Note

Analysis of *N*-glycan profile of *Arabidopsis alg3* cell culture

Ratna Sariyatun¹, Hiroyuki Kajiura^{1,2}, Juthamard Limkul¹, Ryo Misaki^{1,2},
Kazuhiro Fujiyama^{1,2,3,*}

¹International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan; ²Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University, Suita, Osaka 565-0871, Japan; ³Osaka University Cooperative Research Station in Southeast Asia (OU: CRS), Faculty of Science, Mahidol University, Bangkok 10400, Thailand

*E-mail: fujiyama@icb.osaka-u.ac.jp Tel: +81-6-6879-7453 Fax: +81-6-6879-7454

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Abstract *N*-Glycosylation is essential for protein stability, activity and characteristics, and is often needed to deliver pharmaceutical glycoproteins to target cells. A paucimannosidic structure, Man₃GlcNAc₂ (M3), has been reported to enable cellular uptake of glycoproteins through the mannose receptor (MR) in humans, and such uptake has been exploited for the treatment of certain diseases. However, M3 is generally produced at a very low level in plants. In this study, a cell culture was established from an *Arabidopsis alg3* mutant plant lacking asparagine-linked glycosylation 3 (ALG3) enzyme activity. *Arabidopsis alg3* cell culture produced glycoproteins with predominantly M3 and GlcNAc-terminal structures, while the amount of plant-specific *N*-glycans was very low. Pharmaceutical glycoproteins with these characteristics would be valuable for cellular delivery through the MR, and safe for human therapy.

Key words: *alg3*, *Arabidopsis*, *N*-glycosylation, paucimannose.

Plant cell cultures have emerged as a potential alternative production system for recombinant biopharmaceuticals. Plant cell cultures require only simple medium, free of animal-derived products, and thus offer better cost-effectiveness and safety than eukaryotic platforms (Hellwig et al. 2004; Santos et al. 2016; Xu et al. 2011). Plant cells are capable of performing post-translational modifications in a manner similar to mammalian cells, thereby allowing recombinant proteins to be properly folded and assembled (Moustafa et al. 2016). On the other hand, plant cell cultures share characteristics with microbial and mammalian systems, such as allowing contained, controlled, and sterile conditions that meet the criteria for Good Manufacturing Practice (GMP) of biopharmaceutical production (Santos et al. 2016). Notably, plant cell cultures have a good track record for the production of biopharmaceuticals, as evidenced by the approval of carrot cell-produced β -glucocerebrosidase (Elelyso) by the US Food and Drug Administration (FDA), as well as the numerous plant cell-produced recombinant therapeutic glycoproteins currently in clinical trials. Compared to the whole-plant systems, plant cell cultures offer a major advantage in enabling the secretion of products into the culture

medium, thus mediating easier and more cost-effective downstream purification.

N-Glycosylation is essential for the stability, activity, and pharmacodynamics of glycoproteins (Seeberger and Cummings 2015). To date, differences between human and plant *N*-glycosylation have been viewed as a hurdle in the use of plant cells for the production of pharmaceutical glycoproteins. Plants generate β 1,2-xylose (Xyl) and core α 1,3-fucose (Fuc) residues on *N*-glycans, which are absent in humans, thus raising concerns about the potential for immunogenic reaction upon parenteral administration of plant-made biopharmaceuticals in humans (Gomord et al. 2010). Elimination of plant-specific *N*-glycans (β 1,2-Xyl and α 1,3-Fuc) is necessary to ensure the safety of plant-produced biopharmaceuticals. Moreover, minimizing the glycan heterogeneity is important for obtaining consistent product efficacy. The latter has remained a hurdle even in the well-established mammalian cell expression system (Goochee et al. 1991; Sethuraman and Stadheim 2006). Therefore, it is necessary to develop a plant cell line capable of producing homogenous *N*-glycans that lack plant-specific structures in order to optimize the use of plant cell cultures for the production

Abbreviations: ALG3, asparagine-linked glycosylation 3; Fuc, fucose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GMP, Good Manufacturing Practice; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Man, mannose; MR, mannose receptor; MS, mass spectrometry; MS, Murashige and Skoog; PA, 2-pyrimidinamine; RP, reversed-phase; SF, size-fractionation; Xyl, xylose.

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Figure 1. (A) Confirmation of T-DNA insertion in the *alg3* gene in an Arabidopsis *alg3* cell culture by PCR. (B) CBB staining as a loading control. (C) Western blot for the detection of plant-specific N-glycans using anti-HRP antibody. (D) CBB staining of extracellular proteins from the Arabidopsis wild-type (WT) and *alg3* cell cultures. The letters a, b, and c indicate the excised protein bands for characterization using nanoLC-MS/MS and MASCOT search (Supplementary Table S2).

of pharmaceutical glycoproteins.

Previous studies showed that the Arabidopsis *asparagine-linked glycosylation 3* (*alg3*) plant produced more homogenous N-glycans with predominantly unique mannose (Man)-terminal structures and lower amounts of plant-specific N-glycans compared to the wild-type (WT) plant (Henquet et al. 2008; Kajiura et al. 2010). This mutant plant lacks the activity of ALG3, a membrane-bound α 1,3-mannosyltransferase which transfers a Man from a sugar nucleotide dolichol-Man to lipid-linked $\text{Man}_5\text{GlcNAc}_2$ (M5^{ER} ; GlcNAc, N-acetylglucosamine) on the ER luminal side. The N-glycan characteristics of the *alg3* plant could also be passed down to the recombinant protein produced therein (Henquet et al. 2011). Notably, the *alg3* plant also produced a considerable amount of the paucimannosidic structure, $\text{Man}_3\text{GlcNAc}_2$ (M3; 30.7–31.1%), which is normally lacking in plants (Kajiura et al. 2010). M3 could enable the cellular delivery of glycoproteins via the Man receptor (MR) in humans, with the binding of M3 to MR outperforming other structures with more Man residues (Shen et al. 2016; Van Patten et al. 2007). Therefore, the ability of Arabidopsis *alg3* cells to produce M3 could be valuable for the production of therapeutic glycoproteins requiring MR for delivery, such as in the enzyme replacement therapies for Gaucher disease, Wolman disease, and cholesteryl ester storage disease (Du et al. 2005; Limkul et al. 2016). Meanwhile, the lower amount of plant-specific N-glycans is essential to prevent potential immunogenic reaction upon administration of the therapeutic glycoproteins in humans.

In this study, an Arabidopsis *alg3* suspension cell culture was generated from the Arabidopsis *alg3* plant, and the N-glycan profile was characterized. Briefly, the leaves from the Arabidopsis *alg3* plant were sterilized, sectioned, and transferred onto Murashige and Skoog (MS) medium plates for callus induction. After about a month in the dark at 25°C, the calli were transferred into liquid MS medium and incubated in the dark at 25°C on a rotary shaker at 120 rpm until becoming a suspension

cell culture. The suspension cell culture was maintained by weekly transfer of a 20 ml aliquot into 100 ml of fresh liquid MS medium.

To confirm the *alg3* mutation, plant genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Aboul-Maaty and Oraby 2019). T-DNA insertion in the *alg3* gene was then checked by polymerase chain reaction (PCR) using primers 6 (5'-TCTCTTTAATGATTGTTTTGCCAT-3') and Lbd1 (5'-CCACGTTCTTTAATAGTGGACT-3') (Kajiura et al. 2010). The T-DNA insertion in Arabidopsis *alg3* cell culture was confirmed (Figure 1A). Seven-day-old cells were harvested from Arabidopsis WT and *alg3* cell cultures, then dissolved in 20 mM Tris-HCl pH 7.5 containing 10 mM EDTA, sonicated for 1 min, and centrifuged at $12,000\times g$ for 20 min at 4°C. The supernatant was collected as the intracellular protein extract for the detection of plant-specific N-glycans by Western blot using anti-horseradish peroxidase (HRP) antibody (Limkul et al. 2016). Compared to the WT culture, the Arabidopsis *alg3* cell culture contained a very low amount of plant-specific N-glycans (Figure 1C).

Intracellular N-glycans of the *alg3* cell culture were deduced by a combination of high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Briefly, 7-day-old cells were harvested, then ground in liquid N_2 . The thawed cell homogenate was centrifuged at $12,000\times g$ for 20 min at 4°C. Glycoproteins in the supernatant were precipitated using acetone, then lyophilized. Glycans from glycoproteins were released by hydrazinolysis at 100°C for 10 h, and subsequently labelled with 2-pyrimidinamine (PA) (Misaki et al. 2001). The PA-labelled glycans were purified using the phenol-chloroform method to remove excessive PA reagents. The purified PA-labelled N-glycan sample was then separated by reversed-phase (RP)- and size-fractionation (SF)-HPLCs, followed by MALDI-TOF MS analysis (Kajiura et al. 2010). The intracellular N-glycans of Arabidopsis

Table 1. Relative amounts of intracellular *N*-glycans of Arabidopsis wild-type (WT) and *alg3* cell cultures.

Structure	Ratio (%)			
	WT plant*	<i>alg3</i> plant*	WT cell culture	<i>alg3</i> cell culture
M3	—	30.7	0.8	28.5
M4	—	—	5.7	—
M4 ^{ER}	—	17.8	—	—
M5 ^{ER}	—	9.4	—	0.7
GlcM5 ^{ER}	—	2.4	—	—
Gn ¹ M3	—	—	—	14.7
Gn ₁ M3	—	—	—	22.1
Gn2M3	—	—	—	34.0
High-Man				
M5	15.2	—	10.1	—
M7A	4.2	—	4.0	—
M8A	6.2	—	8.2	—
M9	3.0	—	4.1	—
Plant-specific				
M2X	—	—	5.4	—
M2XF	—	—	11.9	—
M3X	6.7	17.5	9.7	—
M3XF	53.6	12.7	2.5	—
Gn ₁ M3X	—	—	10.1	—
Gn ₁ M3XF	1.0	0.1	22.5	—
Gn ¹ M3XF	8.9	4.9	5.0	—
Gn2M3XF	1.1	4.4	—	—

*Previously reported data from the *N*-glycan profiles of Arabidopsis WT and *alg3* plants (Kajiura et al. 2010). Graphical structure of each *N*-glycan is described in Supplementary Table S1.

WT cell culture contained predominantly plant-specific (67.1%) and high-Man (M5, M7A, M8A, M9; 26.4%) structures, similar to the WT plant (plant-specific: 71.4%; high-Man: 28.6%). On the other hand, the *alg3* cell culture produced mainly M3 (28.5%) and GlcNAc-terminal structures without plant-specific *N*-glycans, including Gn¹M3 (14.7%), Gn₁M3 (22.1%), and Gn2M3 (34.0%) (Table 1, Figure 2, Supplementary Table S1). Similar to the *alg3* plant, no high-Man structures were detected in the *alg3* cell culture. While M4^{ER}, M5^{ER}, and GlcM5^{ER} were found in the *alg3* plant, only M5^{ER} (0.7%) was detected in the *alg3* cell culture. Interestingly, plant-specific *N*-glycans were absent in the *alg3* cell culture, whereas they were produced in considerable amounts in the *alg3* plant (39.7%).

Due to the low amounts of secreted glycoproteins in the Arabidopsis WT and *alg3* cell cultures, analyses of the extracellular *N*-glycans were performed using different approach. The media of 7-day-old cell cultures were collected and glycoproteins were precipitated using acetone. The proteins were separated by SDS-PAGE and stained using CBB (Figure 1D). Protein bands were excised and destained, followed by reduction, alkylation, and trypsin digestion prior to *N*-glycan analysis using nanoLC-MS/MS (Limkul et al. 2016). Protein identification was conducted using MASCOT ([https://](https://www.matrixscience.com/index.html)

Figure 2. Reversed-phase high-performance liquid chromatography (RP-HPLC) of *N*-glycan samples from intracellular glycoproteins of Arabidopsis *alg3* cell culture. *N*-glycans were labelled with 2-pyrimidinamine (PA). RP-HPLC was performed using a Cosmosil 5C18-AR-II column (6.0×250 mm; Nacalai Tesque). Numbers at the top represent the elution positions of glucose units (GU) based on the elution times of PA-isomaltoligosaccharides with degrees of polymerization from 3 to 20. Underlined peaks were PA-sugar chain fractions.

www.matrixscience.com/index.html). Glycoproteins secreted from Arabidopsis WT and *alg3* cell cultures were identified (Supplementary Table S2), and the *N*-glycans were characterized (Table 2, Supplementary Table S3). WT cell culture carried predominantly plant-specific structures (41.6–100%), mainly M3XF, GnM3XF, and Gn2M3XF. Smaller amounts of plant-specific *N*-glycans were observed at site N547 of GDPDL4, which might have been due to the protein conformation, which does not allow efficient attachments of the xylosyltransferase (XylT) and fucosyltransferases (FucTA and B). On the other hand, secreted glycoproteins from the Arabidopsis *alg3* cell culture carried mostly M3 (24.5–62.0%) and GnM3 (24.3–70.7%). The GnM3 was the sum of both Gn₁M3 and Gn¹M3, since the nanoLC-MS/MS analysis could not distinguish between these two isomers. Notably, plant-specific *N*-glycan was not observed among the glycoproteins secreted from the *alg3* cell culture.

It is noteworthy that the *N*-glycan profiles between Arabidopsis WT plant and cell culture as well as between the *alg3* plant and cell culture are rather different (Table 1). This may occur because of dedifferentiation process from whole plant into suspension-cultured cells. Similar phenomena were observed in analyses of *Ricinus communis* seeds and callus (Kimura and Takagi 1998) and also between tobacco plant and BY2 cell culture (Oxley et al. 1996; Palacpac et al. 1999).

Table 2. Relative amounts of extracellular N-glycans of Arabidopsis wild-type (WT) and *alg3* cell cultures.

Structure	WT (%)					<i>alg3</i> (%)				
	GDPDL3			GDPDL4	ACO3	GDPDL3			GDPDL4	ACO3
	N251	N528	N242	N547	N510	N251	N528	N242	N547	N510
M1	—	—	—	—	—	3.2	—	—	—	—
M2	—	—	—	—	—	10.5	4.5	6.6	4.8	6.8
M3	—	—	—	5.6	2.6	62.0	34.7	35.9	24.5	48.2
GnM2	—	—	—	1.7	—	—	—	3.0	—	3.5
GnM3	—	—	—	33.9	—	24.3	54.3	44.8	70.7	41.6
Gn2M3	—	—	—	16.3	—	—	6.5	9.6	—	—
GnM4	—	—	—	1.9	—	—	—	—	—	—
Plant-specific										
MXF	23.5	—	—	—	—	—	—	—	—	—
M2X	—	—	—	1.5	2.4	—	—	—	—	—
M2F	—	—	—	—	3.4	—	—	—	—	—
M2XF	—	—	—	—	14.1	—	—	—	—	—
M3X	—	—	—	7.7	7.9	—	—	—	—	—
M3F	—	—	—	—	8.2	—	—	—	—	—
M3XF	44.6	32.4	76.3	—	35.6	—	—	—	—	—
M4F	—	—	—	—	1.7	—	—	—	—	—
GnM2X	—	—	—	1.4	—	—	—	—	—	—
GnM2XF	—	—	—	—	3.3	—	—	—	—	—
GnM3X	—	—	—	23.7	4.8	—	—	—	—	—
GnM3F	—	—	—	1.8	1.3	—	—	—	—	—
GnM3XF	31.8	24.0	23.7	—	12.4	—	—	—	—	—
Gn2M3X	—	—	—	4.5	—	—	—	—	—	—
Gn2M3XF	—	43.6	—	—	2.3	—	—	—	—	—
Total	100	100	100	100	100	100	100	100	100	100

GDPDL3, GDPDL4, and ACO3 are secreted glycoproteins from the Arabidopsis WT and *alg3* cell cultures (Supplementary Table S2). Graphical structure of each N-glycan is described in Supplementary Table S3.

Previously, we observed that the Arabidopsis *alg3* plant produced a lower level of plant-specific N-glycans (39.7%) compared to the WT (71.4%) (Kajiura et al. 2010). This is because N-acetylglucosaminyltransferase-I (GnT-I) has a lower affinity for the M3 (Strasser et al. 2005) to produce Gn₁M3, a substrate for XylT and FucTs to generate plant-specific N-glycans. Surprisingly, once the *alg3* plant was developed into a suspension cell culture, plant-specific N-glycans were hardly detected (Tables 1, 2). Instead, GlcNAc-terminal structures (Gn¹M3, Gn₁M3, and Gn2M3) were produced at much amount. It is intriguing to understand the lack of plant-specific N-glycans in the *alg3* cell culture despite the substantial amounts of GlcNAc-terminal structures, which are substrates for XylT and FucTs. One possible explanation is that the majority of Gn₁M3 might be processed by N-acetylglucosaminyltransferase-II (GnT-II), rather than XylT and FucTs, because GnT-II localizes in the *cis*-medial Golgi, while XylT and FucTs reside in the *medial* and *medial-trans* Golgi, respectively (Strasser 2016; Yoo et al. 2015). This suggests that in the *alg3* cells, GnT-II acts early in the *cis* Golgi on the Gn₁M3 resulting from GnT-I activity on M3, whereas in the WT cells the substrate for GnT-II is available later, after GnT-I generates a GnM5 structure and Golgi- α -mannosidase II (GM-II) cleaves the terminal Man to generate Gn₁M3

(Supplementary Figure S1). The action of GnT-II would inhibit further attachment of plant-specific N-glycans (Yoo et al. 2015), which agrees well with our previous study showing lower XylT activity towards Gn2M3, and lower FucT activity towards N-glycans without the β 1,2-Xyl (Kajiura et al. 2012). However, the remarkable reduction of plant-specific N-glycans was not observed in the *alg3* plant, probably because GnT-II activity in the plant is lower than XylT and FucTs (Yoo et al. 2015). Elucidation of the molecular mechanisms (e.g., gene expression and enzyme activity analyses) responsible for this difference would be necessary.

Our analyses of intra- and extracellular glycoproteins of Arabidopsis *alg3* cell culture indicated that M3 and GlcNAc-terminal structures were predominant. It is known that Man and GlcNAc are recognized by MR, and indeed M3 and Gn2M3 have been shown to enable cellular uptake of therapeutic glycoprotein in vitro (Hintze et al. 2020). Thus, the Arabidopsis *alg3* cell culture should be a suitable host for producing biopharmaceuticals with cellular delivery through the MR pathway.

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