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Remodeling of the Glycosylation Pathway in the Methylotrophic Yeast *Hansenula polymorpha* to Produce Human Hybrid-Type *N*-Glycans[§]

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As a step forward to achieve the generation of human complex-type *N*-glycans in the methylotrophic yeast *Hansenula polymorpha*, we here report the modification of the yeast glycosylation pathway by heterologous expression of the human gene encoding β-1,2-*N*-acetylglucosaminyltransferase I (GnTI). For the optimal expression of human GnTI in the yeast Golgi compartment, the catalytic domain of the GnTI was fused to various N-terminal leader sequences derived from the yeast type II membrane proteins. The vectors containing GnTI fusion constructs were introduced into the *H. polymorpha och1*Δ single and *och1*Δ*alg3*Δ double mutant strains expressing the ER-targeted *Aspergillus saitoi* α-1,2 mannosidase, respectively. Both of the glycoengineered *Hpoch1*Δ and *Hpoch1*Δ*Hpalg3*Δ strains were shown to produce successfully the hybrid-type glycans with a monoantennary *N*-acetylglucosamine ($GlcNAc_1Man_5GlcNAc_2$ and $GlcNAc_1Man_3GlcNAc_2$, respectively) by *N*-glycan profile analysis of cell wall proteins. Furthermore, by comparative analysis of byproduct formation and the glycosylation site occupancy, we propose that the *Hpoch1*Δ strain would be more suitable than the *Hpoch1*Δ*Hpalg3*Δ strain as a host for the production of recombinant proteins with humanized glycans.

Keywords: *Hansenula polymorpha*, humanized hybrid-type *N*-glycans, *ALG3*, *OCH1*

Introduction

The early stages of protein *N*-glycosylation are highly conserved from yeast to human in eukaryotes until the synthesis of the core form of *N*-oligosaccharide, $Man_8GlcNAc_2$, in the endoplasmic reticulum (ER). However, the subsequent maturation of the *N*-linked glycans assembled on proteins, which occurs in the Golgi apparatus, varies considerably depending on the species and cell type. In mammalian cells, some mannose residues in the core form $Man_8GlcNAc_2$ are trimmed during protein transport from the ER to the Golgi apparatus. Subsequently, complex *N*-glycans are generated by the addition of *N*-acetylglucosamine (GlcNAc), galactose, sialic acid, and/or fucose during transit through secretory vesicles in the Golgi. In contrast, the processing of *N*-glycans in the yeast Golgi often leads to hypermannosylated glycoproteins, by further addition of several mannose or mannosylphosphate residues without mannose trimming (Jigami and Odani, 1999). These different features of *N*-glycan maturation between yeast and mammalian cells have been a major limitation in the use of yeast as host systems for the development of recombinant therapeutic glycoproteins. Therefore, several attempts have been made to overcome these problems in various yeast species by deleting genes responsible for the yeast specific outer chain biosynthesis and then by subsequently introducing heterologous genes involved in the synthesis of complex- type *N*-glycans, such as genes encoding *N*-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases (Choi *et al.*, 2003; Hamilton *et al.*, 2003; Vervecken *et al.*, 2004; Wildt and Gerngross, 2005). Recently, the production of glycoprotein with fully sialylated biantennary *N*-glycans was reported in the glycoengineered *Pichia pastoris* (Hamilton *et al.*, 2006). With the increasing demand on the production of recombinant glycoproteins with therapeutic potential, glycoengineering for humanized glycosylation pathways has been a major issue in the development of various yeast species as cost-effective expression systems (De Pourcq *et al.*, 2010).

The thermotolerant methylotrophic yeast *Hansenula polymorpha* serves as a useful host system for the high level production of recombinant proteins, from industrial enzymes to therapeutic proteins (Kim *et al.*, 2004, 2006; Kang and Gellissen, 2005). We had previously shown that *H. polymorpha* is advantageous over the traditional yeast *Saccharomyces cerevisiae* as a host for the production of humanized glycoproteins, in that most *N*-linked glycans of *H. polymorpha* are much less hypermannosylated and devoid of hyper-immunogenic terminal α-1,3-linked mannose residues (Kim *et al.*, 2004). In an effort to engineer this yeast to produce human-type glycoproteins, we had previously con-

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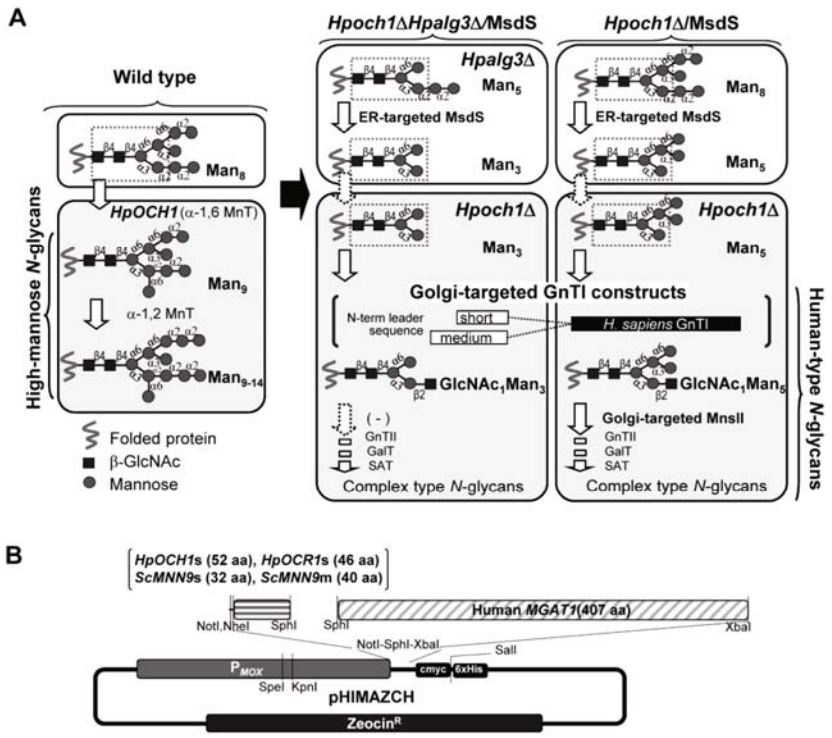

Fig. 1. Schematic representation for the engineering of *N*-glycosylation pathway in *H. polymorpha*. (A) The *N*-glycosylation pathway and strategies of glycoengineering for the production of human complex-type sugar in *H. polymorpha*. *ALG3* (α-1,3-mannosyltransferase), *OCH1* (α-1,6-mannosyltransferase), MsdS (ER-targeted *A. saitoi* α-1,2-mannosidase-HA-HDEL), MnsII (mannosidase II), GnTI (β-1,2-*N*-acetylglucosaminyltransferase I), GnTII (β-1,2-*N*-acetylglucosaminyltransferase II), GalT (galactosyltransferase), SAT (sialyltransferase). (B) *H. polymorpha* vectors for Golgi-targeted GnTI expression. The short leader sequences containing the N-terminal cytoplasmic tail and the transmembrane domain (*HpOCH1*s, *HpOCR1*s, and *ScMNN9*s), or the medium leader sequence (*ScMNN9*m) containing half of the stem region in addition to the short leader sequence derived from HpOch1, HpOcr1, and ScMnn9 proteins were fused with the catalytic domain of human GnTI encoded by the *MGAT1* gene (GenBank number NM_002406), consisting of 407 amino acids without its own N-terminal leader sequence. The Golgi-targeted GnTI fusion constructs were cloned into pHIMAZCH backbone vector as described in 'Materials and Methods'.

structed a *Hpoch1*Δ single mutant strain by deleting the *HpOCH1* gene encoding the yeast specific α-1,6-mannosyltransferase responsible for the outer chain initiation of *N*-glycans in the Golgi (Kim *et al.*, 2006). We had subsequently constructed a *Hpoch1*Δ*Hpalg3*Δ double mutant strain with the additional deletion of *ALG3* encoding a dolichyl-phosphate-mannose-dependent α-1,3-mannosyltransferase, which mediates the conversion step of $Man_5GlcNAc_2$-dolichyl pyrophosphate (Dol-PP) to $Man_6GlcNAc_2$-Dol-PP in the ER (Oh *et al.*, 2008). Then, for the trimming of mannose residues in *N*-glycans, we introduced the ER-targeted expression of the *Aspergillus saitoi* α-1,2-mannosidase (*MsdS*) gene using the C-terminal motif HDEL as a targeting signal into the *Hpoch1*Δ single and the *Hpoch1*Δ*Hpalg3*Δ double deletion strains. The glycoengineered *H. polymorpha* strains, *Hpoch1*Δ/MsdS and *Hpoch1*Δ*Hpalg3*Δ/MsdS, were shown to produce human-type pentamannosyl *N*-glycans ($Man_5GlcNAc_2$) and core trimannosyl *N*-glycan ($Man_3GlcNAc_2$), respectively (Kim *et al.*, 2006; Oh *et al.*, 2008). The yeast strains can be directly used for the production of glycoproteins with human-compatible high mannose type oligosaccharides, but also can be efficiently employed for the further humanization of the glycosylation pathway toward human complex-type *N*-glycans (Fig. 1A). The first common step forward in the generation of complex-type *N*-glycans in both the *H. polymorpha* glycoengineered strains is the heterologous expression of β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) to add a first GlcNAc to $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$, respectively. In contrast to the *Hpoch1*Δ strain, the *Hpoch1*Δ*Hpalg3*Δ strain is blocked in an early step of lipid-linked *N*-oligosaccharide formation in the ER. Thus the *Hpoch1*Δ*Hpalg3*Δ/MsdS strain does not require a subsequent modification step for the introduction of the Golgi mannosidase II (MnsII) to generate $GlcNAc_1$-$Man_3GlcNAc_2$, a productive substrate of β-1,2-*N*-acetylglucosaminyltransferase II (GnTII). The addition of a second GlcNAc residue by GnTII leads to the formation of $GlcNAc_2$-$Man_3GlcNAc_2$, which is the first intermediate for the production of complex type *N*-glycans.

Here, we report the modification of the yeast glycosylation pathway in the glycoengineered *Hpoch1*Δ and *Hpoch1*Δ-*Hpalg3*Δ strains, respectively, to synthesize the human hybrid-type *N*-glycans with a terminal *N*-acetylglucosamine. We also evaluated the engineered yeast strains as hosts for the production of recombinant glycoproteins by accessing glycosylation occupancy of a reporter glycoprotein.

Materials and Methods

Strains, plasmids, and primers

The *H. polymorpha* strains and plasmids used and constructed in this study are listed in Table 1. The primers used in this study are listed in Supplementary data Table S1. *H. polymorpha* strains were maintained and/or selected in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose) medium or YPD+Zeocin^{TM} (100 μg/ml, pH 7.5, Invitrogen, USA), if necessary. Strains containing the *Hptrp1* mutation were maintained in YPD supplemented with 90 mg/L tryptophan. *Escherichia coli* DH5α used for plasmid propagation was cultured in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% sodium chloride) with ampicillin (100 μg/ml) or 2× YT medium (1.6% Bacto-tryptone, 1% Bacto-yeast extract, 0.5% sodium chloride) with Zeocin^{TM} (25 μg/ml) at 37°C.

The *H. polymorpha* expression vector pHIMAZCH con-

Table 1. ***H. polymorpha*** **strains and plasmids used in this study**

Strain name	Genotype	Reference
H. polymorpha		
DL1-LdU	*leu2 ura3Δ::lacZ*	Kang *et al.* (2002)
DL1-Hpalg3	*leu2 ura3Δ::lacZ alg3Δ::lacZ*	Oh *et al.* (2008)
DL1-g15	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ*	Cheon *et al.* (2009)
DL1-g16	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ* pDTMOX-msdS	This study
DL1-g17Ss	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ* pDTMOX-msdS pHIMAZC6-SN9sHG1	This study
DL1-g17Sm	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ* pDTMOX-msdS pHIMAZC7-SN9mHG1	This study
DL1-g17Hs	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ* pDTMOX-msdS pHIMAZ-HH1G1-CH	This study
DL1-g17Rs	*leu2 ura3Δ::lacZ och1Δ::lacZ alg3Δ::lacZ trp1Δ::lacZ* pDTMOX-msdS pHIMAZ-HR1G1-CH	This study
DL1-g22	*leu2 ura3Δ::lacZ och1Δ::lacZ trp1Δ::lacZ*	This study
DL1-g23	*leu2 ura3Δ::lacZ och1Δ::lacZ trp1Δ::lacZ* pDTG-msdS	This study
DL1-g24Sm	*leu2 ura3Δ::lacZ och1Δ::lacZ trp1Δ::lacZ* pDTG-msdS pHIMAZC7-SN9mHG1	This study
S. cerevisiae		
BY4742	*MATα his3 leu2 lys2 ura3*	Open Biosystems
13108	*MATα his3 leu2 lys2 ura3 alg3::KanMX*	Open Biosystems
Plasmid name	**Description[a]**	**Reference**
pHIMAZCH	P_{MOX}-T_{AOX}, Zeocin^R	This study
pHIMAZ-SN9G1-CH	P_{MOX}- *ScMNN9*m-*MGAT1*-c-myc-6xHis-T_{AOX}, Zeocin^R	This study
pHIMAZ-HH1G1-CH	P_{MOX}- *HpOCH1*s-*MGAT1*-c-myc-6xHis-T_{AOX}, Zeocin^R	This study
pHIMAZ-HR1G1-CH	P_{MOX}-*HpOCR1*s-*MGAT1*-c-myc-6xHis-T_{AOX}, Zeocin^R	This study
pHIMAZC6-SN9sHG1	P_{MOX}-*ScMNN9*s-*MGAT1*-T_{AOX}, Zeocin^R	This study
pHIMAZC7-SN9mHG1	P_{MOX}-*ScMNN9*m-*MGAT1*-T_{AOX}, Zeocin^R	This study
pDTMOX-msdS	P_{MOX}-*MsdS*-HA-HDEL, *HpTRP1-HARS36*	Cheon *et al.* (2009)
pDTG-msdS	P_{GAP}-*MsdS*-HA-HDEL, *HpTRP1-HARS36*	This study
pDLGAP-ScCPY	P_{GAP}-*ScCPY*, *HpLEU2-HARS36*	This study

[a] *MGAT1* contains the DNA fragment of 407 amino acids from the C-terminus of human GnTI (GenBank number NM_002406); *ScMNN9*s and *ScMNN9*m contain the DNA fragment encoding 32 amino acids and 40 amino acids from its N-terminus, respectively; *HpOCH1*s and *HpOCR1*m contain the DNA fragment encoding 52 amino acids and 46 amino acids from its N-terminus, respectively.

taining the methanol-inducible *MOX* promoter and the Zeocin resistance marker (Fig. 1B) was created by replacing the BglII-XbaI *GAP* promoter fragment in pGAPZαA (Invitrogen) with the 1.5 kb *MOX* promoter fragment, which was PCR-amplified with a primer set HP01/HP02 from *H. polymorpha* genomic DNA. For targeting of human GnTI to the early secretary pathway of *H. polymorpha*, various N-terminal leader sequences from yeast type II glycosyltransferases, including ScMnn9p, HpOch1p, and HpOcr1p, were fused to GnTI as follows. The 120 bp N-fragments of the *ScMNN9* gene (*ScMNN9*m) was amplified by using a primer set HP03/HP04 from *S. cerevisiae* genomic DNA. The 156 bp N-fragment of the *HpOCH1* gene (*HpOCH1*s), and the 138 bp N-fragment of the *HpOCR1* gene (*HpOCR1*s), were amplified by using primer sets HP05/HP06, and HP07/HP08, respectively, from *H. polymorpha* genomic DNA. The 1,239 bp fragment encoding 407 amino acids corresponding to the C-terminus catalytic domain of human *MGAT1* was amplified by using a primer set HP09/HP10 from the human clone hMU007066 containing the full length cDNA of *MGAT1* (21C Frontier Human Gene Bank, KRIBB, Korea). The expression vector pHIMAZ-SN9G1-CH, containing the *ScMNN9*m fragment fused to GnTI tagged with c-myc and 6x His, was constructed by three-piece ligation with a NotI-SphI *ScMNN9*m fragment, a SphI-XbaI *MGAT1* fragment, and a NotI-XbaI pHIMAZCH fragment. Next, the Nhe1-SphI *ScMNN9*m fragment in pHIMAZ-SN9G1-CH was replaced with the Nhe1-SphI *HpOCH1*s or the *HpOCR1*s fragment, resulting in pHIMAZ-HH1G1-CH, and pHIMAZ-HRG1-CH vectors, respectively. To make the *MGAT1* constructs without C-terminal epitopes, the P_{MOX}-*ScMNN9*s and P_{MOX}-*ScMNN9*m fragments containing the *MOX* promoter fragment fused to the *ScMNN9*s and *ScMNN9*m fragments, respectively, were generated by fusion PCR with the primers listed in Supplementary data Table S1. The 1,245 bp *MGAT1* cDNA fragment was amplified by using a primer set HP09/HP16. The plasmid pHIMAZC6-SN9sHG1 was constructed by three-piece ligation with a KpnI-SphI P_{MOX}-*ScMNN9*s fragment, a SphI-SalI *MGAT1* fragment, and a KpnI-SalI pHIMAZCH fragment. The KpnI-SphI fragment of P_{MOX}-*ScMNN9*m was inserted into the corresponding sites of pHIMAZC6-SN9sHG1, generating a pHIMAZC7-SN9mHG1 vector.

To construct a *ScCPY* overexpression vector under the control of the *H. polymorpha GAP* promoter, a 0.75 kb *GAP* promoter fragment was amplified with primers HP17/18 from the pGA-GOD vector (Kim *et al.*, 2002), and a 1.6 kb *ScCPY* fragment was amplified with the primers HP19/20 from *S. cerevisiae* genomic DNA. The pDLGAP-ScCPY vector was constructed by three-piece ligation with a HpaI-HindIII *GAP* promoter, a HindIII-ClaI *ScCPY* fragment, and a ClaI-HapI pGA-GOD vector fragment.

N-glycan structural analysis of yeast cell wall glycoproteins

The glycoengineered *H. polymorpha* strains were cultivated

in YPM (1% Bacto-yeast extract, 2% Bacto-pepton, 1% methanol) for 48 h with feeding of 1% methanol at 24 h. The yeast cell wall proteins (CWPs) were extracted by a slightly modified method using hot citrate buffer (20 mM sodium citrate buffer, pH 7.0) as previously described (Vervecken *et al.*, 2004; Li *et al.*, 2007). *N*-glycans released from CWPs using PNGase F (New England Biolabs, UK) were labeled by 8-aminopyrene-1,3,6-trisulfonic acid (APTS, Invitrogen), purified and analyzed using capillary electrophoresis equipped with an ABI3131 sequencer (Applied Biosystems, USA) as previously described (Vervecken *et al.*, 2007; Lee *et al.*, 2009). *In vitro* α-1,2 mannosidase and/or β-*N*-hexosaminidase (Prozyme, USA) treatments were carried out as previously described (Laroy *et al.*, 2006).

Western blot analysis of CPY

Yeast cells were cultivated in YPD liquid medium at 37°C for *H. polymorpha* and 30°C for *S. cerevisiae.* Cells were harvested, washed with cold water and 1× G7 buffer (50 mM sodium phosphate, pH 7.5), and then lysed in 1× G7 buffer containing protein inhibitor cocktails (Sigma-Aldrich, USA) and 1 mM PMSF (Sigma-Aldrich) by vortexing with glass beads. After centrifugation at 14,240×*g* for 10 min at 4°C, total intracellular lysates were obtained, and 30 μg of proteins were separated in SDS–PAGE for western blotting with anti-HpCPY antibody (kindly provided by Dr. M.O. Agaphonov) and anti-ScCPY antibody (Invitrogen), respectively.

Results and Discussion

Construction of the *H. polymorpha* strains harboring the Golgi targeted GnTI expression vectors

The first step in the processing of *N*-glycans into the human-complex type is the addition of a GlcNAc residue by *N*-acetylglucosaminyltransferase I. To achieve the heterologous expression of a human β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) at the yeast Golgi apparatus, we constructed a set of GnTI expression vectors using several N-terminal leader sequences derived from yeast glycosyltransferases (Fig. 1B). Most of the type II membrane glycosyltransferases are localized to the early to medial Golgi apparatus, which is largely mediated by their N-terminus consisting of a short cytoplasmic tail, a hydrophobic transmembrane domain, and a part of the luminal stem region (Czlapinski and Bertozzi, 2006). Thus, for efficient targeting to the yeast Golgi, the N-terminal portion of the human GnTI was replaced with the short or medium N-terminal leader nucleotide sequences, differing in the length of stem region following the N-terminal cytoplasmic tail and the transmembrane domain, of *S. cerevisiae* Mnn9 (Choi *et al.*, 2003), *H. polymorpha* Och1, and *H. polymorpha* Ocr1 (Kim *et al.*, 2006) proteins. The HpOch1s-, HpOcr1s, ScMnn9s-, and ScMnn9m-GnTI fusion constructs were expressed under the control of a strong methanol-inducible *MOX* promoter in a pHIMAZCH backbone vector containing the Zeocin resistance marker (Fig. 1B). The GnTI expression vectors were digested with SpeI for linearization and then introduced into *Hpoch1*Δ/MsdS (DL1-g23) or *Hpoch1*Δ*Hpalg3*Δ/MsdS (DL1-g16) strains, respectively. The *H. polymorpha* transformants were selected on Zeocin-containing YPD medium at 37°C, and the correct integration into the *MOX* promoter site was confirmed by PCR using a primer set HP21/HP22 (data not shown).

Production of $GlcNAc_1Man_3GlcNAc_2$ *N*-glycans in the *Hpoch1*Δ*Hpalg3*Δ/MsdS strain expressing GnTI

As an initial trial, we first tested the expression of the GnTI fusion constructs in the glycoengineered *Hpoch1*Δ*Hpalg3*Δ/MsdS strain (DL1-g16). The targeted expression of each chimeric GnTI fusion construct at the yeast Golgi was confirmed by Western blotting with anti-c-myc or anti-MGAT antibody (ab38857, Abcam, UK) of the membrane fraction samples obtained by subcellular compartmentalization (data not shown). To confirm the functional expression of the GnTI fusion proteins, we analyzed *N*-glycans of cell wall proteins prepared from the wild-type strain and the transformants of *Hpoch1*Δ*Hpalg3*Δ/MsdS and *Hpoch1*Δ*Hpalg3*Δ/MsdS strains harboring the GnTI fusion constructs (Fig. 2). By capillary electrophoresis analysis, we observed the peaks corresponding to high-mannose type *N*-glycans of $Man_{8\sim12}GlcNAc_2$ in the wild-type strain, while a predominant peak of core trimannosyl ($Man_3GlcNAc_2$, M_3) *N*-glycan with small peaks of tetra- and penta-mannose *N*-glycans in the *Hpoch1*Δ*Hpalg3*Δ/MsdS strain. In all the transformants of

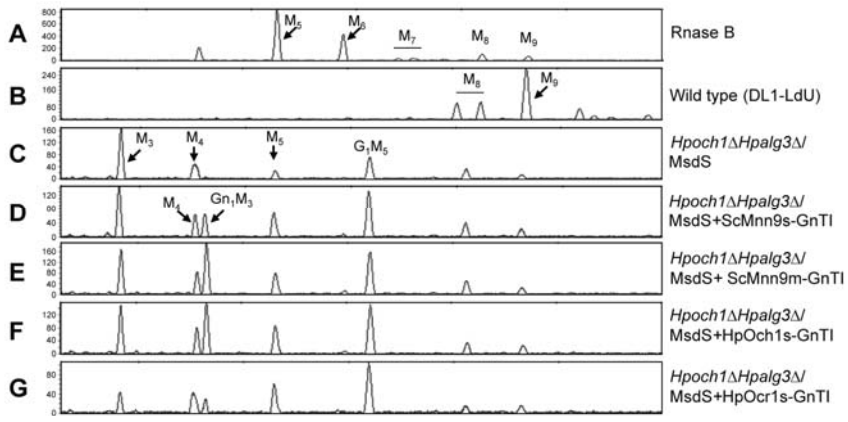

Fig. 2. Comparative analysis of cell wall *N*-glycans from the glycoengineered *Hpoch1*Δ*Hpalg3*Δ strains using capillary electrophoresis. *N*-glycans from bovine RNase B as a reference (A), the *H. polymorpha* wild-type strain (B), the *Hpoch1*Δ*Hpalg3*Δ/MsdS strain (C), the *Hpoch1*Δ*Hpalg3*Δ/MsdS+ScMnn9s-GnTI strain (D), the *Hpoch1*Δ*Hpalg3*Δ/MsdS+ScMnn9m-GnTI strain (E), the *Hpoch1*Δ*Hpalg3*Δ/MsdS+HpOch1s-GnTI strain (F), and the *Hpoch1*Δ*Hpalg3*Δ/MsdS+HpOcr1s-GnTI strain (G). $M_{3\text{-}9}$, Gn_1M_3, and G_1M_5 represent $Man_{3\text{-}9}GlcNAc_2$, $GlcNAc_1Man_3GlcNAc_2$, and $Glc_1Man_5GlcNAc_2$ *N*-glycans, respectively. Y-axis represents relative fluorescence units in capillary electrophoresis analysis. The differences in the scale of fluorescence intensity at the Y-axis reflect the different concentrations of cell wall glycans used for the analysis.

*Hpoch1*Δ*Hpalg3*Δ/MsdS strain harboring each of the GnTI fusion constructs, a newly generated peak was detected just next to the peak of tetramannose *N*-glycans. The new peak still remained after α-1,2-mannosidase treatment, but shifted to the M_3 peak by β-*N*-hexosaminidase treatment, indicating that it may correspond to the peak of $GlcNAc_1Man_3GlcNAc_2$ (Gn_1M_3), a trimannosyl *N*-glycan containing a single residue of GlcNAc (Fig. 3A). The results demonstrated that the GnTI fusion constructs were efficiently localized to the yeast Golgi and functionally expressed to use $Man_3GlcNAc_2$ as a substrate for the addition of a terminal GlcNAc. Interestingly, the N-terminal leader sequences derived from HpOch1s and ScMnn9m displayed an increased conversion efficiency of $Man_3GlcNAc_2$ to $GlcNAc_1Man_3GlcNAc_2$, compared to those from HpOcr1s and ScMnn9s.

Notably, we observed the increase of a peak localized between Man_6 and Man_7 glycans in all the transformants of *Hpoch1*Δ*Hpalg3*Δ/MsdS. This unusual peak is speculated as $Glc_1Man_5GlcNAc_2$ (G_1M_5), a $Man_5GlcNAc_2$ glycan capped with a glucose residue, based on the observation that it still remained after *in vitro* α-1,2-mannosidase and/or β-*N*-hexosaminidase digestion (Fig. 3A). It was previously reported that significant amount of a glucosylated byproduct, $Glc_1Man_5GlcNAc_2$ glycan, was accumulated in *S. cerevisiae* and *P. pastoris alg3* mutant strains, probably due to a reduced efficiency of ER glucosidase I and II on the truncated core oligosaccharides (Verostek *et al.*, 1993; Davidson *et al.*, 2004). Moreover, a recent study on a *Arabidopsis alg3* mutant also reported the accumulation of a rare α-glucosylated $Glc_1Man_5GlcNAc_2$ glycan (Kajiura *et al.*, 2010).

Generation of $GlcNAc_1Man_5GlcNAc_2$ *N*-glycans in the *Hpoch1Δ*/MsdS strain expressing GnTI

To investigate whether the generation of byproducts such as glucosylated glycans is also detected in the *HpOCH1* single mutation background, the yeast Golgi-targeted human GnTI constructs were introduced into the *Hpoch1*Δ/MsdS stain. Based on the high efficiency in the addition of GlcNAc in the *Hpoch1*Δ*Hpalg3*Δ strain, the ScMnn9m- and HpOch1s-GnTI fusion constructs were introduced into the *Hpoch1*Δ/MsdS. The *N*-glycan analysis of cell wall proteins from the transformants of *Hpoch1*Δ/MsdS harboring the ScMnn9m-GnTI construct (*Hpoch1*Δ/MsdS+ScMnn9m-GnTI) showed a newly synthesized peak localized just after the position of $Man_6GlcNAc_2$ (M_6) (Fig. 3B). The transformants of *Hpoch1*Δ/MsdS expressing HpOch1s-GnTI (*Hpoch1*Δ/MsdS+HpOch1s-GnTI) also showed the appearance of the same new peak (data not shown). In the detailed analysis with *in vitro* exoglycosidase digestions, the new peak was resistant to α-1,2-mannosidase treatment but shifted completely to the M_5 peak by β-*N*-hexosaminidase treatment, indicating that the peak corresponds to the pentamannose *N*-glycan containing a terminal *N*-acetylglucosamine residue, $GlcNAc_1Man_5GlcNAc_2$ (Gn_1M_5). In addition, in the *Hpoch1*Δ/MsdS+ScMnn9m-GnTI strain, all the peaks corresponding to Gn_1M_5 and *N*-glycans

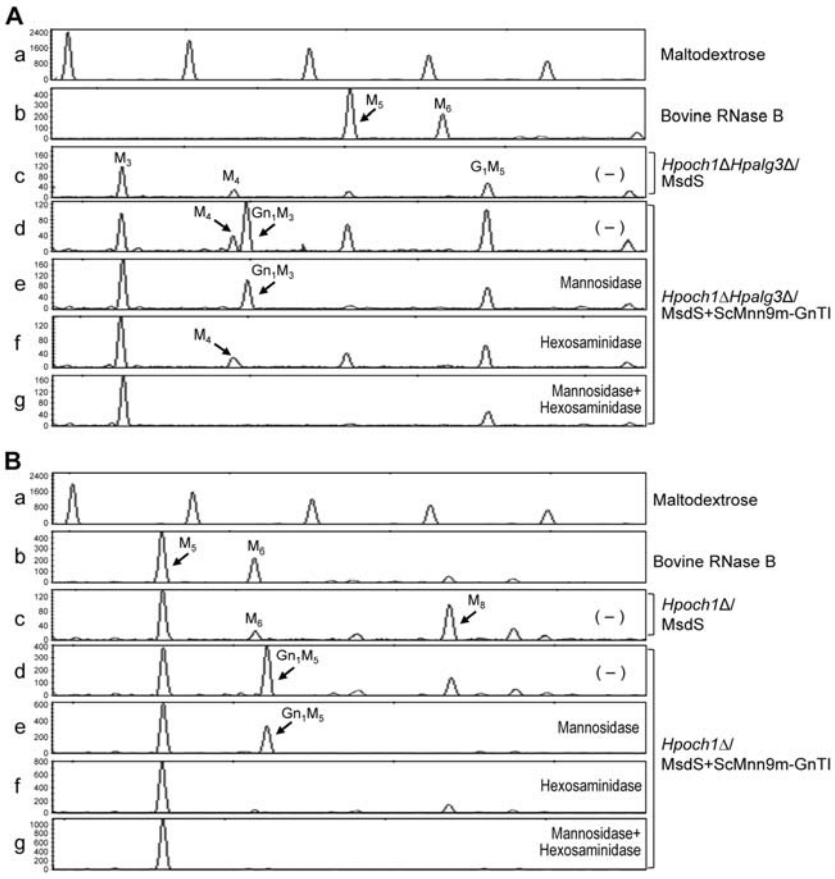

Fig. 3. Analysis of *N*-glycan profiles after exoglycosidase treatment in the *Hpoch1Δalg3*Δ/MsdS+GnTI (A) and the *Hpoch1*Δ/MsdS+GnTI strains (B), respectively. Row a, Reference of maltodextrose; Row b, Reference *N*-glycans from bovine RNase B; Row c, *N*-glycans from *Hpoch1*Δ*Hpalg3*Δ/MsdS (A) and *Hpoch1*Δ/MsdS (B) strains, respectively; Rows d-g, *N*-glycans from *Hpoch1*Δ*Hpalg3*Δ/MsdS+HpOch1s-GnTI (A) and *Hpoch1*Δ/MsdS+ScMnn9m (B) strains, respectively; Row e, Treatment with α-1,2-mannosidase; Row f, Treatment with α-1,2-mannosidase; Row g, Treatment with β-*N*-hexosaminidase after α-1,2-mannosidase treatment. $M_{3\text{-}8}$, Gn_1M_3, and G_1M_5 represent $Man_{3\text{-}8}GlcNAc_2$, $GlcNAc_1Man_3GlcNAc_2$, and $Glc_1Man_5GlcNAc_2$ *N*-glycans, respectively. Y-axis represents relative fluorescence units in capillary electrophoresis analysis. The differences in the scale of fluorescence intensity at the Y-axis reflect the different concentrations of cell wall glycans used for the analysis.

with higher molecular weight were converted completely to the $Man_5GlcNAc_2$ (M_5) form by sequential treatment of β-*N*-hexosaminidase and mannosidase (Fig. 3B), indicating no generation of the byproduct $Glc_1Man_5GlcNAc_2$ *N*-glycan in the *HpOCH1* single deletion background. Thus, considering the high conversion efficiency (~50%) of $Man_5GlcNAc_2$ to a human hybrid-type $GlcNAc_1Man_5GlcNAc_2$ and the absence of byproduct formation, the glycoengineered *Hpoch1*Δ/MsdS+ScMnn9m-GnTI strain would be a good starter strain for further optimization of glycoengineering to achieve the synthesis of the biantennary complex *N*-glycans in *H. polymorpha*.

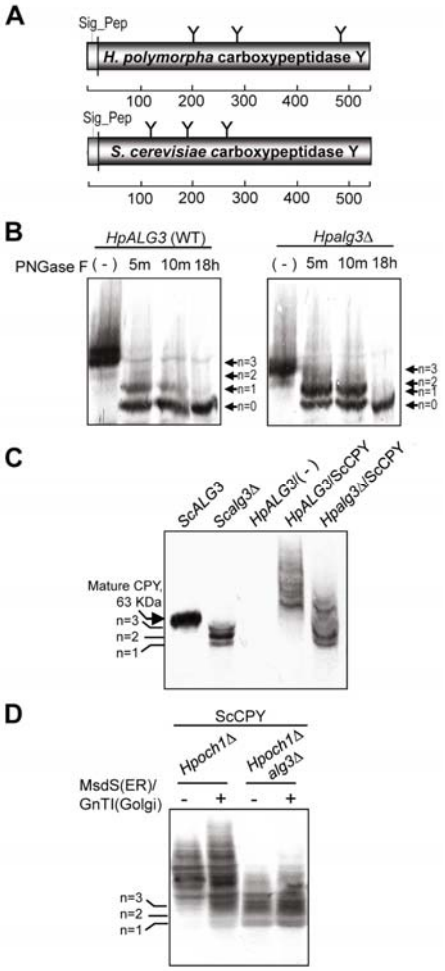

Fig. 4. Comparative analysis of glycosylation occupancy of *H. polymorpha* glycoengineered strains. (A) Structural organization of *H. polymorpha* and *S. cerevisiae* carboxypeptidase Y (HpCPY and ScCPY, respectively). 'Y' indicates *N*-glycans (B) Analysis of glycosylation occupancy of HpCPY proteins by partial deglycosylation with PNGase F. Intracellular proteins were extracted from yeast strains cultivated in YPD liquid medium at 37°C for 16 h and treated partially with PNGase F (1 U/μg of protein) for indicated times and analyzed by western blotting with anti-HpCPY antibody. 'n' indicates the number of *N*-glycosylation sites. (C) Western blot analysis of ScCPY proteins expressed in the wild-type and *alg3*Δ mutant strains of *S. cerevisiae*. Intracellular proteins were extracted from *S. cerevisiae* strains cultivated at 30°C for 12 h and analyzed by immunoblotting with anti-ScCPY antibody. (D) Western blot analysis of ScCPY proteins expressed in the glycoengineered *H. polymorpha* strains harboring P_{GAP}-ScCPY. Intracellular proteins were obtained from yeast cells cultivated in YPD at 37°C for 12 h and analyzed by immunoblotting with anti-ScCPY antibody.

In the present study, the production of *N*-glycans with a terminal GlcNAc in *H. polymorpha* glycoengineered strain was achieved without introduction of any heterologous UDP-GlcNAc transporter. This suggests that a substantial portion of UDP-GlcNAc can be transported into the Golgi of *H. polymorpha* using endogenous transporters for sugar-nucleotides. It is intriguing that *H. polymorpha* has a putative *MNN2-2* gene encoding a protein homologous to the *K. lactis* UDP-GlcNAc transporter (Park *et al.*, 2011). However, the deletion of *HpMNN2-2* did not affect the conversion efficiency to $GlcNAc_1Man_5GlcNAc_2$ (Park and Kang, unpublished data), implying that UDP-GlcNAc might be transported non-specifically via other Golgi sugar transporters in *H. polymorpha*. It was previously reported that the introduction of the *Kluyveromyces lactis* UDP-GlcNAc transporter into the Golgi apparatus improved the conversion efficiency to $GlcNAc_1Man_5GlcNAc_2$ in a glycoengineered *P. pastoris* (Choi *et al.*, 2003). For the implementation of the complete humanized glycosylation pathway in *H. polymorpha*, future work would be necessary to optimize the reaction by GnTI.

Accessing glycosylation occupancy efficiency in the *Hplag3*Δ and *Hpoch1*Δ*Hplag3*Δ mutant strains

Although the deletion of *HpALG3* did not affect normal growth or cell wall integrity (Oh *et al.*, 2008), we observed that the *Hpalg3* mutation caused hypersensitivity to tunicamycin, an inhibitor of lipid-linked oligosaccharide synthesis (data not shown). This implies the potential presence of uncharacterized defects of the *N*-glycosylation process caused by the *Hpalg3* mutation. Based on the previous report on the decreased efficiency of *N*-glycan occupancy in the *S. cerevisiae alg3* mutant strains (Aebi *et al.*, 1996), we examined the mobility pattern of an endogenous vacuolar carboxypeptidase Y protein carrying three putative *N*-glycosylation sites (Fig. 4A) in the *H. polymorpha* wild-type and *Hpalg3Δ* strains. Compared to the wild-type strain, the *Hpalg3*Δ strain showed the increased electrophoretic mobility of endogenous *H. polymorpha* carboxypeptidase Y (HpCPY), which is mainly due to the attachment of shorter oligosaccharides. When digested partially with PNGase F, the similar mobility patterns of CPY occupied in zero to three *N*-glycosylation sites were detected in both the wild-type and *Hpalg3*Δ strains (Fig. 4B), indicating that the *Hpalg3*Δ strain does not have a problem with the *N*-glycosylation occupancy. However, in the case of overexpression of the heterologous *S. cerevisiae* CPY (ScCPY) protein with three *N*-glycosylation sites under the control of a strong *GAP* promoter, the *Hpalg3* mutation apparently generated underglycosylated ScCPY proteins with reduced extent of occupancy at their *N*-glycosylation sites, as detected in the *Scalg3Δ* strain (Fig. 4C). Finally, we compared the glycosylation pattern of ScCPY expressed in the glycoengineered *Hpoch1*Δ/MsdS+ScMnn9m-GnTI and *Hpoch1*Δ*Hpalg3*Δ/MsdS+ScMnn9m-GnTI strains constructed in this study (Fig. 4D). The glycoengineered *Hpoch1*Δ*Hpalg3*Δ/MsdS+ScMnn9m-GnTI strain clearly demonstrated the decreased efficiency of glycosylation occupancy with a similar extent

as shown in the *Hpalg3*Δ strain. However, compared to the *Hpoch1*Δ strain, the glycoengineered *Hpoch1*Δ/MsdS+ ScMnn9m-GnTI strain also generated underglycosylated ScCPY proteins with reduced glycosylation occupancy. It is possible that the trimming of glycans from $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ in the ER by the introduced α-1,2-mannosidase might decrease the transfer efficiency of oligosaccharide moieties to proteins, since uncompleted lipid-linked oligosaccharides in the ER are reported to generate insufficient *N*-glycosylation occupancy (Freeze, 2006). The results imply that the decreased efficiency of glycosylation occupancy in the glycoengineered host strains, designed for the generation of truncated *N*-glycans in the ER, may be problematic for their exploitation as hosts for the high level production of recombinant glycoproteins.

In conclusion, the glycoengineered *H. polymorpha Hpoch1*Δ and *Hpoch1*Δ*Hpalg3*Δ strains expressing the yeast ER-targeted *A. saitoi MsdS* and the Golgi-targeted human GnTI were shown to successfully produce human hybrid-type *N*-glycans with a terminal *N*-acetylglucosamine, $GlcNAc_1Man_5GlcNAc_2$, and $GlcNAc_1Man_3GlcNAc_2$, respectively. However, with respect to the byproduct $Hex_6GlcNAc_2$ formation and the glycosylation site occupancy, we propose the use of a *Hpoch1*Δ deletion strain as a more suitable strategy for the subsequent glycoengineering toward the production of human complex-type *N*-glycans compared to the exploitation of the *Hpoch1*Δ*Hpalg3*Δ double deletion strain.

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