



Title	CRISPR/Cas9-mediated knockouts of ALG3 and GNTI in <i>Nicotiana benthamiana</i> lead to predominant mannosidic N-glycans lacking plant-specific epitopes
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Doctoral Dissertation

CRISPR/Cas9-mediated knockouts of *ALG3* and *GNTI* in *Nicotiana benthamiana* lead to predominant mannosidic *N*-glycans lacking plant-specific epitopes

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September 2025

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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."

.....

BATAA DOLGORMAA

2025

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List of abbreviations

ALG1	β 1,4-Mannosyltransferase
ALG10	α 1,2-Glucosyltransferase
ALG11	α 1,2 Mannosyltransferase
ALG12	α 1,6-Mannosyltransferase
ALG13/14	UDP- <i>N</i> -acetylglucosamine transferase
ALG2	α 1,3/1,6-Mannosyltransferase
ALG3	α 1,3-Mannosyltransferase
ALG6	α 1,3-Glucosyltransferase
ALG8	α 1,3-Glucosyltransferase
ALG9	α 1,2-Mannosyltransferase
BA	6-Benzylaminopurine
BEV	Baculovirus expression vector
BSA	Bovine serum albumin
Cas9	CRISPR-associated protein 9
CBB	Coomassie brilliant blue
CD	Cluster of Differentiation
CDG	Congenital disorder of glycosylation
CHO	Chinese hamster ovary
ConA	Concanavalin A
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DNA	Deoxyribonucleic acid
DPAG1	UDP- <i>N</i> -acetylglucosamine-dolichyl-phosphate <i>N</i> -acetylglucosaminophosphotransferase
EDTA	Ethylenediaminetetraacetic acid
EMA	European medicines agency
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ERT	Enzyme replacement therapy

FDA	Food and Drug Administration
FUCT	α 1,3-Fucosyltransferase
GA	Gibberellic acid
GALT	β 1,3-Galactosyltransferase
GCase	β -Glucocerebrosidase
GCSI	Glucosidase I
GCSII	Glucosidase II
GM-II	Golgi α -mannosidase II
GNTI	<i>N</i> -acetylglucosaminyltransferase I
GNTII	<i>N</i> -acetylglucosaminyltransferase II
gRNA	Guide RNA
HEK	Human embryonic kidney
hGH	Human growth hormone
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
LB	Left border of T-DNA
LB medium	Luria-Bertani medium
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LeA	Lewis A epitope
LSD	Lysosomal storage disorder
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MNS3	Endoplasmic reticulum mannosyl-oligosaccharide α -1,2-mannosidase
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MNS1/2	α 1,2 Mannosidase 1/2
MS medium	Murashige and Skoog medium
NAA	Naphthaleneacetic acid
NK cells	Natural killer cells
NP-40	Nonyl phenoxypolyethoxylethanol
OST	Oligosaccharyltransferase

PA	2-Aminopyridine
PDI	Protein disulfide isomerase
PER C6	Primary human embryonic retinal cell
PMB	Plant-made biopharmaceutical
PNGase F	Peptide- <i>N</i> -glycosidase F
PPM TM	Plant preservative mixture
PVDF	Polyvinylidene fluoride
RB	Right border of T-DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed phase high performance liquid chromatography
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
T-DNA	Transferred-DNA
TFA	Trifluoroacetic acid
tracrRNA	Trans-activating CRISPR RNA
VLP	Virus-like particles
WT	Wild-type
XYLT	β 1,2-Xylosyltransferase

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Chapter One: General Introduction

1.1 Historical key points in the biopharmaceutical industry

The biopharmaceutical industry has revolutionized healthcare and medicine, providing innovative therapies that have improved the lives of millions worldwide. The roots of the biopharmaceutical industry can be traced back to the late 19th and early 20th centuries when pivotal scientific discoveries took place. Louis Pasteur and Robert Koch were the influential figures of the medical microbiology in late 1800s. Their work led to the first commercial production of vaccines for diseases like rabies and diphtheria, marking a significant step towards the industrialization of biopharmaceuticals (Lakhtakia, 2014; Pearce, 2002). The huge jump in the biopharmaceutical industry came in the 1970s with the biotechnology. The discovery of recombinant DNA technology by Paul Berg and the construction of bacterial plasmids by Stanley Cohen and Herbert Boyer opened up entirely new possibilities. These breakthroughs allowed scientists to manipulate DNA, creating genetically engineered organisms capable of producing recombinant biopharmaceuticals (Baltimore, 2023; Cohen *et al.*, 1973). The approval of the first biotechnology-derived drug, human insulin was the next achievement. In 1982, Humulin, the first commercially available recombinant human insulin was introduced, reducing the risk of allergic reactions and enhancing patient outcomes. The success of insulin encouraged the production of the other biopharmaceutical products (Hartman *et al.*, 1996; Lewis and Brubaker). Another advancement was the discovery of hybridoma technology by César Milstein and Georges Köhler in 1975, which allowed the production of monoclonal antibodies (mAbs). mAbs revolutionized diagnostics and therapeutics, leading to the approval of drugs like Rituximab, Trastuzumab, and Infliximab for various diseases, including cancer and autoimmune disorders (Leavy, 2016). As the pharmaceutical industry flourished, regulatory agencies were established to assess pharmaceutical safety and efficacy.

The main examples of these regulatory agencies are The U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), Canada's Food and Drugs Act and Regulations and Pharmaceutical and Medical Devices Agency in Japan. As the industry grows, they face new challenges to adapt their protocols for novel products and platforms.

1.2 Current trends of biopharmaceutical industry and its production hosts

Biopharmaceutical production is a major area in biotechnology, involving the use of various host organisms to produce biopharmaceuticals. These proteins are essential for treating numerous diseases and conditions, and their efficient production is vital for meeting fast expanding global healthcare needs. Different host systems, including mammalian cells, microbial cells, insect cells, plants, plant cells and transgenic animals, have been utilized to produce these proteins, each with its own advantages and challenges. According to the latest Biopharmaceutical benchmarks 2022, total 435 distinct active biopharmaceuticals which are approved in the United States or European Union are currently on the market (Walsh and Walsh, 2022). Between 2018-2022, 188 new biopharmaceuticals entered the market which counts to more than 2/5 of the total. This highlights the rapid research efforts aimed at understanding the mechanisms of various diseases and host immunology, as well as the continuous advancements in production host development.

Biopharmaceutical benchmark 2022 highlighted that mammalian cell systems continue to be the most commonly used expression platforms for biopharmaceuticals, accounting for 67% of the 159 approved recombinant products. Among mammalian systems, CHO cells being the most prevalent choice, producing 95 out of 107 mammalian-derived products (Walsh and Walsh, 2022). They are favored for their ability to perform complex post-translational modifications necessary for the functionality of many biopharmaceuticals. Other mammalian expression systems include NS0 mouse myeloma cells, baby hamster kidney (BHK) cells,

human embryonic kidney (HEK) cells SP2/0 mouse myeloma cells, and PER C6 immortalized primary human embryonic retinal cells. The use of bioreactors for culturing mammalian cells has significantly increased production yields, with ongoing improvements in gene delivery methods and bioprocess design expected to further enhance its productivity. However, due to the complexity of the mammalian cell culture medium, and scale-up limitations, the final product cost is still expensive on the market. In order to provide accessible and affordable biologics to the worldwide, development of alternative expression host is consistently in need (Stefanie Dietmair *et al.*, 2012, 2011; Thomas Jostock and Jostock, 2011).

Production of many recently approved biopharmaceuticals that do not require complex post-translational modifications can be produced more cost-effectively in non-mammalian systems such as bacteria and yeast. As of 2009, 151 recombinant biopharmaceuticals produced in microbial cell had had a approval for human use by FDA and/or by the EMA (Neus Ferrer-Miralles *et al.*, 2009). Particularly *Escherichia coli*, which has produced 36 approved products since 2018 is the primary microbial host for protein-based drugs, with the yeast *Saccharomyces cerevisiae* as a leading microbial host for the production of recombinant vaccines. However, the lack of post-translational modifications and proteolytic stability limit the potential of microbial host expression (Laura Sánchez-García *et al.*, 2016).

Insect cell-based systems, particularly those utilizing the baculovirus expression vector (BEV) system, have emerged as a new platform for the production of recombinant proteins. This technology has been adopted for the manufacture of biopharmaceuticals, including vaccines. So far, following two pharmaceutical products produced in insect cell have been approved by international regulatory agencies. The vaccine Cervarix, used for cervical cancer, was approved by the EMA in 2007 and is produced using the BEV/insect cell culture system. The other is influenza vaccine FluBlok, which is based on the influenza virus hemagglutinin protein, is the first FDA-approved product produced in insect cells (Thomas A. Kost *et al.*,

2016; Wilfried Weber *et al.*, 2009). In summary, despite advances across various expression hosts, a sustainable solution is still required to meet the increasing demands of future biopharmaceuticals.

1.3 Plant as an expression host for biopharmaceuticals

The first study that producing recombinant protein in plant was published in 1986. In this study, human growth hormone (hGH) gene with nopaline synthase gene promoter and poly-adenylation site at the end was introduced into tobacco plant and sunflower cells (Barta *et al.*, 1986). Since this trial of expressing human gene in the plant cells, the potential of plants to be a host for biopharmaceutical production has extensively been studied. The key component of plant-based expression system is *Agrobacterium*-mediated genetic transformation. It is a safe and efficient method to transfer genetic information to plant cells. In nature, *Agrobacterium* transfers transferred-DNA (T-DNA) segment carrying two oncogenes from its tumor inducing (Ti) plasmid to plant tissue. In biotechnology, this feature is engineered and utilized for expressing recombinant proteins in plants (Tzfira and Citovsky, 2006). Based on the number of publications investigating the plant as an expression host for biopharmaceuticals, the plant has become a competitive host among others. Specially, the growth rate from 1994 to 2024 of the plant expression system related studies was the highest, highlighting the rising trend of plant-made biopharmaceutical (PMB) production (Figure 2). As demand increases, the diversity of the plant-based expression system has broadened as well (Figure 3).

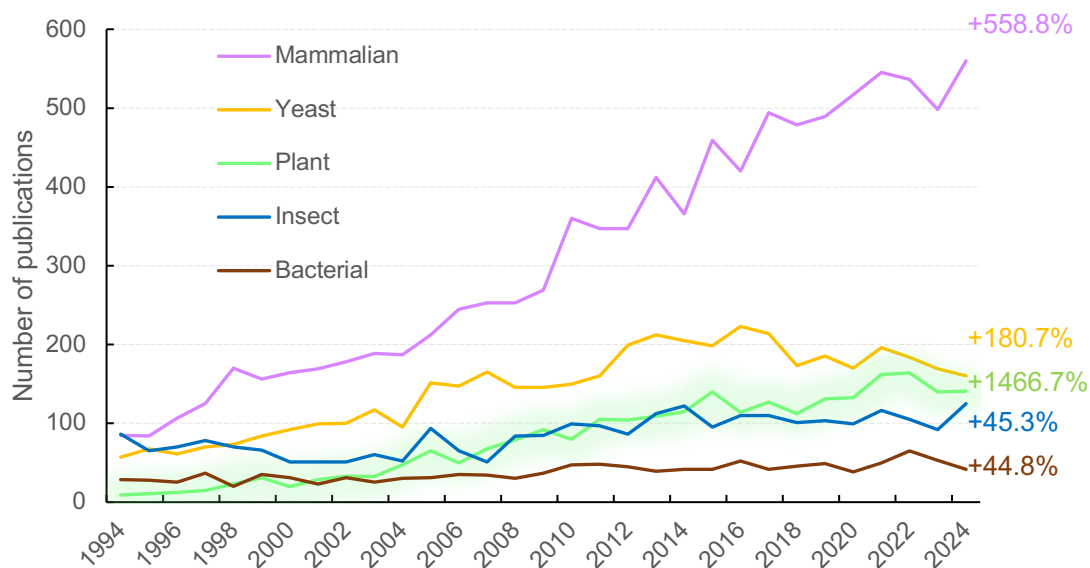


Figure 2. Number of PubMed publications related to biopharmaceutical production across five expression systems over the last 30 years. The growth rate of the publication number on each host is displayed on the right. Data was searched from PubMed (<https://pubmed.ncbi.nlm.nih.gov>) using [Title/Abstract] queries combining platform specific keywords (**Mammalian**: CHO, mammalian expression, HEK293; **Yeast**: *Pichia pastoris*, *P. pastoris*, *S. cerevisiae*, yeast expressed, yeast expression system; **Plant**: plant expression, *Nicotiana*, *N. benthamiana*, plant cell, molecular pharming, plant made, plant produced; **Insect**: baculovirus expression system, Sf9 cells, insect cell, silkworm, *Bombyx mori*; **Bacterial**: *E. coli* expressed, *E. coli* expression system, bacterial expression) and therapeutic keywords (recombinant protein, therapeutic, biologic, vaccine, mAb, antibody, pharmaceutical).

Many species of plants have been utilized as an expression host to produce biopharmaceuticals or other recombinant proteins. The examples are *Oriza sativa* (rice), *Hordeum vulgare* (barley), *Daucus carota* (carrot), *Zea mays* (corn), *Solanum tuberosum* (potato), *Carthamus tinctorius* (safflower), *Lemna minor* (duckweed), *Solanum lycopersicum* (tomato), *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Nicotiana benthamiana*. Aside from the leaf expression, other organs including grain, fruit, root and seed can be targeted to produce the recombinant proteins (Park and Wi, 2016; Shanmugaraj *et al.*, 2020).

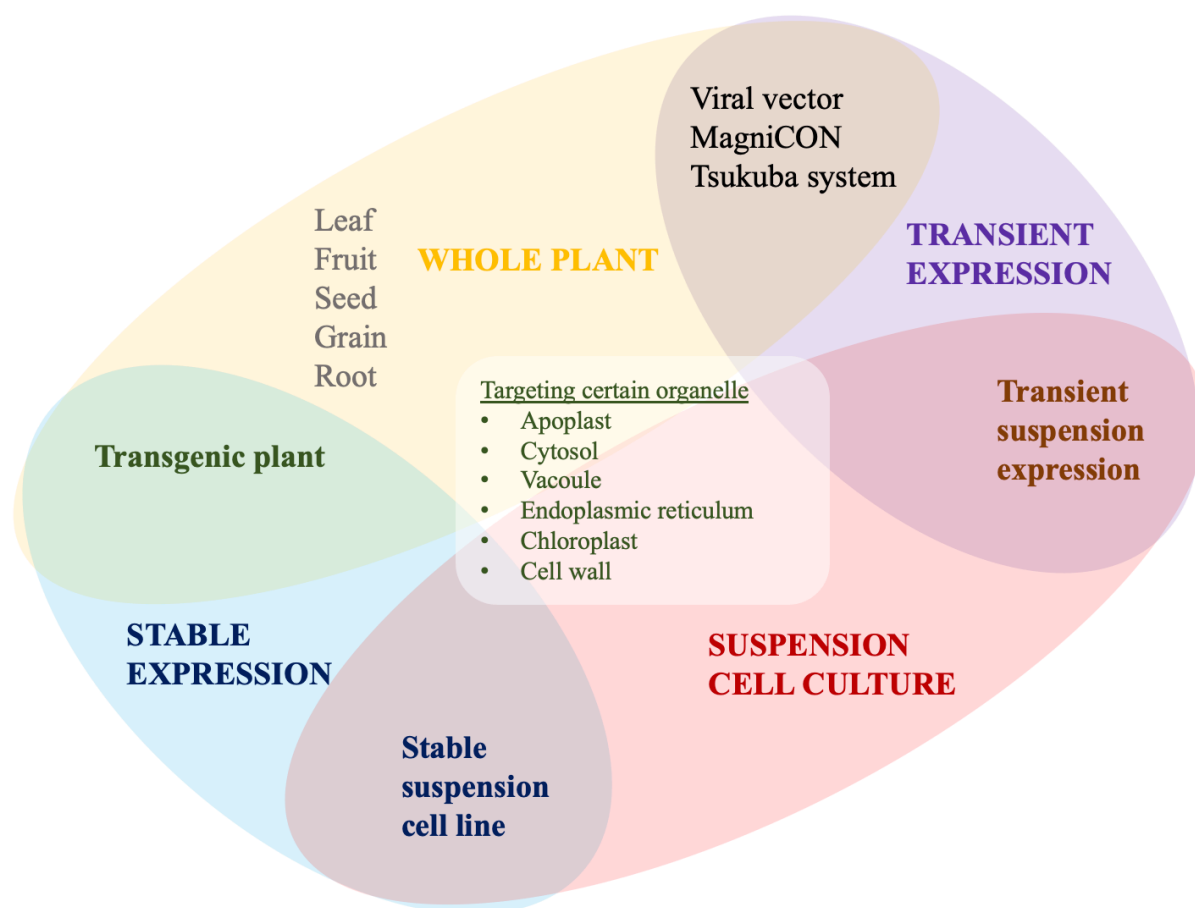


Figure 3. Diversity of the plant-based expression platform.

Among all the platforms, *N. benthamiana* has been extensively investigated for recombinant protein production. Several characteristics of *N. benthamiana* makes it preferable to biopharmaceutical production host. *N. benthamiana* is Australian indigenous species closely related to tobacco plants *N. tabacum* and *N. rustica* (Bally *et al.*, 2018; Goodin *et al.*, 2008). Especially a single strain that has been used as a model plant in the research is adapted to survive in extreme conditions of Australian weather by suppressing the viral defense system in order to grow faster through their evolutionary history (Bally *et al.*, 2015, 2018). This susceptibility to viruses makes *N. benthamiana* good at transiently expressing recombinant proteins. Another advantage of *Nicotiana* family plants is to produce high biomass in a short time. In combination with the *Agrobacterium*-mediated transient expression system, *N. benthamiana* has proven to be a valuable production host for various biopharmaceuticals,

including antibodies, vaccines, and therapeutic enzymes (Beritza *et al.*, 2024; Tzfira and Citovsky, 2006).

1.4 Challenges and prospects of plant-made biopharmaceuticals

The potential of plant-based expression system was based on these main advantages: the low cost of cultivating plants, the potential for large-scale agricultural production, and free from animal pathogens (prions, viruses, and mycoplasmas), capacity to perform posttranslational modifications. As of today, all of the scientific discoveries made not only in plant science, but also in genetic and molecular biology influenced the growth of plant-based expression system. The plant-made biopharmaceuticals (PMBs) mentioned in Table 1 shows the capacity of plant-based expression system to produce various type of biopharmaceuticals including vaccines, enzymes, antibodies, etc. The fact that ten out of the seventeen PMBs were transiently expressed in *N. benthamiana* demonstrates the ease and robustness of *N. benthamiana* as an expression host. The companies or institutes leading the PMB industry are from USA, Germany, Japan, Canada and Israel. However, the number of PMBs that has reached clinical trial step remains limited. To date, only three PMBs have been approved and commercialized for human use. These include Elelyso®, a recombinant β -glucocerebrosidase (GCase) produced in carrot cell suspension culture; Elfabrio®, a recombinant pegunigalsidase alfa produced in *N. tabacum* BY-2 cell suspension culture; and Covifenz®, a COVID-19 virus-like particle (VLP) vaccine produced transiently in *N. benthamiana* plants (Table 1). Later, Covifenz® was discontinued following the shutdown of its producer Medicago Inc, Canadian biotechnology company specializing in *N. benthamiana*-based production. This suggests that there are barriers to bringing plant-based expression systems to industrial application. Since the biopharmaceutical industry operates at the intersections of multiple domains including science, technology, regulation, and finance, the barriers plant-based expression system is

facing are multilayered. This section focuses specifically on the quality of PMBs and the biological challenges associated with the plant host systems used for their production. One of these challenges is downstream processing of the PMBs. Depending on the product and the host, it requires tailored purification method which could remove the plant secondary metabolites and other compounds at industrial level. If it is an edible vaccine produced in plant, batch to batch consistency of the expression level becomes a main concern (Jian Yao *et al.*, 2015). Another hurdle is high proteolytic activity in plants. Normally, large quantity of proteolytic enzymes are present in intra and extracellular compartments in plant cells and it could lead to degradation of the target protein and low yield (Liu and Timko, 2022). Lastly, plant-specific *N*-glycosylation remains one of the most critical concerns in the development of PMBs, due to its potential impact on protein structure, kinetic, and efficacy, immunogenicity, and therapeutic compatibility. This issue is explored in detail in the following section.

Table 1. Summary of clinical trials involving plant-made pharmaceuticals registered globally. Data was collected from ClinicalTrials.gov, UMIN-CTR (Japan), and peer-reviewed literatures (Lee *et al.*, 2023).

Pharmaceutical product	Expression System	Clinical trial status	Sponsor/ Company	Reference
ElELYso (prGCD) (Enzyme replacement for Gaucher's disease)	Carrot cell suspension culture	Phase III completed (2012); FDA approved (NCT00376168)	Protalix BioTherapeutics, Israel	(Shaaltiel <i>et al.</i> , 2007)
CoVLP / Covifenz® (COVID-19 VLP vaccine)	Transient in <i>N. benthamiana</i>	Approved in Canada (2022); later discontinued by Medicago	Medicago Inc., Canada	(Hager <i>et al.</i> , 2022)
Elfabrio (PRX-102) (Enzyme replacement for Fabry disease)	Tobacco cell suspension culture (BY-2)	Phase II completed (2016) (NCT01769001); EMA approved	Protalix BioTherapeutics, Israel	(Schiffmann <i>et al.</i> , 2019)
ZMapp (Ebola antibody cocktail)	Transient in <i>N. benthamiana</i>	Phase II (2017); compassionate/emergency use advisory from FDA	Mapp Biopharmaceutical, USA	(Oldstone and Rose Oldstone, 2017)
KBP-201 (RBD-based COVID vaccine)	Transient in <i>N. benthamiana</i>	Phase II (2021) (NCT04473690)	KBio Inc., USA	(Maharjan and Choe, 2021)
OPRX-106 TNF fusion protein (for ulcerative colitis)	Tobacco cell suspension culture (BY-2)	Phase IIa completed(2021) (in Israel)	Hebrew University-Hadassah Medical Center, Israel	(Almon <i>et al.</i> , 2021)
HAI-05 (H5N1 vaccine)	Transient in <i>N. benthamiana</i> (MagnICON)	Phase I completed (2011) (NCT01250795)	Fraunhofer, Center for Molecular Biotechnology, USA	(Chichester <i>et al.</i> , 2012)
P2G12 Antibody (HIV neutralizing antibody)	Transgenic <i>N. tabacum</i>	Phase I completed (2011) (NCT01403792)	University of Surrey, Guilford, UK	(Julian K-C. <i>et al.</i> , 2015)
Cedar pollen peptides (Immunotherapy for allergy)	Stable rice grain expression	Phase I completed (2013) (UMIN000011086)	The Jikei University, Japan	(Endo <i>et al.</i> , 2021)
MucoRice-CTB vaccine	Stable rice grain expression	Phase I completed (2013) (UMIN000009688)	The University of Tokyo, Japan	(Yuki <i>et al.</i> , 2021)

H5-VLP+GLA-AF (H5N1 VLP vaccine)	Transient in <i>N. benthamiana</i>	Phase I completed (2014) (NCT01657929)	Access to Advanced Health Institute (AAHI), USA	(Carter <i>et al.</i> , 2018)
Autologous FL vaccine	Transient in <i>N. benthamiana</i> (MagnICON)	Phase I completed (2015) (NCT01022255)	Icon Genetics , Munchen, Germany	(Tusé <i>et al.</i> , 2015)
Pfs25 VLP-FhCMB (malaria vaccine)	Transient in <i>N. benthamiana</i>	Phase I completed (2015) (NCT02013687)	Fraunhofer, Center for Molecular Biotechnology, USA	(Chichester <i>et al.</i> , 2018)
PA83-FhCMB (anthrax vaccine)	Transient in <i>N. benthamiana</i>	Phase I completed (2015) (NCT02239172)	Fraunhofer, Center for Molecular Biotechnology, USA	(Paolino <i>et al.</i> , 2022)
PRX-105 (hAChE-R) Acetylcholinesterase splice variant (treatment for organophosphorus poisoning)	Tobacco cell suspension culture (BY-2)	Phase I completed (2015) (in Israel)	Protalix BioTherapeutics, Israel	(Atsmon <i>et al.</i> , 2015)
Non-Hormonal Contraceptive (KB15A) HC4 Antibody	Transient in <i>N. benthamiana</i>	Phase I (2021) (NCT04731818)	KBio Inc., USA	(Thurman <i>et al.</i> , 2023)
VRC01-N/ HSV8-N (HIV/HSV mAbs)	Transient in <i>N. benthamiana</i>	Phase I completed (2021) (NCT02579083)	KBio Inc., USA	(Politch <i>et al.</i> , 2021)

1.5 *N*-Glycosylation engineering of *Nicotiana benthamiana*: Pushing the potential further

Glycosylation is the process of covalently attaching the prior synthesized oligosaccharides to the specific amino acid sequence and this essential post translational modification has proven to occur in three domains of life (Dell *et al.*, 2010). These attached oligosaccharides are also referred as glycans. Glycosylated proteins have a various critical role in the intracellular processes as well as cell to cell, cell to matrix, and cell to molecule interactions. The two main glyco-forms present on the proteins are *N*- and *O*-linked glycans. The *N*-glycosylation occurs between *N*-acetylglucosamine of the *N*-glycan core structure (Man₃GlcNAc₂) and the asparagine residue (Asn or N) of the Asn-X-Ser/Thr (where X is any amino acid except proline) consensus polypeptide motif. On the other hand, *O*-glycans added to the serine (Ser or S) or the threonine (Thr or T), sometimes to hydroxyproline residues, without a consensus sequence in plants (Yang *et al.*, 2017).

Protein expression machinery is highly conserved process among all organisms, unlike of the *N*-glycosylation of the proteins. Initial *N*-glycosylation synthesis in the endoplasmic reticulum (ER) of plant and mammalian is relatively well conserved. However, the final *N*-glycosylation of plant has a unique profile due to its specific glycosyl transferases in the Golgi apparatus. The main differences of plant *N*-glycosylation from mammalian are addition of β 1,2-xylose and α 1,3-fucose on the core structure, deficiency of highly branched, sialylated, and β 1,4-galactosylated structures. Especially the β 1,2-xylose and α 1,3-fucose on the core can result in immunogenic reaction and the antibodies against those structure can limit the efficacy

of plant produced biopharmaceuticals and accelerate the clearing from the body (Bardor *et al.*, 2003).

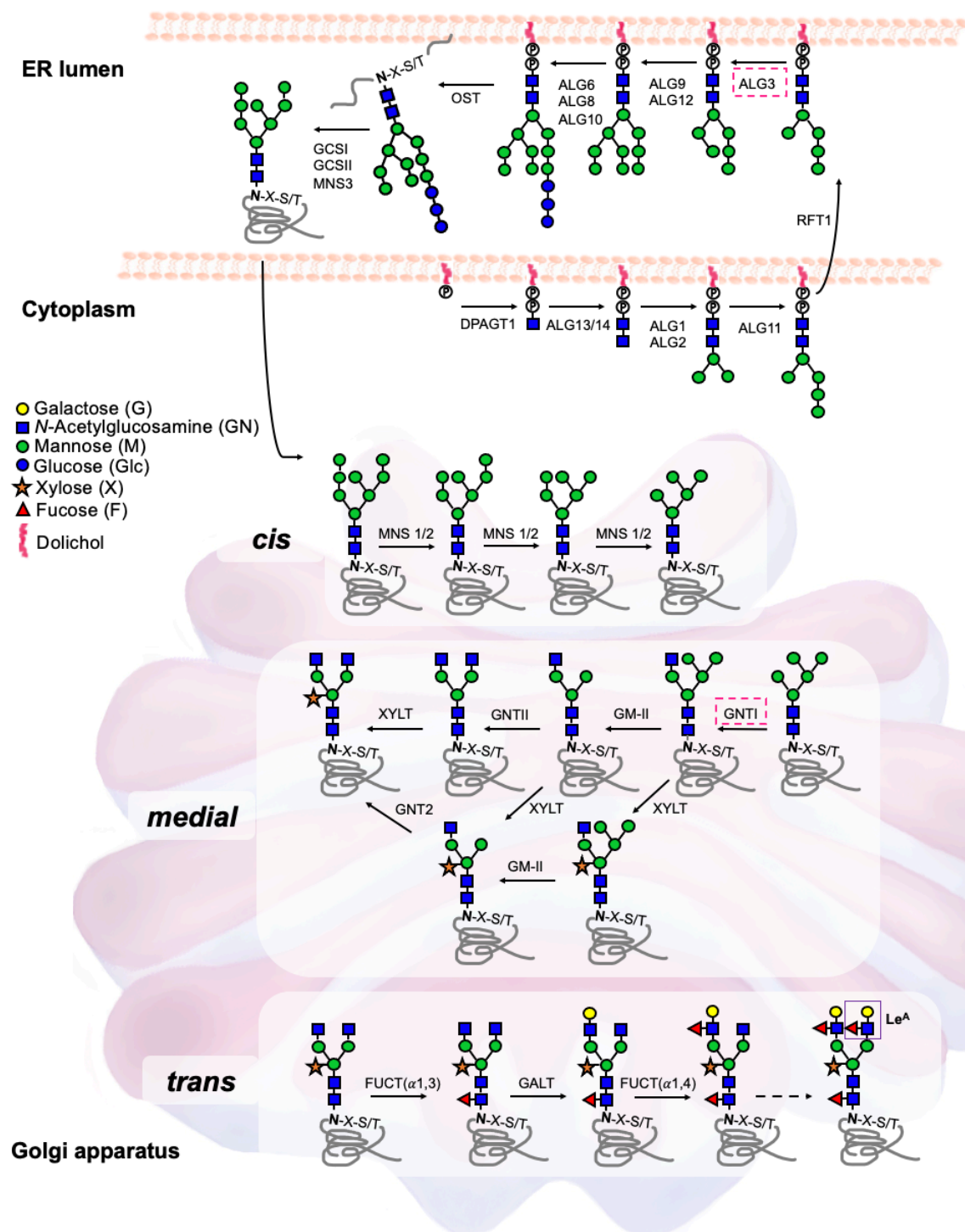


Figure 4. N-Glycosylation pathway in plants. Enzymes targeted in this study are highlighted with pink dashed boxes. The figure is adapted from Essentials of Glycobiology, fourth edition (p. 207-213), 2022, Cold Spring Harbor Laboratory Press and Strasser *et al.*, (2021) Cracking

the “Sugar Code”: A Snapshot of *N*- and *O*-Glycosylation Pathways and Functions in Plants Cells

In plant, biosynthesis of lipid-linked oligosaccharides starts on the cytoplasmic side of ER membrane. First stage of the biosynthesis starts from dolichyl pyrophosphate (Dol-P-P) in the cytoplasmic side of the ER and is carried by series of asparagine-linked glycosylation (ALG) enzymes including ALG 13, 14, 1, 2, and 11. Afterward $\text{Man}_5\text{GlcNAc}_2(\text{M5}^{\text{ER}})\text{-Dol-P-P}$ is flipped into the ER lumen by RFT1 enzyme. Subsequent ALG enzymes (ALG3, 9, 12, 6, 8, and 10) add four mannoses (Man) and three glucoses (Glc) to the M5^{ER} . The $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2(\text{Glc3M9})$ oligosaccharide is transferred to asparagine (Asp) residue of the polypeptide chain by the OST complex. Further trimming of the three glucose residues carried out by α -glucosidases I and II (GCSI and GCSII), and this process is deeply linked to *N*-glycosylated protein folding. Before translocating into Golgi apparatus, one mannose residue is removed by ER α -mannosidase I (MNS3), creating $\text{Man}_8\text{GlcNAc}_2(\text{M8})$. In *cis*-Golgi compartment three terminal mannose residues are trimmed by Golgi class I mannosidases (MNS1/2). Biosynthesis of hybrid and complex *N*-glycans is initiated by *N*-acetylglucosaminyltransferase I (GNTI) creating $\text{GlcNAc}_1\text{Man}_5\text{GlcNAc}_2(\text{GNM5})$ in the *medial*-Golgi. This step is critical for further trimming of the mannose residues by Golgi class II mannosidase (GMII). β 1,2-Xylosyltransferase (β 1,2-XYLT) also requires preceding action of GNTI. After the mannosidase, *N*-acetylglucosaminyltransferase II (GNTII) adds another GlcNAc residue to the $\text{GlcNAc}_1\text{Man}_3\text{GlcNAc}_2(\text{GNM3})$, generating biantennary *N*-glycan. The major core modification of plant *N*-glycan is addition of α 1,3-fucose to the core GlcNAc by α 1,3-fucosyltransferase (FUCT) in the *trans*-Golgi. Each GlcNAc branch can be elongated by galactosyltransferases (GALT) and α 1,4-fucosyltransferases (FUCT(α 1,4)). This glycan motif is called Lewis A (Le^{A}) structure(Strasser *et al.*, 2021) (Figure 4).

As the popularity of *N. benthamiana* rises among the biopharmaceutical production field, post translational modification, especially *N*-glycosylation engineering of the *N. benthamiana* became one of the main contributions for the development of PMB production. Early efforts in plant *N*-glycoengineering primarily focused on eliminating plant-specific glycan epitopes by downregulating the enzymes XYLT and FUCT. In *N. benthamiana*, RNA interference (RNAi) was used to suppress XYLT and FUCT expression, resulting in the elimination of β 1,2-xylose and α 1,3-fucose residues on the monoclonal antibody 2G12 (Strasser *et al.*, 2008). Based upon this foundation, mammalian-like *N*-glycosylation patterns were achieved by expressing human glycosyltransferases. For instance, stable expression of human β 1,4-galactosyltransferase (GALT) yielded β 1,4-galactosylated *N*-glycans in *N. tabacum* BY-2 suspension cell culture and in *N. benthamiana* (Palacpac *et al.*, 1999; Strasser *et al.*, 2009). Additionally, more complex mammalian-type *N*-glycan structures such as bisected and tri-/tetra-antennary forms were generated through the expression of human enzymes including GNTIII, GNTIV, and GNTV (Castilho *et al.*, 2011; Nagels *et al.*, 2011). Furthermore, co-expression of six mammalian genes enabled successful α 2,6-sialylation in *N. benthamiana* (Castilho *et al.*, 2010).

In parallel, the emergence of CRISPR/Cas9 technology enabled precise genome editing in plants (Jinek *et al.*, 2012; Li *et al.*, 2016). This approach was applied to *N. benthamiana* to simultaneously knockout four FUCT isoforms and two XYLT isoforms, further streamlining the *N*-glycoengineering process (Jansing *et al.*, 2019). Despite these advancements, it is important to note that complex mammalian *N*-glycan structures are not always optimal for therapeutic function. For instance, lysosomal enzyme replacement therapies (ERT) rely on high-mannose *N*-glycans, which are essential for recognition and uptake via the mannose-6-phosphate receptor pathway (Kornfeld, 1987). To meet this requirement, researchers have employed inhibitors or gene knockdowns targeting early *N*-glycosylation enzymes such as ER

α -mannosidase I (MNS3) and *N*-acetylglucosaminyltransferase I (GNTI), thereby generating proteins with high-mannose or pauci-mannose *N*-glycans (Choi *et al.*, 2018; Limkul *et al.*, 2016). Beyond structural modification, *N*-glycan heterogeneity remains a major quality concern. Notably, both the FDA-approved placental-derived β -glucocerebrosidase (GCase) and the recombinant GCase produced in carrot cell culture (Elelyso™) exhibit compact, homogeneous *N*-glycan profiles (Friedman *et al.*, 1999; Friedman and Hayes, 1996). This highlights the need for strategies to reduce *N*-glycan variability and favor uniform, terminal mannose-containing structures. Toward this goal, enzymes such as α 1,3-mannosyltransferase (ALG3) and GNTI, which regulate branching steps in the ER and Golgi, have shown to be suitable targets. Gene knockouts of ALG3 and/or GNTI have been shown to produce more homogeneous *N*-glycan profiles lacking plant-specific epitopes, without requiring extensive downstream pathway engineering in *A. thaliana* (Henquet *et al.*, 2008; Kajiura *et al.*, 2010; Strasser *et al.*, 2005). These approaches meet key criteria for producing recombinant lysosomal enzymes with improved *N*-glycan uniformity and functional efficacy. Although there is a report on knockdown of GNTI (Limkul *et al.*, 2016), these enzyme genes have not completely knocked out in *N. benthamiana*.

1.6 Objective of this study

Nicotiana benthamiana has emerged as a leading plant-based expression system in fast expanding PMB industry. A pivotal point in advancing this platform is the engineering of *N*-glycosylation pathway to produce more homogeneous *N*-glycans which lack plant-specific epitopes. This modification is essential to enhance the therapeutic compatibility and quality of recombinant proteins produced in this host. The primary objective of this study is to investigate the impact of *ALG3* and *GNTI* gene knockouts on the *N*-glycosylation profile of *N. benthamiana* using multiplex CRISPR/Cas9 tool, and to evaluate the potential application of these *N*-glycoengineered lines in biopharmaceutical production.

Chapter 2 demonstrates the identification of the *N. benthamiana* *GNTI* and *ALG3* genes, followed by their targeted knockouts using the multiplex CRISPR/Cas9 gene editing tool. Mutant lines were screened, genotyped, and selected. *N*-glycan profiles of the selected mutants were analyzed thoroughly in comparison with WT plants. In addition, phenotypic assessments were performed to evaluate any developmental or morphological effects associated with the *N*-glycosylation profiles.

Chapter 3 explores the practical application of the engineered lines by expressing two representative biopharmaceuticals: Varlilumab, anti-CD14 mAb, and GCase, lysosomal enzyme for enzyme replacement therapy. These proteins were purified and analyzed to investigate the effects of host *N*-glycosylation on their *N*-glycan structures.

Chapter 4 concluded the importance and limitations and suggested further investigations.

Chapter Two: Development of *N. benthamiana* with more homogenous *N*-glycan profile by targeting ALG3 and GNT1 enzymes with multiplex CRISPR/Cas9 genome editing tool

1.7 Introduction

N-Glycosylation is an essential post translational modification in all eukaryotic and some procaryotic organisms. The *N*-glycosylation process takes place in the ER and Golgi apparatus. Initially, the synthesis of oligosaccharides is carried out on the cytoplasmic side and lumen side of the ER membrane. ALG3 catalyzes the first step on the ER lumen side, in the elongation of the α 1,3-linked mannose branch, commonly referred to as the left arm of the oligosaccharide precursor. ALG3 transfers the first mannose residue from mannose-Dol-P to the α 1,3 arm of M5^{ER}-Dol-P-P, a key step in forming the mature M9-Dol-P-P structure in the ER (Varki *et al.*, 2022). This step is well conserved across various organisms including yeast, fungi, plant, and mammals. Defects in ALG3 activity result in incomplete *N*-glycan precursors, which impair protein folding and function, and are associated with congenital disorders of glycosylation (CDG) in humans (Daniel *et al.*, 2024). On the other hand, deletion of the *ALG3* in *A. thaliana* resulted in the unique immature structures in total plants and a paucimannosidic structures in cell suspension culture (Kajiura *et al.*, 2010; Sariyatun *et al.*, 2021). Thus, knocking out *ALG3* gene in *N. benthamiana* can be strategic point to engineer simplified or more homogeneous glycan structures on recombinant proteins.

Another key enzyme of the *N*-glycosylation maturation in the *cis*- to *medial*-Golgi is GNTI. GNTI catalyzes the transfer of an *N*-acetylglucosamine residue from UDP-GlcNAc to the α 1,3-linked mannose of the M5 *N*-glycan on the polypeptide. This modification is crucial for the catalytic activity of following *N*-glycosylation enzymes. Thus, it is the essential step for the synthesis of complex or hybrid *N*-glycans. In mammals, GNTI deficiency led to an absence

of complex *N*-glycans and it has a lethal consequence at the embryonic stage. Thus, GNTI is another key point in *N. benthamiana* to block the synthesis of plant-specific *N*-glycans.

To achieve the targeted modification of the *N*-glycosylation pathway, such as knockout of genes encoding ALG3 and GNTI enzymes, precise and efficient genome editing tool is necessary. CRISPR/Cas9 has been the universal tool for various biotechnological applications since its emergence in 2012. The discovery of CRISPR/Cas9 started in 1987 when short DNA repeats were first identified from bacterial genomes by research groups from the University of Osaka (Ishino *et al.*, 1987). In 1995, similar sequences were found in archaea and its possible function was discussed. In 2005, Mojica *et al.*, (2005) discovered that CRISPR was derived from foreign genetic materials and suggested it was possibly a bacterial immune system (Mojica *et al.*, 2005). In the same year, Bolotin *et al.*, (2005) published about CRISPR-associated proteins (Cas) and their nuclease motifs (Bolotin *et al.*, 2005). Then the RNA component of the system, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) were discovered in 2008 and 2011, respectively (Brouns *et al.*, 2008; Deltcheva *et al.*, 2011). Eventually all of these studies led to the innovative application of CRISPR/Cas9 for genome editing (Jinek *et al.*, 2012). Subsequently, CRISPR/Cas9 application in plant system described in 2013 (Shan *et al.*, 2013). Therefore, in our study, knockouts of *N. benthamiana* *ALG3* and *GNTI* genes were carried out by single cassette multiplex CRISPR/Cas9 genome editing tool.

Following the successful generation of *N. benthamiana* *ALG3* and *GNTI* knockout lines using CRISPR/Cas9, it is essential to evaluate the functional consequences of these genetic modifications. This chapter presents a comprehensive analysis of the *N*-glycan structures present in the mutant lines, using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) based total *N*-glycan analysis. The objective is to determine

whether the disruption of these key genes results in the expected simplification or truncation of *N*-glycan chains, and whether plant-specific epitopes are effectively eliminated. A variety of analytical techniques have been developed to profile and characterize *N*-glycans, each offering distinct advantages in sensitivity, resolution, and structural insight. The first step of the *N*-glycan analysis starts with the release of the *N*-glycans from the polypeptide chain. This can be done enzymatically or chemically. Enzymatic reaction commonly uses peptide-*N*-glycosidase F (PNGase F) or PNGase A, which selectively cleave *N*-linked glycans from *N*-glycoproteins (Maley *et al.*, 1989). The chemical method, hydrazinolysis uses anhydrous hydrazine to release *N*-glycans from glycoproteins under elevated temperature and pressure. This process cleaves the amide bond between the asparagine side chain and the *N*-glycan moiety, releasing intact glycans in their reducing form (Takasaki *et al.*, 1982). This requires the re-acetylation step. Next, released *N*-glycans are typically labelled with fluorescent tags such as 2-aminobenzamide (2-AB) or 2-aminopyridine (PA) to enable detection and quantification through high-performance liquid chromatography (HPLC) coupled with fluorescence detection (Bigge *et al.*, 1995; Hase *et al.*, 1981). For detailed structural characterization, mass spectrometry (MS) techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and LC-ESI-MS/MS are widely used due to their high sensitivity and ability to determine glycan mass, composition, and even linkage isomers (Grabarics *et al.*, 2022). Lectin blotting or some western blotting (WB) can offer functional insight into glycan motifs (Varki *et al.*, 2022). In our study, LC-ESI-MS/MS, lectin blotting, and WB methods are used to analyze the total *N*-glycan profile and detect a presence of plant-specific epitopes (β 1,2-xylose and α 1,3-fucose). In parallel, phenotypic analysis was performed to assess any observable developmental or morphological changes associated with the loss of *N*-glycosylation function. Since *N*-glycans are involved in protein folding, cell wall structure, and signaling, mutations in their biosynthetic genes may

lead to altered growth, leaf morphology, or stress responses. Together, the *N*-glycan and phenotype data provide insight into both the biochemical and physiological impact of targeted *N*-glycoengineering in *N. benthamiana*, and establish a foundation for evaluating the suitability of these lines for biopharmaceutical production.

1.8 Materials and methods

1.8.1 Plasmid construction of the multiplex CRISPR/Cas9 targeting *ALG3* and *GNTI* genes

The plant Cas9 expression backbone vector used in this study was kindly provided by Atsushi Takeda (Ritsumeikan University). Guide RNAs (gRNAs) were designed against target sequences using CRISPRdirect tool (Naito *et al.*, 2015) and were cloned separately into pBluescript-based entry vectors under the control of individual Arabidopsis U6 promoters (U6-26, U6-6, U6-29, U6-4, and U6-5). PCR amplifications were performed with KOD Plus Neo polymerase (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. gRNAs were incorporated into pBluescript vectors by PCR amplification downstream of the respective U6 promoter. gRNA plasmids were transformed into *E. coli* DH5 α , selected on LB agar containing ampicillin (100 μ g/mL), and verified by colony PCR and Sanger sequencing. For assembly of the multiplex gRNA expression cassette, each pBluescript–U6-gRNA unit was digested and integrated into the Cas9 backbone by Golden Gate assembly. Briefly, equimolar amounts of each gRNA cassette and the Cas9 destination vector were combined in a reaction mixture containing BsaI-HFv2 (NEB, Ipswich, MA, USA) and T4 DNA Ligase (NEB) in 1 $\times$ CutSmart buffer. Reactions were cycled between 37 °C (digestion) and 16 °C (ligation) for 30 cycles, followed by final incubation at 50 °C for 5 min and 80 °C for 10 min to inactivate the enzymes. The resulting Cas9 plant expression vector contained five gRNA expression cassettes, each driven by a distinct U6 promoter, together with human codon-optimized Cas9 (hCas9) expressed under the RPS5A promoter, nuclear localization signals, and a kanamycin resistance

cassette for plant selection. Correct assembly was confirmed by diagnostic restriction digestion and sequencing.

1.8.2 *Agrobacterium* transformation

Agrobacterium tumefaciens GV3101 strains were used for all agroinfiltration experiments. Electro-competent *Agrobacterium* cells were prepared according to the instruction manual of the MicroPulser Electroporator (BioRad, Hercules, CA, USA). Electro-competent *Agrobacterium* was transformed with the target expression vector by electroporation. The competent cells were thawed on ice and the target expression vector was added (up to 5 μ L). Twenty five μ L cell-vector mixture was added to the pre-cooled 0.2 cm electroporation cuvette (BioRad) and pulsed once with 2.2 kV. Immediately, 1 mL of LB medium was added to the electroporated cells and gently resuspended and transferred to 1.5 mL tube. Then cultured at 30°C for 2-3 h and plated on the LB agar plate containing 50 μ g/mL rifampicin, 40 μ g/mL streptomycin, and expression vector selection antibiotic.

1.8.3 Preparation of the *N. benthamiana* WT plants for transformation with CRISPR/Cas9 cassette

N. benthamiana WT seeds were sterilized by 2% Plant Preservative Mixture (PPM™ by Plant Cell Technology, WA, USA) and germinated on the MS agar plate (MS salts , 0.3% gellan gum, supplemented with x1 plant vitamins (Table 2)). When the seedlings appear after one week, single germinated seed was transferred to sterile closed pot with MS agar. The plants were grown for four to five weeks.

1.8.4 CRISPR/Cas9 cassette transformation of *N. benthamiana* leaves

One day before the transformation, *A. tumefaciens* GV3101 harbouring the CRISPR/Cas9 cassette was inoculated in 5 mL 2 $\times$ YT medium containing 50 μ g/mL kanamycin, 50 μ g/mL rifampicin, 50 μ g/mL spectinomycin and grown at 28°C for overnight.

The next day, OD₆₀₀ of the culture was checked and adjusted to OD₆₀₀ 0.8~1.3. For the transformation, *N. benthamiana* which have grown in sterile pot was used. The leaves were cut into 5 mm² pieces and put into the *Agrobacterium* culture and incubated for 20 min at RT. Then the co-cultured leaf pieces were transferred into new callus medium plate (MS salts, 1× plant vitamins, 0.3% gellan gum, 2 µg/mL naphthaleneacetic acid (NAA) and 0.2 µg/mL 6-benzylaminopurine (BA)) without any antibiotics. The plates were covered with aluminum foil and incubated at 25°C for 3 days. After the co-incubation, *Agrobacterium* was removed by rinsing with MS medium containing carbenicillin three to four times and transferred to new callus medium containing 250 µg/mL carbenicillin. Callus was induced for 14 days and transferred to the shoot induction medium (MS salts, 1× plant vitamins 0.3% gellan gum, 0.1mg/l NAA and 1 mg/l BA, 250 mg/l carbenicillin, kanamycin). The selection antibiotic, kanamycin concentration was increased gradually from 50 to 100 µg/mL over the shoot induction period. The calluses were transferred to new shoot induction medium with increased kanamycin concentration every 2 weeks until the shoot grows. Once the shoot became big enough, it was transferred to the root medium (MS salts, 1× plant vitamin, 0.3% gellan gum, 100 µg/mL kanamycin) in a sterile pot. When the plant grew big enough, it was transferred from MS medium pot to the soil pot. The plants were watered regularly and maintained in a humid condition specially right after transferring from medium to soil (Table 2).

Table 2. Composition of MS medium used for the mutant plant generation.

	MS broth	MS plate	Callus medium	Shoot induction medium	Root medium
MS basal salts, g/L	4.33	4.33	4.33	4.33	4.33
*1000x Plant vitamin stock, mL/L	1	1	1	1	1
Gellan gum g/L	-	3	3	3	3
NAA, mg/L	-	-	2	0.1	-
BA, mg/L	-	-	0.2	1	-
Carbenicillin mg/L	-	-	250	250	-
Kanamycin mg/L	-	-		50 75 100	100

*Plant Vitamin (1000×) contains 0.1 mg/mL thiamine, 0.5 mg/mL nicotinic acid, 0.5 mg/mL Pyridoxal-HCl, 100 mg/mL myoinositol, 2 mg/mL glycine.

1.8.5 Genomic DNA extraction from plant leaves

One cm² fresh leaf sample was homogenized by mortar and pestle while adding liquid nitrogen. 200 µL Genomic DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added and gently mixed. The sample was incubated for 10 min at RT and centrifuged at 10,000× g for 10 min. The supernatant was mixed with equal amount of isopropanol by inverting. The mixture was incubated for 10 minutes at RT and centrifuged at 10,000× g for 10 min at room temperature. Genomic DNA containing pellet were dried and dissolved in 50 µL dH₂O (Chabi *et al.*, 2015)

1.8.6 Amplification and sequencing of the target gene regions

The first round of screening was done by sequencing of the target gRNA regions of the *ALG3* and *GNTI* genes. The target region was amplified by PCR using KOD-Plus-Neo Polymerase (TOYOBO). Forward and reverse primers (Table 3) are designed for each region as shown in the Figure 8 and Table 3. Amplified products were run on 1% agarose gel and the target DNA product was sliced and purified from the gel by using a Gel Advanced™ Gel

extraction system (Viogene, City, Taiwan). The purified DNA product was sequenced by the GeneWiz Sanger sequencing service (Tokyo, Japan).

Table 3. Primers used for PCR amplification and sequencing of the CRISPR/Cas9 targeting region of the *NbALG3* and *NbGNTI* genes. F1 and R1 primers are for amplification shown as yellow arrows in Figure X; F2 and R2 primers are for Sanger sequencing and shown as green arrows.

Name	Sequence 5'-3'
1 <i>NbGNTI</i> -F1	ATGCGGCTTTTTGCGACACAGTCAG
2 <i>NbGNTI</i> -R1	GAGAATGTATGTACACCTGTAC
3 <i>NbALG3</i> -F1	CCTTAGGCAAGGTTTCAGTTGCCTT
4 <i>NbALG3</i> -R1	GAACCAACCATTCTCTCTCAAATC
5 <i>NbGNTI</i> -F2	TGCAGATCGCCTTGCTGCTGCA
6 <i>NbGNTI</i> -R2	CAGACACATTGACACTAACAGATC
7 <i>NbALG3</i> -F2	ATGGCAGCCAAATCAGCTGC
8 <i>NbALG3</i> -R2	TTGAGAGGGAACAGTCAAT

1.8.7 PNGase F digestion of the total soluble protein

The total soluble protein was extracted as mentioned before. The protein concentration was determined by Bradford assay with bovine serum albumin (BSA) as a standard. Then 100 µg total soluble protein was precipitated by adding 4 times volume of ice-cold acetone followed by 10,000× g centrifugation at 4°C for 10 minutes. The protein pellet was resuspended in 20 µL denaturing buffer containing 0.2 M β-mercaptoethanol, provided by the maker and denatured at 100°C for 3 min. The mixture was cooled down on ice. To make the total volume to 100 µL, 20 µL 5% NP-40, 52 µL dH₂O was added. Finally, 4 µL PNGase F (Takara Bio, Shiga, Japan) was added and mixed gently. Enzyme reaction mixture was incubated at 37°C for 24 h. After the reaction, total digested protein was precipitated by ice-cold acetone and resuspended in appropriate amount of 1× SDS-PAGE sample buffer.

1.8.8 Endo H digestion of the total soluble protein

The total soluble protein was extracted as mentioned before. The protein concentration was determined by Bradford assay with BSA as a standard. Then 100 µg total soluble protein

was precipitated by adding 4 times volume of ice-cold acetone followed by $10,000 \times g$ centrifugation at 4°C for 10 min. The protein pellet was resuspended in 50 μL 1 $\times$ Glycoprotein denaturing buffer and incubated at 100°C for 10 min. After cooling down the mixture on ice, 50 μL 1 $\times$ GlycoBuffer-3 and 5 μL Endoglycosidase H (Endo H) (Takara Bio). Enzyme reaction was obtained at 37°C for 24 h. After the reaction, total digested protein was precipitated by ice-cold acetone and resuspended in appropriate amount of 1 $\times$ SDS-PAGE sample buffer.

1.8.9 Anti-HRP WB and ConA Lectin blotting

After separating the protein samples with SuperSep™ Ace 10-20% gradient gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by SDS-PAGE electrophoresis, the protein was transferred to the Immobilon®-P polyvinylidene difluoride (PVDF) membrane (Merck Millipore, MA, USA) by Trans-Blot semi-dry transfer machine (BioRad) at constant 15V for 40 minutes. The membrane was blocked by 0.5% BSA in PBS-T overnight. For concanavalin A (Con A) lectin blotting, the membrane was transferred to 2 $\mu\text{g/mL}$ HRP-conjugated Con A in 0.5% BSA containing PBS-T and incubated for 1 h at RT with shaking. The membrane was washed four times by PBS-T for 5 min each with shaking. For anti-HRP WB, the membrane was transferred to 1:12000 diluted anti-HRP rabbit antibody (Sigma-Aldrich, St. Louis, MO) in 0.5% skim milk containing PBS-T and incubated for 1 h at RT with shaking. The membrane was washed as described above and transferred to 1:5000 diluted secondary anti-rabbit goat antibody in 0.5% skim milk containing PBS-T and incubated for 1 h at RT with shaking. The membrane was washed as described above and visualized by Immobilon® Classico Western HRP substrate (Merck Millipore) and incubated for 3 min. All the results of the lectin blot and western blot were visualized by iBright™ CL1500 Imaging system (Invitrogen, Waltham, MA).

1.8.10 Total *N*-glycan analysis

Five-week-old *N. benthamiana* plant leaves were homogenized by mortar and pestle using liquid nitrogen. The crude sample was centrifuged at $15000\times g$ for 20 minutes at 4°C . Supernatant is transferred to new tube and a total soluble protein is precipitated by acetone. The protein precipitant was separated by centrifugation and lyophilized overnight. *N*-Glycans were released by hydrazination of the lyophilized samples at 90°C for 10 h. All the peptides and glycans are precipitated by acetone. Precipitated sample was by centrifuged at $10,000\times g$ for 20 min at 4°C . Supernatant was removed and the pellet was dried. 2 mL saturated bicarbonate was added to the pellet and mixed well until the pellet was totally dissolved. And 80 μL acetic anhydrous was added and incubated for 20 min at RT and repeat once from adding 2 mL saturated bicarbonate step. pH of the sample was dropped down to pH 2.0 by adding Dowex 50Wx2 100-200 Mesh (H) cation exchange resin (Wako Pure Chemical Industries, Ltd.). The sample was separated from the resin using a gravity column. Further, the sample was concentrated by Eyela N-1110 rotary evaporator until the total volume was less than 1 mL. Concentrated sample was applied to gel filtration column and fractionated. Sugar chain containing fractions were detected by phenol-sulfuric acid method. The selected fractions were combined and concentrated. The concentrated sugar chain containing sample was lyophilized overnight. Next day, sugar chains were fluorescently labelled with 2-aminopyridin (PA). 100 μL PA-labelling coupling reagent was added to the lyophilized samples and incubated at 90°C for 1 h. After the incubation, sample was slightly cooled down to 80°C and 100 μL reducing agent (Dimethylamine-borane in acetic acid) was added and vortexed well. Then the sample was incubated at 80°C for 45 min. 200 μL MQ was added to the sample and excess PA reagents were removed by phenol-chloroform (1:1, v/v) twice followed by chloroform extraction. Purified PA-labelled *N*-glycan sample was desalted by gel filtration column packed with Toyopearl HW-40F resin (Tosoh Corp., Tokyo, Japan). Eluted fractions were checked by

fluorescence reader with excitation at 310 nm, emission at 400 nm. PA-labelled glycan containing fractions were combined and concentrated until the volume is around 200 μ L. PA-labelled glycans are separated using reversed phase-HPLC (RP-HPLC) with COSMOSIL 5C18-AR-II Packed Column (4.6 mm X 250 mm). The mobile phase consisted of A buffer: 0.02% trifluoroacetic acid (TFA) in MQ and B buffer: 0.02% TFA in 20% Acetonitrile. The RP-HPLC run was performed at flow rate 0.7 mL/min and B buffer gradient was increased 0 to 20% from 15 min to 50 min. The eluted fractions were monitored by fluorescence intensity at an excitation wavelength of 310 nm and emission wavelength of 380 nm. All the elution peaks are collected manually and concentrated with vacuum concentrator. The mass spectrophotometer analysis was done by amaZon speed ETD (Bruker) coupled with HPLC (Agilent 1260 Infinity). Concentrated samples are mixed with acetonitrile at 2:8 ratio and applied to Asahipack NH2P-50 2D column (Shodex) for further separation. The mobile phase of the LC was composed of solvent A (2% acetic acid in acetonitrile) and solvent B (3% triethylamine and 5% acetic acid in MQ) with a flow rate at 0.2 mL/min. B buffer gradient was set at 0-20% between 15 min to 50 min. Mass spectrometric detection was conducted in positive ionization mode with the following parameters: mass-to-charge ratio scanning range of m/z 350-2750, nebulizer pressure of 5.0 psi, drying gas flow rate of 3.0 L/min, source temperature of 300°C, ion accumulation target of 200,000 counts, and fragmentation amplitude of 1.0 V for tandem mass spectrometry experiments. Relative *N*-glycan ratio was calculated by the peak area of LC chromatogram.

1.8.11 Leaf weight measurement

N. benthamiana WT and T₃ mutant seeds were sprouted in the soil for about a week then single sprout were transferred to the 12 cm diameter pot and grown for four weeks in controlled environment with a 16 h light and 8 h dark cycle at 22°C with 50% humidity. Plants were watered regularly. Occasionally, HYPONeX® fertilizer was added to the water to maintain the

health of the plants. The individual leaf was cut excluding the petiole and fresh weight was measured.

1.8.12 Root growth assessment

N. benthamiana WT and T₃ mutant seeds were sterilized by 2% PPM™ and germinated on the MS agar plate (MS salts ,3% sucrose, 0.3% gellan gum, supplemented with x1 plant vitamins). When the seedlings appear after one week, germinated seeds were transferred to rectangular MS agar plate with 0%, 1%, or 3% sucrose or 0.3 mM ascorbic acid supplementation and placed vertically in the plant incubator with a 16 h light and 8 h dark cycle at 22°C. After 10 days, the plate pictures were taken and root lengths were measured by ImageJ software.

1.8.13 Statistical analysis

Statistical analyses and graphical visualization were performed using R statistical software ver. 4.4.3 (R Core Team, 2023). Differences between multiple groups were evaluated using one-way analysis of variance (ANOVA), followed by post hoc testing. For pairwise comparisons, Tukey's honestly significant difference (HSD) test was applied.

1.9 Results

1.9.1 Identification of the GNTI and ALG3 in *N. benthamiana*

GNTI and *ALG3* genes have not yet identified in *N. benthamiana*. Therefore, candidate genes were searched using a Sol Genomics Network: *N. benthamiana* draft genome v2.6.1. database with Arabidopsis *ALG3* and *GNTI* as queries. The search result led to the discovery of one candidate for *ALG3*: Niben101Scf01521g16013.1 and two homolog candidates for *GNTI*: Niben101Scf04294g06042.1 and Niben101Scf01176g01032.1, designated as *NbGNTI-A* and *NbGNTI-B* in this section (Table 4).

Table 4. Candidate genes encoding *N. benthamiana* ALG3 and GNTIs based on Sol Genomics Network database

	Gene ID	Location	Encoding amino acids
<i>NbGNTI-A</i>	Niben101Scf04294g06042.1	Chromosome 10	421
<i>NbGNTI-B</i>	Niben101Scf01176g01032.1	Chromosome 9	401
<i>NbALG3</i>	Niben101Scf01521g16013.1	Chromosome 18	434

The amino acid alignment showed 68% identity between NbALG3 and AtALG3. Moreover, NbALG3 had a C terminal KKXX signal that is important for ER transmembrane protein trafficking from Golgi to ER (Jackson *et al.*, 2012). This suggested that candidate NbALG3 encodes an α 1,3-mannosyltransferase which functions in the ER. (Figure 5).

NbGNTI-A and NbGNTI-B alignment showed 88% amino acid identity suggesting they are isoforms of GNTI. Additionally, critical motif for catalytic activity, DxD motif which binds to Mn^{2+} , donor and ligand was detected (Figure 6). However, there was a missing motif in NbGNTI-B in comparison to NbGNTI-A. In order to examine the potential function of the missing motif, NbGNTI-A amino acid sequence was searched from the AlphaFold 3 Protein Structure Database (Jumper *et al.*, 2021; Varadi *et al.*, 2024) and 100% identical match was found as *N. benthamiana* GNTI (AF-Q8GUA5-F1-v4). The 3D structural data showed the motif missing in NbGNTI-B was located adjacent to the substrate binding DxD motif (Figure 7). Any deletion near the catalytically active site could potentially affect the enzyme's catalytic activity or stability (Kim *et al.*, 2007). Therefore, NbGNTI-B is possibly a pseudoenzyme (Goldberg and Sreelatha, 2023) (Figure 7).

(A)

NbALG3	1	FQLPYPGTALAAAKSAAAIRKNSHRSDHFLQKLIKHP--KLPPAFALLFAD	58
AtALG3	1	---MAGASSPASLRASRSRLGKETNRSD-----LFKKP--AVPPAFALILADAILVALI	50
HsALG3	1	MAAGLRKRGRSGSAAQAEGLCKQWLQRAWQERRLLREPRYTLLVAACLCLAEVGITFWV	60
NbALG3	59	IAYVPYTKIDWDAYMSQVTFLEGERDYSNLIKGDGTLVYPAGFLYIYSAIQYVT--GGQ	116
AtALG3	51	IAYVPYTKIDWDAYMSQVSGFLGGERDYGNLKGDGTLVYPAGFLYVYSAVQNLIT--GGE	108
HsALG3	61	IHRVAYTEIDWKAYMAEVEGVINGTYDYTQLQGDGTLVYPAGFVYIFMGLYYATSRGTD	120
NbALG3	117	VYPAQILFGFLYMLDLAIVFITYLKTDPVPMWALSLLSLS--KRHSIFVLRFLNDC	175
AtALG3	109	VYPAQILFGVLYIVNLGIVLTIYVKTDVPMWALSLLCLS--KRHSIFVLRFLNDC	167
HsALG3	121	IRMAQNIHAYLYLATLLVFLITYHOTCKVPPFVFFMCCASYRHSIFVLRFLNCPVAMV	180
NbALG3	176	LLHAALVSIIYQKWHGLVTFSCAVSTIKMNVLLYAPPLLLLMVKAMDIVGVISALAGAA	235
AtALG3	168	LLHASMAFLYRKWHGLMLVFSQAVSVKMNVLLYAPTLILLKAMNIIGVVSALAGAA	227
HsALG3	181	LLFLSINLLAQRWGWCFFSLAVSVKMNVLFPAGLLFLLLTQFGFRGALPKLGICAG	240
NbALG3	236	VQIVIGLPFVLSHPASYISKAFDLGRVFIHFWSVNFKFVPEEIFVSKAFALSLLVAHLSL	295
AtALG3	228	VQILVGLPFLITYPVSYIANAFDLGRVFIHFWSVNFKFVPERVFSKEFAVCLLI	287
HsALG3	241	LQVVLGLPFLLENPSGYLSRSEDLGRQFLFHWTVNWRFLEALFLHRAFLHALLT	300
NbALG3	296	LLIFAHYRRCRHEGGLFAVHSHKINQLKLG--VAHRNPSTNRVLQTDHIVTTFVGNFI	353
AtALG3	288	LVAFANYKCKHEGGIIGFMRSRHFFLTLPSSLSFSDVSAS--RIITKEHVVTAMFVGNFI	346
HsALG3	301	LLLFALCRWHRTGESILSLLRD-----PSKRKVPQ--PLTPNQIVSTLFTSNFI	348
NbALG3	354	GIICARSLHYQFYSWYFSLPYLLWKT---FPTLLRLLFAAVEFCWNVFPNSFS	410
AtALG3	347	GIVFARSLHYQFYSWYFSLPYLLWRT---FPTWLRLIMFLGTELCWNVYPSTPSS	403
HsALG3	349	GICFSRSLHYQFVWYFHTLPYLLWAMPARWLTHLRLVLGLTELSWNTYPTSCSSAA	408
NbALG3	411	LLCVHLLTLGGLWISSPEYPIVGKITTYKSSFEKKAR	446
AtALG3	404	LLCLHLITLVGLWLAPSDPYQLKEHPKSIQHKKA-	438
HsALG3	409	LHICHAVTLLQLWLGPQFP---KSTQHS---KKAH	438

(B)

	AtALG3	HsALG3
NbALG3	68	43
AtALG3		44

Figure 5. Homology between candidate *N. benthamiana*, *A. thaliana* and human ALG3s
 (A) Amino acid alignment of ALG3. The alignment was constructed using *A. thaliana* ALG3 (AtALG3, At2g47760), and *N. benthamiana* ALG3 (NbALG3, Niben101Scf01521g16013.1) and human ALG3 (HsALG3, CAA70220.1) using CLUSTALW (GENETYX version 22.0.5). ER retention signal, KKXX was highlighted in pink. (B) Amino acid sequence identity of ALG3s. The identical amino acid percentage was calculated by GENETYX

(A)

NbGNT1-A	1	-----MYLDSLVIHTGNLSTAD-----TEAENHCTSQTRLIDHISQ	38
NbGNT1-B	1	-----MCLDSSSHPPNRKSNID-----TEAENHCTSQTRLIDQISQ	38
AtGNT1	1	MARISCDLRFLIPAAFMFIYIQMRLFQTQSQYADRLSSAIESENHCTSQMRGLIDEVSI	60
HsGNT1	1	MLKKQSAGLVWGAILFVANNALLFFWTRPAPGRPPSVSALDGDPAQLTREVIRLAQD	60
NbGNT1-A	39	QQGRIVALEEQMKRQDQECRQLRALVQDLESKGIKKLIQNVQMPVAAVVVMACSRADYLE	98
NbGNT1-B	39	QQGRIVALEEQMKRQDQECRQLRALVQDLESKGIKKLIQNVQMPVAAVVVMACNRADYLE	98
AtGNT1	61	KQSRIVALEDMKNRQDEELVQLKDLIQTFEKKGIAKLTQGGQMPVAAVVVMACSRADYLE	120
HsGNT1	61	AEVELERQRLQLQIGDALSSQGRVPTAAPPAQPRVPVTPAPAVIPILVIACDRS-TVR	119
NbGNT1-A	99	KTIKSMKKYQVSVASKYPLFTSQDGSHPDVRKLALSY-DQLTYMQHLDPEPVHTE-RPGE	156
NbGNT1-B	99	KTIKSMKKYQISVASKYPLFTSQDGSHPDVRKLALSY-DQLTYMQHLDPEPVHTE-RPGE	156
AtGNT1	121	RTVKSVLITYQTPVASKYPLFTSQDGSQAVKSKSLSY-NQLTYMQHLDPEPVTE-RPGE	178
HsGNT1	120	RCLDKLLHYRP-SAEFPITIVSQDCGHEETAQAIASVGSAVTHIRQPLSSIAVPPDHRK	178
NbGNT1-A	157	LIAYYKIARHYKWARDQLFYKHNFSSRVILLEDMEIAPDFFDFEAGATLLDRDKSIMAI	216
NbGNT1-B	157	LIAYYKIARHYKWARDQLFYKHNFSSRVILLEDMEIAPDFFDFEAGATLLDRDKSIMAI	216
AtGNT1	179	LTAYYKIARHYKWARDQLFYKHNFSSRVILLEDMEIAPDFFDFEAAASLMDRDKTISMA	238
HsGNT1	179	FQGYKIARHYRWALGVFRQFRFPAAVVVEDLEIAPDFFDFEYFRATYPLLKADPSLWCV	238
NbGNT1-A	217	SSWNDNGQMVFQD--PYALYRSDFFPGLGWLKSKSTWDELSPKWPKAYWDDWLRLKENH	274
NbGNT1-B	217	SSWNDNGQMVFQD--PYALYRSDFFPGLGWLKSKSTWDELSPKWPKAYPFELI-LKENH	273
AtGNT1	239	SSWNDNGQMVFQD--PYALYRSDFFPGLGWLKSKSTWDELSPKWPKAYWDDWLRLKENH	296
HsGNT1	239	SAWNDNGKQVVDASRPPELLYRTDFFPGLGWLKLAELMAELSPKWPKAFWDDWMRRPEQR	298
NbGNT1-A	275	RGRQFIRPEVCRTYNFGEHGSSLGQFFKQYLEPIKLNVDVVDWKSMDLSYLLLEDNYVKHF	334
NbGNT1-B	274	RGRQFIRPEVCRTYNFGEH-----VDWKSMDLSYLLLEDNYVKHF	312
AtGNT1	297	KGRQFIRPEVCRTYNFGEHGSSLGQFFSQYLEPIKLNVDVVDWKAQDLGYLTFEGNYTKYF	356
HsGNT1	299	QGRACIRPEISRTMTFCRKGVSHGQFFDQHLKFIKLNQQFVHFTQLDLSYLQREAYDRDF	358
NbGNT1-A	335	GNLVKKAKPIHGADAVLKAFNIDGVRIOYRDQLEFEDIARQFGIFEWKDGVPRAAAYKG	394
NbGNT1-B	313	GDLVKKAKPIHGADAVLKAFNIDGVRIOYRDQLEFEDIARQFGIFEWKDGVPRAAAYKG	372
AtGNT1	357	SGLVROARPIQGSIDLKAKNIKDDVRIYRDQVEFERIAGEFGIFEWKDGVPRATAYKG	416
HsGNT1	359	LARVYGAPQLQVEKVRTNDRKELGEVRVOYTGRDSFKAFKALGVMDDLKSGVPRAGYRG	418
NbGNT1-A	395	IVVFYQTSRRVFLVGHDSLQQLGIEDT	422
NbGNT1-B	373	IVVFRYQTSRRVFLVGPDLSLQQLGIEDT	400
AtGNT1	417	VVVFRIQTTRRVFLVGPDVSMQLGIRNS	444
HsGNT1	419	IVTFQFRG-RRVHLAPPLTWEGYDPSWN	445

(B)

	NbGNT1-B	AtGNT1	HsGNT1
NbGNT1-A	88	76	38
NbGNT1-B		71	32
AtGNT1			34

Figure 6. Homology between candidate *N. benthamiana*, *A.thaliana* and human GNTIs
 (A) Amino acid alignment of GNTI. The alignment was constructed using *A. thaliana* GNTI (AtGNTI, AT4G38240), and *N.benthamiana* GNTIs (NbGNTI-A, Niben101Scf04294g06042.1; NbGNTI-B, Niben101Scf01176g01032.1) and human GNTI (HsGNTI, P26572) using CLUSTALW (GENETYX version 22.0.5), substrate binding, DxD motif was highlighted in green and the missing region of NbGNTI-B in pink (B) Amino acid sequence identity percentage of GNTIs. The identical amino acid percentage was calculated by GENETYX.

NbALG3, NbGNTI-A and NbGNTI-B enzyme activities were examined using heterologous expression system (Hiroyuki Kajiura, Appendix 1-2). The activity of NbALG3 was confirmed using a *Saccharomyces cerevisiae* $\Delta alg3$ temperature-sensitive mutant (YG170) under the GAL1 promoter. NbALG3 complemented the growth defect of YG170 in presence of galactose. Further analysis demonstrated that expression of NbALG3 restored synthesis of the final Glc3M9 (Appendix 1). These results identified NbALG3 as *N. benthamiana* α 1,3-mannosyltransferase. Recombinant NbGNTI-A was functional and converted M5 to GNM5 successfully. On the contrary, NbGNTI-B was successfully expressed in *E. coli*. However, the crude extract of NbGNTI-B did not show any enzymatic activity (Appendix 2).

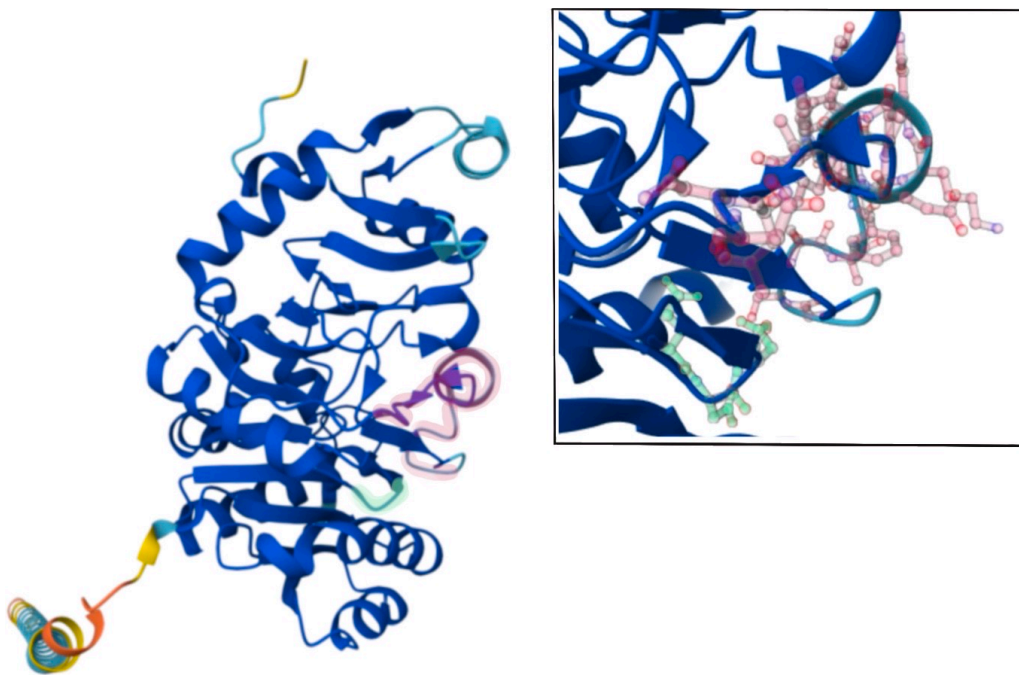


Figure 7. Predicted 3D structural model of *N. benthamiana* GNTI enzyme generated by AlphaFold 3. The structure prediction is based on the amino acid sequence of NbGNTI-A, and colored by per-residue confidence score (pLDDT), with blue indicating high confidence and red indicating low confidence. The region highlighted in green is to the substrate binding DxD motif and in pink is the missing motif in NbGNTI-B. Close up amino acid spatial location is shown in the right.

1.9.2 CRISPR/Cas9- mediated knockout of *N. benthamiana* *ALG3* and *GNTI*

To successfully engineer *N*-glycan structures in *N. benthamiana* using CRISPR/Cas9, two to three gRNA target sites per gene were carefully selected after comprehensive screening, considering their genomic positions and minimizing off-target potential as predicted by the CRISPRdirect tool (Naito *et al.*, 2015) (Figure 8). Total five gRNAs targeting the *NbALG3* and *NbGNTI-A*, *NbGNTI-B* genes were incorporated into single multiplex CRISPR/Cas9 expression vector by golden gate cloning (Figure 9). This CRISPR/Cas9 cassette was constructed by Yukino Yamashita. After the confirmation of the construct by Sanger

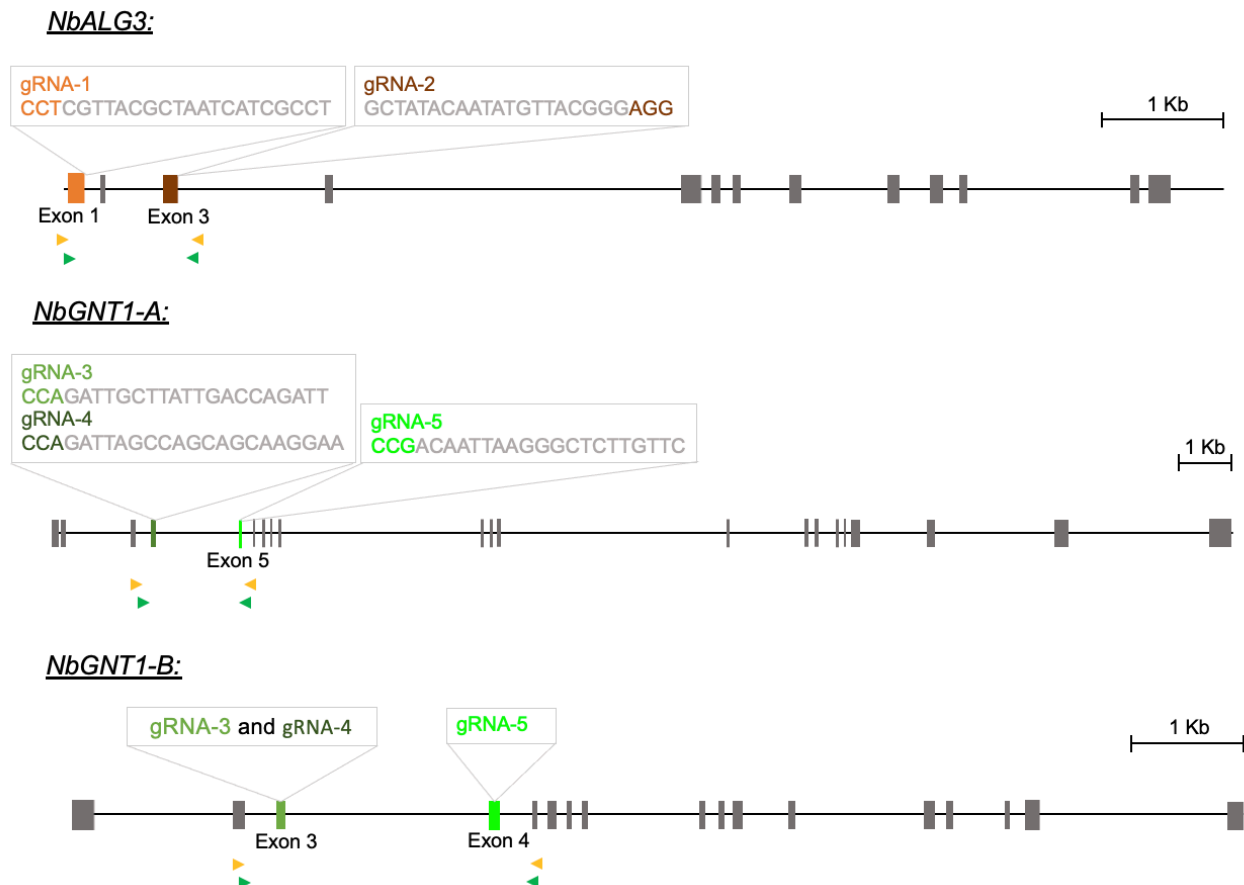


Figure 8. Schematic representation of CRISPR/CAS9 target sites in selected genes. The gRNAs targeting exons are shown as a colored box, untargeted exons; gray boxes, introns; grey line. A total of five gRNAs are designed and PAM sequence on the gene is highlighted as colored nucleotides. Two sets of primers for each gene are designed and shown as arrowheads. Yellow primers are used for PCR amplification and green primers are used for sequencing (Table 1).

sequencing, the vector was introduced into *Agrobacterium tumefaciens* GV3101 and used for stable transformation of WT *N. bentamiana* plants. Since a single cassette for the knock-out was used, a three-step screening method was applied to all T₀, T₁, T₂ and backcrossed plants to distinguish the single (*alg3* or *gntI*) and double (*alg3gntI*) knockouts. Previous studies strongly suggested the loss of GNTI activity eliminated the plant specific β 1,2-xylose and α 1,3-fucose epitopes (Herman *et al.*, 2021; Limkul *et al.*, 2016; Strasser *et al.*, 2005). Therefore, disruption of GNTI activity was examined by anti-HRP western blot (Wuhrer *et al.*, 2005). Then *NbALG3* target amplicon of selected plants was sequenced. Lastly the sequence of *NbGNTI-A* and *-B* genes were checked by Sanger sequencing. Total 17 kanamycin resistant T₀ generation plants were generated and five putative mutant plants were selected through the three-step screening. Subsequently, a total of 120 T₁ plants were grown from those selected plants and screened.

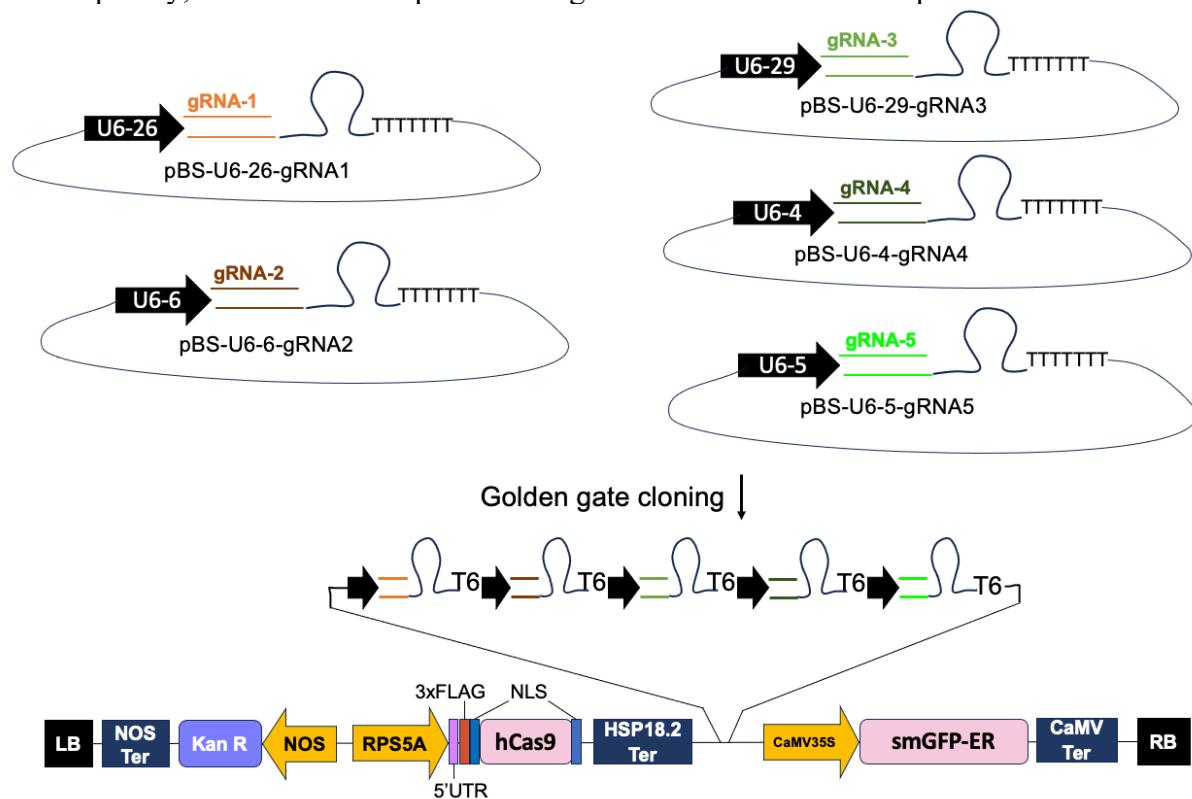


Figure 9. Multiplex CRISPR/Cas9 plant expression cassette. The human codon-optimized Cas9 with N-terminal 3 × FLAG and SV40 nuclear localization signal (NLS) and C terminal nucleoplasmin NLS was constructed under the control of the *A. thaliana* Ribosomal protein S5 A (RPS5A) promoter. Total of five spacer RNA sequences (illustrated as colored parallel lines) were inserted into pBluescript (pBS)-U6 based plasmids harboring tracer RNA scaffold (illustrated as a black loop). The U6-sgRNAs were inserted into multiplex CRISPR/Cas9 expression cassette at *Bsa*I restriction enzyme site by golden gate cloning. NOS Ter: Nopaline

synthase gene terminator, KanR: Kanamycin resistant gene, NOS: Nopaline synthase gene promoter, HSP18.2 Ter: Heat shock protein 18.2 terminator from *A. thaliana*, LB: T-DNA left border, RB: T-DNA right border.

From the screening, mutant 6-8 was identified as T₁ *alg3* and mutant 11-11 was identified as T₁ *alg3gntI* plant. Seeds from the mutant plants were collected and used to grow subsequent T₂ and T₃ generations. The gene mutations were verified in each generation. The T₃ *N. benthamiana alg3* mutant contained a 7-base pair deletion positioned 3 nucleotides downstream of the “NGG” protospacer adjacent motif (PAM) sequence within the exon 1. The T₃ *N. benthamiana alg3gntI* double mutant exhibited multiple mutations: the *NbALG3* gene had deletions of 4 nucleotides in exon 1 and 7 nucleotides in exon 3, both located 4 bases downstream from the PAM site; *NbGNTI-A* gene had a single nucleotide insertion 4 positions downstream of the PAM in exon 5, and *NbGNTI-B* gene had a 5-nucleotide deletion 3 positions from the PAM in exon 3. All mutations resulted in frameshifts that disrupted the reading frame (Table 5).

gntI mutant was not generated among the T₀ plants. One possible explanation is that the gRNAs targeting *NbALG3* had stronger editing activity than those targeting *NbGNTIs*, which may have reduced the likelihood of recovering *gntI* mutants when using the multiplex cassette. Therefore, the *gntI* mutant was generated through cross pollination between WT and T₂ *alg3gntI N. benthamiana*. Seeds from the cross-pollinated nectar were collected and used to grow F₁ plants. A total of 225 F₁ plants were grown and screened. As a result, the 11-11-21 strain was identified as a *gntI* mutant carrying the same frameshift mutations in *NbGNTI-A* and *NbGNTI-B* as the *alg3gntI* mutant, but with a WT *NbALG3* gene (Table 5).

Table 5. Genotype of the T3 generation *N. benthamiana* *alg3*, *gntI*, and *alg3gntI* plants. The occurring homologous mutations are shown in the indels column. Insertions are highlighted in red and deletions are shown as hyphens.

Mutant line	Clone number	Target gene	Position	Nucleotide sequence	Indels
<i>alg3</i>	6-8	<i>NbALG3</i>	Exon 1	ATTCCATCCTCGTTACGCTAATCATCGCCTACGT ATTCCATCCTCG - - - - - AATCATCGCCTACGT	-7 bp
<i>alg3gntI</i>	11-11	<i>NbALG3</i>	Exon 1	ATTCCATCCTCGTTACGCTAATCATCGCCTACGT ATTCCATCCTCGT - - - - CTAATCATCGCCTACGT	-4 bp
			Exon 3	ACTCTGCTATACAATATGTTACGGGAGGTCAAG ACTCTGCTATACAAT - - - - - GGGAGGTCAAG	-7 bp
		<i>NbGNTI-A</i>	Exon 5	GAGTGCCGACAATTAAGGGCTCTTGTTTCAGGAT GAGTGCCGACATATTAAGGGCTCTTGTTTCAGGAT	+1 bp
		<i>NbGNTI-B</i>	Exon 3	TCAGACCAGATTGCTTATTGACCAGATTAGCCA TCAGACCAGA- - - - TATTGACCAGATTAGCCA	-5 bp
<i>gntI</i>	11-11-21	<i>NbGNTI-A</i>	Exon 5	GAGTGCCGACAATTAAGGGCTCTTGTTTCAGGAT GAGTGCCGACATATTAAGGGCTCTTGTTTCAGGAT	+1 bp
		<i>NbGNTI-B</i>	Exon 3	TCAGACCAGATTGCTTATTGACCAGATTAGCCA TCAGACCAGA - - - - TATTGACCAGATTAGCCA	-5 bp

1.9.3 Total *N*-glycan profiles of the *N. benthamiana* WT, *alg3*, *gntI*, and *alg3gntI*

In the preliminary analysis of the total *N*-glycan profile of the WT, *alg3*, *gntI*, and *alg3gntI* plants, total soluble protein extracts were extracted and digested with two glycosidases, PNGase F and Endo H. The result was visualized by lectin blotting with ConA lectin. Generally, ConA binds to the non-reducing α -mannose residues of *N*-glycans (Faye and Chrispeels, 1985). PNGase F is discovered from *Elizabethkingia meningoseptica* and cleaves high mannose, hybrid, and complex oligosaccharides. The cleavage site is between the asparagine and innermost GlcNAc residues. However, PNGase F cannot cleave *N*-glycan that have α 1,3-fucose linked residue on the innermost GlcNAc residue. This makes it unable to cleave plant-specific complex *N*-glycans (Tretter *et al.*, 1991). On the other hand, Endo H is an endoglycosidase discovered from *Streptomyces plicatus* that cleaves the glycosidic bond between the two core GlcNAc residues of high mannose or hybrid *N*-glycans (Tarentino *et al.*, 1974). However, this activity is exceptional to the ManGlcNAc₂, Man₂GlcNAc₂, and

Man₃GlcNAc₂ (M3) structures (Tretter *et al.*, 1991; Trimble and Tarentino, 1991). Therefore, combination of these glycosidase enzymes and ConA lectin blotting was utilized to investigate the total *N*-glycan profiles of the mutants (Figure 10A). The binding of the ConA lectin with WT and *alg3* samples was low regardless of the glycosidase digestions. This suggests that *N*-glycan profile of both the WT and *alg3* plants consisted of mainly complex plant-specific *N*-glycans and only a small amount of mannosidic *N*-glycans (Figure 10B). On the other hand, ConA showed high affinity to the protein samples of the non-digested *gntl* and *alg3gntl* mutants, indicating that high amount of the mannosidic *N*-glycans. The binding of ConA to the *gntl* sample was substantially reduced after the digestion with PNGase F and Endo H.

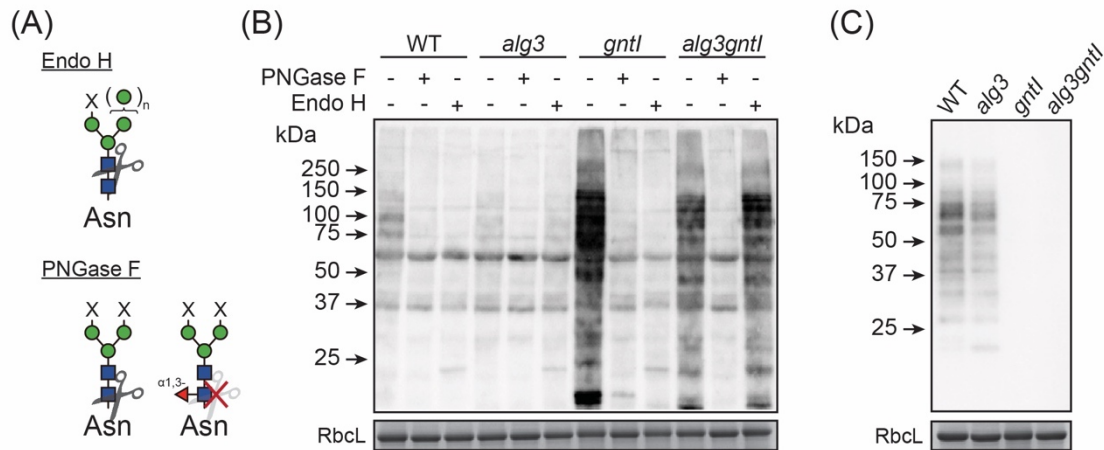


Figure 10. Preliminary analysis of the *N*-glycan profiles in the *N. benthamiana* WT, T₃ generation *alg3*, *gntl*, and *alg3gntl*. (A) Schematic representations of the substrate specificities of Endo H and PNGase F; X can be single mannose/ *N*-acetylglucosamine residue or elongated *N*-glycan arm. (B) Total soluble protein extracts were digested with PNGase F and Endo H and separated by SDS-PAGE. The results for the the ConA-probed lectin blot and the CBB-stained large subunit of RuBisCO (RbcL) used as a loading control are shown. (C) Total soluble protein extracts were separated by SDS-PAGE and the presence of plant-specific-type *N*-glycans is visualized by anti-HRP western blot.

On the other hand, the binding of the ConA to the *alg3gntl* sample was not lost after Endo H digestion. This result demonstrates *gntl* and *alg3gntl* mutants have high amount of mannosidic *N*-glycan profile. But the *N*-glycans in *alg3gntl* mutant could be mostly M3 structure. At the same time, the presence of plant-specific *N*-glycan epitopes in WT, *alg3*, *gntl*, and *alg3gntl*

plants were checked by anti-HRP western blot. Anti-HRP antibody bound only with the WT and *alg3* samples (Figure 10C). The result shows that the plant-specific β 1,2-xylose and α 1,3-fucose epitopes were completely absent in the *gnt1* and *alg3gnt1* plants.

To analyze the *N*-glycosylation profiles of the WT, *alg3*, *gnt1*, and *alg3gnt1* plants in detail, total *N*-glycans were chemically released from *N*-glycoproteins and fluorescently labelled with 2-aminopyridine (PA). The PA-labelled *N*-glycans were collected and concentrated. Prepared PA-labelled *N*-glycans were separated by RP-HPLC with a C18 column (Figure 11), glucose oligomers were used as a standard. The peaks expected to be *N*-glycans were collected and concentrated, and the structures were further examined by the LC-ESI-MS/MS.

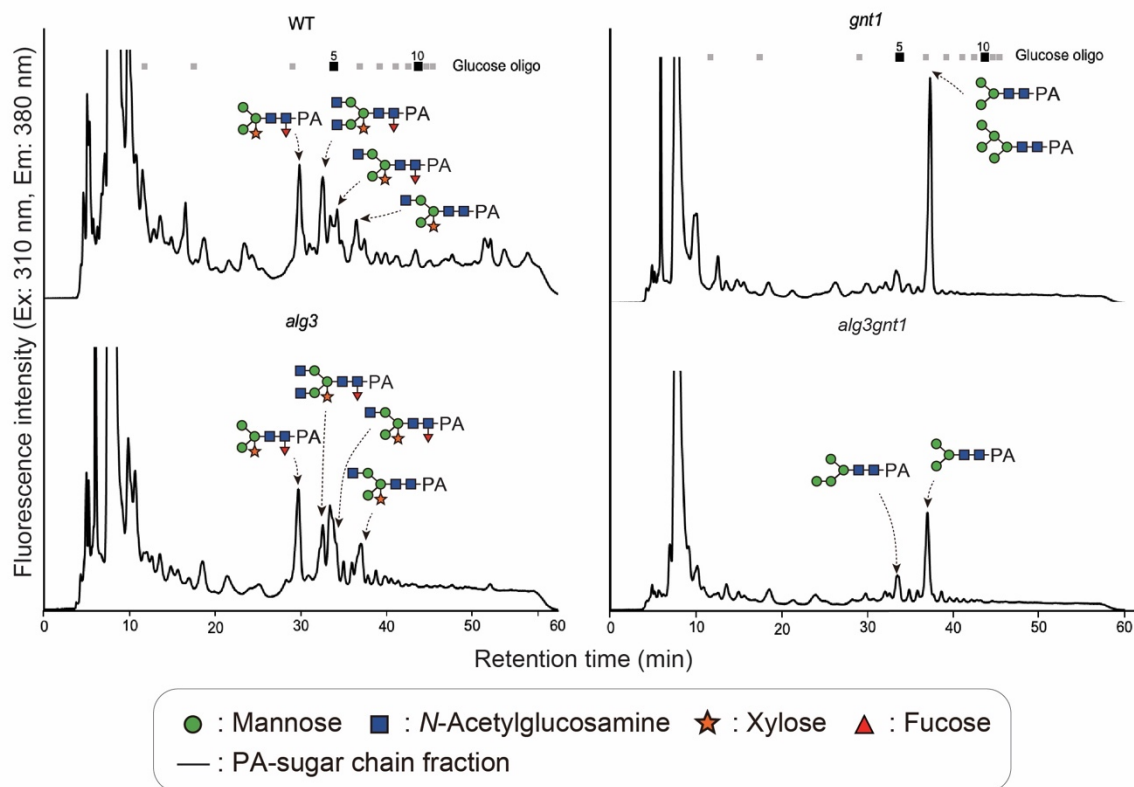


Figure 11. Total *N*-glycan profiles in the WT and T₃ *N. benthamiana* mutants. Total *N*-glycans were extracted, hydrolyzed, PA-labelled and analyzed using RP-HPLC. Glucose oligo standards (Glucose oligo) are shown at the top of the chromatogram. The peaks appeared between 28 minutes and 48 minutes were collected and subjected to LC-ESI-MS/MS analysis. The main *N*-glycans identified from the peaks are shown.

Conforming the lectin and western blotting results, WT and *alg3* mutant produced similar profiles of *N*-glycans: 27.0% and 24.4% mannosidic *N*-glycans, and 73.0% and 73.8% plant-specific *N*-glycans, respectively. The main difference in the composition of the *N*-glycans of *alg3* and WT indicated the loss of the α 1,3-mannosyltransferase enzyme activity in *alg3* mutant. *alg3* mutant produced ER-derived *N*-glycan types: M5^{ER}-PA, GlcM5^{ER}-PA, and M4^{ER}-PA at levels of 6.8%, 1.9%, and 8.4%, respectively (Table 6). As expected, disruption of *N*-acetylglucosaminyltransferase I gene in *gntI* mutant led to a uniform high-mannose *N*-glycan composition consisting of 100% mannosidic type, with 76% of these being M5-PA.

Table 6. Relative amounts of *N*-glycan structures detected in WT and T3 generation *alg3*, *gntI* and *alg3 gntI* *N. benthamiana*

			Ratio (%)			
			WT	<i>alg3</i>	<i>gntI</i>	<i>alg3 gntI</i>
Mannosidic type	M3		0.4	9.2	9.3	77.0
	M4 ^{ER}		-	8.4	-	11.1
	M4		-	-	5.3	-
	M5 ^{ER}		-	6.8	-	7.6
	M5		-	-	76.3	-
	M6		9.7	-	2.3	-
	M7A		2.9	-	1.6	-
	M7B		6.4	-	1.3	-
	M8A		6.6	-	2.3	-
	M8B		-	-	-	-
	M9		1.0	-	1.6	-
Glucosylated type	GlcM5		-	1.9	-	3.4
	Glc2M5		-	-	-	0.8
Plant-specific type	M3X		-	2.2	-	-
	M2FX		2.7	3.1	-	-
	M3FX		33.3	33.8	-	-
	Termnal					
	GlcNAc residue(s)					
	GNM3X		0.4	2.4	-	-
	GNM3FX		20.4	13.2	-	-
	GN2M3FX		14.2	19.0	-	-
	Galactosylated type					
	GalFGN2M3FX		2.0	-	-	-
Total	Manosidic type		27.0	24.4	100	95.8
	Glucosylated type		-	1.9	-	4.2
	Plant-specific type		73.0	73.8	-	-

Lastly, double disruption of both *ALG3* and *GNTI* genes resulted uniquely uniform *N*-glycan composition with 77.0% M3-PA, 11.1% M4-PA, and 7.6 % M5-PA. It can be concluded that

loss of both the ALG3 and GNTI enzymes completely arrested the *N*-glycan maturation in the ER and Golgi, and led to accumulation of mainly trimannosyl *N*-glycans in *N. benthamiana*.

1.9.4 Phenotypic analysis of single and double mutants

N-Glycosylation plays important roles in various physiological and cellular processes. Change in the *N*-glycosylation composition possibly cause some physiological or morphological defect(s). To address this possibility, visible morphological characteristics including overall health, leaf weight, and root growth were assessed. Five-week-old WT and T₃ mutant plants were evaluated by measuring leaf weight. While the *alg3* and *gntI* plants showed no difference in leaf weight compared to the WT, *alg3gntI* leaves were significantly smaller. In accordance with the leaf size, the *alg3* and *gntI* mutants displayed normal growth (Figure 12A, B). In contrast, *alg3gntI* showed dwarf phenotype suggesting the double disruption of *GNTI* and *ALG3* caused severe growth impairment. Additionally, defective root development (Figure 12C) and curled leaves were observed in *alg3gntI* mutant. To further investigate the root development defect in mutants, four different conditions were applied: MS medium with 0%, 1%, or 3% sucrose, and MS medium supplemented with 0.3 mM ascorbic acid to modulate the possible ER stress-derived oxidative stress. The root growth responses of the WT, *alg3*, and *gntI* plants were not significantly different from each other across all conditions. However, the *alg3gntI* exhibited impaired root growth in MS medium with sufficient sucrose (1% and 3%). Notably, the addition of ascorbic acid partially rescued this defective root phenotype in *alg3gntI* (Figure 12D). These results show how the certain changes in *N*-glycosylation led to growth defects in certain species, highlighting the intricate relationship between plant *N*-glycosylation machinery and physiology.

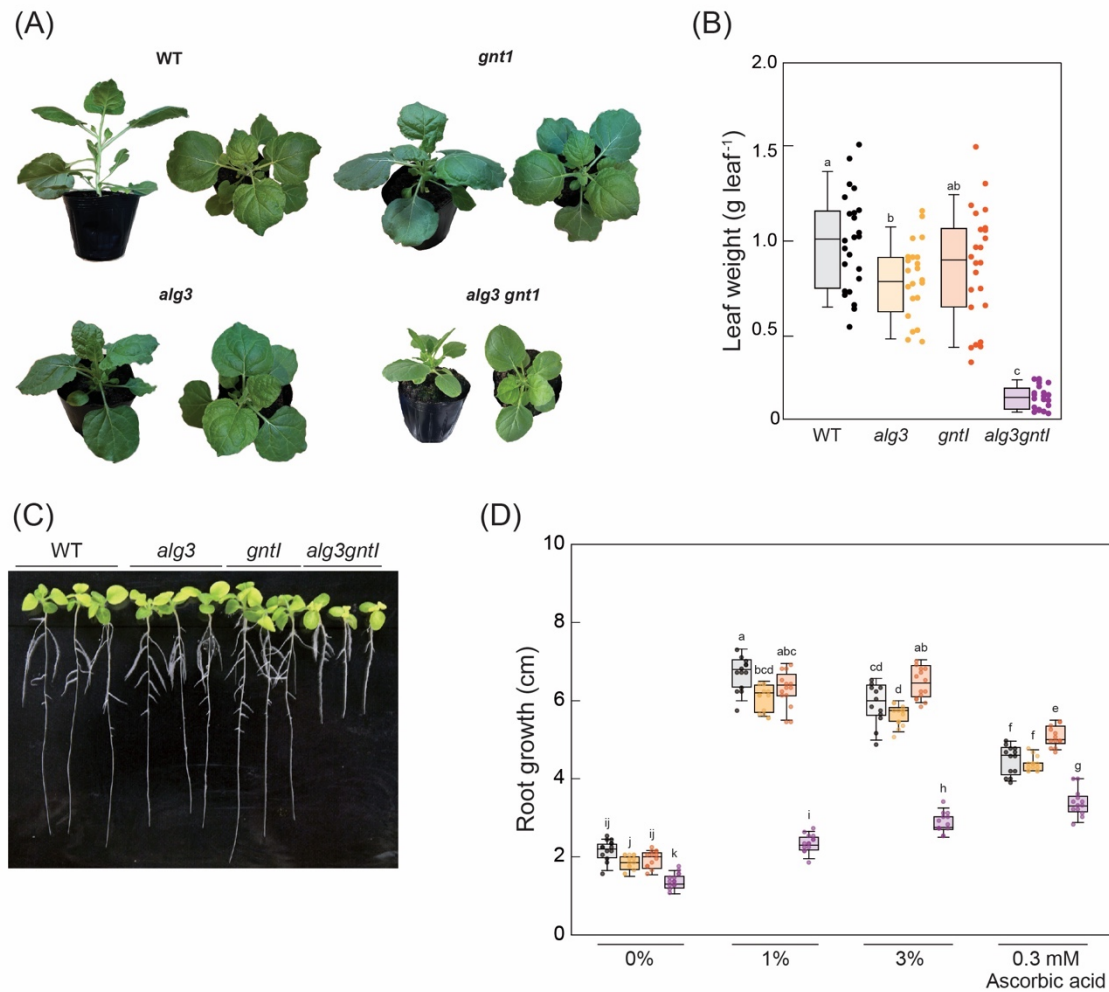


Figure 12. Phenotypic analysis of *N. benthamiana* *alg3*, *gnt1*, and *alg3gnt1*. (A) Top and side image of the 5 week-old WT and T₃ *N. benthamiana* mutants. (B) Leaf weight Measurement of the WT and mutant plants; median and individual values were displayed. (C) Root growth comparison of the WT and mutant seedlings. Seedlings were generated on MS plates and transferred to the MS plate with 3% sucrose and grown for 10 days. (D) Root length measurement of the WT and mutant seedlings in 0%, 1%, or 3% sucrose and 0.3 mM ascorbic acid supplement; median and individual values are plotted. The letters above each plot indicate statistically significant differences between groups. A one-way ANOVA was used to evaluate differences in group means ($p < 0.05$). Pairwise comparisons were then assessed using Tukey's HSD post-hoc test.

1.10 Discussion

Over the past two decades, studies decoding the plant *N*-glycosylation pathway have granted us with a broad understanding of the *N*-glycan processing in plant. The identification of numerous *N*-glycosylation enzymes has proven it is well conserved in plant and mammalian system, with some key differences in the later processing steps in Golgi (Strasser *et al.*, 2021).

Trending interest in the use of plants for biopharmaceutical production urged numerous studies to focus on *N*-glycoengineering to eliminate plant-specific epitopes and produce biopharmaceuticals with humanized *N*-glycans (Castilho *et al.*, 2011; Castilho and Steinkellner, 2012; Jansing *et al.*, 2019; Kallolimath *et al.*, 2016). In the last decade, CRISPR/Cas9 technology has been employed to modify *N*-glycosylation related genes with precision in various plant expression hosts, further boosting the potential of plant biofactories for recombinant biopharmaceuticals with desired *N*-glycan profile (Kao *et al.*, 2024). The first study using CRISPR/Cas9 gene editing tool in *N. benthamiana* was published in 2013. This study demonstrated use of the single guide RNA (sgRNA) to successfully target the phytoene desaturase (*PDS*) gene. Cas9 variant with a eukaryotic nuclear localization signal and sgRNA cassettes were transiently co-expressed in *N. benthamiana* by *Agrobacterium*-mediated infiltration method. The target gene sequence from agroinfiltrated leaves harbored 1 nucleotide insertion to 9 nucleotides deletion modification at the upstream of PAM sequence (Nekrasov *et al.*, 2013). Knockout of *PDS* leads to loss of chlorophyll, resulting albino phenotype. Thus, *PDS* genes are targeted in the earlier method development studies because the loss of function of this gene is visually visible. Protocol to generating genome edited tomato, *A. thaliana* and *N. benthamiana* plants by co-cultivation method were described later using the same target gene, *PDS* with single Cas9-sgRNA cassette by Komatsu *et al.*, (2020). In their study, 10% of the T₀ plants showed double mutation of *NbPDS1* and *NbPDS2* with single sgRNA (Komatsu *et al.*, 2020). Quantifying the efficiency of our CRISPR/Cas9 tool was not in the scope of this study. Total 17 plants were generated then based on the initial anti-HRP WB result and health of the plants next screening was done on only selected plants. Therefore, the mutation frequency could not be accurately calculated. However, all five selected plants showed mutations in Exon 1 of *ALG3*, three had additional mutations in Exon 3 of *ALG3*, and only two exhibited mutations in Exon 5 of *GNTI-A*, one had mutations in Exon 3 and Exon 4 of *GNTI-*

B. All the mutations occurred two to three nucleotides upstream from PAM sequence. This result is consistent with the findings of Jinek et al. (2012), who demonstrated that Cas9-induced mutations typically occur within the three nucleotides immediately upstream of the PAM sequence. The first application of CRISPR/Cas9 for *N*-glycoengineering in *N. benthamiana* was published by Jansing *et al.* in 2020. In their study, a single construct was used to simultaneously target four *FUCT* genes and two *XYLT* genes. A key feature of their design was the use of a polycistronic gRNA/tRNA array, in which four gRNAs targeting *FUCT* and three targeting *XYLT* were expressed under a single promoter and terminator. Additionally, loxP sites were inserted on both sides of the cassette to enable its later removal from the genome. The construct was introduced into the plant by *Agrobacterium*-mediated infiltration, and after five days of cultivation, the tissues were harvested and used for plantlet regeneration. They designed three separate constructs: one targeting only *FUCT*, one targeting *XYLT*, and one targeting both. The mutation frequency varied significantly depending on the construct, particularly within the *FUCT* loci. This variation was attributed to the position of the gRNAs within the polycistronic gRNA/tRNA array, which likely led to differences in expression levels (Jansing *et al.*, 2019). In our study, a single cassette was also used for mutant generation. Interestingly, although more gRNAs were designed to target *GNTI*, the overall mutation frequency was higher for *ALG3*. This suggests that factors such as gRNA binding strength and the chromatin accessibility of the target loci may have influenced editing efficiency in our system (Appendix 3).

The development of recombinant proteins with uniform, mannosylated *N*-glycans without plant-specific epitopes represents a significant advancement in plant-based pharmaceutical production. Through multiplex CRISPR/Cas9 genome editing, we successfully targeted *NbALG3* and *NbGNTI* genes in *N. benthamiana* for the first time to create single and double mutants, each producing distinct *N*-glycan profiles with potential applications in biopharmaceutical expression. The knockout of *NbGNTI* in *N. benthamiana* yielded highly

uniform M5 *N*-glycan structures while completely eliminating plant-specific epitopes. This outcome aligns with previous research in *A. thaliana*, where *GNTI* knockout similarly resulted in the absence of β 1,2-xylose and α 1,3-fucose epitopes (Strasser *et al.*, 2005), confirming the consistency of this genetic modification across different plant species. In our study, disruption of *GNTI* resulted not only elimination of plant-specific epitopes but also 76.3% homogenic M5 structure. On the other hand, CRISPR/Cas9-mediated targeting of six glycosyltransferase genes in *N. benthamiana* successfully eliminated the plant-specific epitope, but the most abundant structure was GN2M3 at 37.0%. Moreover, deletion of *ALG3* in *A. thaliana* was noted to produce rare *N*-glycan structures including M3, M4^{ER}, M5^{ER} and GlcM5^{ER} (Kajiura *et al.*, 2010). This result was confirmed in *N. benthamiana* by our total *N*-glycan analysis of *alg3* mutant. The *alg3gntI* double mutant demonstrated greater uniformity, producing 77.0% M3 *N*-glycan structures with complete elimination of plant-specific epitopes. In previous study, *GNTI* knockdown *N. benthamiana* plant produced 82.4% homogenous M5 *N*-glycans (Limkul *et al.*, 2016). However, *gntI* and *alg3gntI* mutants generated in this study offer more stable phenotype and complete absence of plant-specific epitopes due to complete knockout of the genes. This represents a significant step toward achieving the homogeneous glycan profiles desired for biopharmaceutical production (Table 7). However, the *alg3gntI* mutant exhibited severe phenotypic abnormalities including impaired root development, dwarf morphology, and compromised seed maturation. These developmental defects are consistent with previous studies in *A. thaliana* demonstrating that disrupted *N*-glycosylation caused by the loss of specific OST subunits leads to multiple physiological impairments including growth inhibition, abnormal cell differentiation, reproductive failure, and reduced stress tolerance (Farid *et al.*, 2013; Hoeberichts *et al.*, 2008; Jeong *et al.*, 2018; Koiwa *et al.*, 2003; Lerouxel *et al.*, 2005; Müller *et al.*, 2016). Our investigation into potential interventions revealed that 0.3 mM

ascorbic acid supplementation partially rescued root growth defects in *N. benthamiana* *alg3gntI* mutant. As a potent antioxidant, ascorbic acid indirectly

Table 7. Comparison of total *N*-glycan composition ratios (%) observed in this study with ratios reported in previous *N*-glycoengineered plant systems.

		This study			(Kajiura <i>et al.</i> , 2010)	(Strasser <i>et al.</i> , 2005)	(Limkul <i>et al.</i> , 2016)
		<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>	<i>At-alg3</i>	<i>At-cgl</i>	<i>NbGNTI-RNAi7</i>
Mannosidic type	M3	9.2	9.3	77.0	31.1	-	-
	M4 ^{ER}	8.4	-	11.1	20.7	-	-
	M4	-	5.3	-	-	1.1	-
	M5 ^{ER}	6.8	-	7.6	12.0	-	-
	M5	-	76.3	-	-	74.5	82.4
	M6	-	2.3	-	-	7.1	-
	M7	-	2.9	-	-	7.2	4.1
	M8	-	2.3	-	-	6.7	4.4
	M9	-	1.6	-	-	3.4	-
Total Mannosidic type		24.4	100.0	95.8	63.8	100.0	90.9
Glucosylated type		1.9	-	4.2	2.4	-	-
Plant-specific type		73.8	-	-	34.6	-	9.1

alleviated the ER stress and improve cellular function despite the underlying *N*-glycosylation effect. However, the same treatment proved ineffective in *A. thaliana cglalg3* mutants (*cgl* is a *gntI* mutant of *A. thaliana*), implying species specific responses to oxidative stress (in a parallelly conducted experiment; results are not included in this work). Additionally, a study in *A. thaliana* have shown that GNTII mutants lacking specific GlcNAc residues exhibit increased sensitivity to various stress conditions, accelerated leaf senescence, and altered phytohormone responses, particularly affecting cytokinin and auxin transport pathways (Yoo *et al.*, 2021). Based on previous reports, physiological impact of losing plant-specific epitopes on *N*-glycosylation varied significantly across plant species. While the absence of plant-specific *N*-glycans in *A. thaliana* and *N. benthamiana* produces minimal phenotypic changes (Jansing *et al.*, 2019; Strasser *et al.*, 2004), the same modifications in *Oryza sativa* (rice) result in severe developmental defects (Fanata *et al.*, 2013; Jung *et al.*, 2021). Our study confirmed that the absence of plant specific *N*-glycans are not the cause of phenotypic change in *N. benthamiana*. The dwarf phenotype observed in the *alg3gntI* mutant may be partly explained

by ER stress-induced oxidative stress. Disruption of early *N*-glycan processing in the ER could overload the ER quality control system and cause ER stress (Cao *et al.*, 2022). The direct connection between ER stress and increased reactive oxygen species (ROS) levels can be explained by an imbalanced oxidative protein folding process, which creates a disulfide bond by protein disulfide isomerase (PDI) family proteins and produces H₂O₂ as a byproduct (Urade, 2019). Excessive ROS accumulation can impair cellular redox homeostasis and negatively affect growth and development (Ozgur *et al.*, 2014). The observation that supplementation with ascorbic acid, a major antioxidant, can partially rescue the impaired root growth supports this interpretation, as it suggests that ROS detoxification alleviates the physiological burden caused by ER stress. However, *alg3* mutant, which has the same *ALG3* mutation as the *alg3gntI* mutant did not show any developmental defect. It suggested that ER stress could not be the sole reason for the dwarf phenotype and impaired root growth in *alg3gntI*. The observed phenotypes may be better explained by other reports in *Nicotiana* plants showing similar growth defects. For instance in *N. benthamiana*, over activation of immune system by silencing of certain immune receptor network led to dwarf phenotype (Adachi *et al.*, 2023). In another study, constitutive suppression of gibberellic acid (GA) signaling in *N. benthamiana* showed a dwarf phenotype with decreased sensitivity to GA (Xu *et al.*, 2013). Similarly, overexpression of GA deactivating enzymes in *N. tabacum* reported to cause dwarf phenotype as well (Suo *et al.*, 2012; Xiao *et al.*, 2016). Overall, it remains difficult to pinpoint the precise mechanism underlying the observed phenotype in the *alg3gntI* mutant. The uncertainty arises from whether (i) altered *N*-glycosylation pathway in ER and Golgi causes organelle-specific stress and broadly imbalances cell physiology, for example by affecting endomembrane transport system, (ii) the presence of M3 *N*-glycans on specific proteins such as receptors or transcription effectors interferes with their maturation, folding, or localization, thereby impairing their biological function, or (iii) a combination of these factors collectively contributes to the mutant

phenotype. The successful generation of the mutants not only produced potential alternative expression host for lysosomal enzymes, but also provided some insight into relationship between *N*-glycosylation and physiological phenotypes of *N. benthamiana*.

1.11 Summary

In this chapter, multiplex CRISPR/Cas9-mediated genome editing was utilized to generate knockout lines of *Nicotiana benthamiana* targeting two key genes involved in *N*-glycosylation: *ALG3* and *GNTI*. Firstly, candidate genes were searched and identified from Sol Genomics Network: *N. benthamiana* draft genome v2.6.1. database and identified. Recombinant enzyme activity of those genes was parallelly assessed (Kajiura, private communication). *N. benthamiana* possessed single active *ALG3* and *GNTI* enzymes and NbGNTI-B was proven to be nonfunctional pseudoenzyme based on theoretical 3D structure model and *in vitro* enzyme activity result. CRISPR/Cas9 construct was successfully delivered by *Agrobacterium* co-cultivation method, and genotyping of selected T₀ plants confirmed successful mutagenesis at

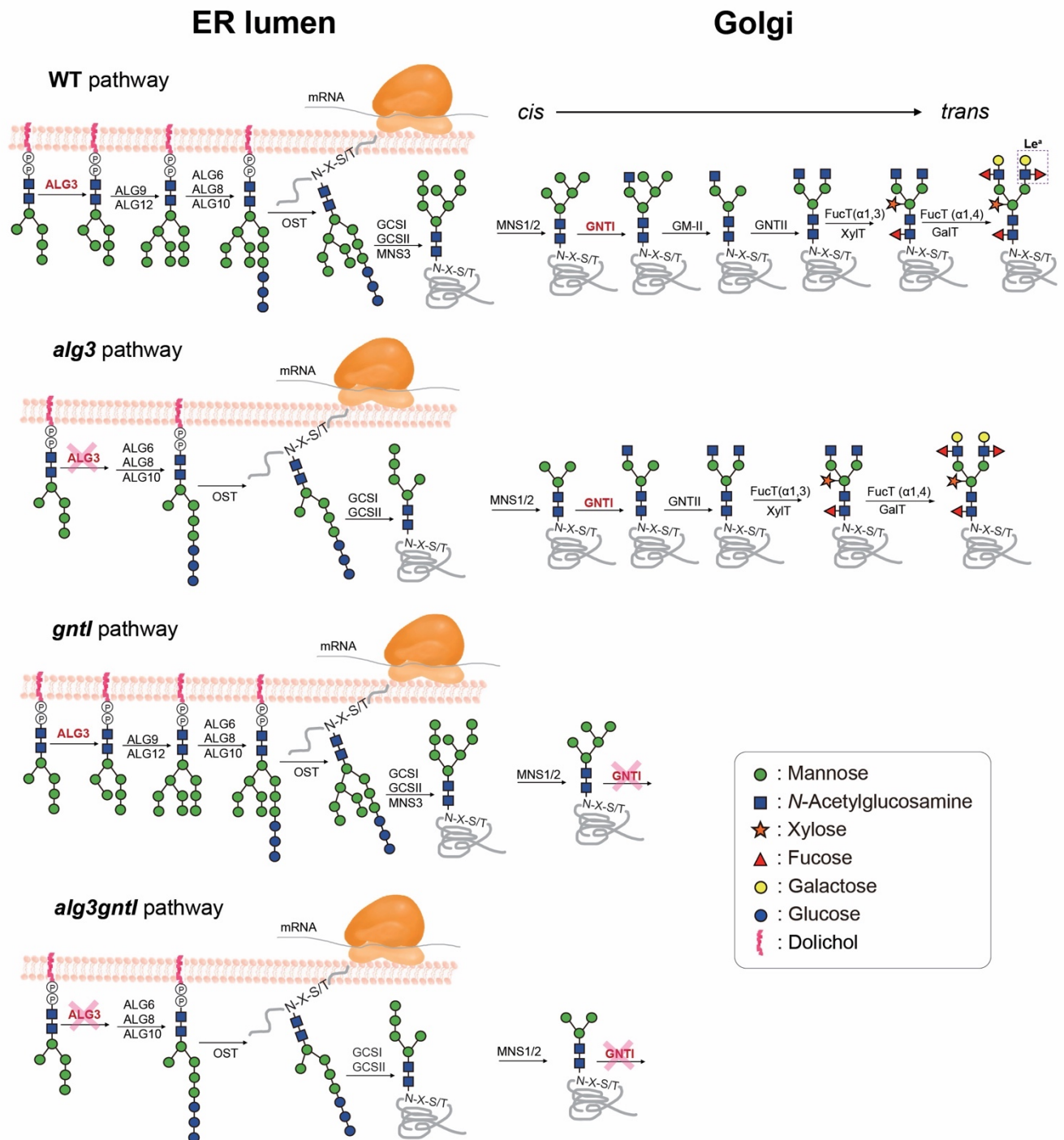


Figure 13. Predicted *N*-glycosylation pathway in the *N. benthamiana* WT and *alg3*, *gntI*, and *alg3gntI*. The *N*-glycosylation pathway is illustrated starting from the intermediate M5^{ER}. Dolichol-2 phosphate (PP)-linked oligosaccharides were subsequently synthesized by asparagine-linked glycosylation (ALG) enzymes. The final Glc3M9/5-Dol-PP was transferred to the Asn (N) residues of the polypeptide chain by OST complex. Glycosyltransferases targeted in this study are written in pink. Specific symbols representing the sugar residues are displayed in the box.

the expected loci. Mutations occurred predominantly within three nucleotides upstream of the PAM site, led to frame shift mutations. Interestingly, although more gRNAs were designed for *GNTI*, *ALG3* exhibited a higher mutation frequency. This is likely due to differences in gRNA efficiency, or chromatin accessibility. *N*-Glycosylation profile and the phenotype of those mutant lines were thoroughly studied in the next chapters.

This chapter examined the functional impact of CRISPR/Cas9-mediated knockouts of *ALG3* and *GNTI* in *N. benthamiana*. *N*-Glycans were chemically released from total soluble proteins, fluorescently labelled, and analyzed using LC-ESI-MS/MS. The *N*-glycan profiles of the *alg3* mutant showed some immature ER resident *N*-glycans. Disruption of *GNTI* eliminated plant-specific epitopes, with a corresponding increase in mannosidic structures in both *gntI* and *alg3gntI* mutants (Figure 13). These results confirm the the potential of these lines for producing recombinant proteins with more homogenous and human-compatible *N*-glycan structures. In parallel, phenotypic analysis was conducted to determine whether loss of function in *N*-glycosylation enzymes resulted in visible developmental changes. While most mutant lines showed normal growth under standard conditions, double disruption of *ALG3* and *GNTI* led to mild alterations in leaf morphology and dwarf phenotype, and root growth abnormality.

Chapter Three: Production of recombinant biopharmaceuticals in *N. benthamiana* *alg3*, *gntI*, and *alg3gntI* mutants

1.12 Introduction

Recombinant protein glycosylation plays an important role in the pharmacological function, stability, and bioavailability of many biotherapeutics. In particular, monoclonal antibodies (mAbs) and ERT enzymes require specific *N*-glycan structures for optimal therapeutic efficacy. The Fc region of IgG antibodies depends on conserved *N*-glycans for mediating effector functions such as antibody-dependent cellular cytotoxicity (ADCC), and a heterogeneity in *N*-glycosylation results in multiple *N*-glycoforms that can cause efficacy variance (Jefferis, 2009). Likewise, most lysosomal enzymes used in ERTs rely on high-mannose or mannose-6-phosphate motifs for efficient cellular uptake and localization via mannose receptors (Ohashi, 2012; Zeng *et al.*, 2019). Consequently, producing these enzymes with well defined *N*-glycosylation is a major goal in biopharmaceutical development. In the last decade, market value of the antibody related drugs is constantly increasing. Besides, monoclonal antibodies are suitable for investigating *N*-glycosylation profile in plant expression system because it can be easily purified and most IgGs have biantennary *N*-glycans with β 1,2-xylose and/or α 1,3-fucose residues, such as GN2M3FX (Chen *et al.*, 2022). Varlilumab is an agonist, human IgG1k anti-CD27 monoclonal antibody that shows T cell activation and antitumor activity. Antibodies are *N*-glycosylated at the Asn297, fragment crystallizable (Fc) domain of the heavy chain and it affects the structure, effector function and the pharmacokinetics of the antibody (Higel *et al.*, 2016). There are several studies focusing on the different types of *N*-glycosylation influence on the binding affinity between Fc region of antibody and Fc-gamma IIIa (Fc γ RIIIa) receptor of T cell (Cambay *et al.*, 2020; Subedi and Barb, 2015).

Lysosomal Storage Diseases (LSD) are toxic accumulation of certain metabolic product in the lysosome caused by hydrolyzing enzyme deficiencies (70%) or defects in enzyme

activators/associated proteins, resulting from mutations (Rajkumar and Dumpa, 2025). Seventy LSDs are described so far. ERT has received worldwide approval, including Japan, for treating six types of lysosomal storage disorders: Gaucher disease, Fabry disease, three forms of mucopolysaccharidosis (types I, II, and VI), and Pompe disease (Ohashi, 2012). β -glucocerebrosidase (GCase) is a lysosomal enzyme responsible for breaking down glucosylceramide into glucose and ceramide. Genetic mutations that affect this enzyme lead to the development of Gaucher's disease (Beutler, 1988). GCase has five potential *N*-glycosylation sites located at Asn58, Asn98, Asn185, Asn309, and Asn501; however, studies have shown that the glycan at Asn501 is typically not occupied (Brumshtein *et al.*, 2010; Limkul *et al.*, 2016).

Based on the nature of *N*-glycan profiles of the mutants and the trend of biopharmaceutical industry, Varlilumab and GCase were chosen as representative pharmaceuticals. In this chapter, Varlilumab and GCase were transiently expressed in WT and CRISPR/Cas9-edited *N. benthamiana* *alg3*, *gntI*, and *alg3gntI* lines. Both proteins were purified and *N*-glycosylation was thoroughly analyzed by nanoLC-MS/MS. This strategy allowed us to reveal the impact of early *N*-glycosylation pathway editing on the quality and homogeneity of recombinant *N*-glycoproteins in two distinct biopharmaceuticals.

1.13 Materials and methods

1.13.1 Plant preparation

Seeds were sprouted in the soil for about a week then single sprout were transferred to the 12 cm diameter pot and grew 4 more weeks until the plants become ready to be agroinfiltrated. The plants were grown under fully controlled environment (16 h light/8 h dark cycle at 22°C with 50% humidity). Plants were watered regularly. Occasionally, HYPONeX® fertilizer was added to the water to maintain the health of the plants. Before the agroinfiltration,

the top small leaves and bottom old leaves were trimmed to ensure a uniform expression of the target protein.

1.13.2 Varlilumab and GCase expression vectors

A. tumefaciens GV3101 strains harboring the target biopharmaceutical expression vectors were obtained from the lab stock. Varlilumab expression vector were designed and constructed by Nguyen Kim Dua (Nguyen *et al.*, 2023) and GCase expression vector were designed and constructed by Juthamard Limkul (Limkul *et al.*, 2016).

1.13.3 Agroinfiltration

Expression vector harboring agrobacterium were streaked on the 2xYT agar plate containing 50 µg/mL Rifampicin, 40 µg/mL Streptomycin and 50 µg/mL Kanamycin. The single colony was picked and cultured in 5 mL at 30°C with shaking for 1 day. Then the culture was transferred to 200 mL 2xYT medium and cultivated for 2 more days. On the day for agroinfiltration, agrobacterium cells were harvested by centrifugation 4000× g for 10 min and resuspended in agroinfiltration buffer (10 mM MgSO₄, 10 mM MES, pH 5.7). The OD₆₀₀ was adjusted to 0.5 for each agrobacterium. If more than one construct is used for agroinfiltration, each agrobacterium was resuspended in the buffer first and mixed according to a selected ratio. Plants were dipped in the agrobacterium solution in vacuum chamber and vacuum was applied for 1 min then air gauge was gradually opened. Infiltration of the agrobacterium solution to the plant leaves was carefully observed and non-infiltrated leaves were removed instantly. Agroinfiltrated plants were brought back to the controlled environment. And the target protein expressing leaves were harvested 5-7 days post infiltration (dpi) depending on the protein. When harvesting the leaves, midrib parts were removed to facilitate better homogenization.

1.13.4 GCase purification

GCase transiently expressed plant leaves were stored at -80° until the time of total soluble protein extraction. Leaf sample was homogenized by mortar and pestle while adding liquid nitrogen to facilitate a plant cell wall disruption. Four mL extraction buffer (20 mM Tris-HCl pH 7.0, 150 mM NaCl, 0.5% Taurocholic acid) was added to per gram of homogenized leaf sample and vortexed vigorously. Then the sample was incubated on ice for 10 min followed by 5 min vortex and these steps were repeated twice. The crude sample was centrifuged at $10,000\times g$ for 20 min. The supernatant was directly used for purification step. For the purification of GCase, two step purification was performed. Firstly, the sample is applied to Concanavalin A (ConA) agarose bound (Funakoshi, Tokyo, Japan) column and the elution fraction was subsequently applied to Phenyl 650M (Tosoh Corp., Tokyo, Japan) column. ConA column was equilibrated by 5 column volume (CV) ConA buffer (20 mM Tris-HCl pH 7.0, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$) prior to sample application. Crude sample (15-20 mL) was applied to 1 mL ConA column and followed by washing with 15 CV ConA buffer. The GCase was eluted by 10 CV elution buffer (50 mM phosphate buffer pH 7.0, 4 mM β -mercaptoethanol, 1.3 mM EDTA, 0.15% Triton X-100, 0.125% taurocholic acid, and 300 mM Methyl- α -D-mannopyranoside). Before applying to the Phenyl 650M column, NaCl was added to adjust the NaCl concentration of ConA elution fraction to 2.0 M. One mL Phenyl 650M column was equilibrated with 5CV Phenyl equilibration buffer (50mM phosphate buffer pH 7.0, 2.0 M NaCl). Con A elution fraction was applied to the column. Then washed by 5 CV Phenyl equilibration buffer. GCase was eluted with a stepwise gradient of 50 mM phosphate buffer (pH 7.0) of increasing hydrophilicity, containing sequentially 1.5 M NaCl, 1.0 M NaCl, 0.5 M NaCl, 20% (v/v) ethylene glycol, 30% (v/v) ethylene glycol, and 50% (v/v) ethylene glycol. The elution was fractionated by 1 mL/fraction and checked by GCase enzyme activity assay. The fractions with an enzyme activity were pooled and dialyzed against 20 mM Tris pH

7.0 overnight. Dialyzed samples were concentrated by Amicon® Ultra Centrifugal device (Merck Millipore) according to the instruction provided.

1.13.5 IgG purification

IgG transiently expressed plant leaves were stored at -80°C until the time of total soluble protein extraction. Leaf sample was homogenized by mortar and pestle while adding liquid nitrogen to facilitate a plant cell wall disruption. Two mL extraction buffer (100 mM Sodium dihydrogenphosphate, 100 mM NaCl, 40 mM Ascorbic acid pH 7.0) was added to per gram of homogenized leaf sample and vortexed vigorously. Then the sample was incubated on ice for 10 min followed by 5 min vortex and these steps were repeated twice. The crude sample was centrifuged at 10,000× *g* for 20 min. IgG was purified from the supernatant using Monospin ProA spin column (GL Sciences Inc., Tokyo, Japan) according to the protocol provided.

1.13.6 In-gel digestion with trypsin and *N*-glycopeptide analysis

Purified proteins were separated by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. Destained gel region containing target band was cut into 1.5 mm³ pieces and placed in 1.5 mL tube. The gel pieces were destained by 1 mL of 0.1 M ammonium bicarbonate and acetonitrile (1:1, v/v) mix until the gel pieces completely turn transparent. Destaining solution was changed 3-4 times. After the destaining, gel pieces were dehydrated with pure acetonitrile twice for 10 min each. The gel pieces were rehydrated in trypsin solution containing 10 ng/μL Trypsin gold (Promega, WI, USA), 1% ProteaseMax™ (Promega) surfactant, 100 mM ammonium bicarbonate. The trypsin digestion was carried out at 50°C for 1 h followed by additional 10 ng Trypsin gold and 1 h incubation at 50°C to ensure the digestion at the difficult positions. Digested peptide containing liquid phase was transferred to new 1.5 mL tube and 0.2 μL TFA was added to stop the digestion. nanoLC-MS/MS analysis was carried out by ESI-Qq-TOF mass spectrometer (microTOF-Q II; Bruker Daltonics, Bremen,

Germany) coupled with nanoLC system (1200 series; Agilent Technologies, CA, USA) incorporating a trap column (5 μm , 0.3 $\times$ 5 mm) and analytical column (3.5 μm , 0.075 $\times$ 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 $\mu\text{L}/\text{min}$ for 5 min. Elution was performed at a flow rate of 0.6 $\mu\text{L}/\text{min}$ using a 2% to 8% gradient of solvent B over 5 min followed by a linear increase of solvent B to 30% for 60 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 m/z 50–4500, nebulizer flow 1.0 psi, dry gas flow rate 5.0 l/min, dry temperature 180°C, and collision 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. The software applied Maximum Entropy deconvolution to collapse all charge states into a single neutral mass, generating an assembled spectrum that represents one molecular species. The ratios of the different *N*-glycoforms were calculated from the intensities of the corresponding peaks.

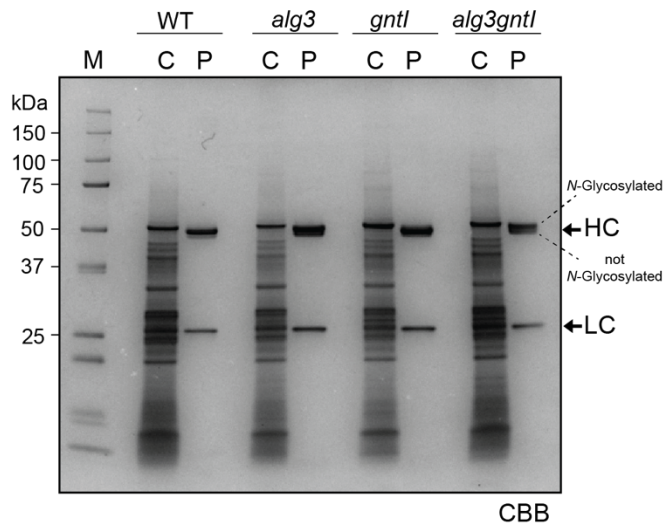
1.14 Results

1.14.1 *N*-Glycan analysis of the Varlilumab expressed in *N. benthamiana* WT, *alg3*, *gntI*, and *alg3gntI* plants

To examine the *N*-glycosylation of the WT, *alg3*, *gntI*, and *alg3gntI* lines, we chose the Varlilumab (CDX-1127). Varlilumab contains a single *N*-glycosylation site in the Fc region of

the heavy chain, typically at Asn299 (²⁹¹TKPREEQYNSTYR³⁰³, *N*-glycosylation site is underlined). Varlilumab was transiently expressed in *N. benthamiana* plants by vacuum agroinfiltration method and successfully purified by a MonoSpin ProA spin column (Figure 14A). Among the mutant lines, the *alg3gntI* mutant achieved the highest purification yield of Varlilumab (17.4 µg per mg of total soluble protein), followed by the *alg3* mutant (10.3 µg/mg). In contrast, the WT and *gntI* plants produced similar but lower yields (6 µg/mg and 6.1 µg/mg, respectively) (Appendix). Notably, the amount of the non-*N*-glycosylated Varlilumab heavy chain amount was higher in the *ALG3* disrupted mutants (11.1% in *alg3* and 27.6% in *alg3gntI*) compared to the WT (5.6%) and *gntI* (4.7%) plants (Figure 14A, Table 7).

(A)



(B)

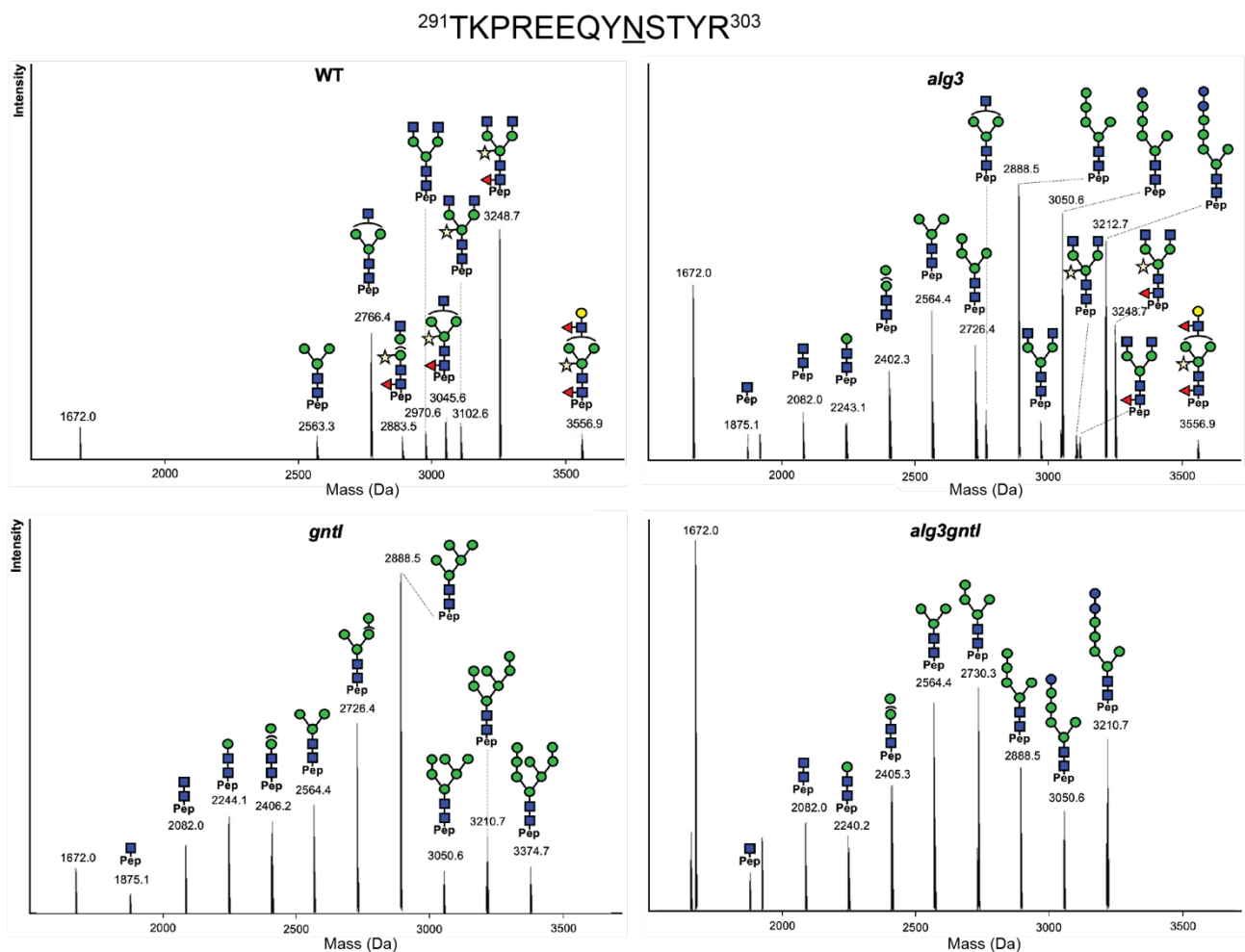


Figure 14. Production and N-glycan analysis of Varlilumab in *N. benthamiana* WT, *alg3*, *gntI*, and *alg3gntI* plants. (A) CBB stained SDS-PAGE gel of transiently expressed Varlilumab C: crude extract; P: Purified, Heavy chain (HC) and light chain (LC) are indicated with arrows. N-Glycosylated or non-N-glycosylated fractions are indicated with dashed lines (B) Analysis of N-glycosylation of the heavy chain glycopeptide (Asn299) of Varlilumab.

Trypsin-digested heavy chain Varlilumab expressed in WT plant contained 66.5% plant-specific *N*-glycans, with the complex biantennary structure GN2M3FX, being the most abundant *N*-glycoform at 42.0% of the total profile (Table 8). Besides, Varlilumab produced in *gntI* displayed 100% mannosidic *N*-glycans: 12.2% M3, 20.9% M4, 38.4% M5, 4.8% M6, 8.4% M7, and 5.3% M8. On the other hand, the one produced in the *alg3* mutant mainly carried the ER-derived immature *N*-glycans, with a distribution comprising 8.9 % M4^{ER}, 20.1% M5^{ER}, 17.9% GlcM5^{ER}, and 16.1% Glc2M5^{ER} structures. Only 13.2% of the *N*-glycans carried plant-specific epitopes. Furthermore, Varlilumab produced in *alg3gntI* plants had mostly paucimannosidic *N*-glycans: 21.6% M3, 22.9% M4^{ER}, 15.1% M5^{ER}, and some ER-derived immature *N*-glycan profile similar to that of *alg3*, 10.4% GlcM5 and 17.4% Glc2M5. Notably, nanoLC-MS/MS analysis showed that the amounts of the non-*N*-glycosylated heavy chains were highest in *alg3gntI* plants. To conclude based on the *N*-glycosylation profile of Varlilumab, disruption of the *GNTI* intercepted the action of plant-specific glycosyltransferases. Moreover, disruption of *ALG3* led to considerably high level of immature ER-derived *N*-glycosylation of Varlilumab and possibly cause underglycosylation in proteins.

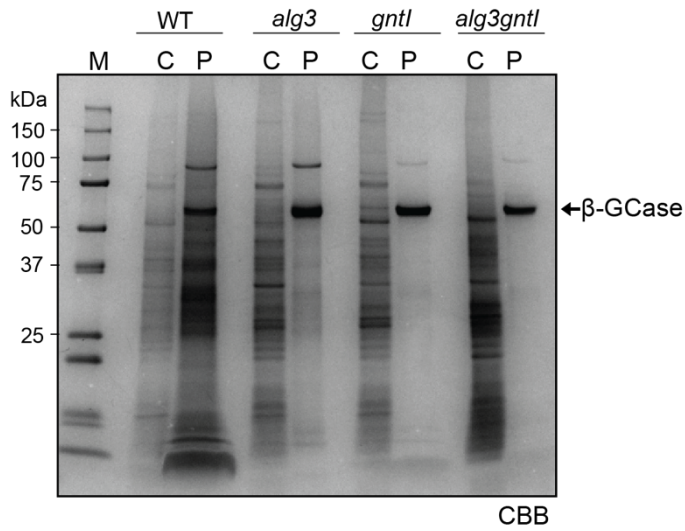
Table 8. *N*-Glycosylation compositions on the heavy chain *N*-glycopeptide (Asn299) of Varlilumab. Relative abundance (%) of the detected *N*-glycoforms are shown.

		WT	<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>	
Non-glycosylated		5.6	11.1	4.7	27.6	
Mannosidic type	M2	-	6.4	9.9	12.7	
	M3	4.4	10.9	12.2	21.6	
	M4	-	-	20.9	-	
	M4ER	-	8.3	-	22.9	
	M5	-	-	38.4	-	
	M5ER	-	20.1	-	15.1	
	M6	-	-	4.8	-	
	M7	-	-	8.4	-	
	M8	-	-	5.3	-	
Glucosylated type	GlcM5	-	17.9	-	10.4	
	Glc2M5	-	16.1	-	17.4	
Termnal GlcNAc residue(s)	Plant-specific type	GNM3	23.8	3.6	-	-
		GN2M3	5.3	2.8	-	-
		GN2M3X	7.0	1.8	-	-
		GN2M3F	-	1.7	-	-
		GNM2FX	4.2	-	-	-
		GNM3FX	8.8	-	-	-
		GN2M3FX	42.0	8.9	-	-
		GalFGNM3FX	4.5	1.4	-	-
Total	Mannosidic type	4.4	45.8	100.0	72.3	
	Glucosylated and terminal GlcNAc	29.1	40.3	0.0	27.8	
	Plant-specific type	66.5	13.9	0.0	0.0	

1.14.2 *N*-Glycan analysis of the GCase expressed in *N. benthamiana* WT, *alg3*, *gntI*, and *alg3gntI* plants

GCase was chosen as a representation of lysosomal enzyme which favors greatly from mannosidic *N*-glycans to express in our *N*-glycoengineered plants. GCase transiently expressed in the *N. benthamiana* WT, *alg3*, *gntI*, and *alg3gntI* plants were purified using two-step purification with ConA column and Phenyl 650M columns (Figure 15A) (Limkul *et al.*, 2016). In this study, *N*-glycan structures were successfully detected on the aforementioned *N*-glycopeptides by nanoLC-MS/MS analysis. The results showed all four *N*-glycopeptides produced in WT and *alg3* plants carried 100% plant-specific *N*-glycans. Although they both produced plant-specific *N*-glycans, the composition shift was apparent. The main *N*-glycan structure detected in WT was GN2M3FX (32.4-50.2%), whereas M3FX (22.9-100%) was predominant in *alg3* across all four *N*-glycan sites. On the contrary, GCase produced in *gntI* mutants contained exclusively mannosidic *N*-glycans, with M5 being the predominant structure (62.9-100%) at all *N*-glycan sites. Similarly, in *alg3gntI* plants, all the *N*-glycosylation sites were occupied with M3 *N*-glycan which ranges from 55.7-100% of total *N*-glycans (Figure 15B-E) (Table 9).

(A)



(B)

⁴⁷SFGYSSVVCVCNATYCDSFDPPTFPALGTFSREYSTRSG⁸⁵

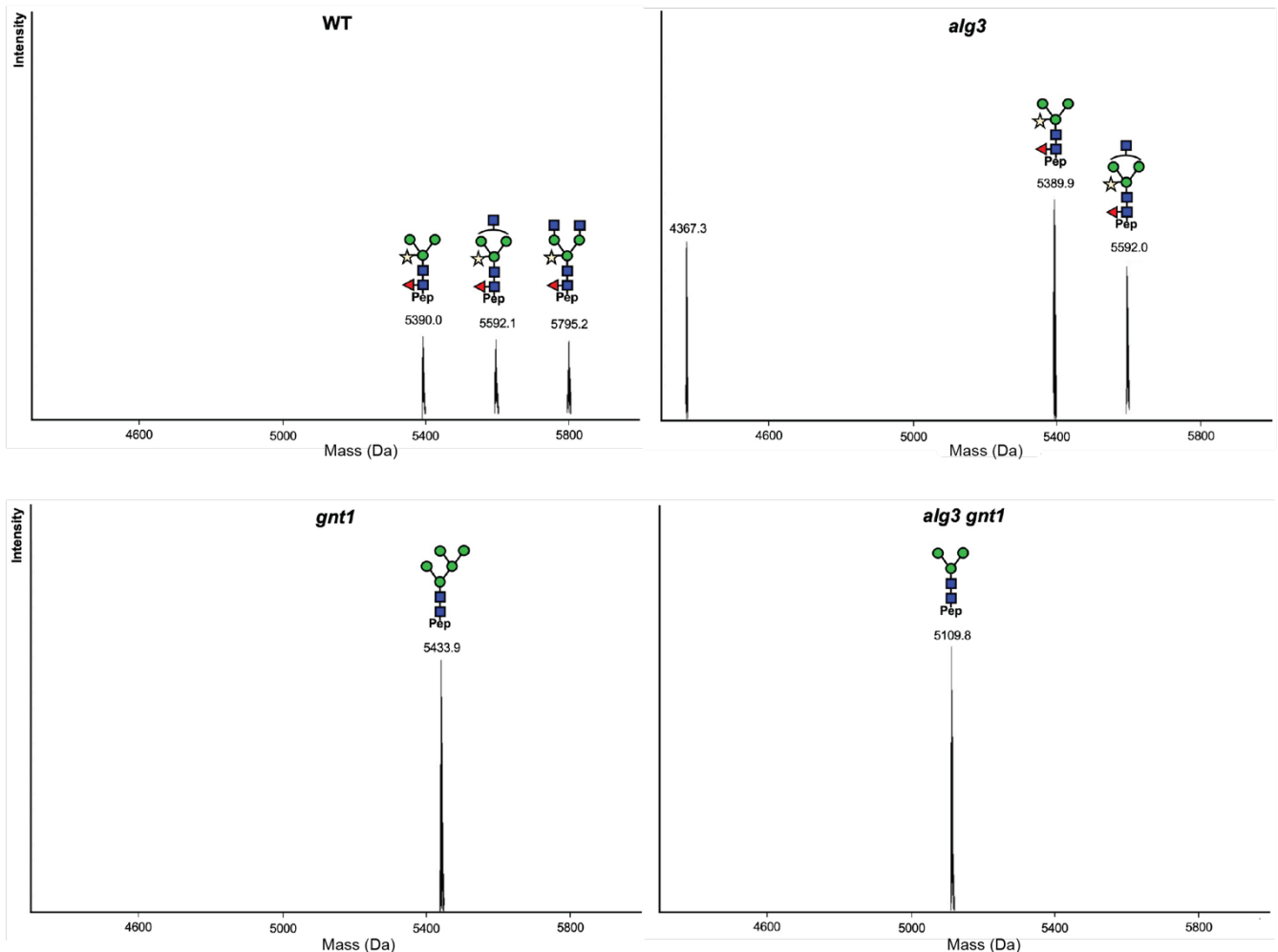
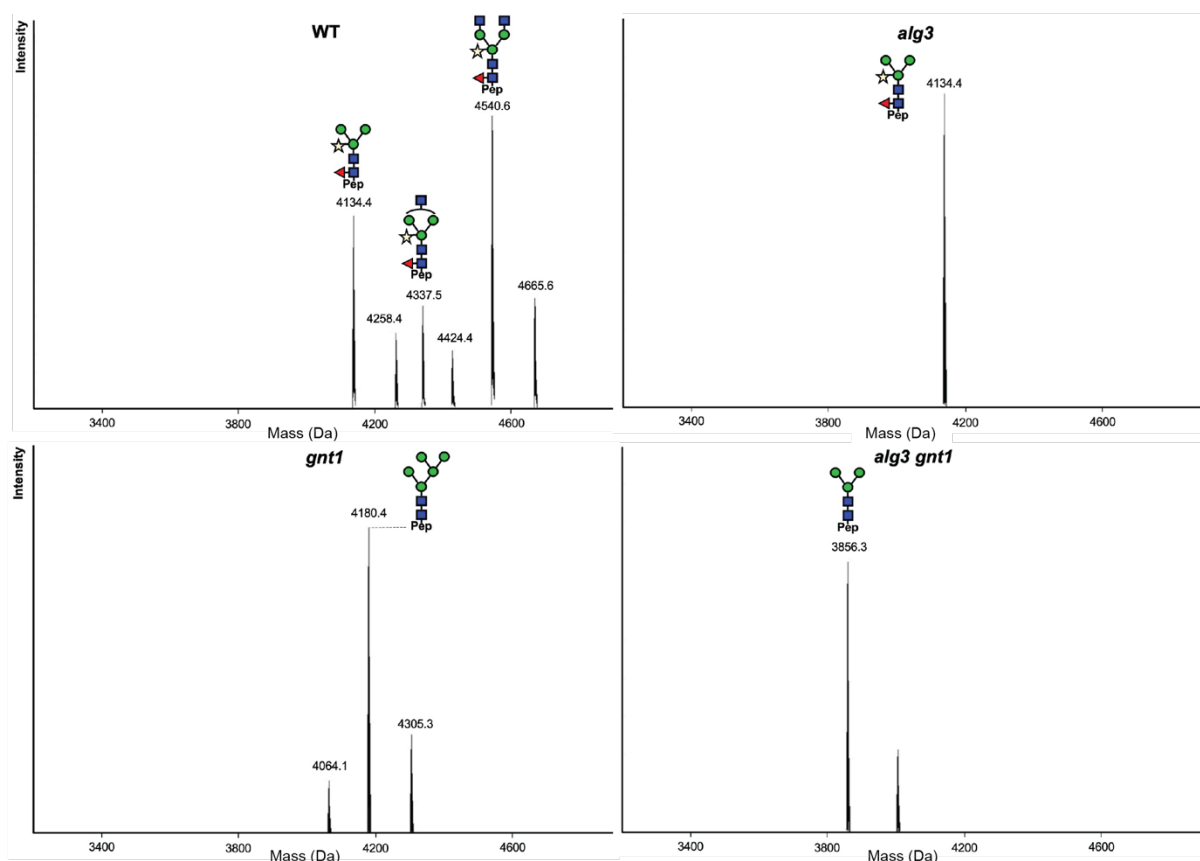


Figure 15. Production and *N*-glycan analysis of GCase in *N. benthamiana* WT, *alg3*, *gnt1*, and *alg3gnt1* plants. (A) CBB stained SDS-PAGE gel of transiently expressed GCase C: crude extract; P: Purified, GCase band was indicated with an arrow. (B-E) nanoLC-MS/MS analysis of *N*-glycopeptides (Asn58, Asn98, Asn185, and Asn309).

(C)

⁸⁷RMELSMGPIQANHTGTGLLLTLQPEQKFQK¹¹⁶

(D)

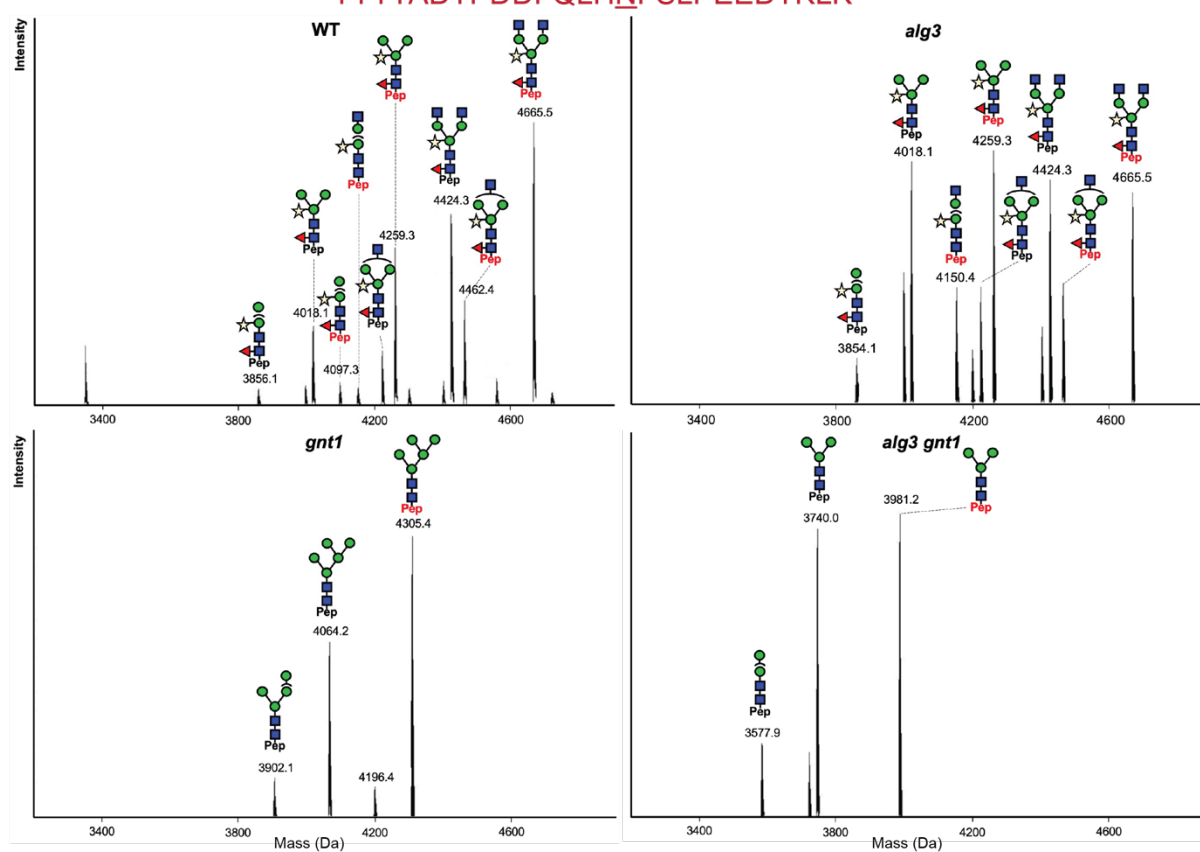
¹⁷¹TYTYADTPDDFQLHNFSLPEEDTK¹⁹⁴¹⁷¹TYTYADTPDDFQLHNFSLPEEDTKLK¹⁹⁶

Figure 15. Continued.

(E)

302DLGPTLANSTHHNVR316

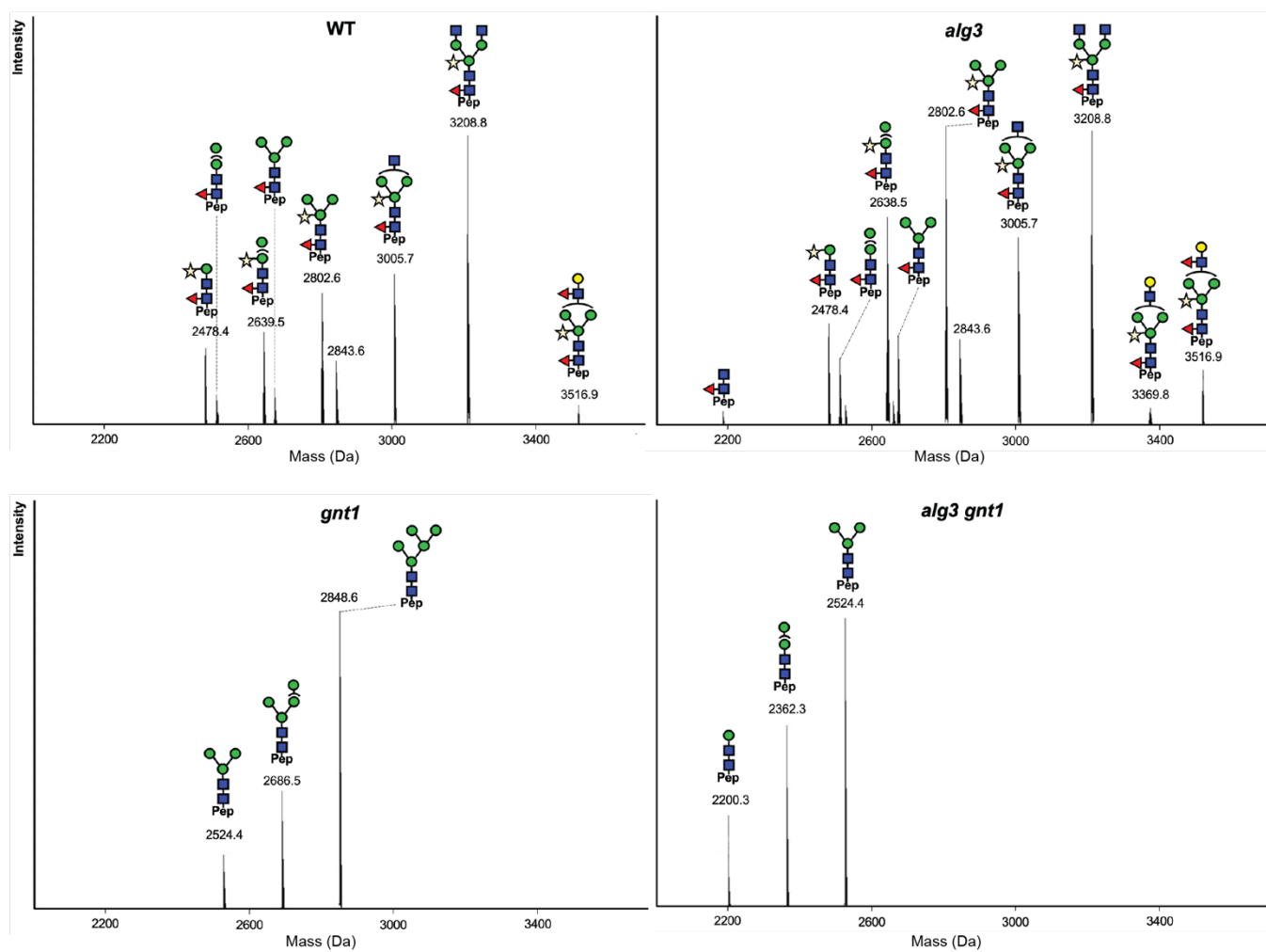


Figure 15. Continued.

Table 9. *N*-Glycosylation compositions of the GCase *N*-glycopeptides. Relative abundance (%) of the detected *N*-glycoforms are shown.

		N58 (S47-G85)				N98(R87-K116)				N185 (T171-K194/K196)				N309 (D302-R316)			
		WT	<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>	WT	<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>	WT	<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>	WT	<i>alg3</i>	<i>gntI</i>	<i>alg3gntI</i>
Mannosidic	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.3
	M2	-	-	-	-	-	-	-	-	-	-	-	9.4	-	-	-	34.0
	M3	-	-	-	100.0	-	-	-	100.0	-	-	-	90.6	-	-	11.9	55.7
	M4	-	-	-	-	-	-	-	-	-	-	7.8	-	-	-	25.3	-
	M5	-	-	100.0	-	-	-	100.0	-	-	-	92.2	-	-	-	62.9	-
Plant-specific type	MFX	-	-	-	-	-	-	-	-	-	-	-	-	9.7	8.1	-	-
	M2F	-	-	-	-	-	-	-	-	-	-	-	-	3.6	5.1	-	-
	M2FX	-	-	-	-	-	-	-	-	4.3	3.8	-	-	11.4	16.1	-	-
	M3F	-	-	-	-	-	-	-	-	-	-	-	-	4.5	6.9	-	-
	M3FX	34.2	59.7	-	-	32.6	100.0	-	-	25.9	40.1	-	-	15.6	22.9	-	-
	GNM2X	-	-	-	-	-	-	-	-	2.0	9.4	-	-	-	-	-	-
	GNM3FX	33.4	40.3	-	-	17.4	-	-	-	17.6	19.8	-	-	17.8	14.5	-	-
	GN2M3FX	32.4	-	-	-	50.0	-	-	-	50.2	26.9	-	-	35.0	22.6	-	-
	GalGNM3FX	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3	-	-
	GalFGNM3FX	-	-	-	-	-	-	-	-	-	-	-	-	2.4	2.6	-	-
Total	Manosidic type	0	0	100	100	0	0	100	100	0	0	100	100	0	0	100	100
	Plant-specific type	100	100	0	0	100	100	0	0	100	100	0	0	100	100	0	0

1.15 Discussion

Plant-based systems like *N. benthamiana* offer a flexible and scalable platform for recombinant protein production, but their native *N*-glycosylation machinery introduces plant-specific sugar residues such as β 1,2-xylose and α 1,3-fucose that may affect efficacy, half-life, or immunogenicity in humans (Gomord *et al.*, 2010; Strasser, 2016). Recent advances in plant *N*-glycoengineering, particularly through the use of CRISPR/Cas9-mediated knockout of key glycosyltransferase genes (*FUCT* and *XYLT*), have enabled the generation of *N. benthamiana* lines that can produce recombinant proteins with humanized *N*-glycan structures or without plant-specific epitopes (Jansing *et al.*, 2019; Pantazica *et al.*, 2023). Previous studies have demonstrated the successful expression of mAbs, lysosomal enzymes, viral epitopes in these lines. Together, these developments underscore the potential of *N. benthamiana* as a platform for the production of recombinant proteins with optimized glycosylation profiles suitable for biopharmaceutical production.

In this study, two key enzymes of *N*-glycosylation in the ER and Golgi were targeted and successfully knocked out by multiplex CRISPR/Cas9 genome editing tool. In this chapter, the possibility to produce recombinant pharmaceuticals in the mutant lines and their *N*-glycosylation were assessed. This work enabled us to see how the same *N*-glycosylation machinery works on the two different proteins. Interestingly, Varlilumab produced in *alg3* and *alg3gntI* carried immature ER *N*-glycans: Glc2M5^{ER}, GlcM5^{ER}. The amount of these *N*-glycans on the Varlilumab was greater than amount detected from the total *N*-glycan analysis. In both cases, Glc3M5^{ER} was not detected. *N*-glycosylated protein folding takes place in the ER after OST complex transfers the *N*-glycan to the polypeptide chain. This process is assisted by the chaperones such as calnexin and calreticulin. These chaperones specifically binds to the monoglucosylated *N*-glycans of the polypeptide (Lederkremer, 2009). Another ER quality

control-associated lectin, malectin, is known to interact with Glc2M9^{ER}-glycosylated proteins (Feng *et al.*, 2022). Therefore, the absence of Glc3M5^{ER} *N*-glycan in *alg3* or *alg3gntI* plant can be explained by the essential trimming the two glucose residues by the GCSI and GCSII. Conversely, these ER immature *N*-glycans were not detected in GCase that produced in *alg3* and *alg3gntI* plants. This can be leading clue why these structures are present in Varlilumab in the first place. ER quality control is a highly complex process in which *N*-glycans guide the folding and processing steps; disruption of *ALG3* further adds to this complexity. It is likely that these immature *N*-glycans were not able to be transported to the Golgi and stayed in the ER. This may result from inefficient ER-to-Golgi trafficking, potentially caused by the ER stress or reduced binding affinity to cargo receptors. In plants, ER-to-Golgi transport remains poorly characterized, and it is still debated whether it occurs primarily through vesicular or tubular mechanisms (Brandizzi, 2018; Fougère *et al.*, 2025; Robinson *et al.*, 2015). In mammalian systems, the ER-Golgi intermediate compartment (ERGIC) is central to the transport of *N*-glycoproteins. Cargo receptors such as ERGIC-53, a mannose-binding lectin, recognize high-mannose-type *N*-glycans and facilitate their trafficking (Appenzeller *et al.*, 1999; Zhang *et al.*, 2023). The absence of glucosylated immature *N*-glycans on GCase could be attributed to the presence of multiple *N*-glycosylation sites, which may enhance the ER exit efficiency, whereas some Varlilumab stays in the calnexin cycle. On the other hand, the *N*-glycans located in the Fc region of IgG are embedded between the two CH2 domains (Russell *et al.*, 2018) This structural shield may restrict the accessibility of ER-resident GCS II once proper folding has occurred. Such a spatial arrangement of antibody *N*-glycans could account for the presence of GlcM5^{ER} and Glc2M5^{ER} structures observed in Varlilumab.

Another finding was the notable underglycosylation of Varlilumab heavy chains observed in the *alg3gntI* mutant. This phenomenon can be attributed to reduced OST efficiency, which has been consistently documented in *ALG3* deletion studies across multiple organisms.

In *S. cerevisiae*, $\Delta alg3$ mutants exhibit similar underglycosylation patterns due to compromised OST recognition of altered *N*-glycan substrates (Aebi *et al.*, 1996; Bailey *et al.*, 2012; Parsaie *et al.*, 2013). The terminal $\alpha 1,2$ -glucose residues of Glc3M9-Dol-P-P serve as key recognition elements for OST substrate binding in *S. cerevisiae* (Burda and Aebi, 1998). Interestingly, our *N. benthamiana alg3*, single mutant, which theoretically possesses the same Glc3M5^{ER}-Dol-P-P substrate as the *alg3gntI*, double mutant, produced less underglycosylated Varlilumab heavy chain. This suggests that the severe phenotypic abnormalities in *alg3gntI* result from a combination of reduced OST-substrate compatibility and additional cellular stresses.

Table 10. Comparison of the relative *N*-glycan profile (%) of antibodies expressed in *N*-glyco-engineered *N. benthamiana* plants (*Jansing *et al.*, 2018)

		Varlilumab		2G12
		<i>gntI</i>	<i>alg3gntI</i>	FX-KO #20-4*
Non-glycosylated		4.7	27.6	15.8
Mannosidic type	M2	9.9	12.7	-
	M3	12.2	21.6	-
	M4	20.9	-	-
	M4ER	-	22.9	-
	M5	38.4	-	-
	M5ER	-	15.1	-
	M6	4.8	-	-
	M7	8.4	-	2.6
	M8	5.3	-	7.8
	M9	-	-	3.7
Glucosylated type	GlcM5	-	10.4	-
	Glc2M5	-	17.4	-
Terminal GlcNAc type	GNM3	-	-	2.8
	GN2M3	-	-	65.4

In previous study, to eliminate plant-specific epitopes, six glycosyltransferase genes were knocked out by CRISPR/Cas9 tool (Jansing *et al.*, 2019). In our study, disruption of *GNTI* eliminated plant-specific *N*-glycan synthesis as well. *N*-glycan profiles of the antibodies

expressed in these plants were compared in Table 9. This comparison revealed the underlying *N*-glycosylation pathway of the mutants. FX-KO mutants produced 65.4% GN2M3 and few high mannosidic *N*-glycans. On the contrary, *gntI* and *alg3gntI* produced Varlilumab mainly carried shorter mannosidic *N*-glycans.

To date, extensive efforts in *N*-glycoengineering from yeast to mammalian systems such as HEK293 cells have aimed to generate lysosomal enzymes with high mannose-type *N*-glycans (Inci *et al.*, 2025; Jin *et al.*, 2018; Kang *et al.*, 2018). These *N*-glycans are critical for efficient lysosomal targeting via the mannose-6-phosphate (M6P) receptor pathway (Kornfeld, 1987) In our study, nanoLC-MS/MS analysis demonstrated that GCase expressed in the *gntI* mutant of *N. benthamiana* contained the mainly M5 structure at all four *N*-glycosylation sites. These findings highlights the potential of the *gntI* mutant as a plant-based production system for lysosomal enzymes used in ERT. In previous studies, human recombinant α -L-iduronidase produced in *A. thaliana cgl* seeds exhibited predominantly M5 *N*-glycans and comparable enzymatic activity to the CHO cell-derived commercial enzyme used to treat mucopolysaccharidosis I, a lysosomal storage disorder. The plant-derived enzyme was subsequently modified *in vitro* to introduce the M6P recognition marker (He *et al.*, 2013). Earlier studies also revealed that GCase chemically conjugated with synthetic trimannosyldilysine had an 11-fold increase in receptor-mediated uptake by rat alveolar macrophages compared to the unmodified form (Doebber *et al.*, 1982). Likewise, the expression of GCase in the *N. benthamiana alg3gntI*, which yields highly uniform M3 *N*-glycans, represents a promising production platform for lysosomal enzyme therapeutics that may offer targeting advantages.

Table 11. Comparison of the relative *N*-glycan profile (%) of GCase produced in *gntI* plants with the knock-down Δ *gntI* line reported in previous studies. (*Limkul *et al.*, 2016; **Uthailak *et al.*, 2021)

		N58 (S47-G85)			N98 (R87-K116)			N185 (T171-K194/K196)			N309 (D302-R316)		
		<i>gntI</i>	Δ <i>gntI</i> *	Δ <i>gntI</i> **	<i>gntI</i>	Δ <i>gntI</i> *	Δ <i>gntI</i> **	<i>gntI</i>	Δ <i>gntI</i> *	Δ <i>gntI</i> **	<i>gntI</i>	Δ <i>gntI</i> *	Δ <i>gntI</i> **
Mannosidic	M2	-	-	ND	-	-	-	-	-	-	-	1.3	1.9
	M3	-	3.2		-	5.1	1.0	-	-	2.0	11.9	8.3	4.9
	M4	-	6.9		-	18.0	9.8	7.8	3.5	13.0	25.3	23.9	20.2
	M5	100.0	63.2		100.0	62.5	89.2	92.2	73.0	85.0	62.9	48.6	73.0
Plant-specific type	M2F	-	-		-	-	-	-	-	-	-	2.7	-
	M2FX	-	-		-	3.4	-	-	-	-	-	0.7	-
	M3X	-	2.5		-	-	-	-	2.3	-	-	0.9	-
	M3F	-	2.5		-	-	-	-	2.5	-	-	2.7	-
	M3FX	-	20.6		-	11.1	-	-	18.6	-	-	9.1	-
	GNM3FX	-	1.1		-	-	-	-	-	-	-	1.9	-
Total	Manosidic type	100.0	73.3		100.0	85.5	100.0	100.0	76.6	100.0	100.0	82.0	100.0
	Plant-specific type	0	26.7		0	14.5	0	0	23.4	0	0	18.0	0

Previously, GCase was expressed stably and transiently in GNTI knockdown (*ΔgntI*) *N. benthamiana* plants and the *N*-glycan profile was investigated (Limkul *et al.*, 2016; Uthailak *et al.*, 2021). Therefore, the *N*-glycan profiles of those GCase were compared with the result of *gntI* mutant of this study. Generally, stable expression of GCase in *ΔgntI* resulted in reduced plant-specific *N*-glycans compared to WT but those structures were still present. However, the one produced transiently in same *ΔgntI* line showed 100% mannosidic *N*-glycans. This difference could be a result of different expression approach or the different RNAi expression in individual plants. But our knockout line, *gntI* produced 100% mannosidic *N*-glycans with more homogenous ratio (Table 11). Our findings contributed to evaluating the utility of *N*-glycoengineered *N. benthamiana* as a platform for producing biopharmaceuticals with improved *N*-glycan.

1.16 Summary

Plant-based expression system has been developed in every aspect to be used for pharmaceutical production. While plant-based expression system, especially *N. benthamiana* offers several advantages including low cost, scalability, absence of animal and human pathogens, ease of handling over other production hosts, the main biological restriction is the presence of plant-specific *N*-glycans. CRISPR/Cas9, robust genome editing tool, has enabled the researchers to engineer the *N*-glycosylation pathway of *N. benthamiana* as desired. In this study, two key enzymes ALG3 and GNTI were targeted by multiplex CRISPR/Cas9 in *N. benthamiana* and *alg3*, *gntI*, and *alg3gntI* mutants have successfully generated. In this chapter, two representative pharmaceuticals, Varlilumab and GCase were expressed in the mutants to assess how the engineered *N*-glycosylation machinery works on two distinct proteins. *N*-Glycan composition of the proteins expressed in *gntI* plant possessed mainly M5 mannosidic *N*-glycans at all sites suggesting that *gntI* can be a well-suited platform for lysosomal enzyme production. On the other hand, both proteins expressed in *alg3gntI* carried M3, shorter

mannosidic *N*-glycans with high uniformity. Additionally, immature ER *N*-glycans, Glc2M5^{ER} and GlcM3^{ER}, were detected only in Varlilumab but not in GCase. This observation has concluded by possible ER stress and inefficient transportation of *N*-glycosylated proteins due to loss of ALG3 function. This has proven that more studies are needed on ER quality control and ER to Golgi transportation studies in plant.

Chapter Four: General conclusion and future perspective

Our study is attempted to explore the target area in the scope of improving the plant-based expression system for future pharmaceuticals. Our main objective was to generate *N. benthamiana* line that produces more homogenous mannosidic *N*-glycans that lack plant-specific epitopes. In this study, we adapted the multiplex CRISPR/Cas9 gene editing tool to manipulate the *N*-glycosylation pathway of *N. benthamiana* by disrupting the *NbALG3* and *NbGNTI* genes. Through extensive selection and identification, I successfully generated T₃ single (*alg3* or *gntI*) and double (*alg3gntI*) knockout mutant plants. The total *N*-glycan analysis revealed several valuable insights related to *N*-glycosylation machinery of these mutants as well as the promising potential of those mutants for pharmaceutical production. *N. benthamiana gntI* predominantly produced M5 *N*-glycans which are optimal for lysosomal enzyme therapeutics due to their M6P tagging and receptor-mediated uptake. Moreover, *ALG3* disrupted mutants produced small amount of immature ER *N*-glycans such as Glc2M5 and GlcM5, hinting toward to increased ER burden. This hypothesis was supported by the increased amount of non-*N*-glycosylated Varlilumab heavy chain in *ALG3* disrupted mutants. Disruption of either *ALG3* or *GNTI* individually did not affect the overall growth and the morphology of *N. benthamiana*. However, loss of the two enzymes severely impaired the development of *N. benthamiana* causing dwarf phenotype, impaired root growth, curled leaves. These phenotypes were partly explained by the *ALG3* knockout-induced ER stress combined with the accumulation of M3 structure in the Golgi.

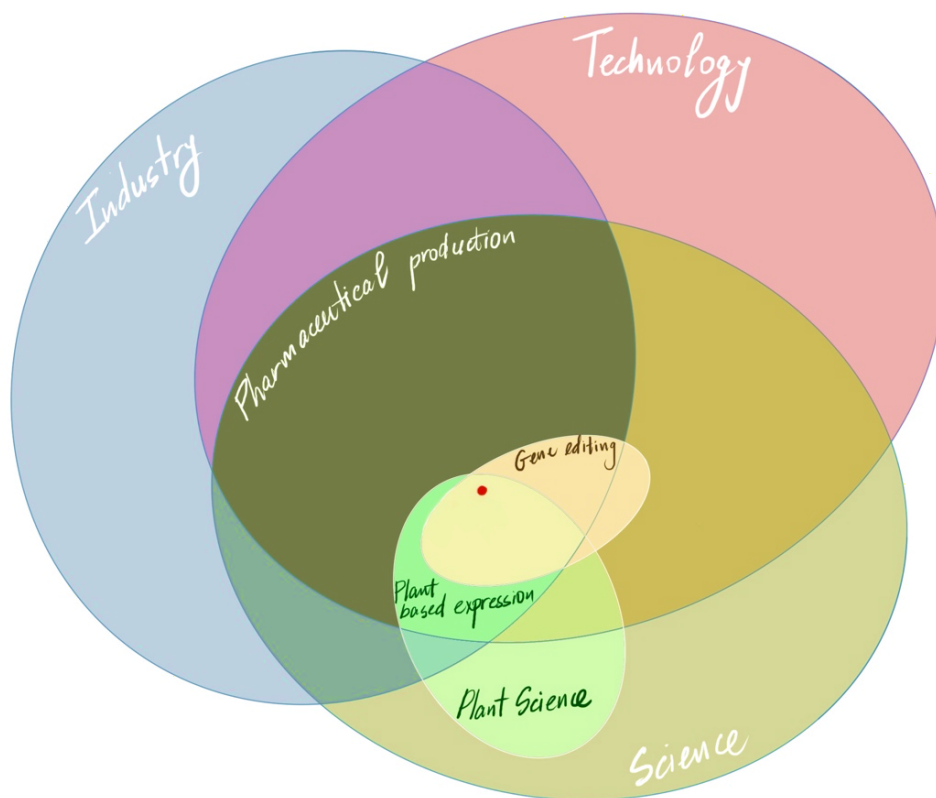


Figure 16. A conceptual overview illustrating the scale and impact of this study. Red dot represents the scope of this study.

As a qualification, two representative biopharmaceuticals were expressed in the mutants and *N*-glycosylation profile of the pharmaceuticals were assessed. Successful purification of the Varlilumab and GCase from the mutants has proved their capacity to produce recombinant proteins. *N*-Glycosylation machinery of the *alg3*, *gntI*, and *alg3gntI* mutants were once again investigated through nanoLC-MS/MS analysis of the trypsin digested *N*-glycopeptides. The result mostly resembled the total *N*-glycan analysis result. Interesting observation was that Varlilumab produced in *alg3* and *alg3gntI* mutant carried the immature ER *N*-glycans, whereas GCase produced in the same mutants did not possess these immature *N*-glycans. We tried to explain this phenomenon based on the number of *N*-glycan sites on each protein and their efficiency to be transported to Golgi. However, these explanations are only theoretical and our experimental approach and scope is limited. Naturally, this study has only initiated the further exploration of the mutants and more fundamental questions.

In the future, generation of the null mutants by cross-pollination with WT *N. benthamiana* are priority. Moreover, the enzymatic or therapeutic activity of the pharmaceuticals produced in these mutants should be validated and compare with other host systems to realistically evaluate these mutant expression platforms.

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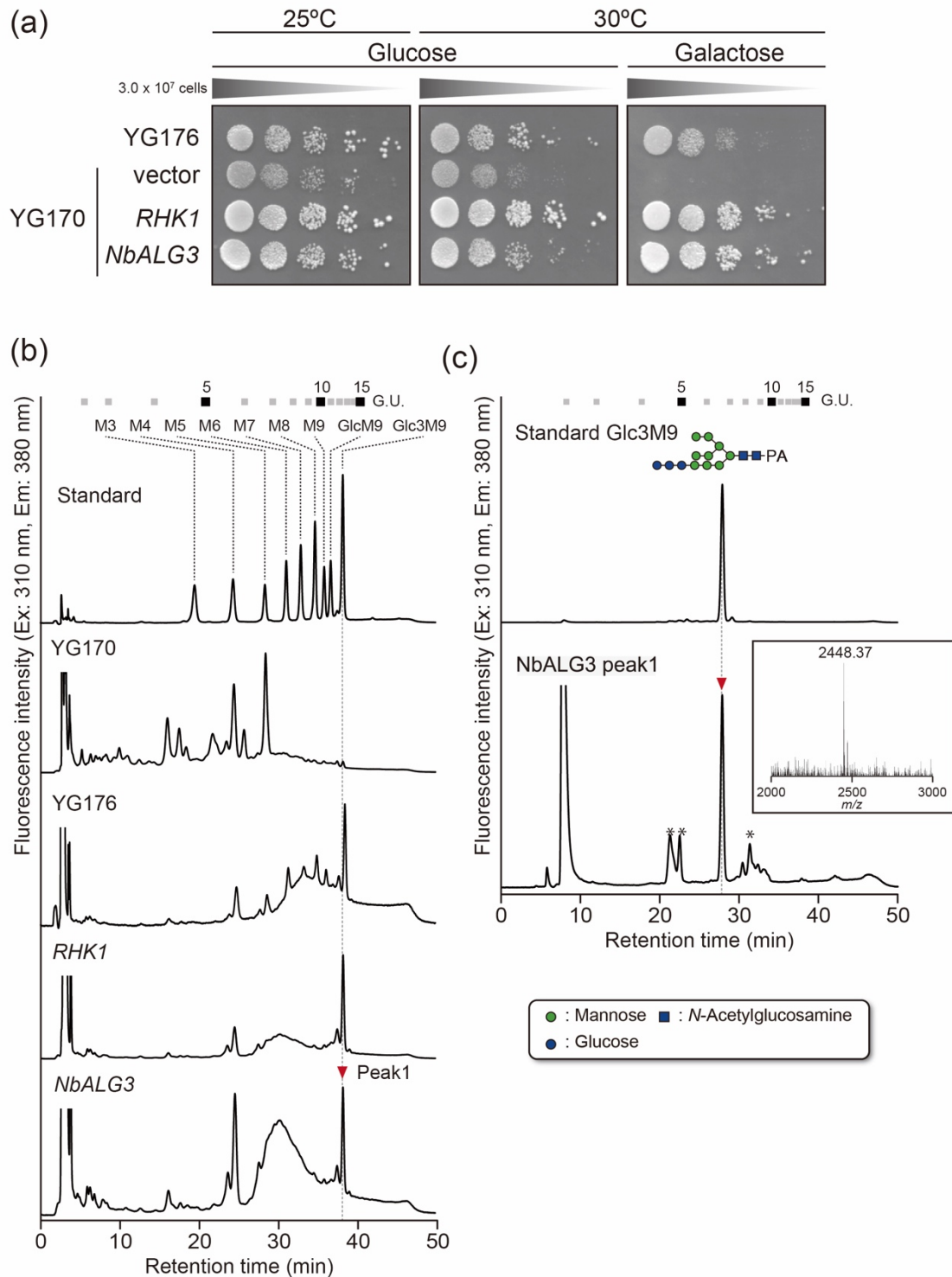
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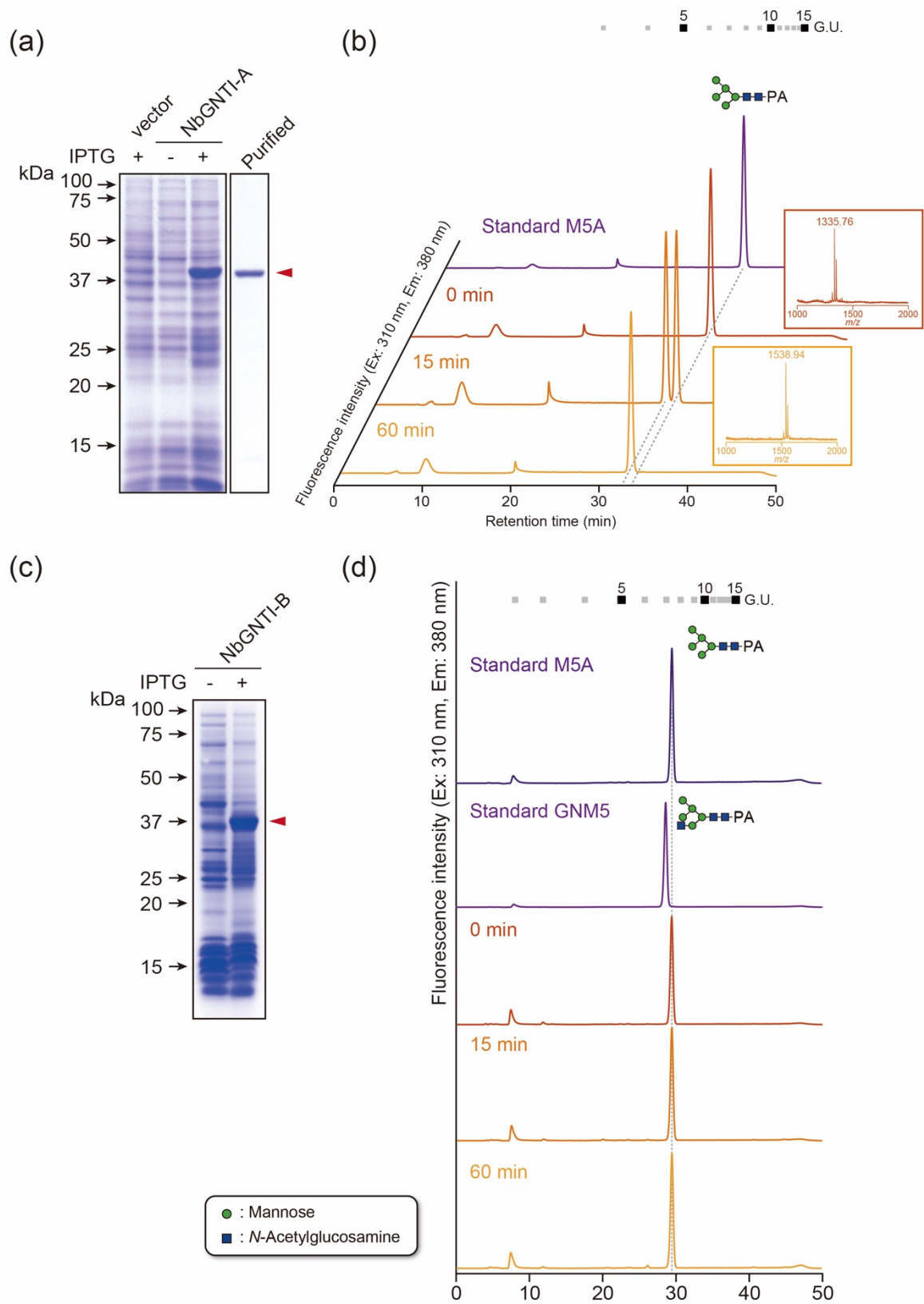
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Appendix



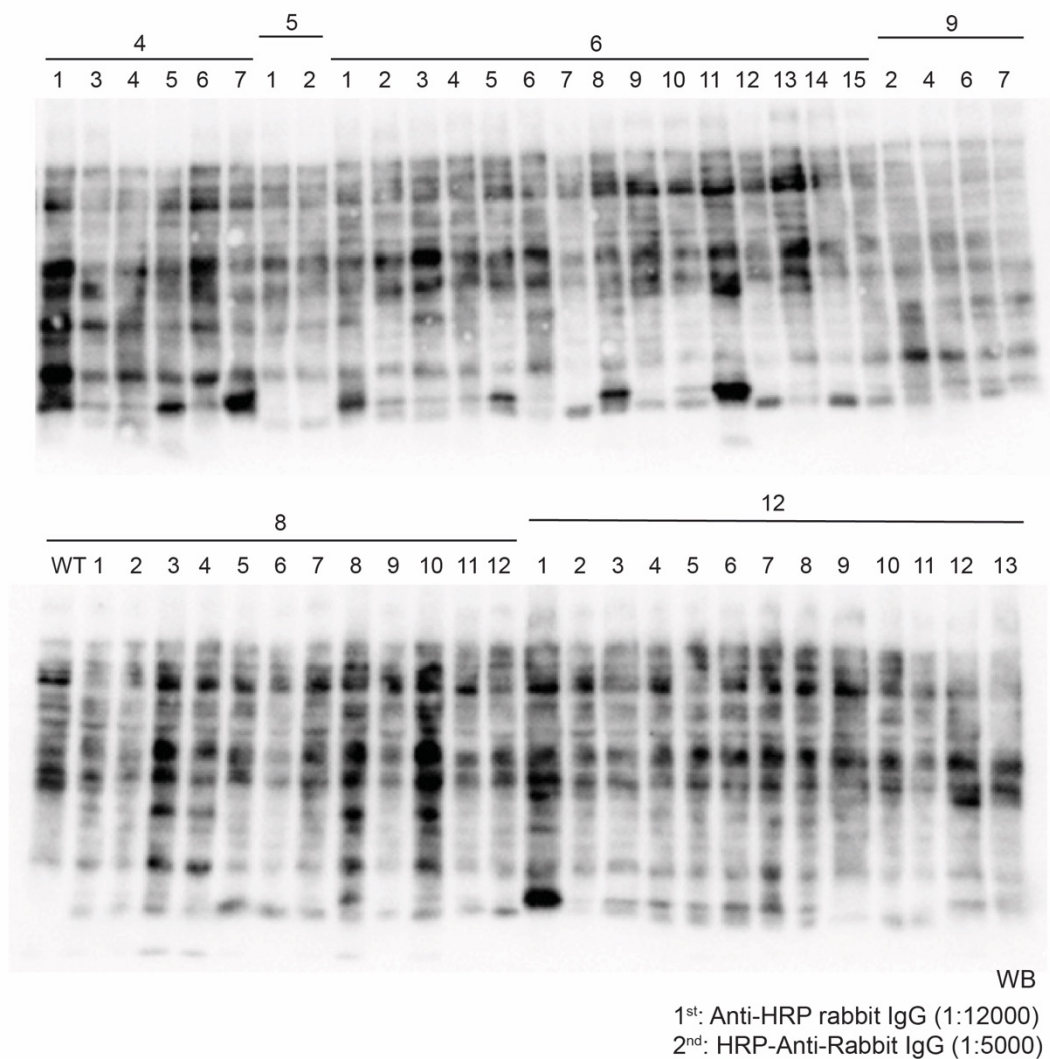
Appendix 1 (a) *In vivo* complementary assay of NbALG3 in a yeast model (b) Analysis of the lipid-linked oligosaccharide biosynthesized in each transformant. (c) Analysis of the peak indicated by the asterisk



Appendix 2 Expression and *in vitro* activity of recombinant NbGNTIs (a, c) CBB staining of recombinant NbGNTI-A and NbGNTI-B. (b,d) The HPLC profile of the recombinant NbGNTI-A and NbGNTI-B enzymatic reaction.

Appendix 3. Screening summary of the T₀ plants. No Mut: no mutation; (-): Not assessed

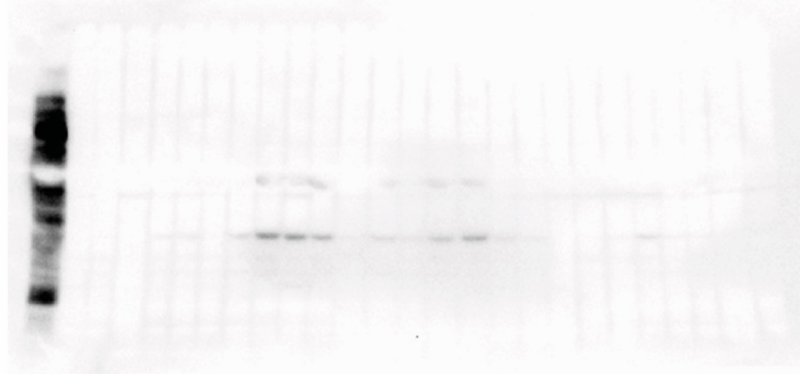
T ₀ plants	anti- HRP WB	Sequencing				
		ALG3		GNTI-A	GNTI-B	
		Exon 1	Exon3	Exon 5	Exon 3	Exon 4
1	Pos	-	-	-	-	-
2	Pos	-	-	-	-	-
3	Died					
4	Pos	-	-	-	-	-
5	Pos	-	-	-	-	-
6	Pos	Hetero (-7 bp)	No Mut	No Mut	No Mut	No Mut
7	Neg	Homo (+1 bp)	Hetero (-5 bp)	Hetero(+11 bp)	No Mut	No Mut
8	Pos	Hetero (-150 bp)	Hetero (+1 bp)	No Mut	No Mut	No Mut
9	Pos	-	-	-	-	-
10	Pos	-	-	-	-	-
11	Neg	Hetero (-4 bp)	Hetero (-12bp; -7 bp)	Homo (+1bp)	Hetero	Hetero
12	Pos	Hetero (-7 bp)	No Mut			
13	Died					
14	Neg	No seed production				
15	Pos	-	-	-	-	-
16	Pos	-	-	-	-	-
17	Pos	-	-	-	-	-



Appendix 4. Anti-HRP Western blot result of the putative T₁ *alg3 N. benthamiana* plants. Wild-type (WT) sample was used as a control. Each lane, 1 µg/lane total soluble protein sample was applied.

7

WT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25



7

WT 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49



11

WT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



WB

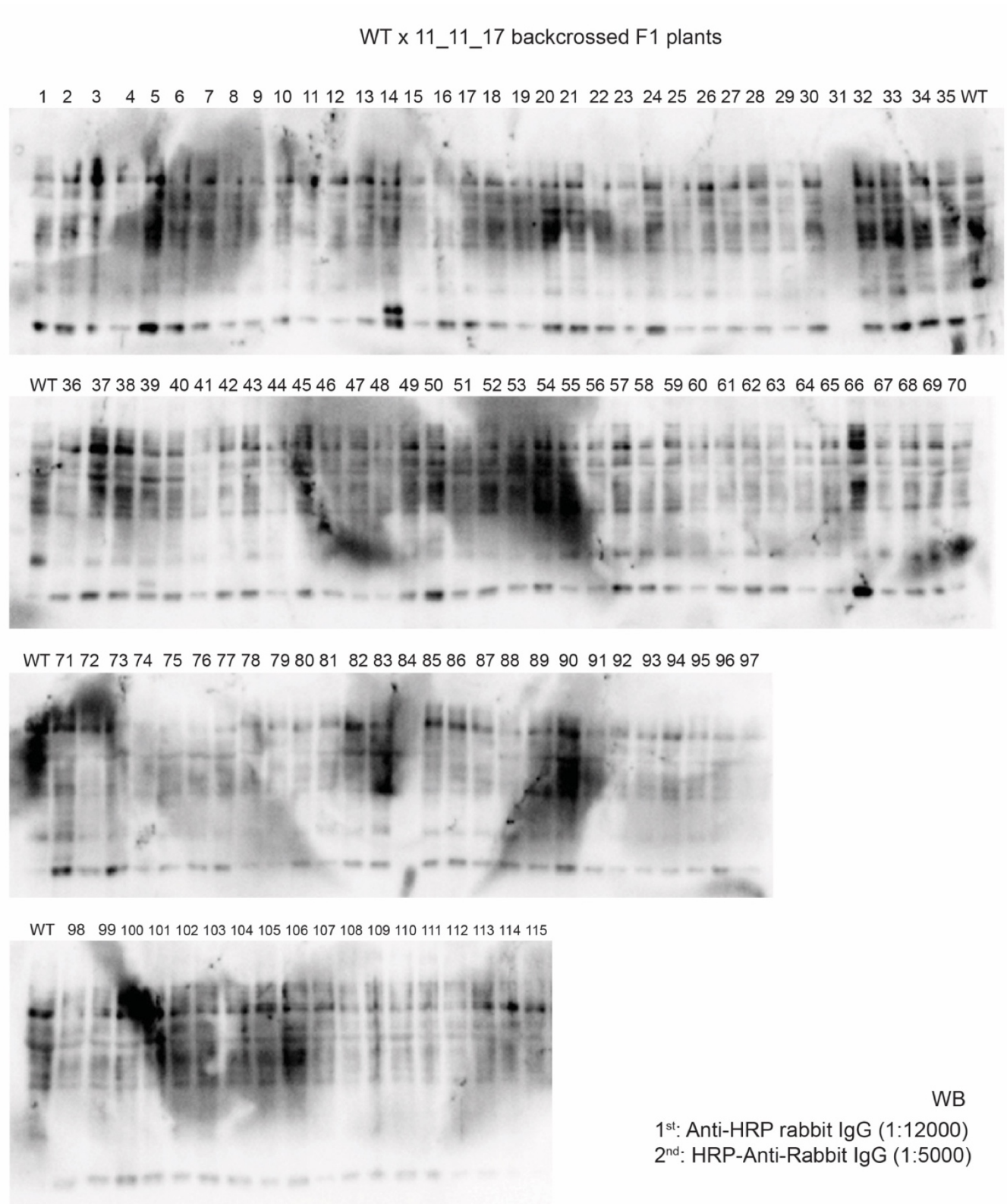
1st: Anti-HRP rabbit IgG (1:12000)

2nd: HRP-Anti-Rabbit IgG (1:5000)

Appendix 5. Anti-HRP Western blot result of the putative T₁ *alg3 gntI* *N. benthamiana* plants. Wild-type (WT) sample was used as a control. Each lane, 1 µg/lane total soluble protein sample was applied.

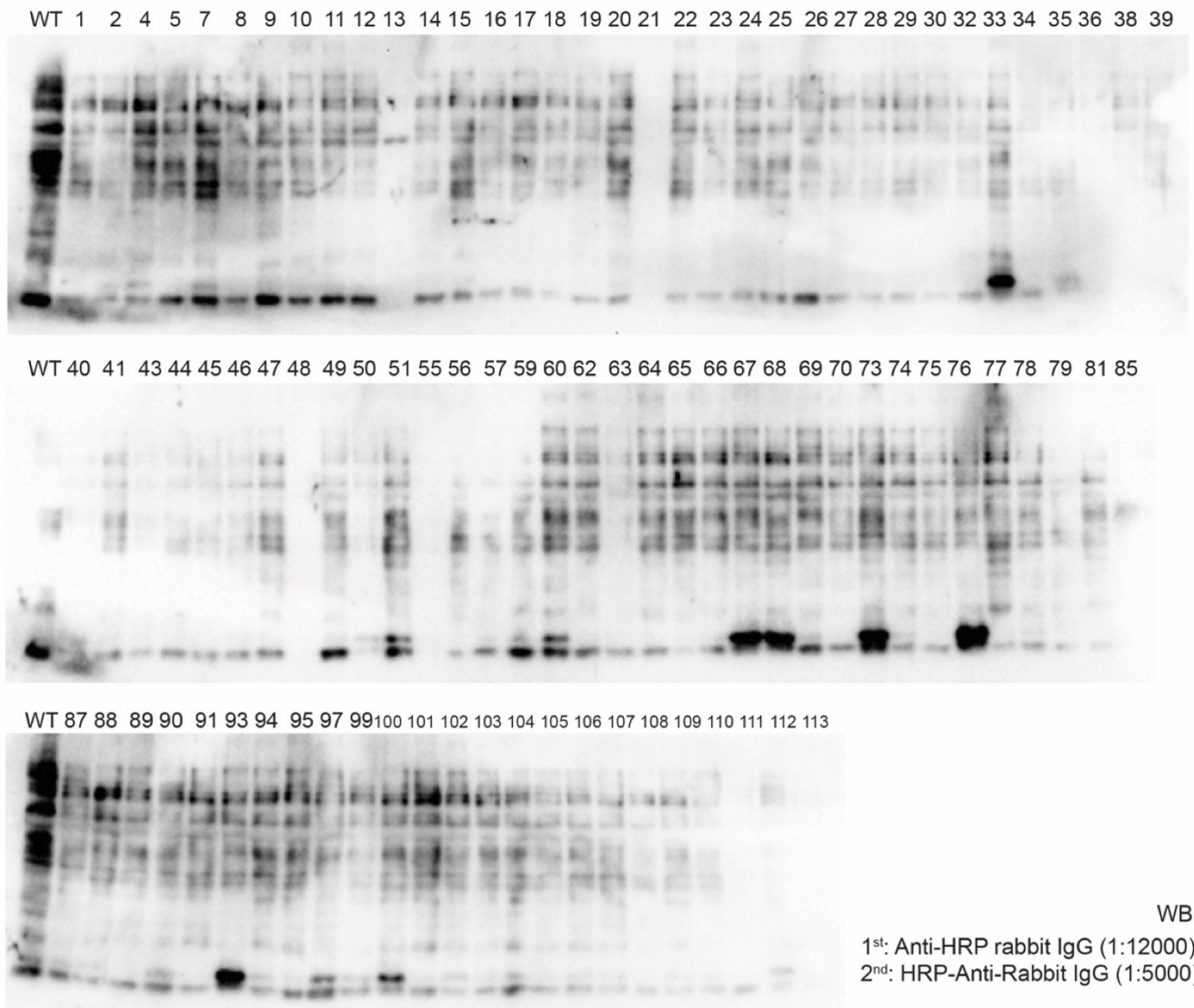
Appendix 6. Screening summary of the T₁ *N. benthamiana* mutant plants. Chosen plants are highlighted. No Mut: no mutation; (-): Not assessed

T ₁ plants	anti-HRP WB	Sequencing				
		ALG3		GNTI-A	GNTI-B	
		Exon 1	Exon3	Exon 5	Exon 3	Exon 4
6 3	Pos	Hetero (-7 bp)	No Mut	-	-	-
6 8	Pos	Homo (-7 bp)	No Mut	No Mut	No Mut	No Mut
6 11	Pos	Hetero (-7 bp)	No Mut	-	-	-
8 3	Pos	Homo (-130 bp)	Homo (+1 bp)	-	-	-
12 9	Pos	Hetero (-7 bp)	No Mut	-	-	-
7 20	Neg	Homo (+1 bp)	-	-	-	-
7 22	Neg		Hetero	-	-	-
7 25	Neg		-	-	-	-
7 26	Neg		Hetero	-	-	-
7 27	Neg		-	-	-	-
7 35	Neg		-	-	-	-
7 36	Neg		Hetero	-	-	-
7 37	Neg		Hetero	-	-	-
7 48	Neg		-	-	-	-
7 49	Neg		-	-	-	-
11 1	Neg	Homo (+4 bp)	-	-	-	-
11 2	Neg		-	-	-	-
11 3	Neg		Homo (-7 bp)	-	-	-
11 4	Neg		Hetero	-	-	-
11 5	Neg		Hetero	-	-	-
11 7	Neg		Homo (+12 bp)	-	-	-
11 9	Neg		Homo (+12 bp)	-	-	-
11 11	Neg		Homo (-7 bp)	Homo (+1 bp)	Homo (-5 bp)	No Mut
11 13	Neg		Homo (-7 bp)	-	-	-
11 18	Neg		Hetero	-	-	-
11 19	Neg		Homo (+12 bp)	-	-	-
11 20	Neg		Hetero	-	-	-
11 21	Neg		Hetero	-	-	-
11 22	Neg		Homo (+12 bp)	-	-	-
11 23	Neg		Hetero	-	-	-
11 24	Neg		Homo (-7 bp)	-	-	-
11 25	Neg		Hetero	-	-	-
11 26	Neg		Homo (-7 bp)	-	-	-
11 27	Neg		Hetero	-	-	-
11 28	Neg		Homo (+12 bp)	-	-	-
11 29	Neg		Hetero	-	-	-
11 30	Neg		Homo (+12 bp)	-	-	-
11 31	Neg		Homo (-7 bp)	-	-	-
11 32	Neg		Hetero	-	-	-
11 33	Neg		Hetero	-	-	-
11 34	Neg		Hetero	-	-	-



Appendix 7. Anti-HRP Western blot result of the F₁ backcrossed *N. benthamiana* plants. Wild-type (WT) sample was used as a control. Each lane, 1 µg/lane total soluble protein sample was applied.

WT x 11_11_21 backcrossed F1 plants



Appendix 8. Anti-HRP Western blot result of the F₁ backcrossed *N. benthamiana* plants. Wild-type (WT) sample was used as a control. Each lane, 1 µg/lane total soluble protein sample was applied.

Appendix 9. Screening summary of the anti-HRP negative putative F₁ *gntI* *N. benthamiana* plants. Chosen plant is highlighted. No Mut: no mutation; (-): Not assessed

F ₁ plants	anti-HRP WB	Sequencing				
		ALG3		GNTI-A	GNTI-B	
		Exon 1	Exon3	Exon 5	Exon 3	Exon 4
11_11_17_31	Neg	Hetero (-4 bp)	No Mut	-	-	-
11_11_17_84	Neg	No Mut	Hetero	-	-	-
11_11_21_13	Neg	Homo (-4 bp)	Homo (-7 bp)	Homo (+1 bp)	Homo (-5 bp)	No Mut
11_11_21_21	Neg	No Mut	No Mut	Homo (+1 bp)	Homo (-5 bp)	No Mut
11_11_21_48	Neg	Hetero	Hetero	-	-	-
11_11_21_55	Neg	Hetero	Hetero	-	-	-
11_11_21_85	Neg	No Mut	No Mut	Homo (+1 bp)	Hetero	-
11_11_21_113	Neg	No Mut	No Mut	Homo (+1 bp)	Hetero	-

Appendix 10. Purification yield of Varlilumab from *N. benthamiana* mutants. This table summarizes the yield of purified Varlilumab expressed in different *N. benthamiana* plants. The yields were calculated based on the amount of antibody purified using Protein A spin column from equal amounts of leaf sample.

	Fresh leaf amount, g	Crude sample volume, μl	Crude sample protein concentration, $\mu\text{g}/\mu\text{L}$	Total eluted antibody, μg	Purification yield per g fresh biomass, $\mu\text{g}/\text{g}$	Purification yield per mg total soluble protein, $\mu\text{g}/\text{mg}$
WT	0.4	1000	3.5	21	52.5	6.0
<i>alg3</i>	0.4	1000	6.6	68	136.0	10.3
<i>gntI</i>	0.4	1000	5.9	36	90.0	6.1
<i>alg3gntI</i>	0.4	1000	3.9	68	195.0	17.4

Publications

Bataa, D., Kajiura, H., Sang Sawada-Choi, R. L., Yamashita, Y., Ishimizu, T., Misaki, R., Takeda, A., & Fujiyama, K. CRISPR/Cas9-Mediated Knockouts of the ALG3 and GNTI in *N. Benthamiana* and Their Application to Pharmaceutical Production. *Plant Biotechnology Journal*. <https://doi.org/10.1111/pbi.70326>