150 mM NH_4HCO_3, pH 7.0, at 30 °C for 16 h. The reactions were performed without (−) or with (+) enzyme. The products were separated by silica gel TLC with EtOAc–MeOH–water (7:2.5:1) as a developing solvent and detected under UV light (A and B) or by exposure to 10% sulfuric acid in ethanol, followed by charring (C and D). G is glucose standard. A and C show reactions with various donors: AF, AraF; FF, FucF; GaF, GalF; GF, GlcF; MF, ManF; and XF, XylF. B and D show reactions with various acceptors: G, glucose; C2, cellobiose; pG, Glc-*p*NP; pC, Glc2-*p*NP; pF, Fuc-*p*NP; pGa, Gal-*p*NP; pM, Man-*p*NP; and pX, Xyl-*p*NP.

and Fuc-*p*NP (75 mg, 1 equiv) and 40 μM of the appropriate enzyme were incubated at 30 °C in 150 mM ammonium bicarbonate, pH 7.0, (2–10 ml total reaction volume) for 24–48 h. The reaction mixtures were filtered and loaded onto a reverse-phase column (Sep-Pak tC18, sorbent weight 2 g, Waters), which was eluted with step gradients of 0–30% methanol in water. Eluted fractions were checked by TLC, as described above. Fractions containing the compounds were pooled, dried on a rotary evaporator, and further purified by TLC. Silica gel containing each compound was scratched from the TLC plate and eluted with 30% MeOH, then the methanol was evaporated and the sample solution was dried by lyophilization. Masses were determined by ESI-MS.

A mixture of pyridine and acetic anhydride (5 ml, $V_{pyridine}$:$V_{acetic\ anhydride}$ = 3:2) was added to the purified glycoside products at room temperature and the reaction mixture was stirred overnight. Ice water was added to quench the unreacted acetic anhydride and the reaction was stirred for another 10 min. After evaporation of the solvent, the residue was dissolved in ethyl acetate and washed successively with ice water, 1 M HCl, $NaHCO_3$, and brine, and dried with $MgSO_4$. The organic solvent was evaporated in vacuo, and the resulting residue was purified by flash column chromatography on silica gel using ethyl acetate/petroleum ether = 1:1 as eluent.

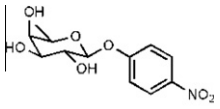
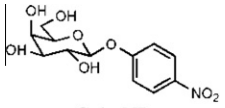
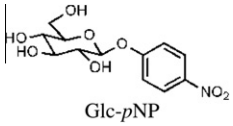
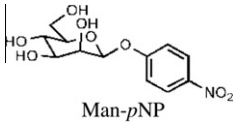
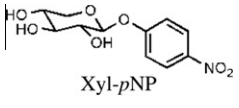
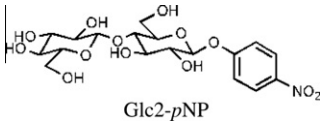
All ^{1}H and ^{1}H COSY nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance-400 inv spectrometer (400 MHz) and chemical shifts were reported in δ units (ppm) and were calibrated based on the deuterium solvent chemical shift ($CDCl_3$). Multiplicity of signals is described as follows: br-broad, s-singlet; d-doublet; t-triplet; m-multiplet.

3.5.1. NMR spectra

3.5.1.1. 4-Nitrophenyl (2,3,4-tri-*O*-acetyl-α-D-fucopyranosyl)-(1→3)-2,4-di-*O*-acetyl-β-D-fucopyranoside (1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H, Ar), 7.05 (m, 2H, Ar), 5.50 (dd, 1H, $J_{1,2}$ 8.1, $J_{2,3}$ 9.9 Hz, H-2), 5.35 (br d, 1H, $J_{3,4}$ 3.4 Hz, H-4), 5.20 (br d, 1H, $J_{3',4'}$ 3.3 Hz, H-4′), 5.11 (dd, 1H, $J_{1',2'}$ 7.9, $J_{2',3'}$ 10.7 Hz, H-2′), 5.07 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1), 4.95 (dd, 1H, $J_{2',3'}$ 10.7, $J_{3',4'}$ 3.4 Hz, H-3′), 4.55 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′), 3.98 (dd, 1H, $J_{2,3}$ 9.9, $J_{3,4}$ 3.4 Hz, H-3), 3.94 (m, 1H, H-5′), 3.76 (m, 1H, H-5), 2.20–1.98 (5s, 15H, 5 × CH_3CO), 1.30 (d, 3H, H6′a, H6′b, H6′c), 1.20 (d, 3H, H6a, H6b, H6c). ESI-MS: *m/z* 454 $[M+Na]^+$.

3.5.1.2. 4-Nitrophenyl (2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→3)-2,4-di-*O*-acetyl-β-D-fucopyranoside (2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H, Ar), 7.06 (m, 2H, Ar), 5.46 (dd, 1H, $J_{1,2}$ 8.0, $J_{2,3}$ 10.0 Hz, H-2), 5.34 (br d, 1H, $J_{3,4}$ 3.4 Hz, H-4), 5.17 (dd, 1H, $J_{3',4'}$ 9.3, $J_{4',5'}$ 9.0 Hz, H-4′), 5.12 (dd, 1H, $J_{2',3'}$ 9.0, $J_{3,'4'}$ 9.3 Hz, H-3′), 5.08 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.93 (dd, 1H, $J_{1',2'}$ 7.8, $J_{2',3'}$ 9.0 Hz, H-2′) 4.65 (d, 1H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.33 (dd, 1H, $J_{5',6'a}$

Table 2
Products of glycosynthase reactions of BGlu1 E386G-2 glycosynthase using α-glucosyl fluoride donor and different acceptors[a]

#	Acceptor	*p*NP-Oligosaccharide product (% yield)								
		Di	Tri	Tetra	Penta	Hexa	Hepta	Octa	Nana	Total yield
4b c	 Fuc-*p*NP	4.2 7.3 (482)	0.040 0.074 (644)	–	–	–	–	–	–	4.24 7.37
5b c	 Gal-*p*NP	9.1 11 (498)	0.15 0.21 (660)	–	–	–	–	–	–	9.25 11.2
6b c	 Glc-*p*NP	3.0 3.4 (498)	0.49 0.55 (660)	0.15 0.14 (822)	0.12 0.13 (984)	–	–	–	–	3.76 4.22
7b c	 Man-*p*NP	0.062 0.068 (498)	0.035 0.032 (660)	0.046 0.039 (822)	–	–	–	–	–	0.143 0.139
8b c	 Xyl-*p*NP	3.1 4.0 (468)	0.1 0.17 (630)	0.052 0.051 (792)	–	–	–	–	–	3.25 4.22
9b	 Glc2-*p*NP		26 (660)	16 (822)	11 (984)	3.5 (1146)	0.47 (1308)	0.03 (1471)	0.002 (1633)	57.0

[a] Preparative reactions in 150 mM ammonium bicarbonate buffer.
*p*NP-glycoside acceptor, and 40 μM E386G-2 concentration for 24 h. The relative percents are in terms of peak area per total 300 nm absorbance in *p*NP-glycoside products separated by HPLC on a ZORBAX carbohydrate column. The molecular mass $[Mass+^{35}Cl]^-$ of each eluted compound was confirmed by mass spectrometry as shown in parenthesis.
[b] pH 7.0, 30 °C, at a 1:1 molar ratio.
[c] 3:1 molar ratios of GlcF donor.

2.6, $J_{6'a,6'b}$ 12.3 Hz, H-6′a), 4.15 (dd, 1H, $J_{5',6'b}$ 3.8, $J_{6'a,6'b}$ 12.3 Hz, H-6′b), 3.97–3.92 (m, 2H, H-3, H-5), 3.69–3.65 (m, 2H, H-5′), 2.18–2.00 (6s, 18H, 6 × CH_3CO), 1.25 (d, 3H, H6a, H6b, H6c). ESI-MS: *m/z* 470 $[M+Na]^+$.

3.5.1.3. 4-Nitrophenyl [(2,3,4-tri-*O*-acetyl-β-D-fucopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-(1→3)-*O*-2,4-di-*O*-acetyl-β-D-fucopyranoside (3). ^1H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H, Ar), 7.06 (m, 2H, Ar), 5.48 (dd, 1H, $J_{1,2}$ 8.0, $J_{2,3}$ 10.0 Hz, H-2), 5.33 (br d, 1H, $J_{3,4}$ 3.0 Hz, H-4), 5.20 (br d, 1H, $J_{3'',4''}$ 3.3 Hz, H-4″), 5.17–5.03 (m, 4H, H-1,H-3′, H-2″, H-3″), 4.89 (dd, 1H, $J_{1',2'}$ 7.9, $J_{2',3'}$ 8.8 Hz, H-2′), 4.58 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′), 4.45 (d, 1H, $J_{1'',2''}$ 8.2 Hz, H-1″), 4.30 (dd, 1H, $J_{5,6'a}$ 3.0, $J_{6'a,6'b}$ 12.1 Hz, H-6′a), 4.14 (dd, 1H, $J_{5',6'b}$ 3.9, $J_{6'a,6'b}$ 12.1 Hz, H-6′b), 3.98–3.91 (m, 2H, H-3, H-5), 3.73–3.63 (m, 3H, H-4′, H-5′, H-5″), 2.19–2.00 (8s, 24H, 8 × CH_3CO), 1.35 (d, 3H, H6a, H6b, H6c), 1.25 (d, 3H, H6″a, H6″b, H6″c). ESI-MS: *m/z* 616 $[M+Na]^+$.

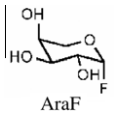
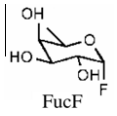
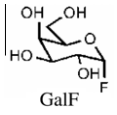
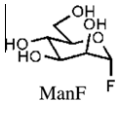
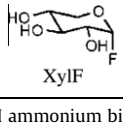
3.5.1.4. 4-Nitrophenyl [(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (4). ^1H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H, Ar), 7.06 (m, 2H, Ar), 5.40 (br d, 1H, $J_{2'',3''}$ 3.0, H-2″), 5.29 (dd, 1H, $J_{2,3}$ 8.5, $J_{3,4}$ 8.5 Hz, H-3), 5.21 (dd, 1H, $J_{1,2}$ 7.3, $J_{2,3}$ 8.5 Hz, H-2), 5.20 (dd, 1H, $J_{3'',4''}$ 10.0, $J_{4'',5''}$ 8.5 Hz, H-4″), 5.17 (d, 1H, $J_{1,2}$ 7.3 Hz, H-1), 5.15 (dd, 1H, $J_{2',3'}$ 9.4, $J_{3',4'}$ 9.7 Hz, H-3′), 5.01 (dd, 1H, $J_{2'',3''}$ 3.0, $J_{3'',4''}$ 10.0 Hz, H-3″), 4.88 (dd, 1H, $J_{1',2'}$ 7.9, $J_{2',3'}$ 9.4 Hz, H-2′), 4.64 (br s, 1H, H-1″), 4.55 (dd, 1H, $J_{5.6a}$ 1.8, $J_{6a,6b}$ 11.8 Hz, H-6a), 4.52 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′), 4.34 (dd, 1H, $J_{6'a,6'b}$ 12.45, $J_{5',6'a}$ 5.2 Hz, H-6′a), 4.31–4.28 (m, 2H, H-6″a, H-6″b), 4.12 (dd, 1H, $J_{6'a,6'b}$ 12.5, $J_{5',6'b}$ 2.1 Hz, H-6′b), 4.11 (dd, 1H, $J_{6a,6b}$ 11.8, $J_{5,6b}$ 4.9 Hz, H-6b), 3.91–3.81 (m, 3H, H-4, H-4′, H-5), 3.64–3.58 (m, 2H, H-5′, H-5″), 2.16–1.99 (10s, 30H, 10 × CH_3CO). ESI-MS: *m/z* 648 $[M+Na]^+$.

3.5.1.5. 4-Nitrophenyl [(2,3,4-tri-*O*-acetyl-β-D-fucopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl]-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (5). ^1H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H, Ar), 7.05 (m, 2H, Ar), 5.29 (dd, 1H, $J_{2,3}$ 8.5, $J_{3,4}$ 8.2 Hz, H-3), 5.23–5.15 (m, 4H, H-2, H-3′, H4″, H-1) 5.08 (dd, 1H, $J_{1''2''}$ 8.0 $J_{2''3''}$ 10.3 Hz, H-2″), 4.94 (dd, 1H, $J_{2''3''}$ 10.3, $J_{3''4''}$ 3.4 Hz, H-3″), 4.87 (dd, 1H, $J_{1'2'}$ 8.1, $J_{2'3'}$ 9.5 Hz, H-2′), 4.55 (m, 1H, H-6a), 4.53 (d, 1H, $J_{1'2'}$ 8.1 Hz, H-1′), 4.43 (d, 1H, $J_{1''2'}$ 8.0 Hz, H-1″), 4.41 (m, 1H, H-6′a), 4.16–4.14 (m, 2H, H-6b, H-6′b), 3.88 (dd, 1H, $J_{3,4}$ 8.2, $J_{4,5}$ 10.1 Hz, H-4), 3.81 (dd, 1H, $J_{3'4'}$ 9.5, $J_{4'5'}$ 9.4 Hz, H-4′), 3.91–3.78 (m, 1H, H-5), 3.76–3.71 (m, 1H, H-5″), 3.65–3.59 (m, 1H, H-5′), 2.20–1.90 (9s, 27H, 9 × CH_3CO), 1.20 (d, 3H, Me). ESI-MS: *m/z* 632 $[M+Na]^+$.

3.6. Generation of E386G-2 mutant of rice BGlu1

BGlu1 E386G-2 was constructed by changing the DNA codon for GAA to GGC by QuikChange mutagenesis (Stratagene, La Jolla, CA, USA) with the primers 5′-G ACA GTC GTC ATA ACT GGC AAC GGA ATG GAT CAA C-3′ and 5′-G TTG ATC CAT TCC GTT GCC AGT TAT

Table 3
Products of glycosynthase reactions of BGlu1 E386G-2 glycosynthase using different donors and Glc2-*p*NP acceptor[a]

#	Donor	*p*NP-Oligosaccharide product (% yield)		
		Tri	Tetra	Total yield
10b	 AraF	25	0.051	25.1
c		59 (630)	0.072 (762)	59.1
11b	 FucF	49	0.011	49.0
c		98 (644)	1.4 (790)	99.4
12b	 GalF	15	0.013	15.0
c		42 (660)	0.018 (822)	42.0
13b	 ManF	19	0.21	19.2
c		71 (660)	7.9 (822)	78.9
14b	 XylF	1.2	0.0091	1.21
c		3.1 (630)	0.013 (762)	3.11

[a] Preparative reactions in 150 mM ammonium bicarbonate buffer.
[b] pH 7.0, 30 °C, at a 1:1.
[c] 3:1 molar ratios of glycosyl fluoride donor:Glc2-*p*NP acceptor, and 40 μM E386G-2 concentration for 24 h. The relative percents are in terms of peak area per total 300 nm absorbance in *p*NP-glycoside products separated by HPLC on a ZORBAX carbohydrate column. The molecular mass $[Mass+^{35}Cl]^-$ of each eluted compound was confirmed by mass spectrometry, as shown by the mass in parenthesis (in amu).

GAC GAC TGT C-3′ and the pET32a/BGlu1 expression vector[11] as template. The transglucosylation activity of the new mutation was compared to the E386G (codon: GGG) toward various donors of AraF, FucF, GalF, GlcF, ManF, and XylF with Glc2-*p*NP acceptor, and GlcF donor with various acceptors of glucose, cellobiose, Glc-*p*NP, Glc2-*p*NP, Fuc-*p*NP, Gal-*p*NP, Man-*p*NP, and Xyl-*p*NP, as described above. Samples (10 μl) of the products from the reactions with 1:1 and 3:1 molar ratios of donor:*p*NP acceptor were analyzed by HPLC on a ZORBAX carbohydrate column as described above.

3.7. Cyclophellitol inhibition

The cyclophellitol inhibition of BGlu1 wild type, E386G, and E386G-2 activities was monitored by analysis of residual enzyme activity as a function of time. Solutions of 0.2 μM BGlu1, 40 μM E386G, and 40 μM E386G-2 in 50 mM sodium acetate, pH 5.0, were pre-incubated with 1 μM cyclophellitol at 30 °C for different times and Glc-*p*NP hydrolysis was assayed as previously described.[11]

Inactivation of BGlu1 by cyclophellitol was performed by incubating the BGlu1 in 50 mM sodium acetate, pH 5.0, at 30 °C in the presence of the cyclophellitol concentrations given in Figure 2B. Aliquots (10 μl) were removed at appropriate time intervals and assayed for hydrolysis of Glc-*p*NP in the standard assay.

The β-glucosidase activity of glycosynthase mutants was inactivated by pre-incubating 0.2 μM BGlu1, 40 μM E386G, or 40 μM E386G-2 with 2 mM cyclophellitol for 2 h, and then transglycosylation activity was tested with 10 mM Fuc-*p*NP and 10 mM GlcF in 150 mM NH_4HCO_3, pH 7.0, at 30 °C for 24 h. The products were analyzed by TLC, using EtOAc–MeOH–water (7:2.5:1) as solvent, as described above.

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