Production of recombinant human acid α-glucosidase with mannosidic N-glycans in α-mannosidase I mutant rice cell suspension culture

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Abstract

High-mannose glycans, containing 6-9 mannose residues, are favorable for *in vitro* mannose phosphorylation to produce Mannose-6-phosphate (M6P) residues. These are important for lysosomal enzyme targeting and essential for uptake. Golgi α-mannosidase-I mediates mannose trimming in the N-glycosylation pathway. In this study, I used mutant rice with a T-DNA insertion in the Os04g0606400 (Δ*α-man*I) gene to produce recombinant human acid α-glucosidase (rhGAA) for enzyme replacement therapy to treat Pompe disease. After characterization of Δ*α-man*I mutant rice, the gene encoding rhGAA was introduced into mutant calli by *Agrobacterium*-mediated transformation. Integration of the target sequence into the rice genome was detected by genomic DNA PCR. Expression of rhGAA from Δ*α-man*I mutant (Δ*α-man*I-GAA) and its secretion into culture media were confirmed by western blot analysis. The N-glycosylation pattern of purified Δ*α-man*I-GAA was analysed by MALDI-TOF mass spectrometry. This showed that the high-mannose type N-glycan with man8 (63.8%) was the most abundant form, followed by Man7 (22.5%), GnGnXF3 (6.7%), Man6 (5.4%), and Man5 (1.6%). The results suggest the successful production of rhGAA with N-glycan containing 6-8 mannose residue in the Δ*α-man*I mutant rice cell suspension culture.

Keywords: Pompe disease, acid α-glucosidase (GAA), rice *α*-mannosidase I, cell suspension culture, *N*-glycosylation

Introduction

Pompe disease is an inherited genetic metabolism disorder and one of several lysosomal storage diseases. Deficiency of recombinant human acid α-glucosidase (rhGAA) causes accumulation of glycogen in muscle cells and varying levels of organ damage and rates of progression to death. Enzyme replacement therapy (ERT) is the current treatment and the enzyme carrying mannose-6-phosphate (M6P) on its N-glycan is taken up into target lysosome by binding to the mannose-6-phosphate receptor [1-3].

Early attempts to use rhGAA purified from Aspergillus niger[4] or human placenta[5] for ERT were unsuccessful, and it might be because of low enzyme dosage, disease stage, and the lack of M6P residues for uptake into muscle cells[1]. Use of rhGAA from the milk of transgenic rabbits showed improved respiratory function and restoration of some muscle function in infants [2, 6]. rhGAA from Chinese hamster ovary (CHO) cells led to the development of a drug called alglucosidase alfa (Myozyme©), and approved by the US Food and Drug Administration (FDA) for the treatment of infants and children with Pompe disease[1]. Uptake efficiency of rhGAA into target muscle cell is highly dependent on the number of M6P moieties and CHO cell-derived rhGAA has 1-2 N-glycans with M6P out of 7 potential N-glycosylation site. More recently, chemical conjugation of M6P onto the periodate-treated rhGAA (neoGAA) was shown to improve its uptake [7-10]. Neo-GAA showed highly improved clearance of lysosomal glycogen in the muscle of GAA knockout mice compared with the unmodified enzyme. Nevertheless, high cost of the ERT enzymes derived from CHO cells approved for treating lysosomal storage disease[11, 12] creates a cost burden for patients (approximately 500,000 USD per year for the life of the adult patient)[13].

Plant cell culture systems have many advantages over animal or bacterial cell culture, such as cost effectiveness, safety and eukaryotic protein processing [14-16]. The simplicity of the culture media, composed of simple carbon sources and hormones, can highly reduce production cost. Although differences in the glycosylation and cellular machinery have been reported [17-20], plant cells have eukaryotic protein processing and post-translational modifications which are essential for glycoprotein production and proper protein folding. Moreover, inadvertent contamination with

animal-derived pathogens can be avoided [21]. In addition, use of RAmy3D promoter and its signal peptide leads to the secretion of the target protein into rice cell culture media which aids downstream purification[22]

Natural mannose phosphorylation in plants has not been reported but recombinant α/β subunits of mammalian UDP-N-acetylglucosamine:glycoprotein N-acetylglucosamine-1-phosphotransferase (GNPTAB) and N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase (NAGPA) from rat liver, simple eukaryotes and humans can mediate this reaction *in vitro* [23-26]. N-glycans that have more than six mannose residues were found to be suitable substrates for this enzyme. In previous attempts to produce rhGAA from rice cell suspension culture, rhGAA was highly expressed in both wild-type and N-acetylglucosaminyltransferase-I (gntI) mutant rice cells and its α-glucosidase activity was highly similar to that of rhGAA from CHO cells [27, 28]. However, its N-glycan pattern, plant-specific or Man5 type, that are the most abundant in each line, were an unfavorable substrate for enzymatic *in vitro* phosphorylation [26].

The α-mannosidase I in ER and/or cis-Golgi apparatus conducts mannose trimming in the early stage of the N-glycosylation pathway [29]. For more detail, sequential removal of glucose residues (Glc) from initial form of N-glycan, $Glc_3Man_9GlcNAc_2Asn$ by α-glucosidases I and II yield $Man_9GlcNAc_2$. The α-mannosidase I in ER remove the terminal α1-2Man from the central arm of $Man_9GlcNAc_2$ and α-mannosidase I in cis-Golgi apparatus two Man residues of the Glcα1-3Manα1-2Manα1-2 moiety from some of N-glycans in the cis-Golgi retain a glucose residue because of incomplete processing in the ER, thereby both generating a Man8GlcNAc2 isomer by using different mechanism. In Arabidopsis, inhibition of ER-type α-mannosidase I or two Golgi-α-mannosidase I blocked N-glycan maturation and resulted in Man9 and Man8 rather than Man5, respectively [30]. Through phylogenetic analysis, Os04g0606400 in rice was expected to encode a putative Golgi-α-mannosidase I. In a more recent study, use of two different inhibitors of α-mannosidase I in the ER and/or cis-Golgi apparatus (kifunensine), and α-mannosidase II in the ER and/or medial-Golgi (Swainsonine) during the production of rhGAA in wild type rice successfully achieved production of rhGAA with Man7-9 type of N-glycan [31]. These studies show that genetic mutation of a-

mannosidase I or pharmacological inhibition results in the production of rhGAA with Man7-9 type N-glycan.

In this study, I screened T-DNA insertional mutant rice expected to be defective in putative Golgi-$α$-mannosidase I genes ($\Delta α$-*man*I). This $\Delta α$-*man*I mutant rice showed highly decreased mannose trimming activity and was used for the production of rhGAA with mannosidic   N-glycans.

1 **Materials and Methods**

2 **Vector construction and Agrobacteria transformation**

3 The rice expression vector pMYD85, carrying rhGAA gene, in which protein expression is

4 induced by the rice α-amylase 3D promoter under the sugar starvation conditions, was constructed

5 in previous study [27, 28]. For the purification, the signal sequence of rice α-amylase 3D (3Dsp),

6 with 8 histidine or FLAG tags and enterokinase cleavage sequence were fused to N-terminus of

7 pre-mature hGAA (57-952 a.a). In detail, 3Dsp was amplified by primer pair 3Dsp F1(XbaI): 5'-

8 TTT CTA GAA TCA GTA GTG GTT AGC -3' and GAA(8His-EK) R1: 5'-CTT GTC ATC ATC

9 GTC GTG GTG ATG GTG ATG GTG ATG GTG GTG AGC TGG GTG AGT CTC CTC CA -3'

10 for the addition of a his tag, primer pair of 3Dsp F1(XbaI) and GAA(FLAG) R1: 5'-CTT GTC

11 ATC ATC GTC CTT GTA GTC GTG AGC TGG GTG AGT CTC CTC CA-3' for FLAG tagging.

12 The partial hGAA sequence was amplified by primer pair EK-GAA F1: 5'- GAC GAT GAT GAC

13 AAG CAG CAG GGA GCC AGC AGA CCA GGG CCC CG -3' and hGAA partial (StuI) R1: 5'-

14 TTA GGC CTG TGA TAT ACT GCG AGG GCA GCG AGG -3'. These DNA fragments were

15 fused by overlapping PCR using primer pair 3Dsp F1 (XbaI) and hGAA partial (StuI) R1 and then

16 N-terminus of pMYD85 was replaced using XbaI+StuI restriction enzyme site to form pMYD532

17 and pMYD534. These vectors were transformed into Agrobacterium EHA-105 for rice

18 transformation using the helper plasmid pRK2013 by tri-parental mating method [32].

19

20 **Screening of *α*-mannosidase I mutant candidates**

21 T-DNA insertional mutant rice seeds of α-mannosidase I candidates for rhGAA expression were

22 obtained from RiceGE (http://signal.salk.edu/cgi-bin/RiceGE). Calli were produced from 16

23 individual T-DNA mutant lines consisting of nine lines for chromosome 1, single lines for

24 chromosome 2, two lines for chromosome 3, three lines for chromosome 4 and a single line for

25 chromosome 5. Cell line showing reduced intensity or no bands in western blot using rabbit anti-

26 horseradish peroxidase antibody (P7899, Sigma-Aldrich) which can recognize core α1,3-fucose

27 and β1,2-xylose were propagated and used for further analysis (supplementary figure 1) [33-36].

28   Genotyping PCR to confirm T-DNA insertion into the gene of Os04g0606400 by using primer

29   pairs LP (5'- TTGCAATGCCTACTGAGTGG-3') and LBP (5'- GTC GAG AAT TCA GTA CAT

30   T-3'), or RP (5'- CTCTCGGGAGTTCTCACTGG-3') and RBP (5'-TTG GGG TTT CTA CAG

31   GAC GTA AC-3') were conducted under the following PCR conditions: 1 cycle at 94 °C for 5

32   min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, followed by 1 cycle at 72 °C

33   for 5 min. Homozygous T-DNA insertion was negatively identified by PCR using primer pair LP

34   and RP under same condition (Figure 1A-B). Cell line shows no band which means homozygous

35   T-DNA insertion was selected and used for further analysis.

36   Again, protein extracts from wild type, *gnt*I mutant (SAI2G12, http://signal.salk.edu/cgi-

37   bin/RiceGE) [27] and homozygous T-DNA insertional mutant rice above were loaded to 10% (w/v)

38   SDS-polyacrylamide gel and separated by electrophoresis. The protein bands were visualized by

39   Coomassie brilliant blue. For western blot, proteins were transferred to Nitrocellulose membrane

40   (Hybond-C, GE healthcare) in transfer buffer (50 mM Tris, 40 mM glycine, and 20% methanol)

41   using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 25 V for 30 min. Membranes were

42   blocked with 10% (w/v) skim milk in Tris-Buffered Saline with Tween-20 on a rotary shaker

43   overnight. α1,3-fucose and β1,2-xylose were detected by 1:5000 dilution of primary antibodies of

44   α1,3-fucose (Agrisera), β1,2-xylose (Agrisera, Sweden), followed by 1/7000 dilution of anti-rabbit

45   IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary antibody. And Lewis a epitope

46   was detected by 1:200 dilution of JIM84 antibody[37], followed by 1/7000 dilution of anti-rat IgG

47   alkaline phosphatase conjugate (Sigma-Aldrich). Color was developed using BCIP/NBT

48   (Millipore). Reaction was stopped within 1 min by washing the membrane with distilled water.

49

50   **Callus induction and rice transformation**

51   Rice seeds were dehusked and washed several times with 70% (v/v) ethanol, 2% (v/v) NaOCl

52   and autoclaved distilled water, respectively. The sterilized seeds were germinated for one week on

53   callus induction agar plate (N6 plates) containing 4 g/L of CHU salts, 30 g/L of sucrose, 2 mg/L of

54   2,4-D, 0.2 mg/L of kinetin, and 4 g/L phytagel with 50mg/L hygromycin B as selection marker.

55   Generated calli were isolated, propagated and used for western blot or genotyping PCR for

56   screening of Δα-*man*I mutants. Putative Δα-*man*I mutant rice calli were further propagated and

57   used for *A.tumefaciens*-mediated transformation with pMYD85, pMYD532 and pMYD534,

58   respectively. Infected calli were cultured on N6 plates without antibiotics for 3 days in dark

59   condition and washed with distilled water containing 500 mg/L of Cefotaxime and transferred to

60   selection media (N6 plates supplemented with DL-phosphinothricin (PPT; 4.4 ppm)) After 3-4

61   weeks, transgenic calli that survived were moved onto new selection media and propagated.

62

63   **Genomic DNA extraction and PCR**

64   To confirm hGAA gene integration into the genome of Δα-*man*I mutant rice, genomic DNA

65   PCR was conducted after extraction of genomic DNA using a Zymobead genomic DNA kit (Zymo

66   Research). Primer pair amplifying hGAA and His-hGAA (GAA gD F1: 5'- GTT CCC CGA GAG

67   CTG AGT GG-3' and GAA gD R1: 5'-GGT AGA AAG GGT GAG ACC CG -3') was used for

68   PCR reaction. Thermal cycling was performed for 30 cycles, consisting of 1 min at 94°C, 1 min at

69   55°C, and 3 min at 72°C. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel,

70   visualized by staining with ethidium bromide, and observed under UV light.

71

72   **Rice cell suspension culture and induction of protein expression**

73   Δα-*man*I rice mutant and Δα-*man*I transformed with individual pMYD85 (D85), pMYD532

74   (D532) and pMYD534 (D534) were cultured in 50 mL N6 medium containing 2 mg/L 2,4-D, 0.02

75   mg/L kinetin, 3% (w/v) sucrose, and 50 mg/L hygromycin B in 300 mL flask at 28 °C in the dark

76   using a rotary shaker with a rotation speed of 100 rpm. 10 mL of Δα-*man*I rice mutant cells,

77   transferred every 3-4 days and spent media were collected and kept in -70 °C for further analysis.

78   To induce target gene expression under control of the Ramy3D promoter, 5 g of fresh cells

79   collected by aspiration, washed with distilled water and inoculated into 50 mL fresh N6 (CHU)

80   medium without sucrose. At 9 days after sugar starvation, spent media was harvested and store in -

81 70 °C for further analysis. The rhGAA expression levels of each cell line were measured by

82 comparing band intensity with that of positive control on SDS-PAGE gel using Image J software.

83

84 **Purification of rhGAA**

85 rhGAA secreted from D532 was purified using 1 mL Ni-NTA agarose resin (Qiagen) according

86 to the manufacturer's instructions and as described previously [27] with slight modification.

87 Briefly, 0.45 µm filtered 50 mL of culture media produced under sugar starvation conditions was

88 loaded. The unbound protein was washed with 10 mL of 1X Phosphate-buffered Saline (PBS), pH

89 7.0, and the bound protein was eluted with 1 mL of PBS (pH 4.3) [27] and neutralized using 25 µL

90 of 1 M Tris-HCl, pH 8.0.

91 rhGAA secreted from D534 was also purified using 1 mL of M2 anti-FLAG affinity column

92 (GE healthcare) according to the manufacturer's instruction. 30 mL of culture media under sugar

93 starvation was loaded to prepared resin and flow-through was collected every 10 mL. The resin

94 was washed with 5 column volumes of TBS (50 mM Tris HCl, with 150 mM NaCl, pH 7.4) to

95 remove unbound protein, and followed by elution using 1 mL aliquots of 0.1 M glycine-HCl, pH

96 3.5, into vials containing 25 µL of 1 M Tris-HCl, pH 8.0.

97

98 **SDS-PAGE and western blot**

99 To detect rhGAA in the samples, SDS-PAGE and western blotting were used. 30 µL of each

100 samples were loaded to 10% (w/v) SDS-polyacrylamide gel and separated by electrophoresis. The

101 protein bands were visualized by Coomassie brilliant blue. For western blot, proteins were

102 transferred to Nitrocellulose membrane (Hybond-C, GE healthcare) in transfer buffer using a

103 Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 25 V for 30 min. Membranes were blocked

104 with 10% (w/v) skim milk in Tris-Buffered Saline with Tween-20 on a rotary shaker overnight.

105 Target protein was detected by 1/2000 dilution of rat anti-GAA polyserum [27], followed by

106 1/7000 dilution of anti-rat IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary

107 antibody. Colour was developed using BCIP/NBT (Millipore). Reaction was stopped within 5 min

108 by washing the membrane with distilled water.

109

110 **MALDI-TOF**

111 The collected media from $\Delta\alpha$-*man*I was centrifuged at 15,000g for 30 min at 4 $^{\circ}$C. The

112 supernatant was lyophilized to reduce the volume and dialyzed in the distilled water. After

113 filtration by 0.45 µm filter, it was applied to a Con A-Sepharose 4 B column (GE Healthcare), and

114 glycoproteins were eluted with 0.5 M α-D-methylglucoside. This sample and purified $\Delta\alpha$-*man*I-

115 GAA (D532) prepared above were lyophilized, each sample (2 mg) was digested with 50 µg

116 trypsin (T0303; Sigma-Aldrich) and chymotrypsin (C4129; Sigma-Aldrich) at 37 °C for 16 h and

117 boiled for 10 min for inactivation of enzyme. N-glycans were released by incubation with 1.0 mU

118 PNGase A (Roche Diagnostics) in 60 µL 0.5 M citrate/phosphate buffer at pH 4.0 for 16 h and

119 purified using a Carbograph Ultra Clean column (Alltech) as described previously [38].

120 Pyridylamination of N-glycans was performed using a pyridylamination manual kit (Takara Bio

121 Inc.) according to the manufacturer's instructions. For further purification of the 2-aminopyridine

122 (PA)-N-glycans, NP-high performance liquid chromatography (HPLC) was performed using an

123 TSK gel Amide-80 column (4.6× 250 mm; Tosoh Co.) and a Waters 2690 Alliance HPLC

124 separation module (Waters Co.) equipped with the 474 Fluorescence Detector (Waters Co.), as

125 described previously [39]. Fluorometric detection was carried out at excitation and emission

126 wavelengths of 310 and 380 nm, respectively. The molecular masses of PA-N-glycans were

127 determined using the 4800 Plus matrix-assisted laser desorption ionization time-of-flight/time-of-

128 flight (MALDI-TOF/TOF) analyzer (Applied Biosystems). The PA-glycans purified from the

129 pooled NP-HPLC fractions (75–130 min) were subjected to MALDI-TOF/MS analysis as

130 described previously [40]. The mass spectra were collected in the reflector positive ion mode using

131 2,5-dihydroxybenzoic acid (10 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid and 10 mM

132 NaCl) as a matrix. Purified PA-N-glycans were mixed with an equal volume of matrix solution,

133 and 2 µL of the mixture were placed on the target plate and air-dried [41]. An m/z range of 1000–

134   2400 was measured and analysed using DATA Explore software (Applied Biosystems). Peaks

135   were assigned according to the calculated molecular masses [M+Na+] of PA-N-glycans, and

136   mannose, galactose, fucose, xylose, and N-acetylglucosamine residues were identified. N-glycan

137   structures were assigned by comparison with the plant N-glycans identified to date using

138   SimGlycan version 2.8 (PREMIER Biosoft Int.). Relative abundances were calculated based on

139   the areas of the corresponding peaks in the mass spectra, as described previously [42].

140

141 **Results**

142 **Identification of Δα-manI mutant**

143   To select the appropriate Δα-manI mutant rice line for expression of rhGAA with high mannose

144 N-glycans, α-mannosidase I in rice known to be localized in the ER or Golgi and containing the

145 glycosyl hydrolase family 47 domain which catalyse mannose trimming reaction were searched

146 from protein database (https://www.uniprot.org/) and rice seeds have T-DNA insertion on those

147 position were purchased through RiceGE: Rice Functional Genomic Express Database

148 (http://signal.salk.edu/cgi-bin/RiceGE) from Kyung Hee University, Republic of Korea and

149 Huazhong Agricultural University, China (supplementary table 1.)[30]. Calli were induced from

150 the mutant rice seeds have T-DNA insertions on five different putative α-mannosidase I gene in

151 rice genome, respectively. Protein extracts from these putative rice calli lines were analysed by

152 western blot using anti-HRP antibody for screening of mutant lacking α1,3-fucose and β1,2-xylose

153 which reflect the blockage of N-glycan maturation. Rice mutant possessing T-DNA insertions in

154 Os04g0606400 (LOC_Os04g51690, PFG-3A-12868) showed highly reduced bands correspond to

155 α1,3-fucose and β1,2-xylose was used for further analysis (supplementary figure 1.). Homozygous

156 and heterozygous T-DNA insertional pattern were observed from two individual Δα-manI mutants

157 by genotyping PCR using LP, RP, LBP and RBP (Fig.1A and 1B). #1 and #2 those are individual

158 rice mutant of Os04g0606400 showed band from PCR using primer pair LP and LBP, or RP and

159 RBP which means T-DNA insertion on expected position of rice genome. Homozygous T-DNA

160 insertion was confirmed by PCR using primer pair LP and RP targeting rice genome flaking T-

161 DNA insertion site. Homozygote (#1) shows no band because of its large length of T-DNA (~8 kb)

162 while wild type and heterozygote (#2) show amplicon. Amplified DNA fragments from #1 were

163 extracted from Agarose gel and analysed by DNA sequencing (data not shown), revealing T-DNA

164 insertion into the intron between the 11th and 12th exon as expected. The growth rate of this

165 homozygous Δα-manI mutant rice callus was similar to that of the wild type without any severe

166 defects even under sugar starvation. For further analysis, western blot using anti-α1,3-fucose, anti-

167 β1,2-xylose and JIM84 antibody showed highly reduced band intensity, respectively (Fig. 1C).

168   This demonstrates that the Δ*α-man*I mutation inhibits N-glycan maturation such as attachment of

169   plant N-glycan species, α1,3-fucose, β1,2-xylose, β1,3-galactose and α1,4-fucose in rice.

170

Expression and purification of Δ*α-man*I-GAA

172   Calli were transformed with Agrobacteria containing the vectors pMYD85 (rhGAA without tag),

173   pMYD532 (8His+EK+rhGAA) or pMYD534 (FLAG-EK-rhGAA) were screened by genomic

174   DNA PCR. Eight or more cell lines from each construct were chosen for propagation and induced

175   to express rhGAA under sugar starvation conditions. D85#7, D532#3 and D534#16 transformed

176   with individual pMYD85, pMYD532 and pMYD534 showed the highest expression of premature

177   form Δ*α-man*I-GAA (~110 kDa) at the 9 days after sugar starvation on SDS-PAGE and Western

178   blot, respectively (Fig. 2). The rhGAA expression levels of each pMYD85#7, pMYD532#3 and

179   pMYD534#16 cell line were approximately 10 mg/L, 21mg/L and 12 mg/L by comparing band

180   intensity with positive control on SDS-PAGE.

181   In the case of D532 and D534, expressed rhGAA was purified by affinity chromatography using

182   the eight histidine residues or FLAG tag on N-terminus (Fig. 3). Histidine tagged Δ*α-man*I-GAA

183   could be purified successfully with protocol used for purification of rhGAA with histidine tag on

184   C-terminus in previous studies [27, 28]. N-terminal FLAG tagged Δ*α-man*I-GAA also could be

185   highly purified using anti-FLAG resin but showed lower binding capacity. Impurities on SDS-

186   PAGE and Western blot might be matured form of rhGAA by acidic condition during purification

187   or host cell protein unspecifically bound. Hence, Histidine tagged Δ*α-man*I-GAA was used for

188   further analysis.

189

N-glycan profiling

191   N-glycan patterns of glycoproteins in culture media of Δ*α-man*I mutant and purified Δ*α-man*I-

192   GAA were analysed and compared to that of wild-type rice (Fig.4 and table.1). N-glycans from

193   Δ*α-man*I mutant and Δ*α-man*I-GAA were almost all high mannose type of N-glycans while wild

194   type cell lines shows typical pattern of plant N-glycan contents. Interestingly, the most abundant

195   N-glycan from Δα-*man*I was Man7 (48.9%), followed by Man8 (33.3%), Man6 (7.0%) while that

196   of Δα-*man*I-GAA was Man8 (63.8%), Man7 (22.5%) and Man6 (5.4%). This difference may be

197   because various host glycoproteins were present in the culture media of the Δα-*man*I mutant while

198   Δα-*man*I-GAA was specifically purified. Most importantly, N-glycan patterns in both Δα-*man*I

199   and Δα-*man*I-GAA reflect that gene disruption of Os04g0606400 highly inhibited N-glycan

200   maturation in rice.

201

202   **Discussion**

203   In this study, I suggest a major role for Os04g0606400 in the early stage of plant N-glycan

204   maturation and the successful expression of Δα-*man*I-GAA. The main N-glycans on this Δα-*man*I-

205   GAA was Man8 which is known as suitable substrate for *in vitro* phosphorylation. Because the

206   production of Δα-*man*I-GAA in our study does not requires any inhibitor treatment, it can greatly

207   reduce production costs and the cost burden for Pompe patients [31]. Although I used rhGAA in

208   this study, other lysosomal enzyme that require M6P such as α-L-iduronidase[24], α-galactosidase

209   A [43] can be produced using same strategy.

210   In screening of Δα-*man*I mutant candidates, only the cell lines that have T-DNA insertions in

211   Os04g0606400 showed highly decreased amounts of plant-specific N-glycans on western blot.

212   However, it does not suggest that Os04g0606400 encodes the only enzyme that conducts N-glycan

213   mannose trimming out of six candidates. Although other cell extracts showed bands corresponding

214   plant specific N-glycans, genotyping PCR of all individual candidates was not conducted in this

215   study. Furthermore, the seeds that I obtained from the library could be a mix of wild type, hetero-

216   and homozygous mutant lines. In this study, I focused on screening for cell lines suitable for the

217   production of rhGAA carrying more than six mannose residues on its N-glycan. Hence, I analysed

218   only the Os04g0606400 mutant in detail and used it as the rhGAA expression host.

219   Because gntI mutant rice was unable to grow or regenerate in previous studies [27, 44], and Δα-

220   *man*I is located upstream of gntI in N-glycosylation pathway, I hypothesized possible lethality of

221   the Δα-*man*I knock-out mutant. Despite more than three attempts at plant regeneration from calli,

222 no regenerated plant of the Os04g0606400 mutant were obtained while wild type callus were

223 regenerated successfully (data not shown). I assume this mutation may causes a problem with plant

224 regeneration and cell differentiation [44].

225 Both Δα-*man*I-GAA tagged with Histidine and FLAG on N-terminus were successfully

226 expressed and purified from transgenic Δα-*man*I mutant rice. Although FLAG tag showed low

227 binding to the affinity column, this can be improved by increasing volume of column. Furthermore,

228 these tags would be be cleaved by enterokinase treatment while or after purification. By adding an

229 enterokinase cleavage site between N-terminus tag and target protein, the heterogeneity of N-

230 terminus, which can be made different signal peptide cleavage in plants and animals, would

231 probably   be improved.

232 Enzymatic activity was not measured in this study but rhGAA with 7-9 mannose residues

233 produced from rice cell suspension culture under presence of mannosidase inhibitor showed

234 similar *in vitro* enzymatic activity to that of CHO cell derived rhGAA in previous study [31].

235 Although this strategy blocks α-mannosidase I function at the protein level while Δα-*man*I acts at

236 the DNA level, both approaches target α-mannosidase I so it seems likely that Δα-*man*I-GAA

237 would probably have similar enzyme activity.

238 In N-glycan profiling, suitable N-glycan substrates for enzymatic *in vitro* phosphorylation,

239 Man8, Man7, andMan6 were detected from rhGAA produced from Δα-*man*I mutant rice.

240 Interestingly, although N-glycan maturation is highly inhibited in this cell line, mannose trimming

241 is not completely terminated with Man8 and processed further. Moreover, plant specific N-glycan

242 species, GnGnXF3 (6.7% from Δα-*man*I-GAA, 2.9% from Δα-*man*I), GnMXF3 (1.9% from Δα-

243 *man*I) and MMXF3 (4.0% from Δα-*man*I) were still detected. The reason of the presence of these

244 unexpected N-glycans may be a result of insufficient knock-out by T-DNA insertion on intron or

245 minor activity of α-mannosidase I isotype. This could be further characterized by using knock-out

246 using other technique such as CRISPR/Cas9 in further studies. Nevertheless, it is clear that

247 Os04g0606400 plays a major role in mannose trimming in N-glycosylation pathway in rice, and

248 rhGAA expressed from this mutant mainly carried Man6-8 that are known as the most favorable

249   substrates of *in vitro* phosphorylation using two enzyme, GNPTAB and NAGPA [45]. Although *in*

250   *vitro* phosphorylation of Man6-8 on Δ*α-man*I-GAA was not tested in this study, studies involving

251   *in vitro* phosphorylation of Man6-9 on the other recombinant lysosomal enzymes for ERT and the

252   presence of Man6-8 on Δ*α-man*I-GAA suggest that Δ*α-man*I-GAA would also probably be

253   phosphorylated via similar approaches [23, 24].

254   Therefore, this study provides knowledge about the early stage of N-glycosylation in plants and

255   the production of rhGAA in Δ*α-man*I mutant rice. I expect that Δ*α-man*I mutant rice can be used

256   for attractive expression host for production of lysosomal enzymes for ERT with cost effectiveness

257   and safety.

258

266   **Reference**

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451

452 **Figure Legends**

453 **Figure 1.** Characterization of Δ*α-man*I mutant rice. (A) Scheme of genotyping PCR. Depends on

454 genome sequence of rice (Acession number. AP014960.1) and information from mutant rice

455 library, genotyping PCR primers were designed. Numbers in the figure mean position on rice

456 chromosome 4 (e.g 30621614-30621633 for Left primer) (B) Agarose gel images of genotyping

457 PCR. Genomic DNA was extracted from putative Δ*α-man*I T-DNA insertional mutants and used

458 for PCR. Lane M, 1kbp plus 100 DNA ladder (Elpis); Lane WT, wild type rice; Lane #1, putative

459 homozygote; Lane #2, putative Heterozygote. (C) SDS-PAGE and Western blot analysis using

460 antibodies against to plant specific N-glycans. Protein was extracted from calli and separated by

461 SDS-PAGE(left upper), and then transblotted to nitrocellulose membrane to predict if plant

462 specific N-glycans such as α1,3-Fucose(right upper), β1,2-xylose (left lower) and Lewis a epitopes

463 (right lower). Lane M, Pre-stained protein ladder; Lane gntI, gntI mutant rice callus extracts; Lane

464 WT, wild-type rice callus extracts; Lane 1, Callus extracts from #1 line in fig.1B.

465

466 **Figure 2.** Expression of rhGAA in Δ*α-man*I mutant rice. (A) Rice expression vectors construction.

467 Rice expression vectors for the human acid α-glucosidase (hGAA) gene (pMYD85), hGAA with

468 six histidine residues (pMYD532), or hGAA with FLAG-tag on N-terminus with the signal peptide

469 of the rice α-amylase 3D gene and its own pro-peptide, located between the rice α-amylase 3D

470 promoter (Ramy3D-p) and the 3′ untranslated region (3′ UTR). LB, T-DNA right border; 35S-p,

471 CaMV 35S promoter; Bar, bialaphos resistance gene; 35S polyA, terminator of 35S gene; RB, T-

472 DNA right border; EK, Enterokinase cleavage sequence between purification tags and hGAA. (B)

473 Agarose gel images of genomic DNA PCR. Lane M, 1kbp plus 100 DNA ladder (Elpis); PC,

474 Plasmid DNA; NC, genomic DNA PCR product of wild type rice; Lane numbered, genomic DNA

475 PCR products of putative transgenic calli. (C) Rice cell suspension culture media 9 days after

476 induction (dai). SDS-PAGE (upper) and Western blot using rat anti-GAA antiserum (lower). Lane

477 M, Pre-stained protein ladder; Lane PC, CHO cell derived rhGAA 500ng; Lane NC, wildtype rice

478   culture media under sugar starvation; Lane numbered, putative transgenic rice culture media under

479   sugar starvation.

480

481   **Figure 3.** Purification of recombinant human acid α-glucosidase (rhGAA) from rice cell

482   suspension culture. SDS-PAGE and Western blot using anti-GAA antibody of purified rhGAA by

483   affinity chromatography using N-terminus histidine tag (A) or FLAG tag (B). Lane M, Pre-stained

484   protein ladder; Lane PC, CHO cell derived rhGAA 500ng; Lane NC, Δ*α-man*I mutant rice cultured

485   media under sugar starvation; Lane C, crude cultured media under sugar starvation; FT, Flow

486   through from the affinity column; Lane W, Wash from the affinity column; Lane E, eluted

487   fractions.

488

489   **Figure   4.**   Matrix-assisted   laser   desorption   ionization   time-of-flight/time-of-flight

490   (MALDITOF)/mass spectra of 2-aminopyridine (PA)-N-glycans released from extra cellular

491   glycoproteins. Peaks from each sample were characterized in the positive reflector ion mode, and

492   detected ions were assigned to Na-adducts (M+Na+) of the PA-N-glycans. Glycoproteins were

493   collected from the culture media from wild-type rice (A), Δ*α-man*I mutant rice (B), and purified

494   recombinant Δ*α-man*I-GAA (C), respectively.

495

496 **Table 1. Comparison of N-glycan patterns among total secreted glycoproteins from**

497 **wild type and Δα-manI mutant rice and purified Δα-manI-GAA.**

			[†] Relative abundance (%)		
			Extracellular glycoprotein		
[a] Peak	Mass	Type of glycan	[b] WT	[b] Δα-*man*I	[c] Δα-*man*I-GAA
a	1143	MMX-PA		0.7		
b	1289	MMXF3-PA	13.3	3.9	
c	1335	Man5-PA		**2**	**1.6**
d	1346	GnMX-PA	1.3		
e	1492	GnMXF3-PA	18.9	1.8	
f	1497	Man6-PA		**6.8**	**5.4**
g	1508	AMX-PA	0.9		
h	1549	GnGnX-PA	0.8		
i	1654	(FA)MX-PA	1.8		
j	1659	Man7-PA		**47.4**	**22.5**
k	1695	GnGnXF3-PA	28.6	2.8	6.7
l	1711	AGnX-PA	1.1		
m	1799	(FA)MXF3-PA	2.4	3.1	
n	1821	Man8-PA		**32.2**	**63.8**
o	1857	(FA)GnX-PA AGnXF3-PA	5.6		
p	2003	(FA)GnXF3-PA	16		
q	2019	(FA)AX-PA AAXF3-PA	0.6		
r	2165	(FA)(FA)X-PA	0.7		
s	2310	(FA)(FA)XF3-PA	7.3		
	Total		100.0	100.0	100.0

498
499 [†] Numbers are the sum of the relative abundances of a specific N-glycan, and MALDI
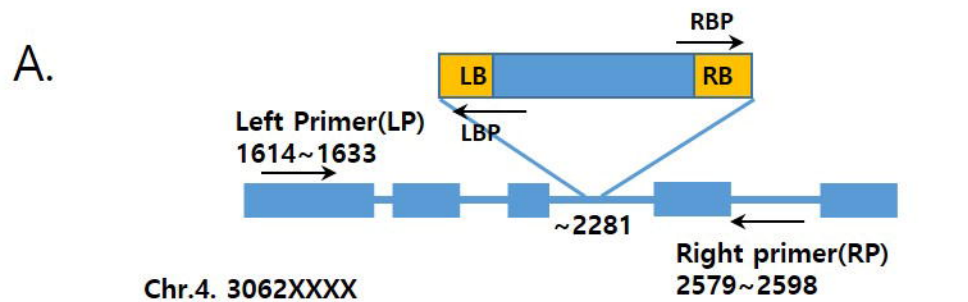500 intensities<1% were omitted.
501 [a] peak: correspond to Fig. 4.
502 [b] Total extracellular glycoproteins derived from wild-type and Δα-*man*I mutant rice cell
503 lines grown in N6 medium with sugar.
504 [c] Recombinant hGAA were induced in N6 medium without sugar.
505

Figure 1.

A.

LB | RB
RBP
Left Primer(LP)
1614~1633
LBP
~2281
Chr.4. 3062XXXX
Right primer(RP)
2579~2598

B.

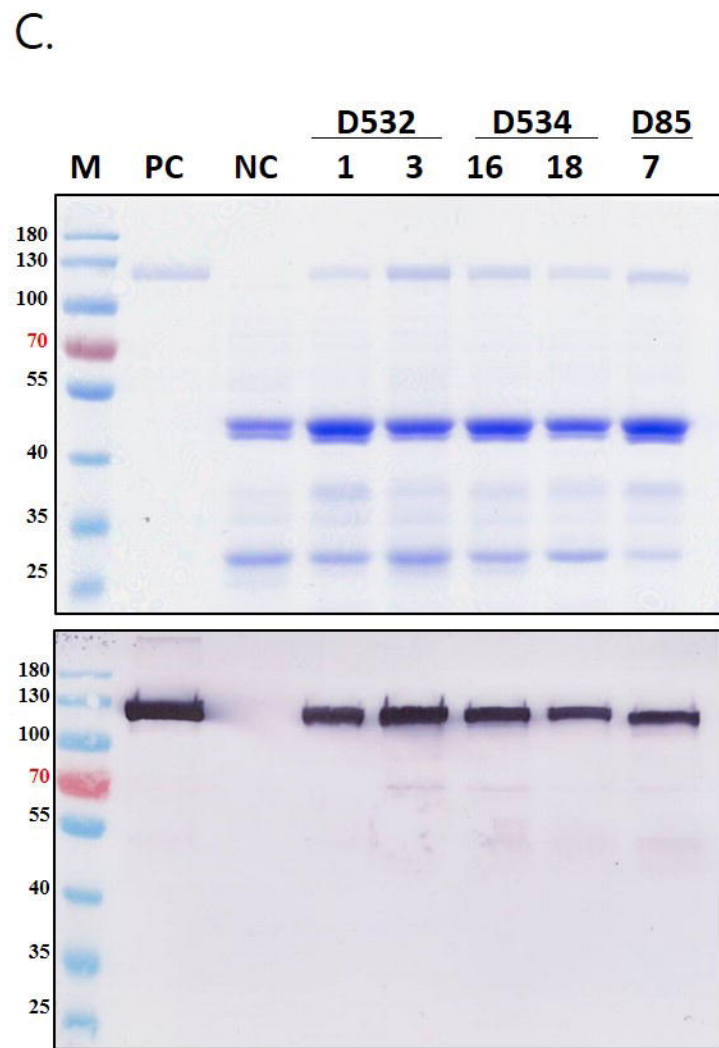| LP&RP | LP&LBP | RBP&RP |
| Expected Size : 985bp | Expected Size : ~900bp | Expected Size : ~600bp |
| M WT #1 #2 | M WT #1 #2 | M WT #1 #2 |

(kb)
3.0
2.0
1.5
1.0
0.5
0.1

C.

M gntl WT #1   M gntl WT #1

180
130
100
70
55
40
35
25
15

M gntl WT #1   M gntl WT #1

180
130
100
70
55
40
35
25
15

Figure 2.

A.

RB	Ramy3D-p.	3Dsp+ hGAA	3'UTR	35S-p.	Bar	35S polyA	LB	pMYD85
RB	Ramy3D-p.	3Dsp-8His+EK+hGAA	3'UTR	35S-p.	Bar	35S polyA	LB	pMYD532
RB	Ramy3D-p.	3Dsp-FLAG+EK+hGAA	3'UTR	35S-p.	Bar	35S polyA	LB	pMYD534

1kb

B.

D85: M PC NC 7
D532: M PC NC 1 3
D534: M PC NC 16 18

(kb)
3.0
2.0
1.5
1.0
0.5
0.1

C.

M PC NC D532(1 3) D534(16 18) D85(7)
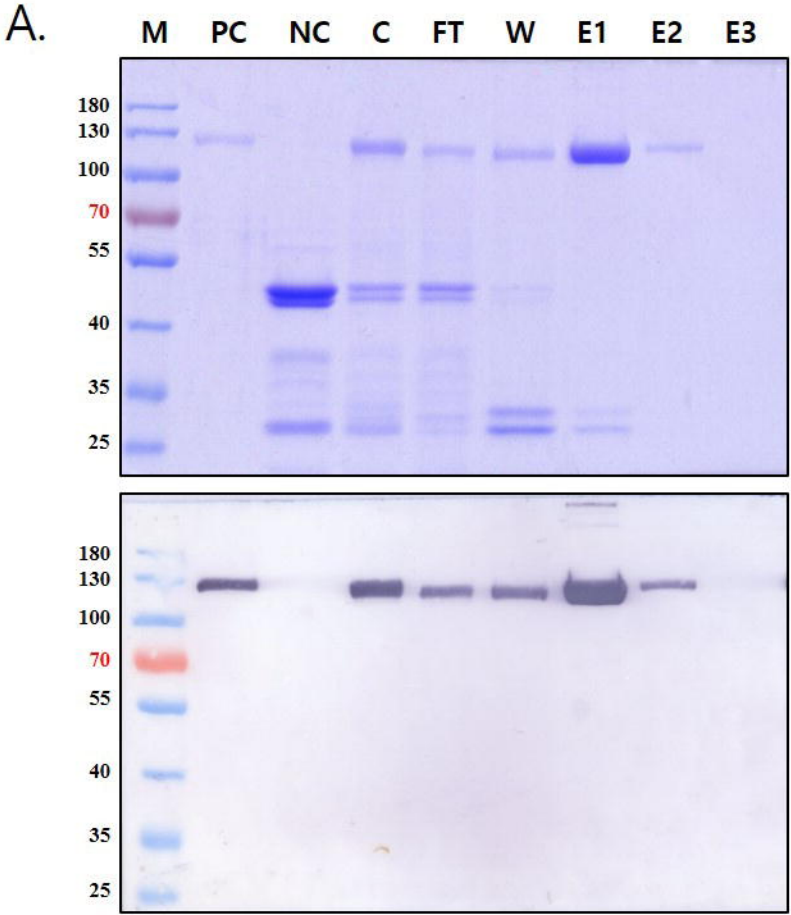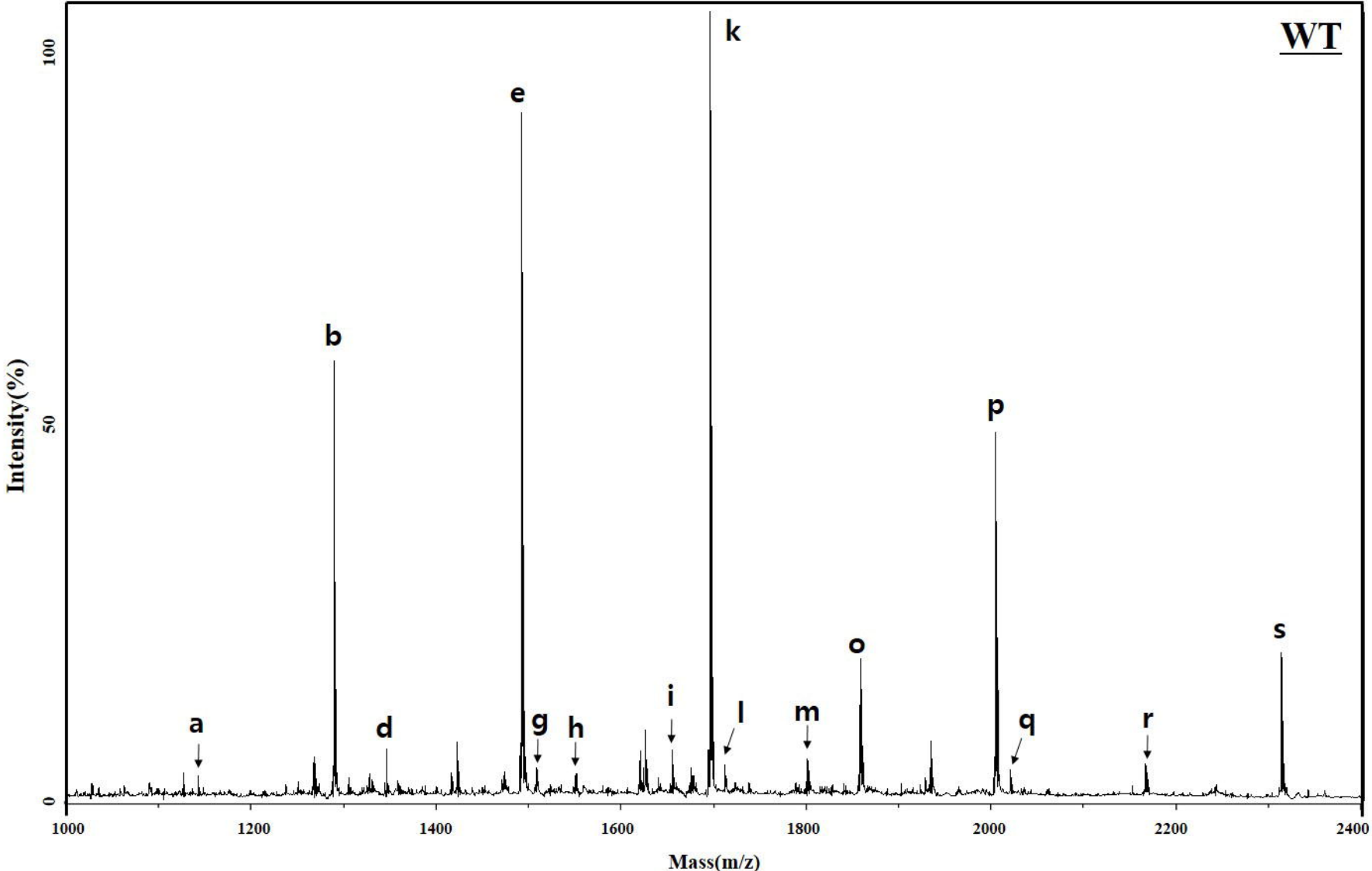
180
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Figure 3.

Figure. 4

A.

Figure. 4

Figure. 4

C.

α-manI-GAA