Doctoral Dissertation

Production of Human Recombinant Acid-Alpha Glucosidase (GAA) With Paucimannose Structure in Arabidopsis *alg3* Cell Culture

Ratna Sariyatun

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Laboratory of Applied Microbiology

International Center for Biotechnology

Department of Biotechnology

Graduate School of Engineering

Osaka University

DECLARATION

"I hereby declare that this dissertation is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which has been used in it. This dissertation has also not been submitted for any degree in university/institution previously."

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Ratna Sariyatun

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Contents

List of abbreviations

ALG3	Asparagine-linked glycosylation 3 (α1,3-mannosyltransferase)
ALG6	Asparagine-linked glycosylation 6 (α1,3-glucosyltransferase)
ALG8	Asparagine-linked glycosylation 8 (α1,3-glucosyltransferase)
ALG9	Asparagine-linked glycosylation 9 (α1,2-mannosyltransferase)
ALG10	Asparagine-linked glycosylation 10 (α1,2-glucosyltransferase)
ALG12	Asparagine-linked glycosylation 12 (α1,6-mannosyltransferase)
ADH	5'-Untranslated region of Arabidopsis alcohol dehydrogenase gene
BAR	Bialaphos resistance gene
CBB	Coomassie Brilliant Blue
CTAB	Cetyltrimethyl ammonium bromide
Dol-PP	Dolichol-pyrophosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ERT	Enzyme replacement therapy
FDA	Food and Drugs Administration
Fuc	Fucose
FucT	α1,3-Fucosyltransferase
FUT13	α1,4-Fucosyltransferase
GAA	Acid-alpha glucosidase
Gal	Galactose
GalT	Galactosyltransferase
GALT1	β1,3-Galactosyltransferase
GCS-I	α-Glucosidases I
GCS-II	α-Glucosidases II

Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GM-II	Golgi-α-mannosidase II
GMP	Good Manufacturing Practice
GnT-I	*N*-Acetylglucosaminyltransferase-I
GnT-II	*N*-Acetylglucosaminyltransferase-II
GnT-IV	*N*-Acetylglucosaminyltransferase-IV
GnT-V	*N*-Acetylglucosaminyltransferase-V
hGM-CSF	Human granulocyte-macrophage colony-stimulating factor
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSP	Terminator of Arabidopsis heat shock protein gene
LB	Left border
LC	Liquid chromatography
MALDI-TOF	Matrix-Assisted Laser Desorption-Ionization-Time of Flight
Man	Mannose
MNS-1/2	Mannosyl-oligosaccharide 1,2-alpha-mannosidase MNS1/2
MR	Mannose receptor
MS	Murashige Skoog, mass spectrometry
M3	$Man_3GlcNAc_2$ or paucimannose
M6P	Mannose-6-phosphate
M6PR	Mannose-6-phosphate receptor
NaCl	Natrium chloride
OST	Oligosaccharyltransferase
P35S	Cauliflower mosaic virus (CaMV) 35S promoter

PA	2-Pyrimidinamine
pAg7	Gene 7 poly(A) signals
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PNOS	Nopaline synthase promoter
PVP	Polyvinylpyrrolidone
PVDF	Polyvinylidene fluoride
RB	Right border
ROS	Reactive oxygen species
RP	Reversed-phase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF	Size-fractionation
TFA	Trifluoroacetic acid
Xyl	Xylose
XylT	β1,2-Xylosyltransferase
5’-UTR	5’-Untranslated region

List of figures

List of tables

Chapter 1 General Introduction

1.1. Plant cell culture as a platform for producing biopharmaceuticals

The market of biopharmaceuticals has been a promptly developing area of economics so that large companies such as Medicago, Ventria, Pfizer, and Greenovation have strong interest and investment in the development of novel platforms for producing pharmaceutical proteins (Zagorskaya and Deineko, 2017). In particular, an enormous attention has been given to the potentials of plant cell cultures because plant cell cultures offer low production cost and better safety than the mammalian platform. The low production cost is a result of less expensive media for growing plant cells, which consists of mainly sucrose and salt and without macromolecules (Kwon *et al*., 2003; Hellwig *et al*., 2004), as well as easier maintenance. Plant cell cultures do not harbour any known human pathogens and bacterial endotoxins, therefore providing better safety for producing biopharmaceuticals (Hellwig *et al*., 2004). On the other hand, plant cell cultures share many similarities to the mammalian-based platform such as capability of performing post-translational modifications similar to those in humans (Moustafa *et al*., 2016) and enabling contained, controlled and sterile environments to meet the criteria of Good Manufacturing Practice (GMP) for producing biopharmaceutical proteins (Santos *et al*., 2016). Due to these features, thus, it is not surprising that the first plant-produced biopharmaceutical approved by the US FDA (Food and Drugs Administration), β-glucocerebrosidase marketed as ELELYSO (Mor, 2015), was produced using carrot cell culture.

A common limitation of using plant cell culture is its low productivity (Su and Lee, 2007; Xu and Zhang, 2014). Some efforts have been done to solve this problem, such as by improving genetic constructions for plant cell transformation, optimizing plant cell cultivation conditions and medium composition, preventing gene silencing, and lowering

protein degradation by knockout of plant proteases or use of protease inhibitors (Boivin *et al*., 2010; Santos *et al*., 2016; Zagorskaya and Deineko, 2017). However, the productivity generally remains lower than mammalian cell platform, thus further research in this area is still needed.

1.2. *N*-Glycosylation and its significance for biopharmaceutical productions

N-Glycosylation is essential for protein folding, stability, activity, and pharmacodynamics so that it is an important factor for manufacturing biopharmaceutical glycoproteins (Seeberger and Cummings, 2015). In fact, control over *N*-glycosylation is a regulatory prerequisite for recombinant protein therapeutics prior to use in patients (Rudge and Nims, 2018). For this reason, differences between human and plant *N*-glycosylation have become a limiting factor in the use of plant cell cultures for producing biopharmaceuticals. Human-type core α1,6-fucose, β1,4-galactose, and sialic acid residues are absent in plant *N*-glycans (Figure 1). Instead, plant cells generate β1,2-xylose (Xyl) and core α1,3-fucose (Fuc) residues on *N*-glycans, which are absent in human, thus raising a concern of potential immunogenic reaction upon parenteral administration of the plant-made biopharmaceuticals to humans (Gomord *et al*., 2010). Moreover, production of biopharmaceuticals with minimal glycan heterogeneity is highly desirable in order to obtain consistent efficacy, which remains a bottleneck even in the well-established mammalian cell expression system (Goochee *et al*., 1991; Sethuraman and Stadheim, 2006). Thus, to generate human-type and homogenous *N*-glycosylation in the plant expression system is necessary.

Several strategies have been done to "humanize" the *N*-glycan profile of plant cells in order to optimize them for producing biopharmaceutical glycoproteins. One approach is to prevent the attachment of plant-specific *N*-glycans by (1) cellular targeting of the

recombinant proteins to the ER by fusion with KDEL/SEKDEL ER-retention signal, (2) knocking-out/down the plant β1,2-xylosyltransferases (XylTs) and α1,3-fucosyltransferases (FucTs), or (3) knocking-out/down *N*-acetylglucosaminyltransferase-I (GnT-I), an enzyme responsible for the initial transfer of GlcNAc to the core mannose (Man) residue necessary for subsequent attachment of the plant specific *N*-glycans. On the other hand, the generation of human-type *N*-glycan structures can be done by co-expressing human *N*-acetylglucosaminyltransferases-IV and -V (GnT-IV and GnT-V), galactosyltransferase (GalT), and sialic-acid synthesizing enzymes (Strasser *et al*., 2014; Rozov *et al*., 2018). Interestingly, introductions of multiple human glycosyltransferases do not affect the plant phenotype, indicating that plant cells harbour high plasticity to tolerate *N*-glycan modifications (Strasser *et al*., 2014).

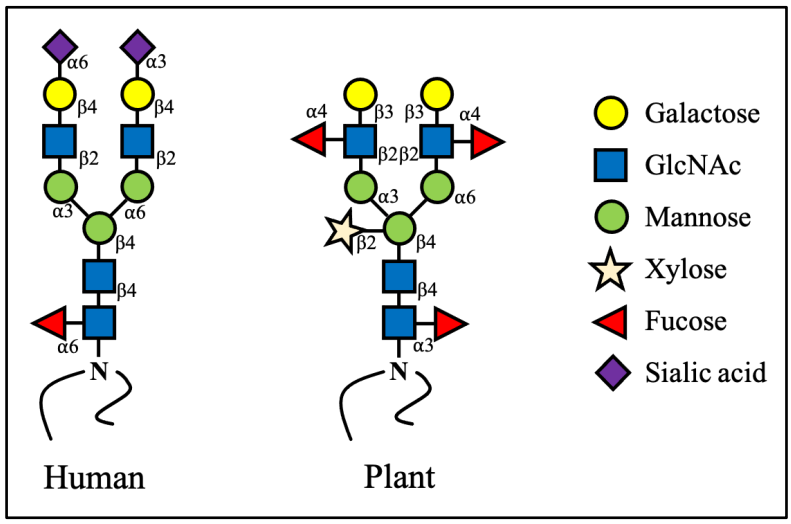


Figure 1. Typical *N*-glycan structures from human and plant. The symbols for the monosaccharides are drawn according to the nomenclature from the Consortium for Functional Glycomics.

1.3. Arabidopsis *alg3* mutant and its potentials as a host for biopharmaceutical productions

During the synthesis of glycan precursor in the ER lumen, asparagine-linked glycosylation 3 (ALG3) acts to transfer a Man residue to $Man_5GlcNAc_2$-PP-Dol (Man:

mannose, GlcNAc: *N*-acetylglucosamine, PP-Dol: pyrophosphate-dolichol) from a sugar nucleotide dolichol-Man, resulting in $Man_6GlcNAc_2$-PP-Dol structure (Figure 2). Previous studies have shown that Arabidopsis plant lacking ALG3 activity, a so called *alg3* mutant, results in more homogenous *N*-glycans than the wild-type (Col-0) and comprises predominantly paucimannose structure, $Man_3GlcNAc_2$ (M3) (Henquet *et al*., 2008, 2011; Kajiura *et al*., 2010). Interestingly, the level of plant-specific *N*-glycans in Arabidopsis *alg3* mutant plant is also lower compared to the wild-type due to low affinity of GnT-I enzyme to attach a GlcNAc residue to the M3 structure (Strasser *et al*., 2005). Meanwhile, no phenotypic change is observed in the *alg3* mutant plant under normal cultivation condition. These characteristics indicate that Arabidopsis *alg3* mutant line can be a suitable host for producing recombinant biopharmaceutical glycoproteins bearing M3 structure and lacking the plant-specific *N*-glycans. The M3 structure is beneficial to direct cellular targeting of the glycoproteins through Man receptor (MR) in humans, while the lack of plant-specific *N*-glycans ensures the product safety by preventing potential immunogenic reaction upon administration to humans.

1.4. Cellular delivery of biopharmaceuticals through Man receptor (MR) in humans

Man receptor (MR) is an endocytic receptor which plays an important role in homeostatic processes, such as clearance of endogenous products and cell adhesion, as well as pathogen recognition and antigen presentation (Taylor *et al*., 2005). It is expressed in numerous cells such as macrophage, hepatic and lymphatic endothelia, glomerular mesangial cells in the kidneys, tracheal smooth muscle cells and retinal pigment epithelium (Shepherd *et al*., 1991; Lew *et al*., 1994; Linehan *et al*., 1999; Taylor *et al*., 2005). MR possess lectin activity towards Man, Fuc, and GlcNAc. Due to this property, MR has been applied to mediate delivery of biopharmaceutical glycoproteins, such as in the treatment of

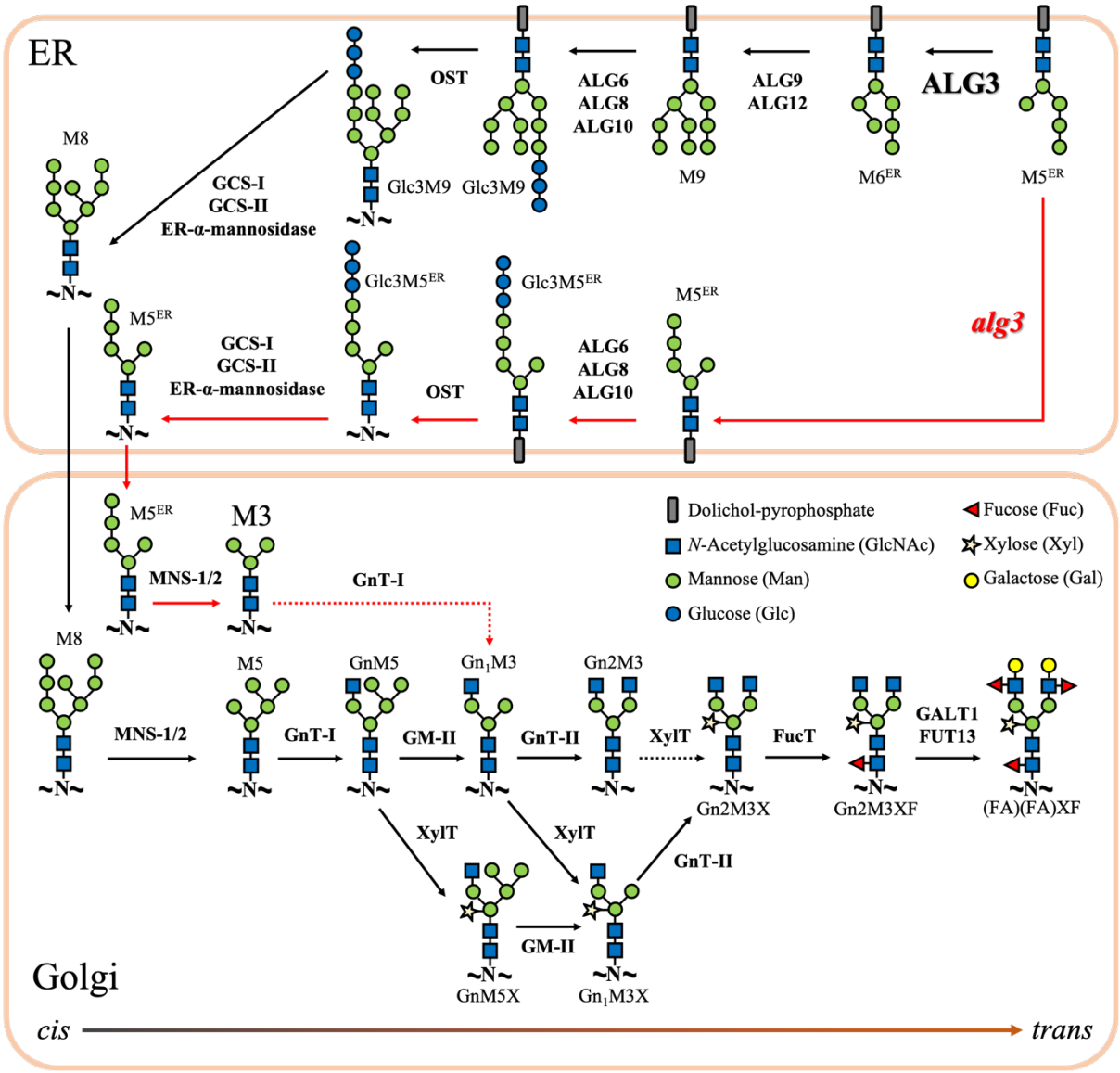


Figure 2. *N*-Glycosylation pathways in Arabidopsis wild-type and *alg3* mutant, starting with the intermediate $Man_5GlcNAc_2$ ($M5^{ER}$) at the ER lumen. Glycosyltransferases involved in these pathways are written in bold, which consist of asparagine-linked glycosylations 3, 6, 8, 9, 10, 12, (ALG3, ALG6, ALG8, ALG9, ALG10, ALG12), oligosaccharyltransferase (OST) complex, α-glucosidases I and II (GCS-I and GCS-II), ER-α-mannosidase, Golgi class I α-mannosidases (MNS-1 and MNS-2), *N*-acetylglucosaminyltransferases-I and -II (GnT-I and GnT-II), Golgi-α-mannosidase II (GM-II), β1,2-xylosyltransferase (XylT), α1,3-fucosyltransferase (FucT), β1,3-galactosyltransferase (GALT1), and α1,4-fucosyltransferase (FUT13). The black and red lines indicate *N*-glycosylation pathways in the wild-type and *alg3* mutant, respectively. Dashed lines indicate that the catalytic activities are less efficient. The generation of this figure is based on Henquet *et al*. (2008), Kajiura *et al*. (2010), and Strasser (2016).

Gaucher disease, Fabry disease, Wolman disease, and cholesteryl ester storage disease (Du *et al*., 2005, 2008; Limkul *et al*., 2016; Shen *et al*., 2016).

The number of exposed terminal Man residues affects the efficacy of MR-mediated delivery. The paucimannosidic structure, $Man_3GlcNAc_2$ (M3), exhibits a higher level of binding with MR than other *N*-glycans bearing more Man-terminal residues/high-Man structures (e.g., M5-M9) (Van Patten *et al*., 2007; Shen *et al*., 2016). On the other hand, M3 has lower binding affinity with Man-binding lectin (MBL), thus, the clearance rate in human body will be lower than the high-Man types (Van Patten *et al*., 2007). To optimize the delivery of biopharmaceutical glycoproteins through MR pathway, therefore, it is preferrable to produce the glycoproteins with M3 structure.

1.5. Recombinant human acid-alpha glucosidase (GAA) for enzyme replacement therapy of people with Pompe disease

Human acid-alpha glucosidase (GAA) is a lysosomal enzyme which hydrolyses glycogen to glucoses in the acidic milieu of the lysosome. Deficiency of this enzyme leads to Pompe disease, which is characterized by lysosomal accumulation of glycogen especially in muscle cells. If untreated, the disease leads to severe metabolic myopathy characterized by cellular damages of cardiac, skeletal, and smooth muscle cells (Kohler *et al*., 2018). Failure of cardiac function causes the incidence of deaths early in life, whereas pathology in skeletal and smooth muscle cells contributes to poor quality of life later in childhood and adult patients.

In humans, GAA is synthesized through multiple proteolytic cleavages and *N*-glycosylation. The enzyme also contains five disulfide bonds (C^{82} – C^{109}, C^{92} – C^{108}, C^{103} – C^{127}, C^{533} – C^{558}, and C^{647} – C^{658}). As described by Moreland *et al*. (2005; 2012) and shown is Figure 3A, GAA is initially translated as a 952-aa protein (~105 kDa). The signal peptide

(aa 1-28) is subsequently cleaved by a cellular signal peptidase, allowing entrance of GAA polypeptide into the ER. In the ER, the *N*-terminus is cleaved and at least six of seven *N*-glycan sites are glycosylated, generating a 110 kDa precursor protein (aa 57-952). Subsequent cleavage occurs in the *N*-terminus generating a 95 kDa intermediate form (aa 122-952) covalently linked via a disulfide bond to the cleaved amino-terminal 3.9 kDa fragment (aa 78-113). Further cleavage occurs in the *C*-terminus generating a 76 kDa mature GAA (aa 122-781) and a cleaved carboxyl-terminal 19.4 kDa fragment (aa 792-952). The 76 kDa GAA contains five *N*-glycosylation sites and remains bound to the 3.9 kDa fragment. Lastly, another cleavage can occur in the *N*-terminus to generate a 70 kDa mature GAA (aa 204-781) which contains four *N*-glycosylation sites and a cleaved amino-terminal 10.3 kDa fragment (aa 122-200) linked to the 3.9 kDa fragment (Figure 3A). These sequential proteolytic cleavages are essential since the 76/70 kDa mature forms show 7-10 times higher affinity to the substrate compared to the 110 kDa precursor (Wisselaar *et al*., 1993; Bijvoet *et al*., 1998). Of note, at each cleavage site, several amino acids are not observed in the final polypeptides (e.g., aa 114-121, 201-203, and 783-791; Figure 3B). Whether this is resulting from two specific proteolytic activities or a single cleavage followed by nonspecific peptidase activity remains unknown (Moreland *et al*., 2005). Moreover, the proteases responsible for these sequential cleavages of GAA have not been reported yet. On the other hand, *N*-glycosylation is important to direct GAA trafficking to the target organelle—the lysosome. The majority of GAA *N*-glycans purified from human placenta is high-Man type (Mutsaers *et al*., 1987), and at least two of the seven GAA *N*-glycan sites contains phosphorylated Man, namely Man-6-phosphate (M6P) residues, for lysosomal targeting via M6P receptor (M6PR) (Hermans *et al*., 1993).

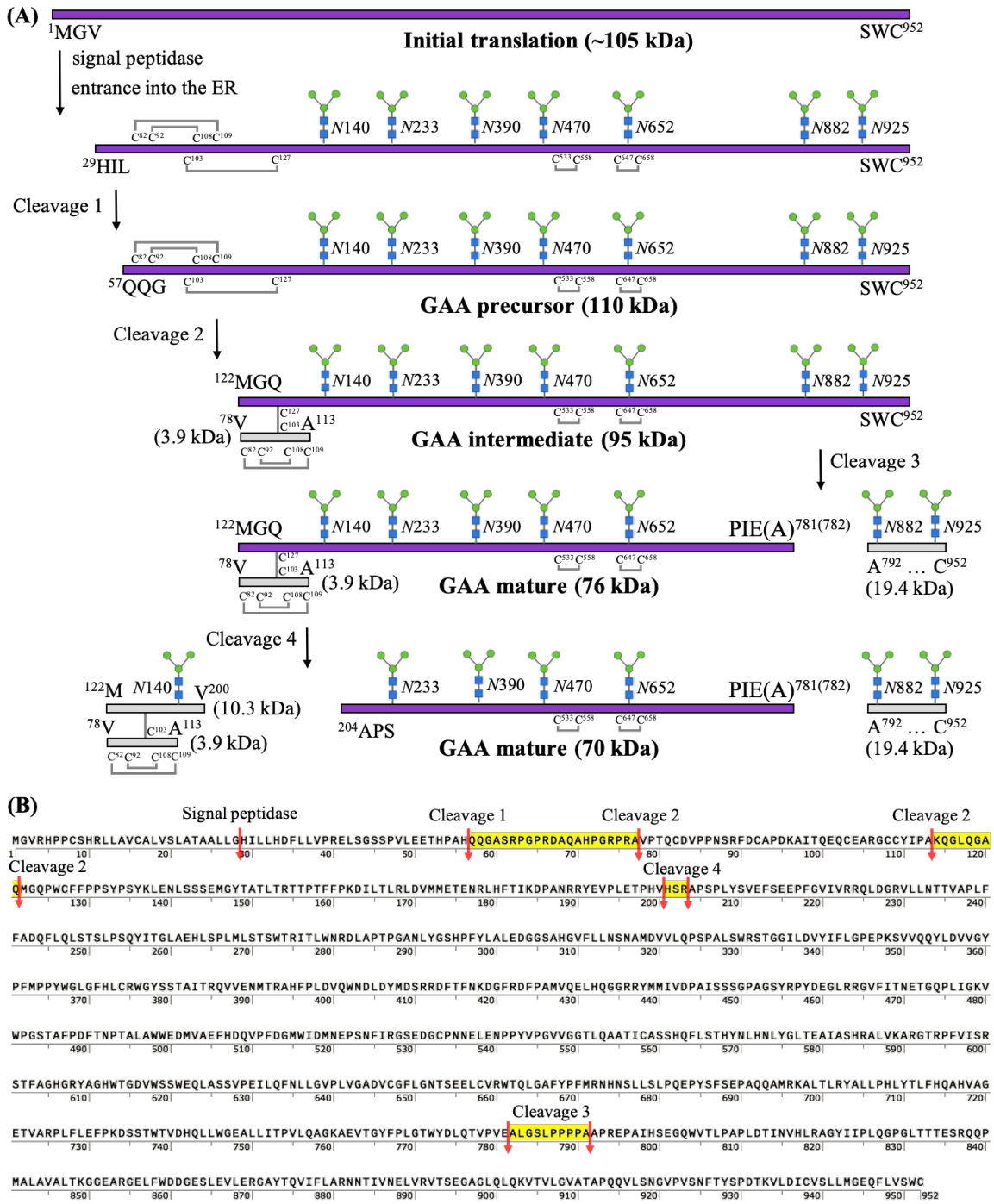


Figure 3. Model for GAA maturation. (A) GAA is translated as a 952-aa polypeptide. The signal peptide is cleaved, allowing entrance into the ER, then the *N*-terminal is cleaved generating a 110 kDa GAA precursor. Further proteolytical cleavages occur in *N*- and *C*-termini generating 95 kDa intermediate, 76 and 70 kDa mature forms. *N*140, *N*233, *N*390, *N*470, *N*652, *N*882, and *N*925 are *N*-glycosylation sites. C^{82} – C^{109}, C^{92} – C^{108}, C^{103} – C^{127}, C^{533} – C^{558}, and C^{647} – C^{658} indicate disulfide bonds. (B) Sequence information of GAA and positions of the cleavages. Red arrows indicate the cleavage points. Yellow squares indicate the missing amino acids in the observed cleavage products (Moreland *et al*., 2005).

Enzyme replacement therapy (ERT) is the only approved treatment for Pompe disease patients by biweekly injection with recombinant GAA. Commercial recombinant GAA for ERT of Pompe disease patients include Myozyme and Lumizyme (a scaled-up product of Myozyme) produced by Genzyme Corporation (Cambridge, MA, USA). Notably, the costs of these enzymes are exceptionally expensive, reaching up to USD 300,000 annually per adult patient (Beasley, 2017). This cost is related to the use Chinese hamster ovary cells for the production platform. To reduce the cost, some alternative GAA has been produced using other production platforms such as tobacco plant (Martiniuk *et al*., 2013), rice cell culture (Jung *et al*., 2016; 2017), and moss cell culture (Hintze *et al*., 2020) (Table 1).

Pompe disease patients under ERT show prolonged lifespan due to restored function of the cardiac muscles. However, the effects of ERT to ameliorate the manifestations related to damaged skeletal muscles (e.g., motor weakness, speech difficulties, dysphagia, osteopenia, and macroglossia) and smooth muscles (e.g., respiratory, vascular, gastrointestinal, genitourinary, ocular, and dermatologic smooth muscle pathologies) has not been satisfying (Kohler *et al*., 2018; Mccall *et al*., 2018). To improve the function of smooth muscles is particularly important because the damages in lower respiratory tract is related to respiratory failure, which leads to fatality in long-onset Pompe disease patients despite use of ERT (Meena and Raben, 2020). Inadequate effect of ERT to restore the functions of skeletal and smooth muscle cells is caused by low number of M6PR on these cells, resulting in insufficient uptake of the enzyme (Wenk *et al*., 1991). Thus, it is important to make a novel GAA that can improve the functions of skeletal and/or smooth muscle tissues more effectively. Notably, MR is known to be expressed on skeletal and smooth muscle cells (Jansen and Pavlath, 2006; Lew *et al*., 1994; Taylor *et al*., 2005). Thereby, producing GAA with M3 structure to direct cellular delivery via MR is expected to provide a better alternative than the commercial GAA to treat skeletal and smooth muscle cells.

Table 1. Comparison of recombinant human GAAs produced in animal and plant cells.

Host	Description	Productivity	*N*-Glycans	References
Animals				
Rabbit	GAA was produced in the milk of transgenic rabbit as a 110 kDa precursor protein starting from aa 67.	8 g/l	Complex, sialylated, and oligo-Man types. The phosphorylated Man were capped by GlcNAc.	Bijvoet *et al*., 1999; Jongen *et al*., 2007; McVie *et al*., 2008
CHO (Myozyme)	GAA was produced as a 110 kDa precursor protein starting from aa 57. This GAA has been used as a standard ERT for Pompe disease patients.	91 mg/l	Hybrid, complex, and sialylated types. M6P glycans comprised 1.0% of the total (~1.2 mol M6P/mol GAA).	Van Hove *et al*., 1996; McVie-Wylie *et al*., 2008; Park *et al*., 2018
CHO	GAA was produced as a 110 kDa precursor protein starting from aa 57. Highly phosphorylated GAA was generated by: (1) supplementing the culture with kifunensine, an inhibitor of MNS-1/2, to generate high-Man glycans, followed by (2) enzymatic reaction to phosphorylate the Man-terminal glycans using GlcNAc-phosphotransferase, and (3) removal of the terminal GlcNAc by *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase.	not reported	Oligo-Man and phosphorylated oligo-Man structures.	Moreland *et al*., 2005; McVie-Wylie *et al*., 2008
Plants				
Tobacco	GAA was produced in seeds of transformed *Nicotiana tabacum L.,* cv. Xanthi plant. GAA signal peptide was	40 μg/g	not reported	Martiniuk *et al*., 2013

	replaced with signal peptide sequence of soybean β-conglycin to allow translocation into the seeds.			
Rice	GAA was produced as a 110 kDa precursor protein. GAA was produced under Ramy3D rice α-amylase expression system induced by sugar starvation. Native GAA signal peptide was replaced with Ramy3D signal peptide to enable secretion. GAA contains His-tag.	37 mg/l	Plant-specific types, mainly Gn2M3XF (40.7%).	Jung *et al*., 2016
Rice *gntI*	As Jung *et al*. (2016), except that the rice cells were *gntI* knockout.	45 mg/l	Oligo-Man types, mainly M5 (89.6%).	Jung *et al*., 2017
Rice	The rice cells were same as Jung *et al*. (2016). Inhibitors of MNS-1/2 (kifunensine) and GM-II (swainsonine) were supplemented into the culture.	24-40 mg/l	High-Man types (81.8%), mainly M9 (60.4%), and reduced plant-type structures.	Choi *et al*., 2018
Rice	Transgenic rice cell suspension cultures were cultivated under eight different conditions: untreated, 5 μM of 2-fluoro-l-fucose (2-FF), 50 μM of 2-FF, 100 μM of 2-FF, 100 μM of 2-FF + 0.5% Pluronic F-68 (PF-68), 100 μM of 2-FF + 0.05% Tween 20 (Tw 20), 0.5% PF-68, and 0.05% Tw 20.	not reported	Plant-specific types, in which lowest amount of core-fucosylation was obtained in 100 μM of 2-FF combined with 0.5% PF-68.	Kim *et al*., 2020
Moss *xylt fuct* Moss *gntI*	GAA was secreted into the culture medium of moss cells (*Physcomitrella patens*). The cells with *xylt* and *fuct* knockouts were used to produce GAA with Gn2M3 structure. β-*N*-Acetylglucosaminidase was used to generate M3 from Gn2M3. The cells with *gntI* knockout were used to produce GAA with M5 structure.	not reported	Gn2M3 in moss *xylt fuct* cells, and M3 after enzymatic cleavage. M5 in moss *gntI* cells.	Hintze *et al*., 2020

1.6. Objectives of this study

The initial objective of this study was to analyze the *N*-glycans of Arabidopsis *alg3* cell culture and its potential for producing glycoproteins with M3 structure and lacking the plant-specific *N*-glycans (Chapter 2). As a proof of the concept, a recombinant human GAA was produced in the Arabidopsis *alg3* cell culture and purified, then the *N*-glycans were analyzed (Chapter 3). Moreover, the effects of additives (polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), gelatin, mannitol, ammonium nitrate (NH_4NO_3), and dimethyl sulfoxide (DMSO)) to increase GAA production in the Arabidopsis *alg3* cell culture was also examined (Chapter 3).

Chapter 2 Analysis of Arabidopsis *alg3* Cell Culture for Producing Glycoproteins With Paucimannose and Lacking Plant-specific *N*-Glycans

2.1. Introduction

Plant cell cultures possess advantageous characteristics as an alternative production system for recombinant biopharmaceuticals, such as (1) plant cell cultures use simple medium free of animal-derived products thus offering cost-effectiveness, (2) the absence of harmful human pathogens and bacterial endotoxins provides safety, (3) plant cells undergo post-translational modifications similar to mammals, thus allowing recombinant proteins to be properly folded and assembled, and (4) allowance of contained, controlled and sterile condition to meet the criteria of Good Manufacturing Practice (GMP) of biopharmaceutical production (Hellwig *et al*., 2004; Moustafa *et al*., 2016; Santos *et al*., 2016). Notably, plant cell cultures have shown a good record as a novel platform of biopharmaceutical production as shown by the commercialization of the carrot cells-produced β-glucocerebrosidase marketed as ELELYSO for the treatment of Gaucher disease (Mor, 2015) and numerous therapeutic glycoproteins currently in clinical trials. Therefore, it is not surprising that plant cell cultures have gained increasing attention as a next generation platform for producing biopharmaceuticals.

N-Glycosylation is not only essential for proper protein folding, but also greatly affecting protein stability, activity, and characteristics (Seeberger and Cummings, 2015). The differences between human and plant *N*-glycosylation have become a hurdle in the use of plant cells for production of biopharmaceutical glycoproteins. Plants generate β1,2-Xyl and core α1,3-Fuc residues on *N*-glycans, which are absent in humans, thus potentially induce an immunogenic reaction upon parenteral administration to humans (Gomord *et al*., 2010). Elimination of plant-specific *N*-glycans is required to ensure the safety of plant-

produced biopharmaceuticals. On the other hand, it is desirable to produce biopharmaceuticals with minimal glycan heterogeneity in order to maintain consistent quality of the product. This has remained a problem even in the well-established mammalian cell expression system (Goochee *et al*., 1991; Sethuraman and Stadheim, 2006). In order to optimize the use of plant cell cultures as a production platform of biopharmaceuticals, it will be necessary to develop plant cell line with homogenous *N*-glycosylation and lacking the plant-specific *N*-glycans.

Previous study showed that Arabidopsis plant lacking the activity of asparagine-linked glycosylation 3 (ALG3), an α1,3-mannosyltransferase which transfers a Man residue from a sugar nucleotide dolichol-Man to lipid-linked $Man_5GlcNAc_2$ on the ER lumen, generated unique Man-terminal *N*-glycans predominantly paucimannosidic structure $Man_3GlcNAc_2$ (M3) (30.7-31.1%) and contained lower amount of plant-specific *N*-glycans compared to the wild-type (Kajiura *et al*., 2010). These properties are beneficial for producing biopharmaceuticals since M3 structure can enable cellular delivery of glycoproteins via Man receptor (MR) in humans (Van Patten *et al*., 2007; Shen *et al*., 2016), while the lower amount of plant-specific *N*-glycans is essential to prevent potential immunogenic reaction in humans. In this study, an Arabidopsis *alg3* suspension cell culture was generated from the Arabidopsis *alg3* mutant plant, then the *N*-glycan profile was characterized.

2.2. Materials and Methods

2.2.1. Checking of T-DNA insertion in *alg3* gene

Arabidopsis *alg3* cell culture was previously developed from the leaves of Arabidopsis *alg3* plant (Kajiura *et al*., 2010) using a standard protocol (Sanchez-Serrano and Salinas, 2014). Plant genomic DNA was extracted from the Arabidopsis *alg3* cell culture by cetyltrimethyl ammonium bromide (CTAB) method (Aboul-Maaty and Oraby, 2019). Then,

the presence of T-DNA insertion in *alg3* gene was checked by polymerase chain reaction (PCR) using primers 6 (5'-TCTCTTTAATGATTGTTTTGCCAT-3') and Lbd1 (5'-CCACGTTCTTTAATAGTGGACT-3') as previously described (Kajiura *et al*., 2010).

2.2.2. Protein extraction and Western blot analysis

Seven-day-old cells were harvested from Arabidopsis wild-type (Col-0) and *alg3* cell cultures. The medium is decanted, then the cells were dissolved in 20 mM Tris-HCl pH 7.5 containing 10 mM EDTA (1 ml/mg of cells) and sonicated for 1 min on ice. Cell debris was separated by centrifugation at 12,000 × *g* for 20 min at 4°C, and crude intracellular protein extract in the supernatant was collected. The protein extracts were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto a PVDF membrane (Merck Millipore) in transfer buffer (50 mM Tris, 40 mM glycine, 20% methanol) using a mini-transblot apparatus (Bio-Rad) for 90 min. To prevent non-specific binding, membranes were blocked with 5% non-fat milk powder in phosphate-buffered saline containing 0.05% Tween 20 buffer with gentle agitation on a rotary shaker at 20 rpm for 1 h. The membrane was incubated with a 1:10,000 dilution of anti-horseradish peroxidase (HRP) polyclonal antibody produced in rabbit (Sigma-Aldrich) for 1 h, followed by a 1:5,000 dilution of anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) for 1 h. For detection, Luminata™ Forte Western HRP substrate (Merck Millipore) was added to the membrane, and luminescent signals on the membrane were detected using the Invitrogen iBright Imaging System 1500 Series (Thermo Fisher Scientific).

2.2.3. *N*-Glycan analysis

Analysis of intracellular *N*-glycan profile of Arabidopsis *alg3* cell culture was performed using a combination of reversed-phase (RP) and size-fractionation (SF) high-performance liquid chromatographies (HPLCs) followed by tandem mass spectrometry

(MS/MS) analysis. For preparation of the sample, 7-day-old cells were harvested and washed in dH_2O. Cells were subsequently ground in the presence of liquid N_2 into fine powder. The thawed cell homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C, and supernatant was collected and precipitated using acetone. The glycoprotein precipitates were lyophilized. Then, the *N*-glycans were released from the proteins by hydrazinolysis at 100°C for 10 h followed by acetylation, desalting, and labelling with 2-pyrimidinamine (PA) (Misaki *et al*., 2001). To remove the excessive PA reagent, the sample was mixed with phenol:chloroform (1:1, v/v) solution, vortexed, and centrifuged at 10,000 × *g*, 1 min, at room temperature. The upper aqueous phase, which contains *N*-glycans, was collected. Then, the residual phenol was removed by chloroform extraction: sample was mixed with chloroform (1:1, v/v), vortexed, and centrifuged at 10,000 × *g* for 1 min, then the upper aqueous fraction was collected. Lastly, a final desalting step was performed using a gel filtration (Toyopearl HW-40F, Tosoh Corporation).

Purified PA-labelled *N*-glycans were separated using RP-HPLC system (Elite LaChrom HPLC System; Hitachi) using a Cosmosil 5C18-AR-II column (6.0 × 250 mm; Nacalai Tesque). The mobile phase for RP-HPLC was composed of solvent A (0.02% trifluoroacetic acid/TFA) and solvent B (20% acetonitrile/0.02% TFA). RP-HPLC was performed at a flow rate of 1.2 ml/min and increased the percentage of solvent B linearly from 0% to 30% over 40 min. The eluted fractions were monitored by fluorescence intensity at an excitation wavelength of 310 nm and emission wavelength of 380 nm. The eluted fractions collected from RP-HPLC were concentrated and analyzed using a LC–MS/MS system (Agilent Technologies) equipped with the amaZon ETD software (Bruker Daltonics). The mobile phase for LC was composed of solvent C (2% acetic acid in acetonitrile) and solvent D (3% triethylamine/5% acetic acid in water). The LC was performed using a Shodex Asahipak NH2P-50 2D column (2.0 × 150 mm; Showa Denko) at a flow rate of 0.2

ml/min, and the percentage of solvent D was increased linearly from 20% to 55% over 35 min. The operating parameters for MS/MS were set as follows: positive-ion mode, mass range 350–2750 m/z, nebulizer flow 5.0 psi, dry gas flow rate 3.0 l/min, dry temperature 300°C, target count 200 000 and MS/MS Frag. Ampl. 1.0 V. The amount of *N*-glycans was quantified by the peak area in LC.

For the analysis of extracellular *N*-glycan profile, media from 7-day-old Arabidopsis wild-type and *alg3* cell cultures were collected and directed to acetone-mediated glycoprotein precipitation. Precipitated glycoproteins were dissolved in 20 mM Tris-HCl pH 7.5 buffer and separated by SDS-PAGE under denaturing condition. The gel was subsequently stained using CBB. Bands of interest were excised from the gel followed by destaining, reduction, and alkylation. In-gel digestion was performed using trypsin at 50°C for 1 h. The digestion was stopped with addition of TFA to a final concentration of 0.5%. The tryptic glycopeptides were dried, dissolved in 0.1% formic acid, then injected to a nanoLC-MS/MS system. The nanoLC-MS/MS analysis was performed on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II; Bruker Daltonics) using a nanoLC system (1,200 series; Agilent Technologies) incorporating a trap column (5 μm, 0.3 × 5 mm) and analytical column (3.5 μm, 0.075 × 150 mm), both packed with Zorbax 300SB C-18 (Agilent Technologies). For the nanoLC system, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The tryptic peptides were trapped in the column at a flow rate of 10 μl/min for 5 min. Elution was performed at a flow rate of 0.6 μl/min using a 2 to 8% gradient of solvent B over 5 min followed by a linear increase of solvent B to 50% for 40 min at 35°C. After elution, the column was washed with 95% solvent B for 5 min before returning to the initial conditions. For MS and MS/MS analyses, the system was operated with automatic switching between MS and MS/MS modes. The operating parameters were set as follows: positive-ion mode, mass range 50–4,500 *m/z*,

nebulizer flow 1.0 psi, dry gas flow rate 5.0 l/min, dry temperature 180°C, and ISCID energy 5.0 eV. The three most abundant signals (absolute threshold >20 counts/s) were selected on each MS spectrum for further isolation and fragmentation. The complete system was fully controlled by micrOTOF control software (Bruker Daltonics). MASCOT search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) was conducted using the MS/MS ion data to allow analysis of the excised proteins. Bruker Compass DataAnalysis (version 4.0) was used for deconvolution of MS spectra and glycan analysis, and BioTools (version 3.2) was used for *de novo* sequencing.

2.3. Results

The Arabidopsis *alg3* suspension cell culture retained a T-DNA insertion in *alg3* gene (Figure 4A), thus confirming the *alg3* mutation. Western blot using an anti-HRP polyclonal antibody, known to bind the plant-specific α1,3-Fuc and β1,2-Xyl residues (Faye *et al*., 1993; Bencúrová *et al*., 2004), showed much weaker signal in crude extract of the *alg3* cell culture compared to the wild-type (Figure 4C). A deeper analysis of the intracellular *N*-glycan profile was performed using RP-HPLC and LC-MS/MS, and the results showed that the *alg3* cell culture produced more homogenous *N*-glycans predominantly M3 (46.5%), followed by GlcNAc-terminal structures including $Gn^{1}M3$ (25.8%), Gn2M3 (15.8%), and $Gn_{1}M3$ (11.9%) (Figures 5 and 6, Table 2). $Gn^{1}M3$ was the most frequent GlcNAc-terminal structure in intracellular *N*-glycan of the *alg3* cell culture. The generation of this structure was a result of trimming of Gn2M3 by β-*N*-acetylhexosaminidases (HEXOs) residing in the vacuoles and apoplast. Interestingly, no plant-specific *N*-glycans could be detected.

Analyses of the media of Arabidopsis wild-type and *alg3* cell cultures revealed the proteins secreted by the cells (Figure 4D, Table 3). The *N*-glycans of three secreted glycoproteins, including GDPDL3, GDPDL4, and ACO3, were characterized (Table 4). In

the wild-type, these secreted glycoproteins carried predominantly plant-specific *N*-glycans (41.6-100.0%), mainly M3FX, GnM3FX, and Gn2M3FX. A lower ratio of plant-specific *N*-glycans was observed at site *N*547 of GDPDL4 protein, which might occur because of the protein conformation to not allow the access of XylT and FucTs to this site. On the other hand, the extracellular *N*-glycans of the *alg3* cell culture consisted of mostly M3 (24.5-62.0%) and GnM3 (24.3-70.7%). The GnM3 is a sum of both Gn_1M3 and Gn^1M3 since the nanoLC-MS/MS analysis could not distinguish between these two isomers. Moreover, no high-Man structure was detected from the extracellular *N*-glycans of both cell cultures because the secreted glycoproteins already underwent multiple maturation processes in the Golgi, including trimming of the Man, prior to secretion (Figure 2). Overall, the relative ratios of each *N*-glycan structure tend to show a substantial degree of variation between the sites and proteins, suggesting that the formation of *N*-glycan structure may be protein- and site-specific.

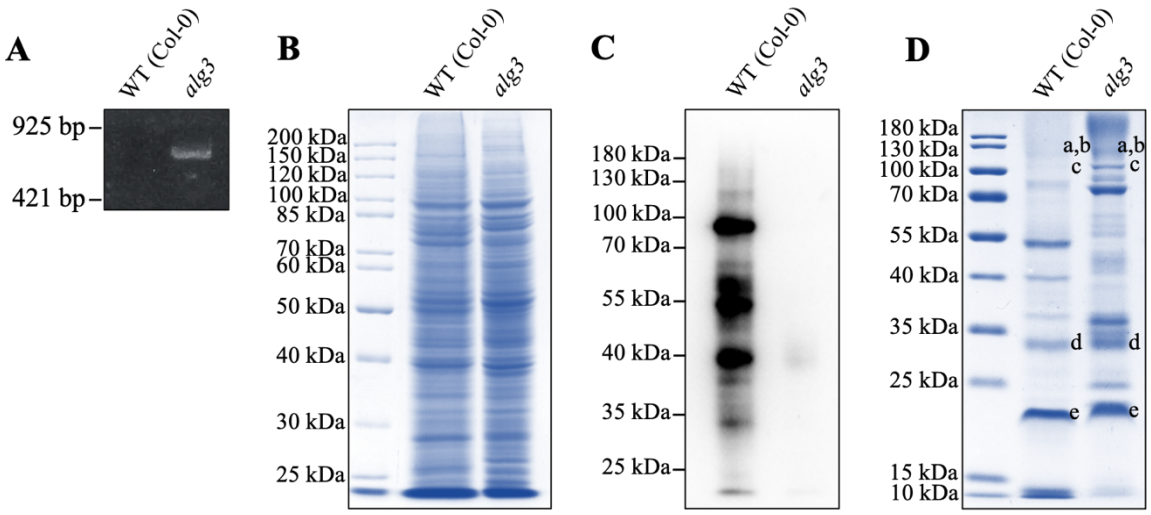


Figure 4. (A) Confirmation of T-DNA insertion in *alg3* gene of Arabidopsis *alg3* cell culture by PCR. (B) CBB staining as a loading control. (C) Western blot for detection plant-specific *N*-glycans using anti-HRP antibody. (D) CBB staining of extracellular proteins from Arabidopsis wild-type and *alg3* cell cultures. Marks a-e indicate the excised protein bands for characterization using nanoLC-MS/MS and MASCOT search.

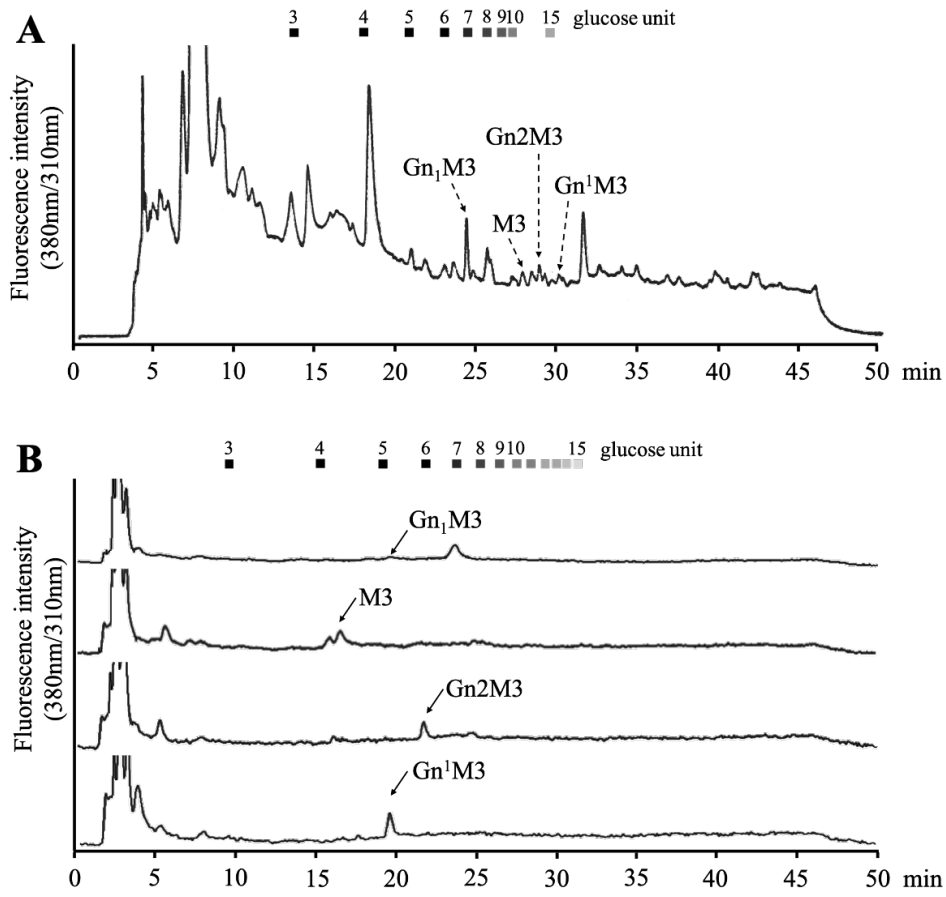


Figure 5. (A) Reversed-phase/RP and (B) size-fractionation/SF high-performance liquid chromatographies (HPLCs) of intracellular *N*-glycan sample from Arabidopsis *alg3* cell culture. *N*-Glycans were labelled with 2-pyrimidinamine (PA). RP- and SF-HPLCs were performed using a Cosmosil 5C18-AR-II column (6.0 × 250 mm; Nacalai Tesque) and a Shodex Asahipak NH2P-50 2D column (2.0 × 150 mm; Showa Denko) columns, respectively. Numbers at the top represent the elution positions of glucose units based on the elution times of PA-isomalto-oligosaccharides with degrees of polymerization from 3 to 15.

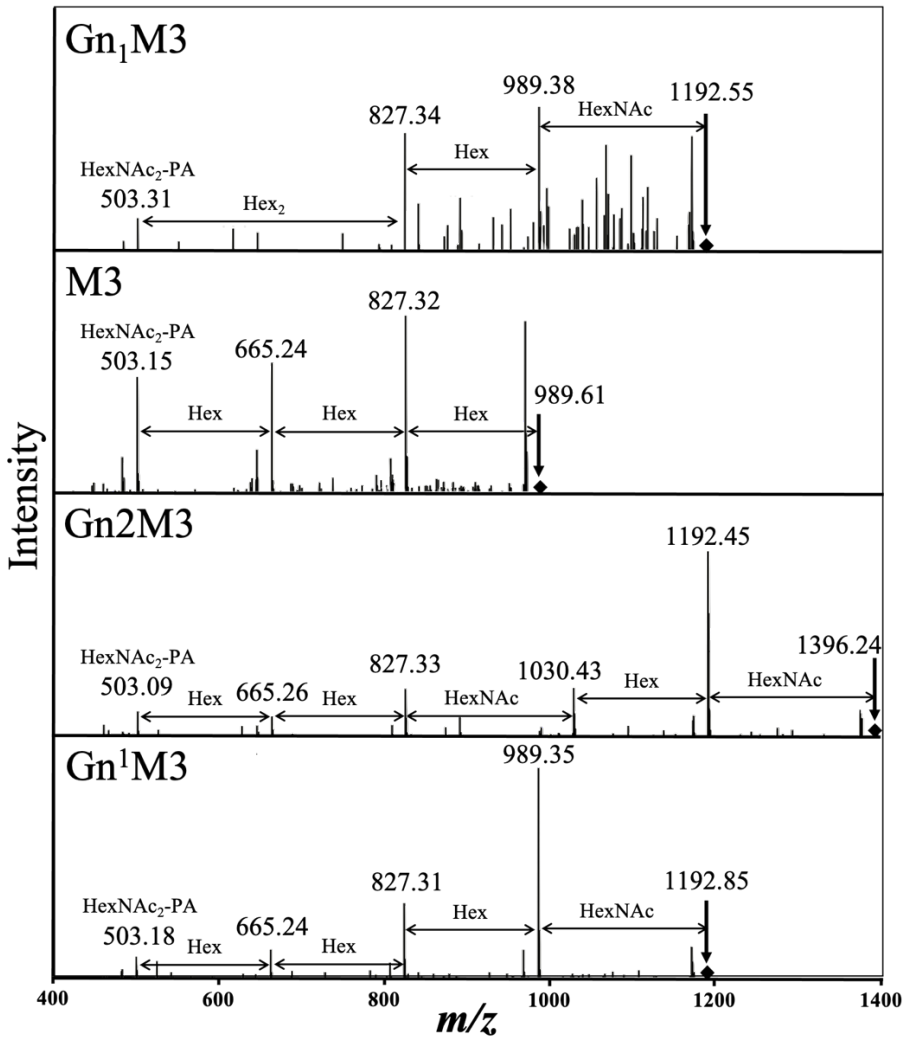
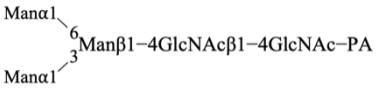
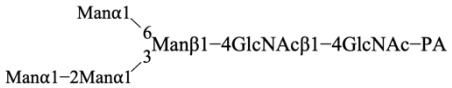
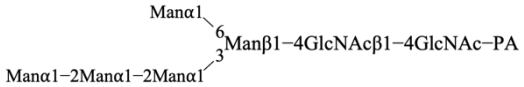
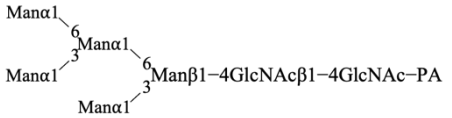
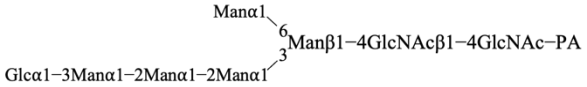
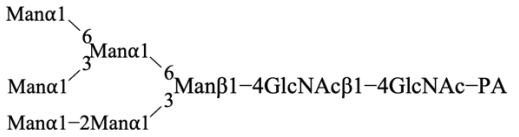
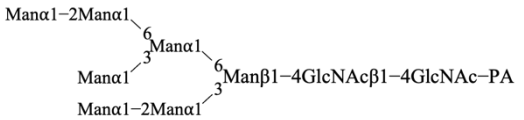
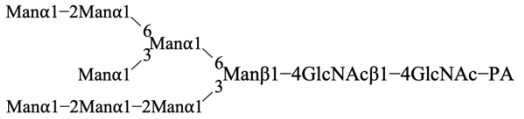
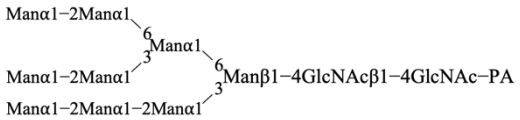
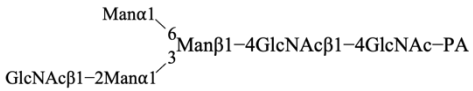
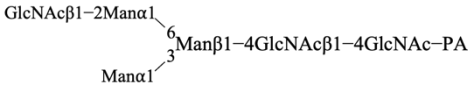
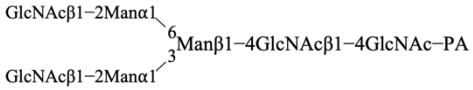
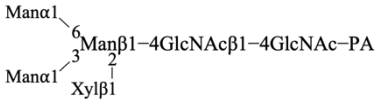
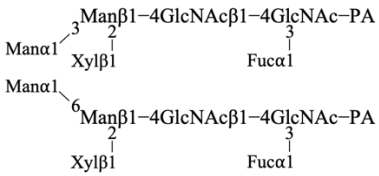
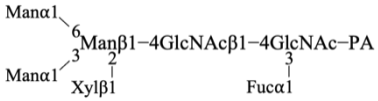
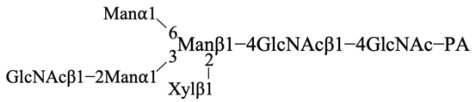
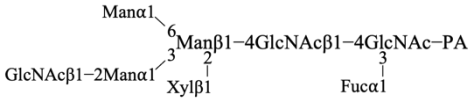
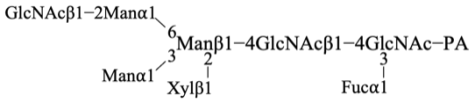
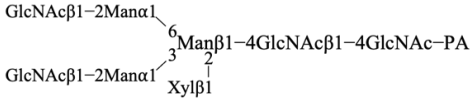
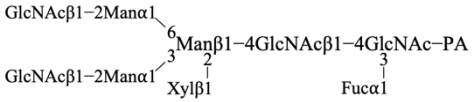


Figure 6. MS/MS analysis of intracellular *N*-glycan from Arabidopsis *alg3* cell culture.

Table 2. Relative amounts of intracellular *N*-glycan structures in Arabidopsis *alg3* cell culture.

Abbreviation	Structure	Relative amounts (%)			
		Wild-type Col-0 plant*	Wild-type T87 cell culture**	*alg3* plant*	*alg3* cell culture
M3		-	-	30.7	46.5
M4^{ER}		-	-	17.8	-
M5^{ER}		-	-	9.4	-
M5		15.2	-	-	-
GlcM5^{ER}		-	-	2.4	-
M6		-	0.6	-	-
M7		4.2	-	-	-
M8		6.2	7.7	-	-

M9		3.0	1.5	-	-
Gn_1M3		-	-	-	11.9
Gn^1M3		-	-	-	25.8
Gn2M3		-	-	-	15.8
Plant-specific					
M3X		6.7	0.7	17.5	-
M2XF		-	0.9	-	-
M3XF		53.6	19.1	12.7	-
Gn_1M3X		-	4.3	-	-
Gn_1M3XF		1.0	18.0	0.1	-

$Gn^{1}M3XF$		8.9	3.6	4.9	-
Gn2M3X		-	1.3	-	-
Gn2M3XF		1.1	42.3	4.4	-

*Data from Kajiura *et al*. (2010), **data from Kajiura *et al*. (2012a).

Table 3. Analysis of secreted proteins from Arabidopsis wild-type (WT) and *alg3* cell cultures by nanoLC-MS/MS analysis followed with analysis using MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS).

Code	Predicted protein	WT		*alg3*		Protein size (kDa)		Potential *N*-glycan site
		Score	Match	Score	Match	Predicted	Observed	
a	Glycerophosphodiester phosphodiesterase GDPDL3	84	4	974	27	84.2	117.9	12
b	Glycerophosphodiester phosphodiesterase GDPDL4	789	22	487	15	82.6	117.9	12
c	Aconitate hydratase 3 (ACO3)	71	4	1491	47	108.2	105.4	6
d	Xyloglucan endotransglycosylase-related protein (XTR6)	703	16	918	22	32.1	30.9	2
e	Osmotin-like protein (OSM34)	590	13	565	12	26.6	20.4	-

Table 4. Relative amounts of extracellular *N*-glycan structures in Arabidopsis wild-type (WT) and *alg3* cell cultures.

Structures*	WT					*alg3*				
	GDPDL3			GDPDL4	ACO3	GDPDL3			GDPDL4	ACO3
	*N*251	*N*528	*N*242	*N*547	*N*510	*N*251	*N*528	*N*242	*N*547	*N*510
M1	-	-	-	-	-	3.2	-	-	-	-
M2	-	-	-	-	-	10.5	4.5	6.6	4.8	6.8
M3	-	-	-	5.6	2.6	62.0	34.7	35.9	24.5	48.2
GnM2	-	-	-	1.7	-	-	-	3.0	-	3.5
GnM3	-	-	-	33.9	-	24.3	54.3	44.8	70.7	41.6
Gn2M3	-	-	-	16.3	-	-	6.5	9.6	-	-
GnM4	-	-	-	1.9	-	-	-	-	-	-
Plant-type										
MXF	23.5	-	-	-	-	-	-	-	-	-
M2X	-	-	-	1.5	2.4	-	-	-	-	-
M2F	-	-	-	-	3.4	-	-	-	-	-
M2XF	-	-	-	-	14.1	-	-	-	-	-
M3X	-	-	-	7.7	7.9	-	-	-	-	-
M3F	-	-	-	-	8.2	-	-	-	-	-
M3XF	44.6	32.4	76.3	-	35.6	-	-	-	-	-
M4F	-	-	-	-	1.7	-	-	-	-	-
GnM2X	-	-	-	1.4	-	-	-	-	-	-
GnM2XF	-	-	-	-	3.3	-	-	-	-	-
GnM3X	-	-	-	23.7	4.8	-	-	-	-	-
GnM3F	-	-	-	1.8	1.3	-	-	-	-	-
GnM3XF	31.8	24.0	23.7	-	12.4	-	-	-	-	-
Gn2M3X	-	-	-	4.5	-	-	-	-	-	-
Gn2M3XF	-	43.6	-	-	2.3	-	-	-	-	-
Total	100	100	100	100	100	100	100	100	100	100

*Graphical structure of each glycoform is shown in Supplementary Table 2.

2.4. Discussion

Previous studies have reported the *N*-glycan profile of Arabidopsis *alg3* plant (Henquet *et al*., 2008; Kajiura *et al*., 2010), however the profile in the form of suspension cell culture had been unknown. In this study, an Arabidopsis *alg3* suspension cell culture was developed. Similar to previous results in the *alg3* plant, the *alg3* cell culture produced high amount of M3 structure (46.5% in intracellular and 24.5-62.0% in extracellular fractions). While the *alg3* plant still produced considerable amount of plant-specific *N*-glycans (39.6% of total *N*-glycans, Table 2), interestingly, the production of plant-specific *N*-glycans in the *alg3* cell culture was very low as indicated by a weak binding of the crude extract to anti-HRP antibody (Figure 4C), and even undetectable in the LC-MS/MS analysis (Table 2). Instead, GlcNAc-terminal structures were generated in much amount, which consisted of Gn_1M3 (11.9%), Gn2M3 (15.8%), and Gn^1M3 (25.8%) (Table 2). On the other hand, Arabidopsis wild-type ecotype Columbia (Col-0) plant and T87 cell culture, which is derived from the Col-0 plant (Axelos *et al*., 1992), produced mainly plant-specific structures. Interestingly, while M3XF is the most abundant structure in the Col-0 plant, the T87 cell culture produced predominantly Gn2M3XF instead (Table 2). This suggests that the activities of HEXOs, responsible for the cleavage of Gn2M3XF into M3XF, in the plant and cell culture system are quite different.

It is intriguing to understand the lacking production of plant-specific *N*-glycans in the *alg3* cell culture despite a substantial production of Gn_1M3 (11.9%), which is a substrate for XylT and FucTs as well as *N*-acetylglucosaminyltransferase-II (GnT-II). The results of this study indicated that Gn_1M3 was used by GnT-II to generate Gn2M3 rather than processed by XylT and FucTs (Figure 7), which is reasonable because GnT-II resides in the *cis-medial* Golgi while XylT and FucTs are localized in the *medial* and *medial-trans* Golgi, respectively (Strasser, 2016; Yoo *et al*., 2015). This indicates that in the *alg3* cells GnT-II acts early in

the *cis* Golgi on Gn_1M3 resulting from GnT-I activity on M3, whereas in the wild-type cells the substrate for GnT-II is available later after GnT-I generated a GnM5 and GM-II cleaves the terminal Man (Figure 7). Moreover, Yoo *et al*. (2015) showed that the act of GnT-II would inhibit further attachment of β1,2-Xyl and α1,3-Fuc. This agrees well with Kajiura *et al*. (2012b) showing that XylT exhibits lower activity towards Gn2M3, and FucT activity is less efficient towards structure without the β1,2-Xyl.

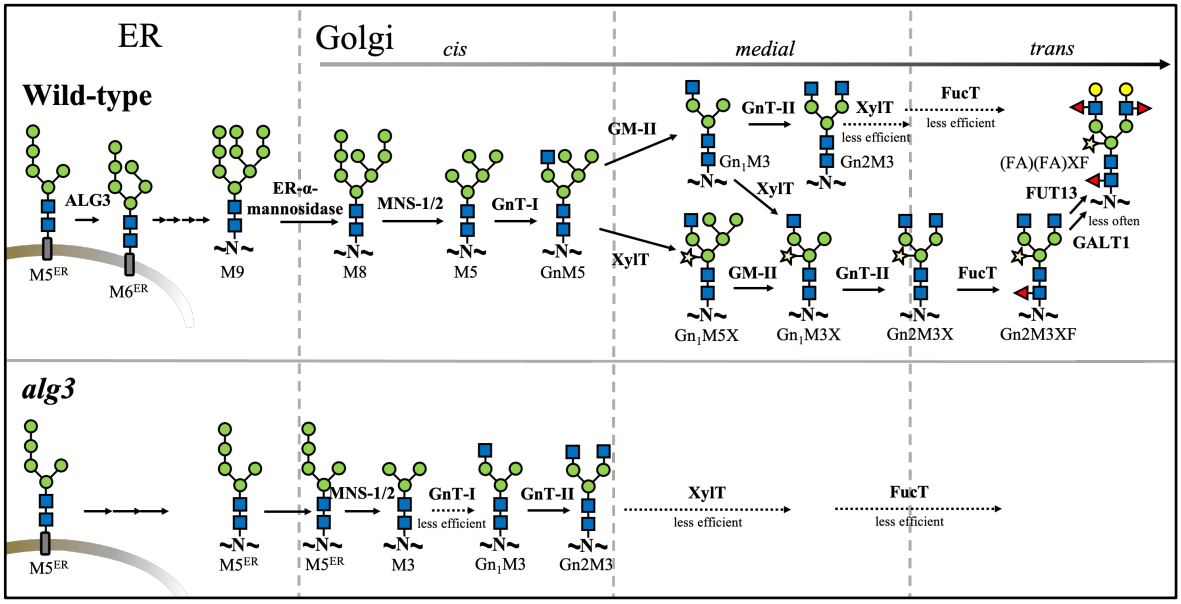


Figure 7. Putative mechanism of lacked formation of plant-specific *N*-glycans in Arabidopsis *alg3* cells as compared to the wild-type. The enzymes involved in the pathways are written in bold, as described in Figure 2. Blue square (■), green circle (●), yellow circle (●), red triangle (◀), and star (☆) indicate GlcNAc, mannose, galactose, fucose, and xylose, respectively.

Overall, the *N*-glycan profiles of intra- and extracellular fractions of Arabidopsis *alg3* cell culture was more homogenous than those of the wild-type and *alg3* plants, lacking plant-specific *N*-glycans, and producing mostly M3 and GlcNAc-terminal structures. Notably, both Man and GlcNAc could be recognized by MR, and indeed M3 and Gn2M3 have been shown enable cellular uptake of therapeutic glycoprotein *in vitro* (Hintze *et al*., 2020). These features imply that the Arabidopsis *alg3* cell culture should be a suitable host for producing biopharmaceuticals with delivery mechanism through the MR pathway and safety over potential immunogenicity induced by the plant-specific *N*-glycans.

2.5. Summary

N-Glycosylation is essential for determining the characteristics and pharmacodynamics of biopharmaceutical glycoproteins upon administration to humans. Certain structures are required to mediate delivery to target cells, such as a paucimannose $Man_3GlcNAc_2$ (M3) to enable cellular uptake via MR in humans. Such structure is normally scarce in plant cells. Further, the generation of plant-specific *N*-glycans is also a problem since it could induce an immunogenic reaction in humans. In this study, a cell culture was established from Arabidopsis *alg3* mutant plant which lacks asparagine-linked glycosylation 3 (ALG3) enzyme activity. Arabidopsis *alg3* cell culture produced more homogenous glycoproteins with predominantly M3 and GlcNAc-terminal structures and lacked the plant-specific *N*-glycans. These characteristics are valuable for producing biopharmaceutical glycoproteins with cellular delivery through MR and safe for human therapy.

Chapter 3 Production of Recombinant Human Acid-Alpha Glucosidase (GAA) in Arabidopsis *alg3* Cell Culture

3.1. Introduction

Plant cell cultures have emerged as a potential bioproduction platform for recombinant biopharmaceuticals which offer greater cost-effectiveness and safety over other eukaryotic systems (Hellwig *et al*., 2004; Xu *et al*., 2011; Santos *et al*., 2016). Compared to the whole-plant system, using plant cell cultures provide an advantage by enabling secretion of the products into the plant cell culture medium, which contains mainly sucrose and salts and no macromolecules (Kwon *et al*., 2003), thus simplifying and cutting the cost of downstream purification step.

Plant *N*-glycosylation profile is one of the limiting factors in using plant cell cultures for producing biopharmaceuticals. Plants are incapable of producing some human-type structures, such as sialic acid and phosphorylated Man, which can be necessary for protein characteristics or delivery to the target cells. Moreover, the appearance of plant-specific *N*-glycans potentially induces immunogenic reactions in humans (Gomord *et al*., 2010). In Chapter 2, an Arabidopsis *alg3* cell culture is shown to generate predominantly paucimannose $Man_3GlcNAc_2$ (M3) structure and lacks the plant-specific *N*-glycans. These characteristics are valuable for producing biopharmaceutical glycoproteins: the M3 structure can mediate cellular delivery via Man receptor (MR), while the lack of plant-specific *N*-glycans ensures its safety upon administration to humans. To date, however, there have been no studies exploring the use of Arabidopsis *alg3* cell culture for the production of biopharmaceuticals. As a proof of the concept, a human glycoprotein acid-alpha glucosidase (GAA) would be produced using the Arabidopsis *alg3* cell culture.

GAA is a human lysosomal enzyme which degrades glycogen into glucoses in the acidic milieu of the lysosome. In human cells, GAA is present as a 110 kDa precursor, 95 kDa intermediate, and 76/70 kDa mature forms (Moreland *et al*., 2005; Figure 3). Lack of GAA activity results in Pompe disease, which is indicated by accumulation of glycogen in the lysosome leading to severe metabolic myopathy (Kohler *et al*., 2018). Enzyme replacement therapy (ERT) by injection with recombinant human GAA has increased the survival of patients with Pompe disease by restoring the function of cardiac muscles. However, the ability of ERT to improve the pathology in skeletal and smooth muscle cells has remained inadequate (Kohler *et al*., 2018; Mccall *et al*., 2018). To restore the function of smooth muscle cells is particularly important because the pathologic smooth muscles of respiratory tract can cause fatality due to respiratory failure, which is not uncommon in late-onset Pompe disease patients despite use of ERT (Meena and Raben, 2020). This inadequacy of ERT to improve the pathologic skeletal and smooth muscle cells is due to the low number of Man-6-phosphate receptors (M6PRs) on cell surface of these cells, resulting in insufficient enzyme uptake and delivery to the lysosome (Wenk *et al*., 1991). Notably, MR is known to be expressed on skeletal and smooth muscle cells (Jansen and Pavlath, 2006; Lew *et al*., 1994; Taylor *et al*., 2005). Using the Arabidopsis *alg3* cell culture, it would be possible to produce an alternative GAA with M3 *N*-glycan for cellular delivery via MR, which potentially outperforms the efficacy of the commercial GAA for targeting skeletal and smooth muscle cells.

A common limitation of plant cell cultures is their low productivity (Su and Lee, 2007; Xu and Zhang, 2014). In plant cell cultures, medium or nutrient engineering can be as important as the expression construct and cell line development for enhancing the protein productivity (Holland *et al*., 2010; Xu *et al*., 2011; Fischer *et al*., 2015). However, because use of plant cell cultures for producing recombinant proteins was developed later than other

well-established platforms (e.g., such as mammalian and microbial cells), there have been fewer knowledge into methods for enhancing protein production and secretion into the culture media. Previous studies have shown the effects of protein stabilizer (e.g., polyvinylpyrrolidone/PVP, polyethylene glycol/PEG, bovine serum albumin/BSA, NaCl, gelatin, and DMSO) to improve recombinant protein production in plant cell cultures (LaCount *et al*., 1997; James *et al*., 2000; Sharp and Doran, 2001; Lee *et al*., 2002). Use of osmotic agent (e.g., mannitol) and modification of nitrogen content have also been shown to increase recombinant protein production (Tsoi and Doran, 2002; Zhang *et al*., 2016). However, none has been tested in Arabidopsis or for GAA production.

In the present study, GAA was produced in Arabidopsis *alg3* cell culture, purified, and the *N*-glycans were characterized. The effects of medium engineering on the GAA production in Arabidopsis cell culture were also examined. The results of this study would contribute to optimization of plant cell cultures to produce recombinant proteins.

3.2. Materials and Methods

3.2.1. Protein extraction and Western blot analysis of GAA-expressing Arabidopsis *alg3* cell culture

Previously, a GAA-producing Arabidopsis *alg3* cell culture had been developed by introducing an expression cassette for GAA expression using *Agrobacterium tumefaciens* LBA4404 into the plant genome. The expression cassette derived from Limkul *et al*. (2015) where the β-glucocerebrosidase (GC) gene was replaced with GAA (Figure 8A). Here, the cells of GAA-expressing Arabidopsis *alg3* cell culture were harvested on 7 days post-cultivation, and dissolved in lysis buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 ml/mg of cells). The mixture was sonicated for 1 min and centrifuged at 12,000 × *g* for 20 min at 4°C, and then the supernatant was collected as the intracellular protein extract. Meanwhile,

analysis of secreted proteins was conducted using the harvested culture media directly. Protein concentration of samples was determined by Bradford assay using pre-determined concentrations of bovine serum albumin to generate the standard curve.

The extracted intracellular and extracellular proteins were separated via SDS-PAGE, then transferred onto a PVDF membrane as described in Chapter 2. Membranes were blocked with 5% non-fat milk powder in phosphate-buffered saline containing 0.05% Tween 20 buffer with gentle agitation on a rotary shaker at 20 rpm for 1 h. The membrane was incubated with a 1:5,000 dilution of anti-GAA monoclonal antibody produced in rabbit (Abcam) or 1:10,000 dilution of anti-HRP polyclonal antibody produced in rabbit (Sigma-Aldrich) for 1 h, followed by a 1:5,000 dilution of anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) for 1 h. A recombinant human GAA (aa 70-952, with an *N*-terminal 6-His tag) derived from human embryonic kidney 293 (HEK293) cells (R&D Systems) was used as a positive control. For detection, Luminata™ Forte Western HRP substrate (Merck Millipore) was added, and luminescent signals on the membrane were detected using Invitrogen iBright Imaging System 1500 Series (Thermo Fisher Scientific).

3.2.2. GAA enzyme activity assay

GAA activity was determined by measuring the hydrolysis of a synthetic substrate *p*-nitrophenyl α-D-glucopyranoside (Santa Cruz Biotechnologies) in 50 mM sodium acetate pH 4.3 containing 0.1% BSA. The assay was performed at 37°C for 30-60 min, and stopped by the addition of 0.87 M sodium bicarbonate pH 11.0. Hydrolysis of the substrate was monitored by measuring the release of the reaction product, *p*-nitrophenol, at an absorbance of 405 nm by using iMark™ Microplate Reader (Bio-Rad). A unit (U) of activity was defined as the amount of activity that resulted in the hydrolysis of 1 μmol of substrate per min at 37°C under the assay condition.

3.2.3. GAA purification

The medium of 11-day-old GAA-producing Arabidopsis *alg3* cell culture was filtered through a Glass Econo-Column (Bio-Rad). Then, NaCl was added to the medium until a final concentration of 4 M. The NaCl-containing medium was subsequently loaded into a hydrophobic interaction chromatography column (Toyopearl Phenyl-650M, Tosoh Corporation) pre-equilibrated in 20 mM Tris-HCl pH 7.5 with 4 M NaCl. After sample loading, the column was washed with 4 M NaCl in 20 mM Tris-HCl pH 7.5 buffer. Then, GAA was eluted by decreasing NaCl concentration (3 M to 0 M). GAA-containing fractions were dialyzed to exchange the buffer into 20 mM sodium acetate pH 4.3, then applied to a cation exchange chromatography column (Toyopearl SP 550C, Tosoh Corporation) pre-equilibrated in 20 mM sodium acetate pH 4.3 buffer. After washing the column, GAA was eluted with 20 mM sodium acetate pH 4.3 containing 0.1-0.5 M NaCl. GAA-containing fractions were concentrated and desalted using a Vivaspin 20 with a 10 kDa cut-off (Sartorius Stedim Biotech GmbH).

3.2.4. Manipulation of medium composition

Stock solutions of 0.125 g/ml PVP (Sigma-Aldrich), 0.2 g/ml PEG (Sigma-Aldrich), and 10% BSA (Nacalai Tesque) were prepared and filter-sterilized using a Millex-GP syringe filter unit (0.22 μm; Merck Millipore) before use, while 5 M NaCl (Wako), 0.5 M EDTA (Dojindo), and 1.0 M ammonium nitrate (NH_4NO_3; Wako) stock solutions were sterilized by autoclaving. Then, proper amounts of PVP, PEG, BSA, EDTA, and DMSO (Nacalai Tesque) stock solutions were added to 100 ml of MS medium to final concentrations of 0.75 g/l, 2 g/l, 0.1%, 1 mM, and 4%, respectively, while NaCl was added to final concentrations of 25 mM, 50 mM, 100 mM, and 150 mM. Meanwhile, media

supplemented with 5% gelatin (Difco Laboratories) and 34 g/l mannitol (186.6 mM; Nacalai Tesque) were prepared by inputting appropriate amounts of stock powders before autoclaving the MS media. These concentrations were chosen on the basis of previous studies to increase recombinant protein production in plant cell cultures (James *et al*., 2000; LaCount *et al*., 1997; Lee *et al*., 2002; Sharp and Doran, 2001; Tsoi and Doran, 2002). Moreover, while MS medium already contains NH_4NO_3 (165 mg/100 ml), in some experiments an additional 330 mg of NH_4NO_3 was added to the 100 ml of MS medium, resulting in final NH_4NO_3 concentration of 495 mg/100 ml (61.8 mM).

In all experiments, the cell cultures were cultivated by transferring 10 ml of fresh cells onto 100 ml of MS medium along with additions of the tested compound. The cell cultures were grown for 14 days from the day of cultivation (day 0). One ml of cell culture was taken on each of days 0, 4, 7, 11, and 14. The cells were separated from the medium for the determination of fresh cell weight and the analysis of intracellular proteins. The medium was also collected for the analysis of secreted proteins. All samples were stored at -20°C until analysis.

3.2.5. *N*-Glycan analysis of GAA produced in Arabidopsis *alg3* cell culture

Purified GAA was run on SDS-PAGE and stained with CBB. The 95 and 76 kDa GAA bands were excised from the gel, sliced into small pieces, then destained with methanol:50mM NH_4HCO_3 (1:1 v/v) for 2 min with intermittent vortex mixing followed by overnight destaining in acetonitrile:50 mM NH_4HCO_3 (1:1 v/v) with intermittent vortex mixing. Then, in-gel GAA digestion was performed using Trypsin Gold (approximately 1 μg per sample; Promega) in ProteaseMAX™ surfactant (Promega) at 50°C for 1 h. The reaction was stopped by addition of trifluoroacetic acid to a final concentration of 0.5%. Tryptic glycopeptides were extracted from the gel, dried, and dissolved in 0.1% formic acid

prior to injection into a nanoLC-MS/MS system. The nanoLC-MS/MS analysis was performed on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II; Bruker Daltonics) using a nanoLC system (1200 series; Agilent Technologies) incorporating a trap column (5 µm, 0.3 × 5 mm) and analytical column (3.5 µm, 0.075 × 150 mm), both packed with Zorbax 300SB C-18 (Agilent Technologies). For the nanoLC system, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The tryptic glycopeptides were trapped in the column at a flow rate of 10 µl/min for 5 min. Elution was performed at a flow rate of 0.6 µl/min using a 2% to 8% gradient of solvent B over 5 min followed by a linear increase of solvent B to 50% for 40 min at 35°C. After elution, the column was washed with 95% solvent B for 5 min before returning to the initial conditions. For MS and MS/MS analyses, the system was operated with automatic switching between MS and MS/MS modes. The operating parameters were set as follows: positive-ion mode, mass range 50–4500 *m/z*, nebulizer flow 1.0 psi, dry gas flow rate 5.0 l/min, dry temperature 180°C, and ISCID energy 5.0 eV. The three most abundant signals (absolute threshold >20 counts/s) were selected on each MS spectrum for further isolation and fragmentation. The complete system was fully controlled by micrOTOF control software (Bruker Daltonics). Bruker Compass DataAnalysis (version 4.0) was used for glycan analysis and BioTools (version 3.2) was used for *de novo* sequencing.

The nanoLC-MS/MS analysis could not distinguish between glycoforms with same masses (such as $M5^{ER}$ from M5, and $GlcM5^{ER}$ from M6). To help speculate the *N*-glycan structures on GAA, the previous characterization of *N*-glycan profiles of Arabidopsis *alg3* plant (Kajiura *et al.*, 2010) was applied as a reference because the culture used in this study was derived from the homozygous *alg3* plant aforementioned.

3.3. Results

3.3.1. GAA production in the transformed Arabidopsis *alg3* cell culture

A GAA expression cassette within pFK1-BAR-GAA was used in this study. The cassette had a GAA sequence downstream of the CaMV 35S promoter and 5'-UTR ADH enhancer, followed by an HSP terminator (Figure 8A). The plasmid was introduced into *Agrobacterium tumefaciens* LBA4404, then the *Agrobacterium* was used to transform the *alg3* cells, generating GAA-producing Arabidopsis *alg3* cells. A suspension cell culture was subsequently generated, and GAA production in the cell culture was confirmed by Western blot (Figures 8B and C). In human cells, GAA polypeptide is initially translated into 952 amino acids (aa) and then processed through sequential cleavages in the *N*- and *C*-termini generating 110 kDa GAA precursor, 95 kDa GAA intermediate, and 76/70 kDa GAA mature forms (Figure 3). In Arabidopsis *alg3* cell culture, GAA was produced in the intracellular fraction as three forms corresponding to the 110, 95, and 76 kDa GAAs (Figure 8C). The 95 and 76 kDa GAAs were also secreted into the plant culture medium. Detection of these proteins indicated that the plant cells were capable of cleaving the GAA similar to that in humans. However, the 70 kDa GAA was not observed in intra- or extracellular fraction of the *alg3* cell culture.

Compared to an untransformed Arabidopsis *alg3* cell culture, the GAA-producing cell culture showed higher GAA-specific activities both intracellularly (9.1 ± 0.7 mU/mg in the wild-type and 35.1 ± 5.2 mU/mg in the GAA-producing cell culture) (Figure 8D) and extracellularly (35.4 ± 1.0 mU/mg in the wild-type and 1423.5 ± 37.4 mU/mg in the GAA-producing cell culture) (Figure 8E). These results indicated that the GAA was functional.

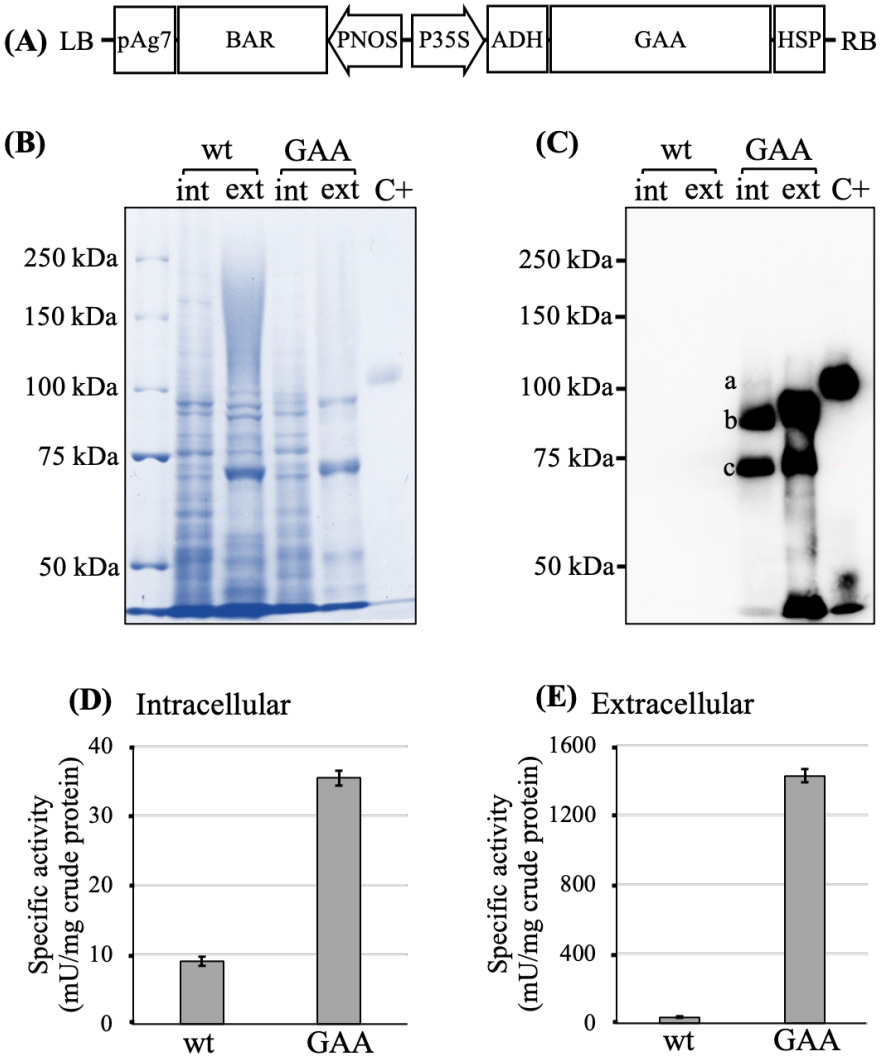


Figure 8. Expression cassette and confirmation of GAA production in the transformed Arabidopsis *alg3* cell culture. (A) Schematic representation of GAA expression construct and antibiotic selection marker. P35S: Cauliflower mosaic virus (CaMV) 35S promoter; ADH: 5'-untranslated region of Arabidopsis alcohol dehydrogenase gene which acts as an enhancer; GAA: cDNA of human acid-alpha glucosidase; HSP: terminator of Arabidopsis heat shock protein gene; PNOS: nopaline synthase promoter; BAR: bialaphos resistance gene as plant selection marker; pAg7: gene 7 poly(A) signals; LB and RB: left and right borders of T-DNA, respectively. (B, C) CBB staining and Western blot using anti-GAA antibody of untransformed (wt) and transformed (GAA) 7-day-old Arabidopsis *alg3* cell culture to confirm GAA production intra (int)- and extracellularly (ext). Marks a, b, and c indicate the 110, 95, and 76 kDa GAAs, respectively. C+ is human embryonic kidney/HEK293-derived recombinant human GAA (100 ng) used as a positive control with an expected molecular size of 95-105 kDa. Precision Plus Protein™ Unstained and All Blue Prestained Protein Standards (Bio-Rad) are used as protein markers in the CBB staining and Western blot analyses, respectively. (D, E) GAA specific activities in crude intracellular and extracellular proteins of untransformed (wt) and transformed (GAA) Arabidopsis *alg3* cell culture.

3.3.2. GAA purification

GAA was purified from the plant cell culture medium using two-step column chromatographies as two forms, the 95 and 76 kDa proteins (Figures 9A and B). Since the 95 kDa GAA contains seven *N*-glycosylation sites (*N*140, *N*233, *N*390, *N*470, *N*652, *N*882, and *N*925) and the 76 kDa GAA contains five *N*-glycosylation sites (*N*140, *N*233, *N*390, *N*470, and *N*652), it was expected to see multiple protein bands due to different *N*-glycosylation on each of these sites. A degraded GAA band was observed between the 95 and 76 kDa (Figure 9A, Supplementary Figure 1 and Supplementary Table 1). The degradation should have occurred near the *N*-terminus, thus causing failed detection by the monoclonal anti-GAA antibody known to bind an epitope near the *N*-terminus of GAA (within aa 150-250).

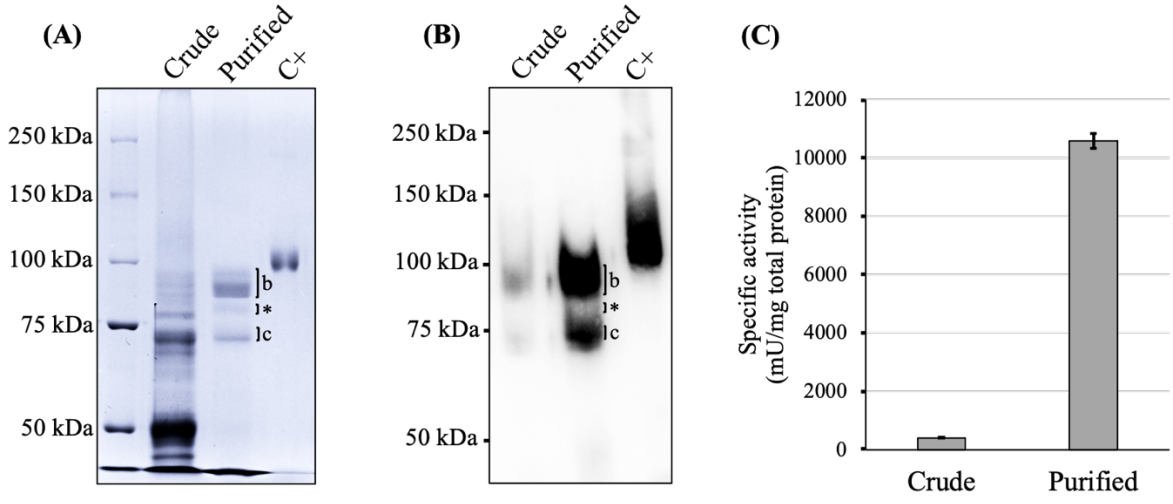


Figure 9. Analysis of GAA purified from GAA-producing Arabidopsis *alg3* cell culture by CBB staining (A), Western blot using anti-GAA antibody (B), and GAA activity assay (C). Marks b and c indicate the 95 and 76 kDa GAAs, respectively, whereas star (*) indicates a degraded GAA (Supplementary Figure 1, Supplementary Table 1). C+ is human embryonic kidney/HEK293-derived recombinant human GAA (100 ng) used as a positive control with an expected molecular size of 95-105 kDa. Precision Plus Protein™ Unstained and All Blue Prestained Protein Standards (Bio-Rad) are used as protein markers in the CBB staining and Western blot analyses, respectively.

After purification, the GAA-specific activity in the purified sample (10583.8 ± 251.7 mU/mg) increased up to 26-times compared to that in the crude sample (408.7 ± 7.9 mU/mg) (Fig. 9C), while the productivity was 60.9 ± 12.4 µg/l or 2.5% of the initial sample (Table 5).

Table 5. GAA purification from the medium of GAA-producing Arabidopsis *alg3* cell culture. Purification was performed using two-step chromatography columns including hydrophobic interaction chromatography column/HIC (Toyopearl Phenyl-650M, Tosoh Corporation) and cation exchange chromatography column (Toyopearl sulfopropy/SP 550C, Tosoh Corporation).

	Volume (ml)	mg/ml	mg	mU/mg	Total U	Purification fold	Recovery (%)
Crude	210	0.089	18.61	408.7	7.61	1	100
HIC	15	0.075	1.12	904.4	1.02	2.2	13.4
SP	0.1	0.18	0.02	10583.8	0.19	25.9	2.5

3.3.3. Effects of PEG, PVP, BSA, EDTA, DMSO, gelatin, mannitol, NH_4NO_3, and NaCl additions to the Arabidopsis *alg3* cell growth and GAA production

The following nine compounds were examined for their effects on the Arabidopsis *alg3* cell growth and GAA production: PVP, PEG, BSA, EDTA, DMSO, NH_4NO_3, mannitol, gelatin, and NaCl. These compounds were reported to improve the production of recombinant proteins in plant cell cultures (James *et al*., 2000; LaCount *et al*., 1997; Lee *et al*., 2002; Sharp and Doran, 2001; Tsoi and Doran, 2002; Zhang *et al*., 2016). In this study, the presence of 0.1% BSA, 4% DMSO, 34 g/l (186.6 mM) mannitol, 100 mM NaCl, 1 mM EDTA and 5% gelatin in the MS medium severely inhibited the Arabidopsis cell growth, whereas adding 0.75 g/l PVP and 2 g/l PEG increased the cell growth. Meanwhile, supplementing the MS medium with an additional 330 mg of NH_4NO_3 (61.8 mM) slightly decreased the cell growth, but lengthened the exponential growth phase (Figure 10A).

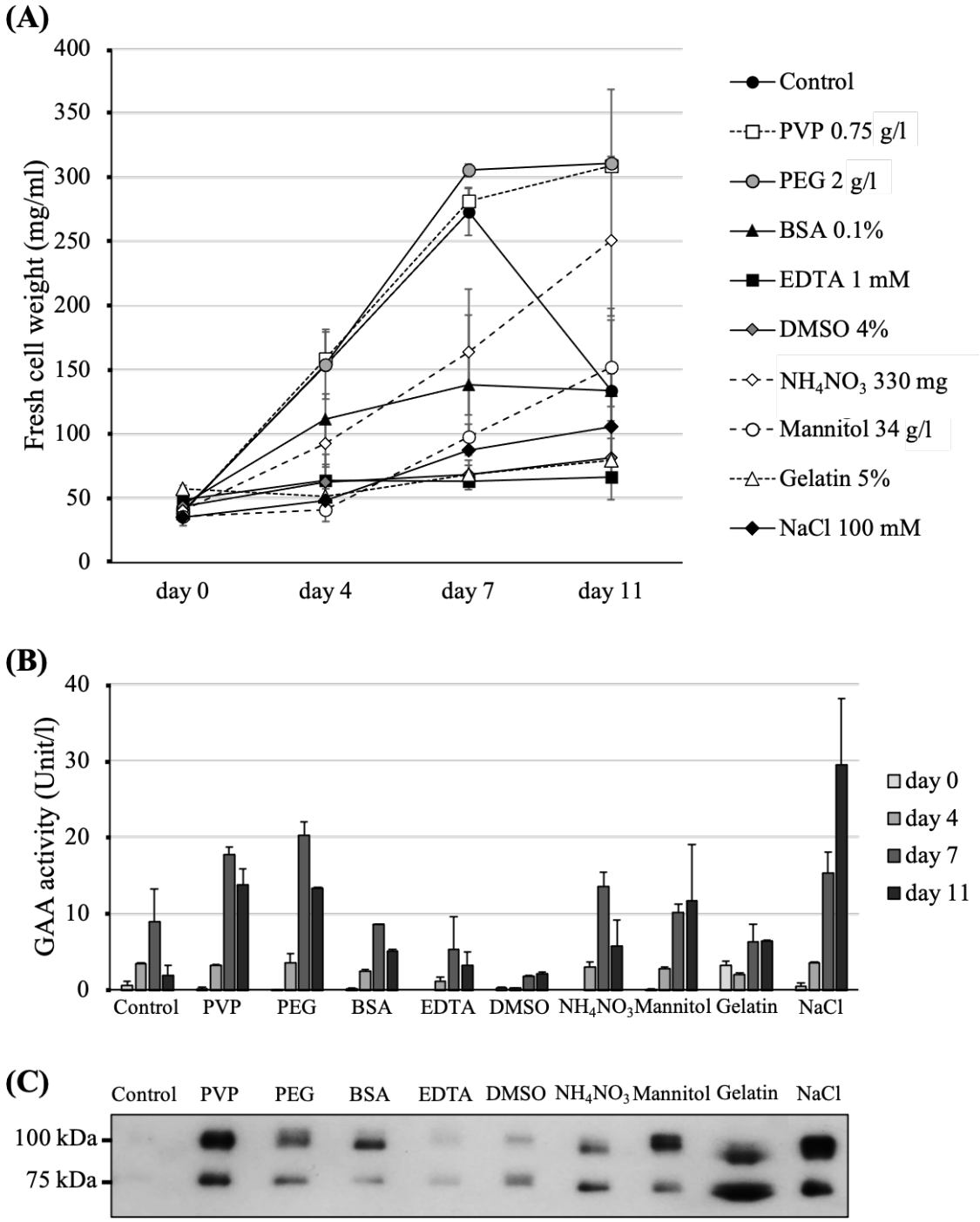


Figure 10. Effects of chemical additives on the cell growth of GAA-producing Arabidopsis *alg3* cell culture (A) as well as on GAA production as evaluated by a GAA activity assay (B) and Western blotting using anti-GAA antibody (C). Data of Fig. 10A are average of three independent replications. The media of 11-day-old cell cultures are used for the Western blot. Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad) is used as a protein marker in the Western blot.

Compared to the control, samples from the media with 0.75 g/l PVP, 2 g/l PEG, additional 330 mg (61.8 mM) NH_4NO_3, 34 g/l (186.6 mM) mannitol, and 100 mM NaCl

exhibited higher GAA activities both at 7 and 11 days of culture. While cell cultures with 0.1% BSA and 5% gelatin showed higher activities than the control only at 11 days of culture. The highest GAA activity was shown in cell culture containing 100 mM NaCl (Figure 10B) at 11 days of culture. These results were confirmed by Western blot analysis showing GAA bands for the 11-day-old culture media (Figure 10C), except in the gelatin-supplemented cell culture. Western blot analysis of the gelatin-supplemented cell culture showed thick band, indicating that gelatin improved the stability of secreted GAA. However, the activity assay showed low activity, suggesting that the presence of gelatin hindered the assay. Because gelatin supplementation greatly inhibited the cell growth, caused viscous medium solution, and would become a contaminant protein in the purification step, gelatin was not used in further optimization. Instead, NaCl was chosen for further optimization to achieve the highest GAA production in the medium.

3.3.4. Selection of an NaCl concentration for optimum GAA production

The presence of 100 mM NaCl in the MS medium dramatically increased GAA accumulation in the medium, but also severely reduced the Arabidopsis cell growth. To identify the optimum NaCl concentration to obtain high GAA production while maintaining the cell growth, the GAA-producing Arabidopsis *alg3* cell culture was grown in MS media containing 0 (control), 25, 50, 100, and 150 mM NaCl. The results showed that cell growth was severely inhibited in the presence of 100 mM NaCl or more, and could be maintained in presence of 25 and 50 mM NaCl (Figure 11A). Meanwhile, the highest GAA activity was observed in the medium sample from 11-day-old culture grown in MS containing 50 mM NaCl (Figure 11B) as confirmed by Western blot data showing the thickest band on this sample (Figure 11C).

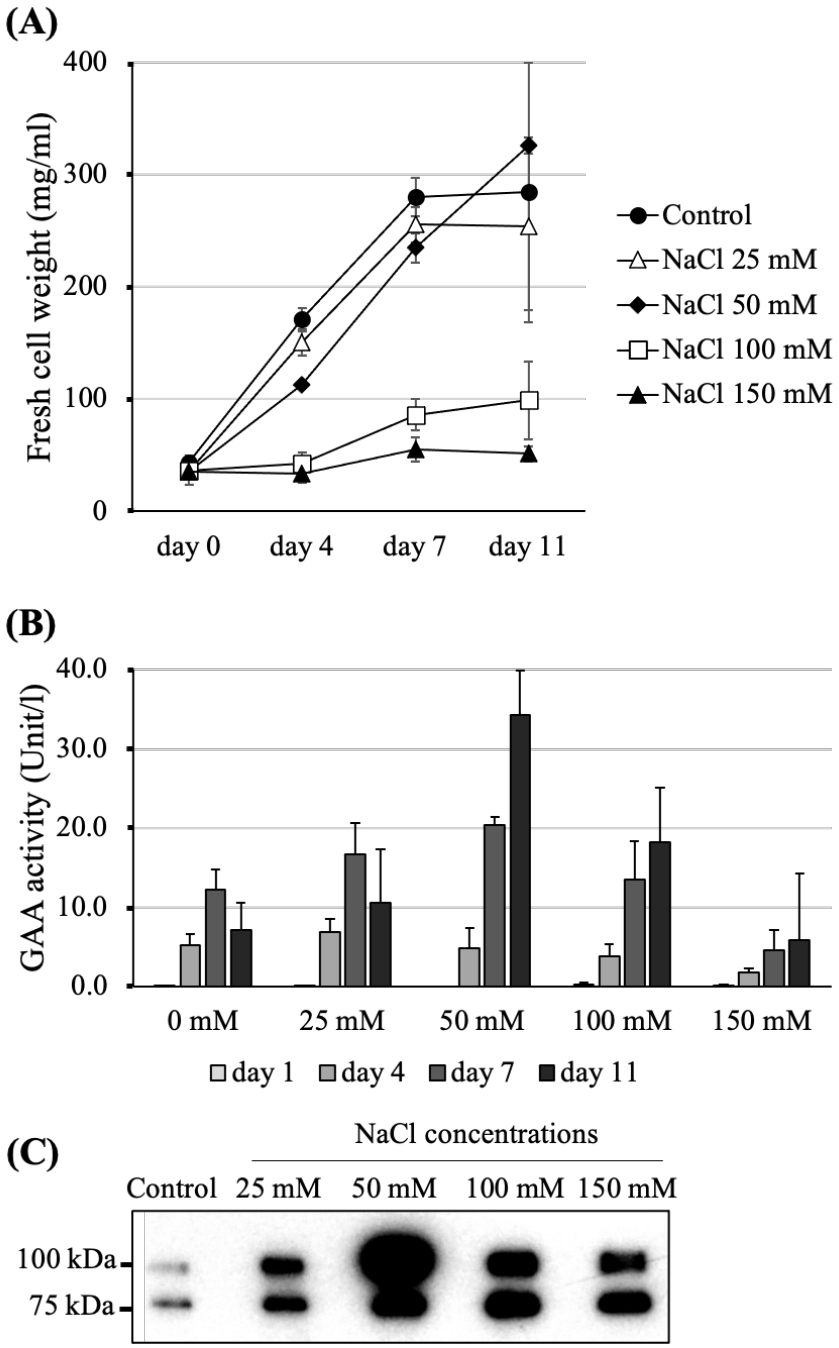


Figure 11. Optimization of the NaCl concentration for highest GAA production while maintaining Arabidopsis *alg3* cell growth. (A) Effects of addition of 0-150 mM NaCl on the cell growth of GAA-producing Arabidopsis *alg3* cell culture. Data are average of three independent replications. (B, C) Evaluation of GAA accumulation in the NaCl-supplemented cell culture medium by GAA activity assay and Western blot using anti-GAA antibody, respectively. The media of 11-day-old cell cultures are used for the Western blot. Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad) is used as a protein marker in the Western blot.

To elucidate the reason for the higher GAA level in the medium of NaCl-supplemented cell culture, the intracellular GAA production was tested. Interestingly, GAA activity assay and Western blot of the intracellular sample indicated increased GAA production with each increment of NaCl concentration (Figure 12). This suggested that the enhanced GAA accumulation in the medium resulted from increased intracellular GAA production. However, because the Arabidopsis cell growth was severely inhibited in the presence of 100 or 150 mM NaCl, the total GAA accumulation in the media of cell cultures supplemented with 100 or 150 mM NaCl was also low.

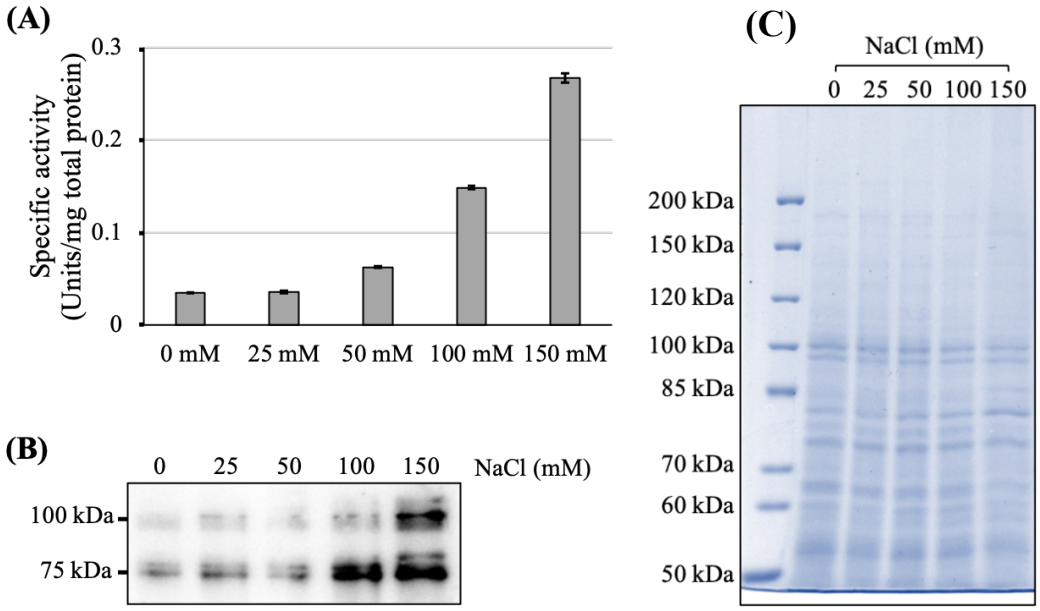


Figure 12. Effects of NaCl supplementation on intracellular GAA production. (A) Intracellular GAA-specific activity in 7-day-old cell cultures supplemented with various amounts of NaCl (0-150 mM). (B) Western blot of crude intracellular proteins of NaCl-supplemented 11-day-old culture cells using anti-GAA antibody. (C) CBB staining as a loading control. Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad) and PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific) are used as protein markers in the Western blot and CBB staining, respectively.

GAA produced in NaCl-supplemented cell culture was purified using the same purification scheme as the control. After purification, the NaCl-supplemented cell culture resulted in 3.8-times higher GAA production (228.15 ± 21.10 μg/l) than the control (60.91

± 12.38 μg/l) (Figure 13A), while the GAA purity was similar to that in the control (Figures 13B and C). Notably, the degraded GAA band that appeared between the 95 and 76 kDa GAAs was less visible in the purified GAA from the NaCl-supplemented cell culture (Figure 13B, marked by an asterisk), suggesting that the NaCl supplementation helped protect GAA from degradation.

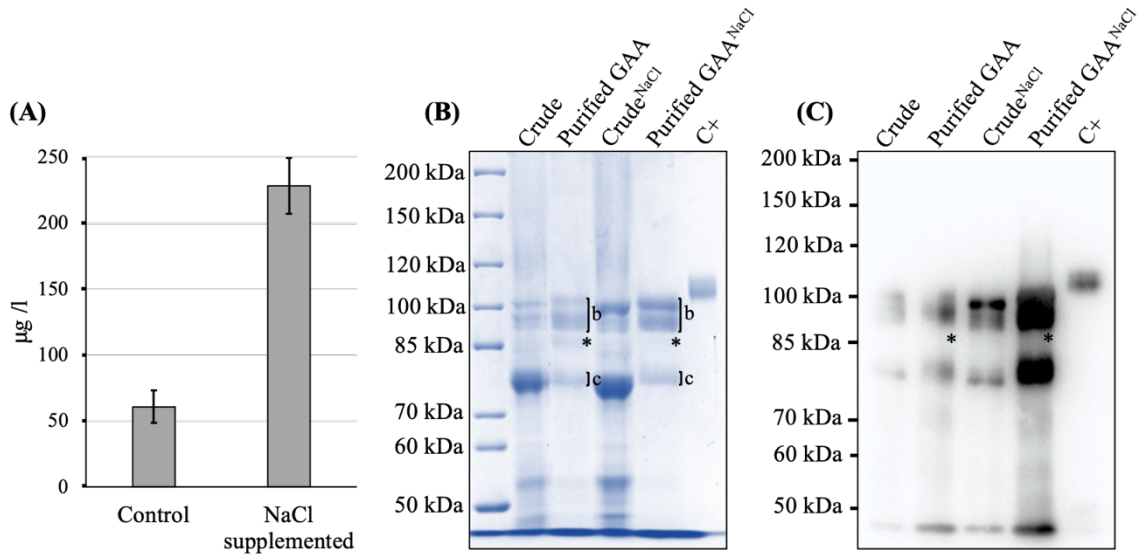


Figure 13. Analysis of GAA purification from NaCl-supplemented cell culture. (A) GAA productivity from cell cultures with and without NaCl supplementation. (B, C) Analysis of purified GAAs from cell cultures with and without NaCl supplementation by CBB staining and Western blot using anti-GAA antibody. Marks b and c indicate the 95 kDa intermediate and 76 kDa mature GAAs. These bands are excised and used for *N*-glycan analysis using nanoLC-MS/MS. An asterisk (*) indicates a degraded GAA, which is also excised and analyzed using nanoLC-MS/MS (Supplementary Figure 1 and Supplementary Table 1). PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific) is used as a protein marker.

3.3.5. *N*-Glycan profiles of GAA produced in Arabidopsis *alg3* cell culture with and without NaCl supplementation

The presence of plant-specific *N*-glycans in the crude and purified GAAs was examined by Western blot using anti-HRP polyclonal antibody, which contains antibodies against peptide epitope of HRP as well as plant complex *N*-glycans (i.e., α1,3-Fuc and β1,2-

Xyl) (Faye *et al*., 1993; Bencúrová *et al*., 2004). The result showed that Arabidopsis *alg3* cell cultures (both GAA-producing and nonproducing) produced only low amount of plant-specific *N*-glycans, similar to the result described in Chapter 2. Notably, the Western blot data showed no signal in the sizes of GAA, suggesting that the GAA lacked neither α1,3-Fuc nor β1,2-Xyl residues (Figure 14).

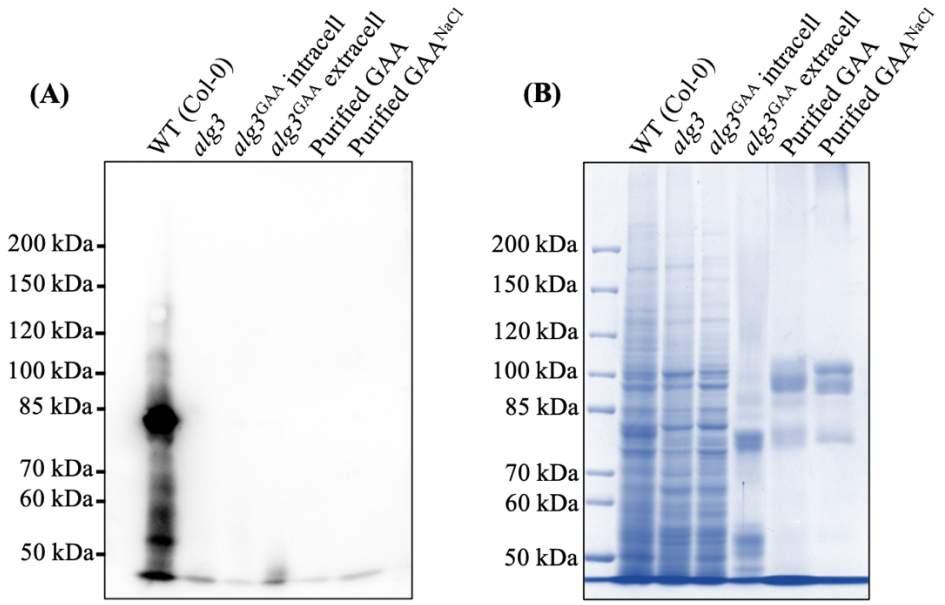


Figure 14. Detection of plant-specific *N*-glycans in Arabidopsis *alg3* cell culture and purified GAA from the cell cultures with and without NaCl supplementation. (A) Western blot analysis using anti-HRP to detect plant specific *N*-glycans. (B) CBB staining as a loading control. PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific) is used as a protein marker.

Constituent glycoforms of each *N*-glycosylation site in GAA were further analyzed by *de novo* sequencing of the tryptic GAA peptides using nanoLC-MS/MS analysis. The glycopeptides bearing the sequence of *N*390 (^{386}QVVENMTR^{393}), *N*470 (^{465}GVFITNETGQPLIGK^{479}), and *N*882 (^{882}NNTIVNELVR^{891}) were successfully characterized. The majority of the *N*-glycans on these sites, both in the 95 and 76 kDa GAAs, were composed of M3 structure which accounted for 51.1-80.1% of total *N*-glycan variants (Figure 15, Table 6). Plant-specific α1,3-Fuc and β1,2-Xyl residues were not observed in any of the sites. Meanwhile, GAA produced in the NaCl-supplemented cell culture also

showed a similar composition, indicating that supplementing the cell culture with NaCl at a concentration of 50 mM did not affect GAA *N*-glycosylation. Moreover, the *N*-glycans of the degraded GAA (marked with a star in Figure 9A) was also characterized, and the profile was similar to the 95 and 76 kDa GAAs (Supplementary Figure 1, Supplementary Table 1).

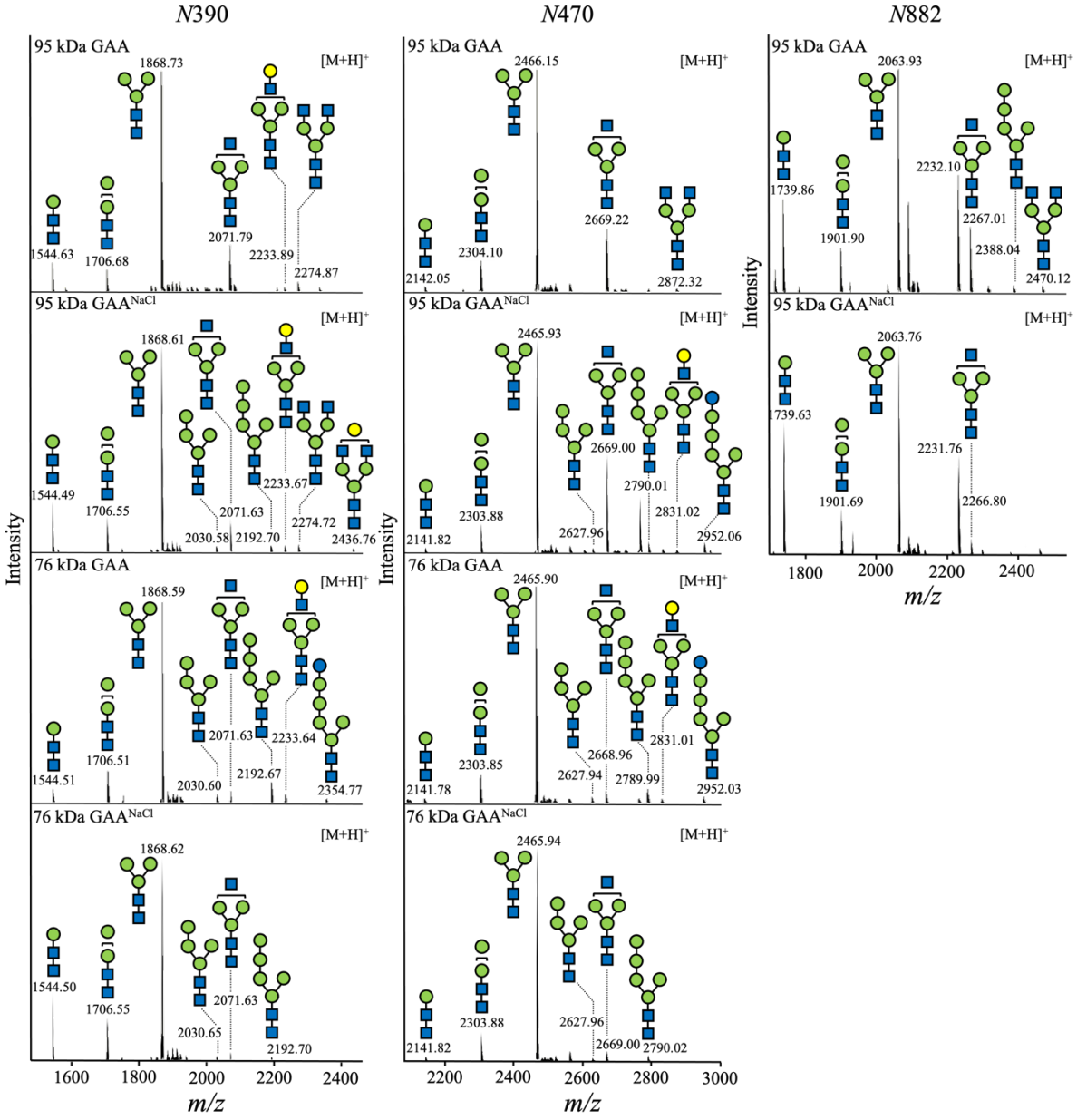
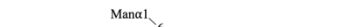
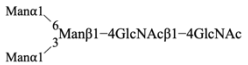
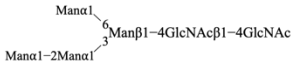
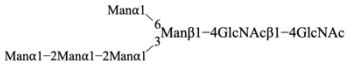
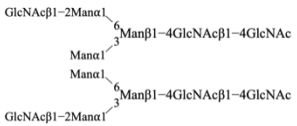
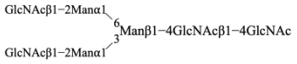
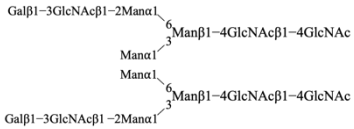
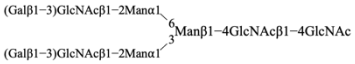


Figure 15. *N*-Glycan profile of GAA purified from the media of 11-day-old GAA-producing Arabidopsis *alg3* cell cultures with and without NaCl supplementation. In the shown deconvoluted MS spectra, the mass $[M + H]^+$ of the tryptic glycopeptides carrying *N*-glycosylation sites (*N*390, *N*470, and *N*882) is depicted. Blue square (■), green circle (●), yellow circle (●), and blue circle (●) indicate GlcNAc, mannose, galactose, and glucose, respectively.

Table 6. Composition of the *N*-glycan structures in GAA produced in Arabidopsis *alg3* cell cultures with and without NaCl supplementation.

Abbreviation	Structure	Relative amount (%)									
		95 kDa GAA			76 kDa GAA		95 kDa GAA^{NaCl}			76 kDa GAA^{NaCl}	
		*N*390	*N*470	*N*882	*N*390	*N*470	*N*390	*N*470	*N*882	*N*390	*N*470
M1		8.7	1.5	21.2	4.4	1.4	14.7	1.2	33.0	30.1	1.6
M2		6.4	9.5	10.1	11.4	9.5	10.2	7.6	11.3	13.2	11.2
M3		68.0	68.2	51.1	67.5	75.5	56.6	57.8	51.8	54.0	80.1
M4^{ER}		-	-	-	2.6	1.8	1.6	1.1	-	0.9	1.1
M5^{ER}		-	-	1.5	6.6	4.5	1.4	3.1	-	0.6	2.3
GlcM5^{ER}		-	-	-	1.2	2.0	-	2.6	-	-	-
GnM3		12.6	20.0	14.3	3.4	4.0	10.6	25.7	3.9	1.2	3.7
Gn2M3		2.7	0.8	1.8	-	-	2.2	-	-	-	-
GalGnM3		1.6	-	-	2.9	1.3	2.0	0.9	-	-	-
GalGn2M3		-	-	-	-	-	0.7	-	-	-	-
	Total	100	100	100	100	100	100	100	100	100	100

3.4. Discussion

The only previous study using *alg3* mutant to produce a recombinant protein was reported a decade ago, where a single chain Fv-Fc antibody MBP10 was produced in an Arabidopsis *alg3* mutant plant (Henquet *et al*., 2011). However, the antibody was fused with a KDEL ER retention signal so that the antibody accumulated in the ER and carried mostly $M5^{ER}$ *N*-glycan. Conversely, in this study, recombinant human GAA was produced in an Arabidopsis *alg3* cell culture without any ER retention signal, and thus the fate of the recombinant protein in the *alg3* mutant cells could be better understood.

GAA is a human lysosomal enzyme known to encounter sequential proteolytic processing and *N*-glycosylation for enzyme maturation (Moreland *et al*., 2005; Figure 3). In humans, GAA is primarily synthesized as a polypeptide of 952 amino acids, which contains an *N*-terminal signal peptide for transport into the ER. The signal peptide is cleaved by the host signal peptidase, generating a 110 kDa GAA precursor, and followed by sequential proteolytic cleavages on the *N*- and *C*-termini to generate the 95 kDa intermediate and 76 kDa mature forms (Figure 3). Finally, a protease cleaves the *N*-terminus, bringing on the 70 kDa mature GAA (Moreland *et al*., 2005). These sequential proteolytic processes are important for GAA activity since the 76/70 kDa mature forms exhibit 7-10 times greater affinity to the substrate than the 110 kDa precursor (Wisselaar *et al*., 1993; Bijvoet *et al*., 1998). Yet, the proteases responsible for these processes are still unknown. In this study, the Arabidopsis *alg3*-produced GAA was present as three forms corresponding to the 110, 95, and 76 kDa GAA forms (Figure 8C), suggesting that the Arabidopsis *alg3* cells were capable of performing similar processing to that in the human cells. However, the 70 kDa form was not observed, indicating that the essential protease is absent in Arabidopsis cells. Furthermore, the amount of the 95 kDa GAA seemed to be higher than the 76 kDa GAA. To optimize the GAA production in plant cell culture, it will be necessary to identify the

proteases responsible for the cleavages so that it will become possible to increase the production of mature GAA by co-expressing the human protease(s) *in vivo* or by performing the protease cleavage *in vitro*.

Previous studies have reported the production of recombinant human GAA in plant cells, including tobacco plant, rice cell culture, and moss cell culture (Table 1). In those studies, however, GAAs were constructed under plant-derived signal peptides to direct plant organ-specific localization or secretion into the cell culture medium. In this study, the native human GAA signal peptide was used. Surprisingly, the results of this study indicated that the Arabidopsis cells were capable of recognizing and processing the human GAA signal peptide, as shown by the presence of the cleaved GAA polypeptide of 110 kDa protein (Figure 8C). This suggests that the human GAA signal peptide could facilitate GAA entrance into the ER, followed by transport into the Golgi apparatus and, finally, secretion into the plant cell culture medium.

The Arabidopsis *alg3*-produced GAAs had a predominantly M3 structure (51.1-80.1%). Moreover, anti-HRP antibody could not detect any plant-specific *N*-glycans in GAA, although a low amount of plant-specific *N*-glycans were detected in the cell culture (Figure 14). This suggests that *N*-glycosylation depends on the protein. The lack of plant-specific *N*-glycans in the GAA would eliminate the risk of allergic reaction upon injection to patients with Pompe disease. Furthermore, galactose-terminal *N*-glycans were also detected (0.7-2.9%). This galactosylation resulted from the activity of β1,3-galactosyltranferase resident in the Golgi (Fitchette *et al*., 1999; Strasser *et al*., 2007). Detection of this residue on GAA *N*-glycans confirms that GAA is transported to the Golgi apparatus prior to secretion into the plant cell culture medium.

Previous studies have demonstrated the benefits of chemical additives to increase the production of recombinant proteins in plant cell culture; however, the outcome seems to be

dependent on the target protein and plant species. For instance, PVP was reported to improve the secretion of recombinant heavy-chain monoclonal antibody in *N. tabacum* NT-1 suspension cell culture (LaCount *et al*., 1997; Magnuson *et al*., 1998; Hellwig *et al*., 2004), however, Lee *et al*. (2002) did not find any effect of PVP in the production of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) in *N. tabacum* cv Havana SR1 suspension cell culture. That is why medium optimization is needed for each product and cell line on a case-by-case basis. In this study, several chemicals were shown to increase GAA production, including PVP, PEG, NH_4NO_3, mannitol, and NaCl. Supplementation with 50 mM NaCl achieved the highest production while maintaining cell growth, and eventually resulting in 3.8-fold higher productivity than the control.

The improved GAA production intra- and extracellularly of NaCl-supplemented cell culture could be attributed to the stabilizing effect of NaCl, which protects GAA from degradation, as indicated by the lower intensity of a degraded GAA band as compared to the control (Figures 13B). The natural environment of GAA in human cells is in the lysosome, which is known to contain ~108 mM chloride ion (Saha *et al*., 2015). Thus, it is possible that GAA is more stable in the NaCl-supplemented culture because that environment more closely resembles *in vivo* lysosomal condition. The similar effect was reported in the production of hGM-CSF (James *et al*., 2000). Moreover, the 3.8-fold increase of GAA accumulation in NaCl-supplemented cell culture medium might result from enhanced GAA secretion. This hypothesis is based on a previous study showing that salt (e.g., NaCl) triggers the generation of reactive oxygen species (ROS), which attacks membrane lipoproteins and causes impaired membrane permeability (Mansour, 2013), thus facilitating the secretion of intracellular proteins. However, it remains unknown whether this mechanism also occurs in the case of GAA secretion. Notably, GAA produced in NaCl-supplemented cell culture carried similar *N*-glycan profiles to those in the control, indicating that NaCl

supplementation to a final concentration of 50 mM does not affect *N*-glycosylation in the Arabidopsis cells. These results indicate that NaCl supplementation can be an economic, easy, and effective way to increase GAA production in the *alg3* cell culture.

The GAA productivity in this study is much lower than previous studies in rice cell cultures (Table 7). Nevertheless, the rice cell-produced GAA contains His-tag, which potentially induces immunogenic reaction upon administration in humans (Singh *et al*., 2020). Thus, although the quantity of GAA production in this study is lower than previous ones, however, the quality of the *alg3* cells-produced GAA is considerably better by offering: (1) absence of potentially immunogenic plant-specific *N*-glycans and purification tag, and (2) M3 structure to mediate alternative cellular delivery through MR. Moreover, the profile of GAA *N*-glycan in this study share some similarities to recent reports of GAA production in glycoengineered moss cells (Table 7). However, detailed information of the productivity and relative ratios of the *N*-glycan structures in moss-produced GAA are not reported, thus it is difficult to compare.

Finally, GAA bearing M3 structure was successfully produced in this study. Previous studies have highlighted the merit of using M3 for MR-mediated cellular delivery (Van Patten *et al*., 2007; Shen *et al*., 2016). The success of this study emphasizes the potential of the Arabidopsis *alg3* cell culture for producing other recombinant proteins for which M3 and/or MR-mediated delivery is required, such as in the enzyme replacement therapies for Gaucher disease, Fabry disease, Wolman disease, and cholesteryl ester storage disease (Du *et al*., 2005; Limkul *et al*., 2016; Shen *et al*., 2016).

Table 7. GAA productions and glycoengineering approaches in plant cell cultures

Cell culture conditions	Productivity		*N*-glycans	Plant-specific *N*-glycans	References
	Crude	Purified			
Arabidopsis					
alg3	0.5 mg/l	60.9 μg/l	Mainly M3 (51.1-75.5%)	not detected	This study
alg3 + NaCl	2.4 mg/l	228.2 μg/l	Mainly M3 (51.8-80.1%)	not detected	This study
Rice					
wild-type	37 mg/l	unknown	Plant-type structures, mainly Gn2M3XF (40.7%)	abundant	Jung *et al*., 2016
gntI	45 mg/l	unknown	Oligo-Man, mainly M5 (89.6%)	not detected	Jung *et al*., 2017
+ kifunensine + swainsonine	24-40 mg/l	unknown	High-Man types (81.8%), mainly M9 (60.4%)	reduced	Choi *et al*., 2018
+ 100 μM 2-FF + 0.5% PF-68	unknown	unknown	Plant-type structures with reduced core fucosylation	reduced	Kim *et al*., 2020
Moss					
xylt fuct	unknown	unknown	Gn2M3 (relative ratio is not reported)	not reported	Hintze *et al*., 2020
xylt fuct + β-*N*-acetylglucosaminidase	unknown	unknown	M3 (relative ratio is not reported)	not reported	Hintze *et al*., 2020
gntI	unknown	unknown	M5 (relative ratio is not reported)	not reported	Hintze *et al*., 2020

3.5. Summary

Plant cell cultures have emerged as a promising platform for the production of biopharmaceuticals due to their cost-effectiveness, safety, ability to control the cultivation and secrete products into cell culture medium. However, the use of this platform is hindered by the generation of plant-specific *N*-glycans, the inability to produce essential *N*-glycans for cellular delivery of biopharmaceuticals, and low productivity. In this study, an alternative acid-alpha glucosidase (GAA) for enzyme replacement therapy of Pompe disease was produced in a Arabidopsis *alg3* cell culture. The *N*-glycan composition of the GAA consisted of a predominantly a paucimannosidic structure, $Man_3GlcNAc_2$ (M3) structure (51.1-80.1%), without the plant-specific *N*-glycans. Supplementing the cell culture medium with NaCl to a final concentration of 50 mM successfully increased GAA production by 3.8-fold. GAA from an NaCl-supplemented cell culture showed a similar *N*-glycan profile, indicating that the NaCl supplementation did not affect *N*-glycosylation. The results of this study highlight the feasibility of using an Arabidopsis *alg3* cell culture to produce recombinant proteins for which M3 or MR-mediated delivery is desired.

Chapter 4 General Conclusions and Perspectives

Plant cell cultures have emerged as a potential platform for production of biopharmaceuticals. However, differences between plant and human *N*-glycans have been the limiting factor in using plant cell cultures for producing biopharmaceutical glycoproteins for human treatments. Whereas the *N*-glycan profile greatly affects protein characteristics and pharmacodynamics upon administration to humans. In this study, an Arabidopsis *alg3* cell culture was shown to produce *N*-glycans with predominantly M3 as well as GnM3 structures, both intra- and extracellularly (Tables 2 and 4). The M3 structure is normally scarce in plants and known to enable cellular uptake of glycoproteins via MR in humans. Interestingly, the amount of plant-specific *N*-glycans in the Arabidopsis *alg3* cell culture was very low (Figure 4C), which was even undetectable in the LC-MS/MS analysis (Tables 2 and 4). The lack of plant-specific *N*-glycans is valuable to prevent potential immunogenic reaction upon administration of the therapeutic glycoproteins to humans.

As a proof of the concept, a human acid-alpha glucosidase (GAA) was produced in the Arabidopsis *alg3* cell culture. As expected, the enzyme carried predominantly M3 structure (51.1-80.1%) and lacked the plant-specific *N*-glycans (Table 6). The production of GAA in the *alg3* cell culture was increased 3.8-fold by simply supplementing the cell culture with 50 mM NaCl. GAA from an NaCl-supplemented cell culture showed a similar *N*-glycan profile, indicating that the NaCl supplementation did not affect *N*-glycosylation. These results highlight the feasibility of using Arabidopsis *alg3* cell culture in producing recombinant proteins for which M3 or MR-mediated delivery is desired.

The level of GAA production in this study was relatively low as compared to previous studies of GAA production in plant cells (Tables 1 and 7). The low level of production could be the result of ER stress as previously reported in Arabidopsis *alg3* plant (Henquet *et al*.,

2008, 2011; Kajiura *et al*., 2010). For further work, it may be possible to increase GAA production by co-expressing chaperon proteins to ameliorate the ER stress (Margolin *et al*., 2020). Moreover, the GAA production may be improved by combining with other methods known to enhance production of recombinant proteins in plant cells, including suppression of gene silencing (Papp *et al*., 2003; Butaye *et al*., 2004; Jeong *et al*., 2018), use of more vigorous expression system (e.g., use of geminiviral replication, double promoter and terminator) (Yamamoto *et al*., 2018), and transient expression approach (e.g., plant-cell pack technology) (Rademacher *et al*., 2019).

Interestingly, GAA produced in Arabidopsis *alg3* cell cultures seems to undergo similar processing to those in the human cells as indicated by generation of the 110, 95, and 76 kDa proteins (Figures 3 and 8C). To confirm this result, *N*-terminal sequencing by Edman chemistry or peptide mapping by LC-MS/MS would be needed.

This study showed that Arabidopsis *alg3* cell culture and a recombinant GAA produced in this cell culture generated more homogenous *N*-glycoforms of predominantly M3 structure and lacking the plant-specific *N*-glycans. However, low amounts of GlcNAc-terminal structures and plant-specific *N*-glycans were still generated. For further work, it would be preferrable to knock-out/down *gnt-I* gene so that the homogeneity of M3 structure will be increased and the generation of plant-specific *N*-glycans can be completely abolished, and therefore ensuring the safety of the glycoproteins produced using this cell culture for human therapy. Moreover, to check whether the enzyme could be delivered through MR, further study will be needed to test the pharmacokinetics of the GAA in Pompe disease cells or animal model, especially to examine the uptake by skeletal and smooth muscle cells.

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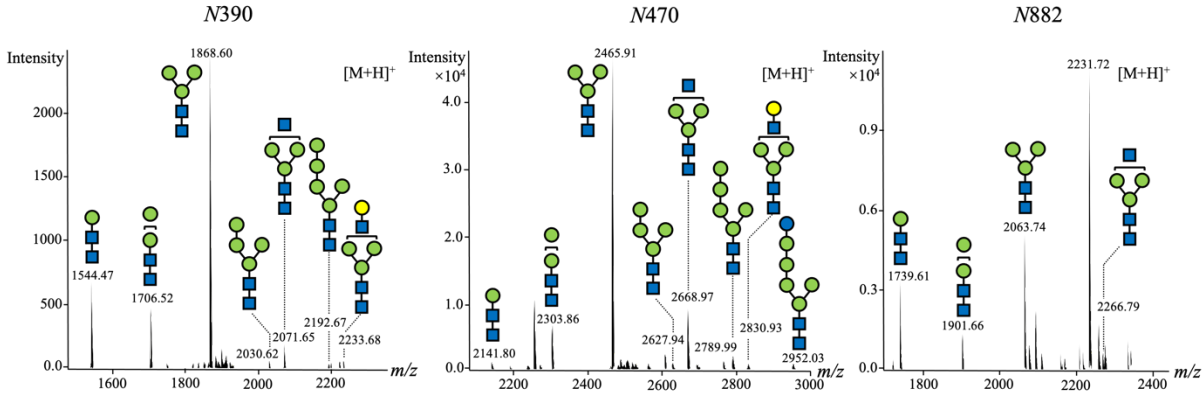
Supplementary Materials

1. Characterization of a degraded GAA fragment

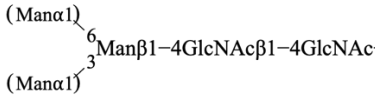
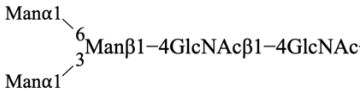
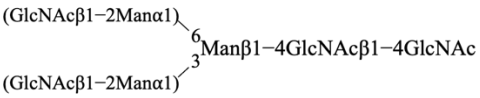
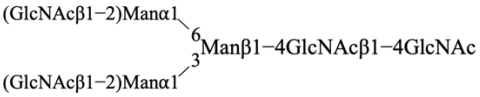
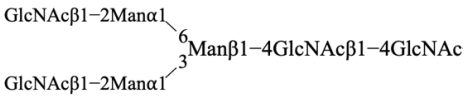
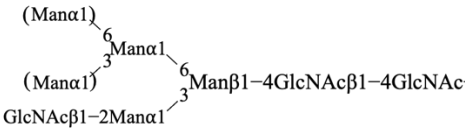
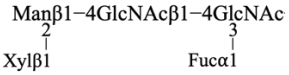
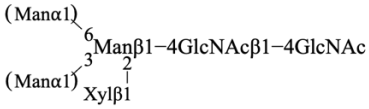
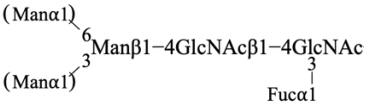
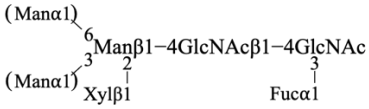
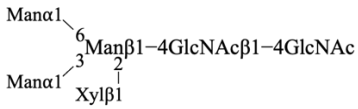
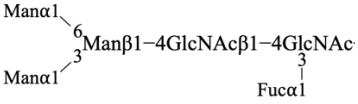
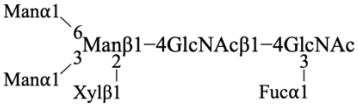


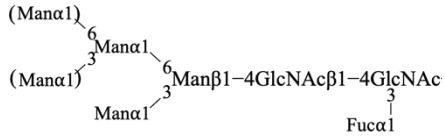
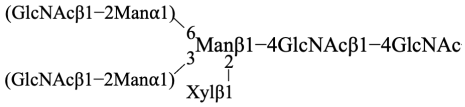
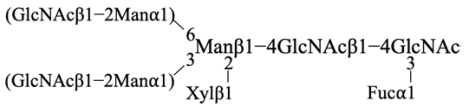
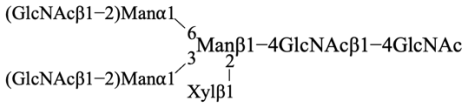
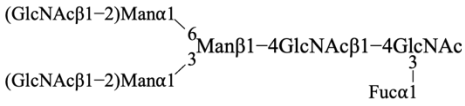
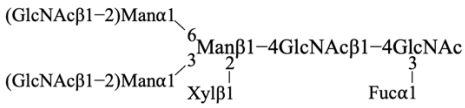
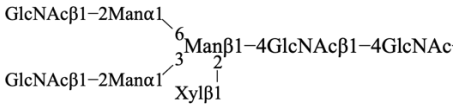
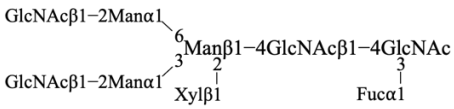
Supplementary Figure 1. Glycoforms of glycopeptides with *N*-glycosylation sites *N*390, *N*470, and *N*882 from the degraded GAA. In the shown deconvoluted MS spectra, the mass $[M + H]^+$ of the tryptic glycopeptides is depicted. Blue square (■), green circle (●), and yellow circle (●) indicate GlcNAc, mannose, and galactose, respectively.

Supplementary Table 1. Composition of *N*-glycans attached on the degraded GAA.

Abbreviation	Structure	Relative amount (%)		
		*N*390	*N*470	*N*882
M1	Manβ1−4GlcNAcβ1−4GlcNAc	16.9	1.7	31.2
M2	Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1 3 Manβ1−4GlcNAcβ1−4GlcNAc	11.7	10.5	12.2
M3	Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1 3	62.9	72.4	50.1
M4^{ER}	Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1−2Manα1 3	1.5	-	-
M5^{ER}	Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1−2Manα1−2Manα1 3	0.9	-	-
GnM3	GlcNAcβ1−2Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1 3 Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc GlcNAcβ1−2Manα1 3	4.5	14.3	6.5
GalGnM3	Galβ1−3GlcNAcβ1−2Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Manα1 3 Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc Galβ1−3GlcNAcβ1 −2Manα1 3	1.6	1.1	-
Gn2M3	GlcNAcβ1−2Manα1 6 Manβ1−4GlcNAcβ1−4GlcNAc GlcNAcβ1−2Manα1 3	-	-	-
	TOTAL	100	100	100

Supplementary Table 2. Graphical structures of *N*-glycans described in Table 4.

Abbreviation	Structure
M1	
M2	
M3	
GnM2	
GnM3	
Gn2M3	
GnM4	
Plant-specific	
MXF	
M2X	
M2F	
M2XF	
M3X	
M3F	
M3XF	

M4F	
GnM2X	
GnM2XF	
GnM3X	
GnM3F	
GnM3XF	
Gn2M3X	
Gn2M3XF	

List of publication

Sariyatun, R., Florence, Kajiura, H., Ohashi, T., Misaki, R., Fujiyama, K. (2021). Production of human acid-alpha glucosidase with a paucimannose structure by glycoengineered *Arabidopsis* cell culture. *Front Plant Sci* 12, 703020. doi: 10.3389/fpls.2021.703020.

Sariyatun, R., Kajiura, H., Limkul, J., Misaki, R., Fujiyama, K.. Analysis of *N*-glycan profile of Arabidopsis *alg3* cell culture. Submitted to *Plant Biotechnol* (Manuscript ID: PB-R-0846).

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