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ORIGINAL ARTICLE

# Glycan structure and serum half-life of recombinant CTLA4Ig, an immunosuppressive agent, expressed in suspension-cultured rice cells with coexpression of human β1,4-galactosyltransferase and human CTLA4Ig

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**Abstract** Human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) is an immunosuppressive therapeutic, and recently produced rice cell-derived hCTLA4Ig ($hCTLA4Ig^P$) reportedly exhibits *in vitro* immunosuppressive activities equivalent to those of Chinese hamster ovary cell-derived hCTLA4Ig ($hCTLA4Ig^M$). However, limitations of $hCTLA4Ig^P$ include shortened *in vivo* half-life as well as the presence of nonhuman *N*-glycans containing (β1-2)-xylose and α1,3-fucose, which cause immunogenic reactions in humans. In the present study, human β1,4-galactose-extended $hCTLA4Ig^P$ ($hCTLA4Ig^P$-Gal) was expressed through the coexpression of human β1,4-galactosyltransferase (hGalT) and hCTLA4Ig in an attempt to overcome these unfavorable effects. The results indicated that both encoding hGalT and hCTLA4Ig were successfully coexpressed, and the analysis of *N*-glycan and its relative abundance in purified $hCTLA4Ig^P$-Gal indicated that not only were the two glycans containing (β1-4)-galactose newly extended, but also glycans containing both β1,2-xylose and α1,3-fucose were markedly reduced and high-mannose-type glycans were increased compared to those of $hCTLA4Ig^P$, respectively. Unlike $hCTLA4Ig^P$, $hCTLA4Ig^P$-Gal was effective as an acceptor *via* (β1-4)-galactose for *in vitro* sialylation. Additionally, the serum half-life of intravenously injected $hCTLA4Ig^P$-Gal in Sprague–Dawley rats was 1.9 times longer than that of $hCTLA4Ig^P$, and the clearance pattern of $hCTLA4Ig^P$-Gal

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was close to that for hCTLA4Ig$^{M}$. These results indicate that the coexpression with hGalT and hCTLA4Ig$^{P}$ is useful for both reducing glycan immunogens and increasing *in vivo* stability. This is the first report of hCTLA4Ig as an effective therapeutics candidate in glycoengineered rice cells.

**Keywords** Plant cell culture · CTLA4Ig · Human β1,4-galactosyltransferase · *N*-glycan · Half-life · Clearance

**Abbreviations**

| | |
|---|---|
| BY2 | *Nicotiana tabacum* L. *cv.* Bright Yellow 2 |
| CHO | Chinese hamster ovary |
| CTLA4 | Cytotoxic T-lymphocyte antigen-4 |
| ELISA | Enzyme-linked immunosorbent assay |
| Fuc | Fucose |
| Gal | Galactose |
| GlcNAc | *N*-acetylglucosamine |
| GU | Glucose unit |
| hCTLA4Ig | Human cytotoxic T-lymphocyte antigen 4-immunoglobulin |
| hCTLA4Ig$^{M}$ | CHO cell-derived human cytotoxic T-lymphocyte antigen 4-immunoglobulin |
| hCTLA4Ig$^{P}$ | Rice cell-derived human cytotoxic T-lymphocyte antigen 4-immunoglobulin |
| hCTLA4Ig$^{P}$-Gal | β1,4-galactose-extended rice cell-derived human cytotoxic T-lymphocyte antigen 4-immunoglobulin |
| hGalT | Human β1,4-galactosyltransferase |
| HPLC | High-performance liquid chromatography |
| HRP | Horseradish peroxidase |
| Hyg$^{R}$ | Hygromycin selection marker |
| MAA | *Maackia amurensis* agglutinin |
| Man | Mannose |
| ODS | Octadecylsilyl |
| PA | Pyridylamino |
| RCA | *Ricinus communis* agglutinin $RCA_{120}$ |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| UTR | Untranslated region |
| Xyl | Xylose |

## Introduction

Transgenic plants and plant cell cultures are becoming more powerful production systems for therapeutic glycoproteins because of their low cost and large-scale production capabilities, low risk of virus and prion contaminations, and the ability to perform most of the posttranslational modifications including glycosylation [1]. However, plants are unable to demonstrate perfect human-type glycosylation in pharmaceutical recombinant proteins [2], and other production systems including transgenic animals, mammalian cells, insect cells, and yeasts have the same problem. Therefore, studying plant glycans is important not only for medical and biotechnological purposes [3], but also for investigation of the role of glycan structures and the *N*-glycosylation pathway in plants [4].

The *N*-glycosylation mechanism in endoplasmic reticulum of mammalian and plant systems has been conserved evolutionarily. However, trimming and further modification of glycans in the Golgi apparatus differ between the two clades [5, 6]. Thus, the high-mannose-type *N*-glycans in plants contain structures identical to those found in other eukaryotic cells, but plant complex-type *N*-glycans differ substantially. In the mammalian system, the tri-mannosyl core structure (mannose $(Man)_3$ *N*-acetylglucosamine $(GlcNAc)_2$) is extended further to contain (β1-4)-galactose (Gal) and terminal sialic acid residues [5]. In contrast, the typically processed *N*-glycans in plants consist mostly of a $Man_3GlcNAc_2$ structure with or without β1,2-xylose (Xyl) and/or α1,3-fucose (Fuc) residues. The presence of β1,2-Xyl and/or α1,3-Fuc residues renders plant-derived therapeutic proteins less desirable. Complementing the *N*-glycan machinery using heterologous glycosyltransferases derived from human may help to achieve the production of glycoproteins with human-compatible glycan structures [1].

*Nicotiana tabacum* L. *cv.* tobacco Bright Yellow 2 (BY2) suspension-cultured cells do not contain any galactosylated *N*-glycan, suggesting that the galactosyltransferase may either be absent or be present only at levels that are too low to be effective in these cells [7]. The expression of human β1,4-galactosyltransferase (hGalT) in BY2 cells confers them with β1,4-GalT activity [8]. The glycoproteins produced from hGalT-expressed BY2 cells were shown to possess glycans that bind to (β1-4)-Gal-specific *Ricinus communis* agglutinin $RCA_{120}$ (RCA) without the dominant xylosylated- and fucosylated glycans [9]. Combining a tobacco plant expressing hGalT and a mouse antibody resulted in the expression of a plantibody with partially galactosylated *N*-glycan [10].

Human cytotoxic T-lymphocyte antigen 4 (CTLA4)-immunoglobulin (hCTLA4Ig) is a soluble recombinant fusion protein that consists of the extracellular domain of hCTLA4 and a human immunoglobulin heavy chain [11]. Mammalian cell-derived hCTLA4Ig was approved by the US Food and Drug Administration in 2005, and abatacept (hCTLA4Ig) is the first selective modulator of a costimulatory signal required for full T-cell activation available commercially for the treatment of rheumatoid arthritis [12]. The hCTLA4Ig has two conserved *N*-glycosylation sites in the hCTLA4 extracellular domain and one in the Fc region [13, 14] in each monomer of the homodimer structure, similar to the heavy chain of antibodies.

The glycans of antibodies are buried between the two heavy chains and exhibit reduced mobility due to the interactions with the protein backbone [15–17]. In particular, the terminal (β1-4)-Gal residues are in close contact with the protein backbone. Therefore, the Gal residues are of broad significance for antibody performance because they are involved in immunological effector functions and complement interactions, as well as correct antibody folding [18, 19]. Thus, the addition of a terminal Gal residue to the glycans of therapeutic antibodies may play a critical role in the pharmacokinetics.

In the present study, the glycosylation machinery of the rice cell was engineered to produce humanized galactosylated glycans in rice cell-derived hCTLA4Ig (hCTLA4Ig$^{P}$). After rice cells were transformed with an hGalT expression vector, the expression of human-type Gal was evaluated in a transgenic rice cell culture. The structure and relative abundance of *N*-glycan, and the *in vitro* sialylation, *in vivo* clearance, and half-life of the newly expressed fusion protein [β1,4-Gal-extended rice cell-derived hCTLA4Ig$^{P}$ (hCTLA4Ig$^{P}$-Gal)] were analyzed, and compared with those of hCTLA4Ig$^{P}$ and mammalian [Chinese hamster ovary (CHO)] cell-derived hCTLA4Ig (hCTLA4Ig$^{M}$).

## Materials and methods

### Cloning of the genes encoding hCTLA4Ig and hGalT and construction of the plant expression vector

The pMYN409 plasmid containing the gene encoding hCTLA4Ig was prepared as described previously [12]. The gene encoding hGalT was amplified by PCR using the method described by Palacpac *et al*. [7] using two sets of primers based on the cDNA sequence reported by Watzele and Berger [20] (GenBank accession no. X55415). The primers GAL N1 (5′-AAA*TCTAGA*GCGATGCCAGGCGCGTCC CT-3′, *Xba*I site in italics) and GAL N2 (5′- AAT*ACTA GT*AGCGGGGACTCCTCAGGGCA-3′, *Spe*I site in italics) were used to amplify a 381-bp fragment of the N-terminal portion of the gene encoding hGalT from human genomic DNA (Clontech, USA) using Ex Taq polymerase (Takara, Japan). For the C-terminal portion of the gene, the primers GAL C1 (5′- AAG*ACTAGT*GGGCCCCATG CTGATTGA-3′, *Spe*I site in italics) and GAL C2 (5′-GTA*GGTACC*GTGTACCAAAAC GCTAGCT-3′, *Kpn*I site in italics) were used to obtain an 811-bp fragment from cDNA synthesized with mRNA of human lung fibroblast MRC-5 cells. The PCR products were cloned in pGEM-T easy vectors (Promega, USA) to generate pGEM-GalN and pGEM-GalC, respectively. The pGEM-GalN vector was digested with *Spe*I and ligated with a *Spe*I/*Kpn*I-cleaved gene fragment from the pGEM-GalC vector to obtain the complete hGalT gene (1.2 kbp). The resulting plasmid, pGEM-hGalT, was digested with *Xba*I and *Kpn*I to excise a 1.2-kbp DNA fragment containing the whole hGalT gene, which was subcloned into the *Xba*I–*Kpn*I site between the *RAmy3D* promoter and 3′-UTR in the pMYN75 vector. The resulting 2.6-kbp fragment, a 3D-hGalT-UTR cassette, was excised with *Hin*dIII and *Eco*RI, and substituted for the CaMV35S-GUS-NOS cassette gene in the binary vector pBI121 (Invitrogen, USA) to construct the hGalT expression vector pBI-3D-hGalT.

### Transformation and screening of transgenic rice calli

The pMYN409 plasmid containing the hCTLA4Ig gene and the pBI-3D-hGalT plasmid were inserted into *Agrobacterium tumefaciens* LBA4404 by triparental mating [21]. Immature embryogenic calli derived from rice seeds (*Oryza sativa* L. *cv.* Dongjin) were cotransformed using an *Agrobacterium*-mediated transformation [22]. Transformants were selected and maintained on N6 medium supplemented with 50 mg/L hygromycin, 50 mg/L G-418, and 250 mg/L cefotaxim. Calli containing the genes encoding hCTLA4Ig and hGalT were selected by genomic DNA PCR in the hygromycin and G418 coresistant transformants.

### Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from transgenic calli using the DNeasy plant mini-kit (Qiagen, USA). The primers GAL N1 and GAL C2, which were used for cloning of hGalT, were prepared to identify the hGalT gene insertion into rice chromosomes. The primers RAmy1A-F (5′-GGATCCGC ATGCAGGTGCTGAA-3′) and hIgG-R (5′-CTCTAGAC TCATTTACCCGGAGAC AGGGAG-3′), and Hyg-F (5′-CTACATGGCGTGATTTCATA-3′) and Hyg-R (5′-CACTATCGGCGAGTACTTCT-3′) were also used to amplify the hCTLA4Ig gene and hygromycin selection marker, Hyg$^{R}$. A thermal cycle consisting of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C was repeated 30 times. The PCR products were separated on a 1.2 % agarose gel and stained with ethidium bromide.

### Establishment, propagation, and induction of transgenic rice cell suspension cultures

Rice calli coexpressing hCTLA4Ig and hGalT (Os/3DhGalT cell line) were used to establish cell suspension cultures, as described previously [12]. Transgenic rice calli were cultured on AA medium containing 2,4-dichlorophenoxyacetic acid (2 mg/L), kinetin (0.02 mg/L), and 3 % sucrose [AAS(+)] [23], and established suspension-cultured cells were subcultured every 10 days. Subcultures were performed with 20 % (v/v) inoculum, and cells were cultured on a shaker at 110 rpm and incubated at 27 °C in the dark.

### Enzyme-linked immunosorbent assay

The hCTLA4Ig$^{P}$-Gal expression levels in transgenic rice cell suspension cultures were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [12]. Briefly, suspension-cultured medium was centrifuged for 10 min at 14,000×*g*. For the sandwich ELISA, 96-well microplates were coated with goat antihuman IgG (Fc; 1:1,000; KPL, USA) and blocked with phosphate-buffered saline containing 2 % fetal bovine serum and 0.05 % Tween 20. Each well was loaded with sample or protein standards at a twofold dilution. The application of horseradish peroxidase (HRP)-labeled goat antihuman IgG (γ; 1:5,000; KPL), used as the detection antibody, was followed by an HRP substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (KPL).

### Purification of hCTLA4Igs

Culture media were harvested and centrifuged for 30 min at 17,000×*g*. The supernatant was purified with Streamline rProtein A resin using the ÄKTA fast protein liquid chromatography system (Amersham Biosciences, USA), as described previously [12].

### SDS-PAGE and immunoblot analysis

Purified hCTLA4Igs (1 μg of each) that had been separated on 10 % SDS-PAGE gels (Invitrogen) were blotted onto nitrocellulose using the iBot Dry Blotting System (Invitrogen). For detection of hCTLA4Ig and core-bound (β1-2)-Xyl and (α1-3)-Fuc, blots of purified hCTLA4Ig were probed with mouse anti-hCTLA4 or rabbit anti-HRP antibodies, respectively. Bound primary antibodies were detected with 3,3′,5,5′-tetramethylbenzidine (KPL) after incubation with HRP-conjugated goat antimouse and goat antirabbit antibodies. For immunological detection of β1,4-Gal on purified hCTLA4Ig, the blot was probed with biotinylated RCA (Vector Laboratories, USA). Detection of bound agglutinin was performed using 3,3′,5,5′-tetramethylbenzidine as a substrate after incubation with HRP-labeled streptavidin (KPL).

### Glycan analysis

Glycan analyses of hCTLA4Igs (100 μg) were performed according to a method that is described in detail in our papers [24–26]. Briefly, the samples were incubated in the presence of trypsin (Sigma, USA) and chymotrypsin (Sigma) in 10 mM Tris–HCl (pH 8.0) at 37 °C for overnight; glycans were released after reaction with 0.1 mU of glycoamidase A (Seikagaku Kogyo, Japan) in 0.5 M citrate-phosphate buffer (pH 5.0) at 37 °C overnight. The released glycans of the reducing ends were reductively aminated with 2-aminopyridine using sodium cyanoborohydride. The PA derivatives of the glycan mixture were applied to HPLC (Waters, USA) onto a Shim-pack CLC-ODS column (Shimadzu, Japan) equipped with a fluorescence detector. The collected fractions were subjected to HPLC analysis using a TSK-GEL Amide-80 column (Tosoh, Japan). The structure of each peak separated on the column was identified as described previously [25]. Each fraction from the ODS column was applied to a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Ultraflex III system, Bruker Daltonik, Germany) using 2,5-dihydroxybenzoic acid as a matrix.

### *In vitro* sialylation and dot-blot analysis

hCTLA4Ig$^{P}$-Gal (50 μM) in 50 mM sodium cacodylate buffer (pH 6.0) was treated with 5.6 mU of recombinant *Spodoptera frugiperda* α2,3-sialyltransferase (Roche Applied Science, USA), 5 mM cytidine monophosphate sialic acid, and 8 mM $MnCl_2$ at 37 °C for 20 h. The sialylated hCTLA4Ig$^{P}$-Gal was purified with a rProtein A resin and used for further analysis. Glycoproteins were spotted onto a nitrocellulose membrane (Whatman, Germany) with a 96-well dot-blot apparatus (Bio-Rad, USA) and subjected to lectin-binding analysis with the digoxigenin Glycan Differentiation Kit according to the manufacturer's protocol (Roche Applied Science, Germany). This lectin digoxigenin conjugate was MAA, which binds α2,3-sialic acid.

### *In vivo* clearance and half-life

Purified hCTLA4Igs were diluted to 1.2 mg/mL in sterile phosphate-buffered saline and injected (2 mg/kg, six animals per sample) intravenously into normal, male Sprague–Dawley rats (SLC, Japan). Blood samples were drawn at 5, 15, 30, 60, 150, 240, 360, 480, 720, and 1440 min by retro-orbital bleeds, and the samples were refrigerated overnight. After spinning for 10 min in a microcentrifuge to remove any clotted material, the sera were assayed using a sandwich ELISA for hCTLA4Ig determinants. The animal experiments were performed in accordance with approval granted by the Institutional Animal Care and Use Committee at Boryung Pharmaceutical Co. Ltd.

## Results

### Construction of the hGalT expression vector and rice transformation

The hGalT vector for expression in rice cells is depicted in Fig. 1a. The hGalT gene was controlled by a rice α-amylase 3D (*RAmy3D*) promoter [12] that induces gene expression under sugar-deprivation conditions in a rice cell culture. The expression cassette of *RAmy3D*/hGalT/3′-untranslated region (UTR) was confirmed by restriction enzyme mapping

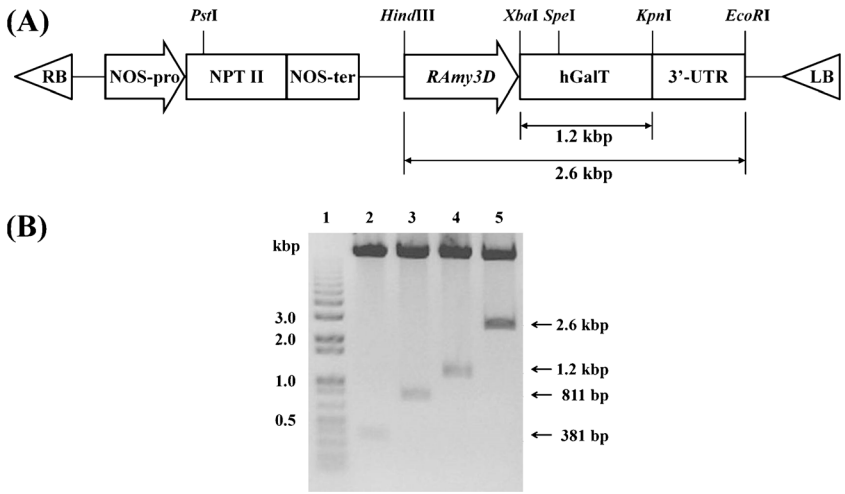



**Fig. 1** Construction of the hGalT expression vector, pBI-3D-hGalT. **a** Schematic diagram of pBI-3D-hGalT. The gene encoding hGalT lies downstream of *RAmy3D* promoter, followed by the 3′-UTR region from pMYN75. RB, T-DNA right border; NOS-pro, nopaline synthase promoter; NPT II, neomycin phosphotransferase II gene; NOS-ter, nopaline synthase terminator; 3′-UTR, 3′-UTR of the rice α-amylase gene; LB, T-DNA left border. **b** Restriction enzyme digestion mapping of pBI-3D-hGalT. Plasmid DNAs were digested with restriction enzymes and separated on 1.2 % agarose gel. Lane 1, 1-kb DNA size marker (Invitrogen, USA); lane 2, *Xba*I+*Spe*I digested vector; lane 3, *Spe*I+*Kpn*I digested vector; lane 4, *Xba*I+*Kpn*I digested vector; lane 5, *Hin*dIII+*Eco*RI digested vector. The numbers on the left-hand side indicate the sizes of the DNA markers. The arrows and numbers on the right-hand side indicate the locations and sizes of the pBI-3D-hGalT DNA fragments digested with the corresponding restriction enzymes

(Fig. 1b). The recombinant vector containing the hGalT gene (pBI-3D-hGalT) also carries a neomycin phosphotransferase expression cassette conferring resistance to G-418, an aminoglycoside antibiotic, in transformed cells. Thus, after cotransformation with pMYN409 [12], which is the hCTLA4Ig expression vector containing hygromycin phosphotransferase genes for hygromycin resistance, the cells coexpressing hGalT and hCTLA4Ig were selected with G-418 and hygromycin B resistance. Nine transformed rice calli exhibited the insertion of two target genes: hGalT and hCTLA4Ig (data not shown). Through polymerase chain reaction (PCR) of genomic DNA obtained from Os/3DhGalT rice cells (cell line #1-2), one of nine transformed cell lines was confirmed by the presence of a 1192-bp hGalT fragment and a 1183-bp hCTLA4Ig fragment derived from the T-DNA of pBI-3D-hGalT and pMYN409, respectively. No PCR product of hGalT was observed in pMYN409 rice cells expressing only hCTLA4Ig (Fig. 2).

## Expression of human-type Gal on hCTLA4Ig in a transgenic rice cell culture

Because the expressions of hGalT and hCTLA4Ig are controlled by the *RAmy3D* promoter under sugar-deprivation conditions, transgenic suspension-cultured rice cells were inoculated in AAS(-) [23] induction medium (Fig. 3). During the induction period, extracellular hCTLA4Ig increased up to day 9, and the maximum productivity of total hCTLA4Ig was 10.2 mg/L in the medium. The intracellular content of hCTLA4Ig was less than 10 % of this amount. The expressions of hGalT and hCTLA4Ig were also confirmed by reverse transcription PCR (RT-PCR) and Western-blot analysis (Fig. 4). mRNA levels of both hGalT and hCTLA4Ig increased rapidly after induction by sugar deprivation (Fig. 4a). The molecular weight of extra- and intracellular hCTLA4Ig was about 50 kDa under reducing conditions, and was similar to that of $hCTLA4Ig^{M}$ (Fig. 4b).

## Glycan analysis

Figure 5 shows the *N*-glycosylation profile of hCTLA4Igs, which was obtained by high-performance liquid chromatography (HPLC) analysis of pyridylamino (PA)-glycans using an octadecylsilyl (ODS) column. Each fraction that was individually separated on the ODS column was further applied to an amide column. The glucose unit (GU) values were determined by comparison against a glucose standard using ODS and amide columns. Using a combination of the GU results on a two-dimensional map and mass (m/z) values of the collected peaks, the structures of PA-glycans of CTLA4Igs were identified by comparison with those in the GALAXY database (http://www.glycoanalysis.info/galaxy2/), and are listed in Table 1. Four glycan structures (P1–P4) of $hCTLA4Ig^{P}$ were analyzed, all of which are both (β1-2)-xylosylated and (α1-3)-fucosylated glycans, and no high-mannose-type glycans were found. The seven glycan structures (G1–G7) of $hCTLA4Ig^{P}$-Gal were analyzed; when compared to the glycans of $hCTLA4Ig^{P}$, (β1-2)-xylosylated and (α1-3)-fucosylated glycans (G5) were markedly reduced, accounting for 2.0 % of the total glycans, and (α1-3)-fucosylated glycans (G4) accounted for 6.8 %. G4, G6,

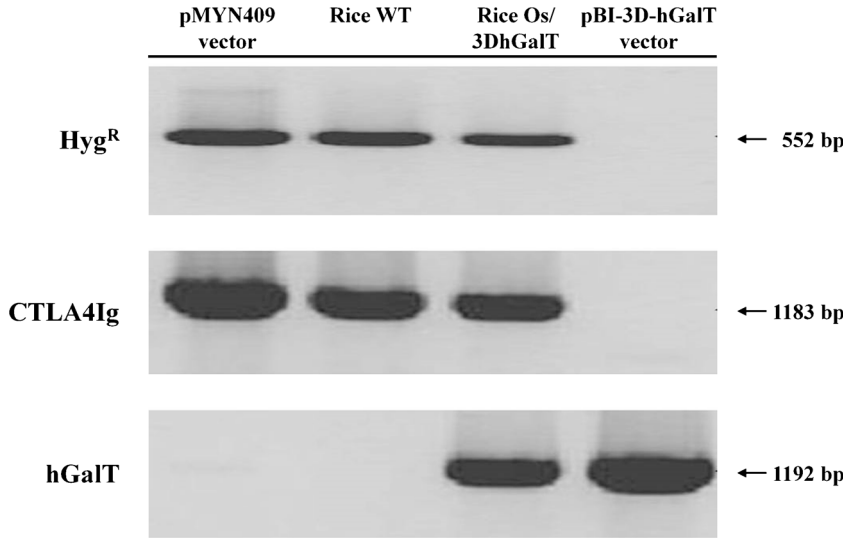



**Fig. 2** Results of genomic DNA PCR of transgenic rice cells. pMYN409 rice cells were transformed with the pMYN409 vector containing the gene encoding hCTLA4Ig, whereas Os/3DhGalT rice cells were cotransformed with pMYN409 and pBI-3D-hGalT containing the gene encoding hGalT. The left side denotes the target sequences of the PCR, and the arrows indicate the amplified gene sizes of each target sequence. WT, wild type

and G7 are hybrid-type glycans, and G5 is a complex-type glycan. The (β1-4)-Gal-extended glycans were G5 and G7 (accounting for 2.0 and 5.5 % of the total glycans, respectively). The G5 and G7 glycans were totally removed by β-galactosidase digestion (data not shown). High-mannose-type glycans (G1, G2, and G3) increased from zero to 82.1 % of the total glycans of hCTLA4Ig$^{P}$-Gal, in contrast to the result for hCTLA4Ig$^{P}$. The glycans of hCTLA4Ig$^{M}$ [27] were also analyzed, and (β1-4)-galactosylated (22.6 %), biantennary (β1-4)-monogalactosylated with monosialylated (6.1 %), disialylated (2.5 %), and high-mannose-type (18.9 %) glycans were found (data not shown).

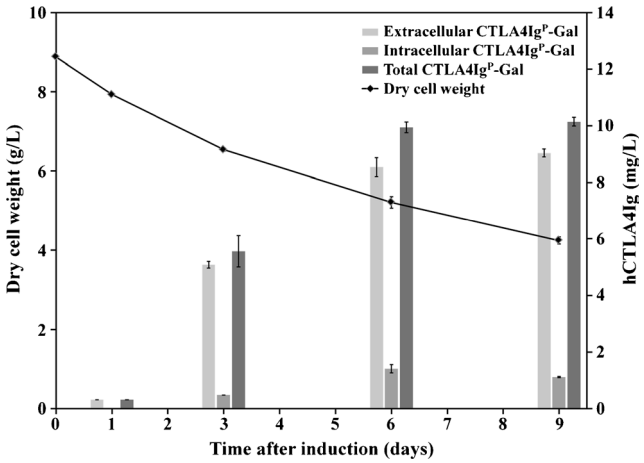



**Fig. 3** Time-course changes in cell growth and hCTLA4Ig production in a transgenic rice cell suspension culture. The rice Os/3DhGalT cell line, was cotransformed with the expression vector pMYN409 and the recombinant vector pBI-3D-hGalT, which contained the genes encoding hCTLA4Ig and hGalT, respectively. Rice cells were inoculated into sugar-deprivation medium and their gene expression was induced for 9 days. The line graph represents cell growth and the bar graph represents hCTLA4Ig expression. The bars at each time point denote extracellular, intracellular, and total hCTLA4Ig, respectively. Data are mean and SD values

### Immunoblot analysis and *in vitro* sialylation

Purified hCTLA4Ig was analyzed on blots with mouse anti-hCTLA4 antibody, (β1-4)-Gal-specific RCA, anti-HRP polyclonal antibody (which also binds to Xyl and Fuc in HRP glycan) [28] (Fig. 6a), and (α2-3)-sialic acid-specific *Maackia amurensis* agglutinin (MAA) (Fig. 6b). The mouse anti-hCTLA4 antibodies bound to all three of the isolated hCTLA4Igs. In contrast, RCA bound to hCTLA4Ig$^{M}$ and hCTLA4Ig$^{P}$-Gal (also fetuin as galactosylated glycoprotein control), but not to hCTLA4Ig$^{P}$. Anti-HRP antibodies detecting plant-specific Xyl and Fuc residues linked to the core glycan recognized both hCTLA4Ig$^{P}$ and hCTLA4Ig$^{P}$-Gal, but differences in band intensities reflecting the contents of the plant-specific glycans were observed (right panel in Fig. 6a, labeled "anti-HRP"). Equal amounts of hCTLA4Ig had been loaded in all lanes (left panel in Fig. 6a, labeled "anti-CTLA4"), and the hCTLA4Ig$^{P}$ stained strongly with anti-HRP, as expected (lane 3 in the "anti-HRP" panel in Fig. 6a). The (β1-4)-Gal residue in hCTLA4Ig$^{P}$-Gal was also identified by *in vitro* sialylation using α-2,3-sialyltransferase. MAA, a α-2,3-sialic acid-specific lectin, was used to confirm the sialylation of hCTLA4Ig$^{P}$-Gal. MAA became bound to hCTLA4Ig$^{M}$ and sialylated hCTLA4Ig$^{P}$-Gal (also fetuin as a sialylated glycoprotein control), but not to hCTLA4Ig$^{P}$ even after *in vitro* sialylation reaction (Fig. 6b).

### Serum *in vivo* clearance and half-life values

Purified hCTLA4Igs were injected intravenously into Sprague–Dawley rats; no adverse effects were seen for any of the hCTLA4Igs. The mean serum concentrations of hCTLA4Ig following the intravenous administrations are illustrated in Fig. 7. The serum concentration–time profiles indicated that the serum

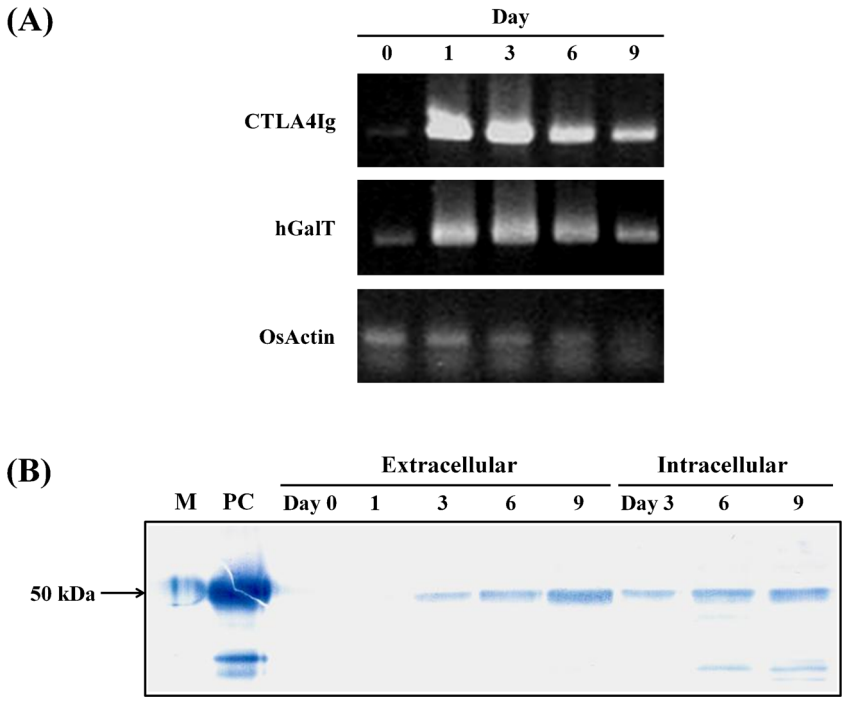



**Fig. 4** Expressions of hCTLA4Ig and hGalT in transgenic rice cell suspension cultures. Their expressions were analyzed by RT-PCR (**a**) and Western-blot analyses (**b**). Rice cells were transformed with the pMYN409 and/or pBI-3D-hGalT vectors. Vacuum-filtered cells (3 g) were inoculated into 30 mL of sugar-deprivation AA medium in a 100-mL flask. The cells were periodically harvested and used to extract total RNA and proteins. In RT-PCR, the gene encoding rice actin (*OsActin*) gene was used as a control. Western blot analysis was performed for intracellular and extracellular hCTLA4Ig proteins, and CHO derived hCTLA4Ig (2 μg) was used as a positive control (PC)

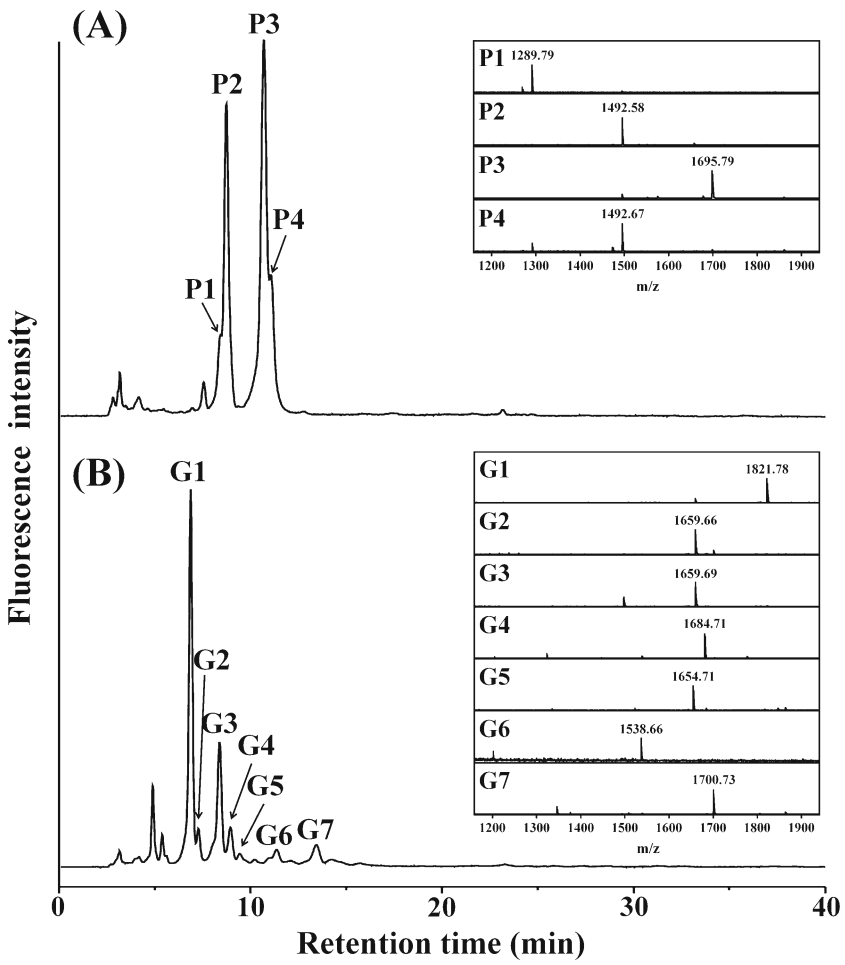
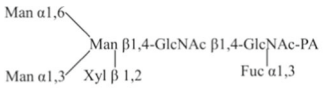
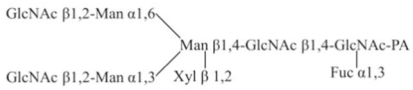
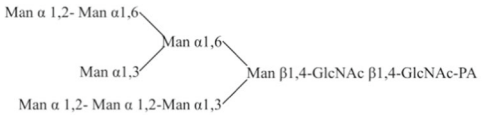
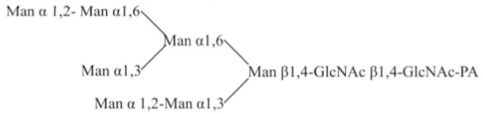
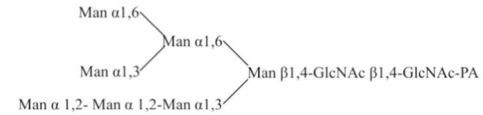
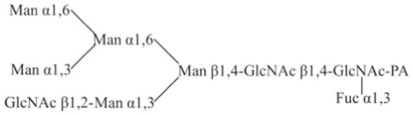
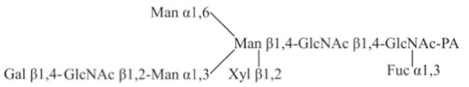
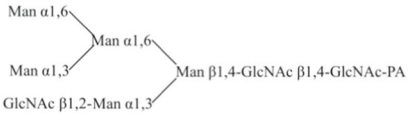
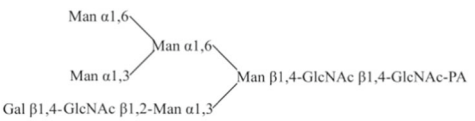



**Fig. 5** *N*-glycan profiles of hCTLA4Ig$^{P}$ (**a**) and CTLA4Ig$^{P}$-Gal (**b**) on an ODS column (inset, MALDI-TOF mass spectra of each fraction collected from the ODS column). Glycans were obtained from hCTLA4Ig$^{P}$ (100 μg) and hCTLA4Ig$^{P}$-Gal (100 μg) by protease (trypsin and chymotrypsin) digestion, and subsequent treatment by 0.1 mU of glycoamidase A at 37 °C overnight. The released glycans were reductively aminated with 2-aminopyridine. The PA-glycans were subjected to HPLC on a Shim-pack CLC-ODS column equipped with a fluorescence detector

**Table 1** Summary of the proposed structure of PA-glycans from hCTLA4Ig$^P$ and hCTLA4Ig$^P$-Gal

| Protein | Peak | Proposed structure | Observed (reported)[b] | | | Relative quantity (%) |
|---|---|---|---|---|---|---|
| | | | ODS (GU)[a] | Amide (GU) | Mass, m/z $[M+Na]^+$ | |
| hCTLA4Ig$^P$ | P1 |  | 5.7 (5.6) | 6.0 (5.7) | 1289.79 (1289.48) | 5.8 |
| | P2 |  | 5.9 (5.8) | 6.3 (6.4) | 1492.58 (1492.56) | 31.8 |
| | P3 |  | 6.8 (7.0) | 6.7 (6.5) | 1695.79 (1695.63) | 49.0 |
| | P4 |  | 7.0 (7.3) | 6.4 (6.2) | 1492.67 (1492.56) | 13.4 |
| hCTLA4Ig$^P$-Gal | G1 |  | 4.6 (4.9) | 9.5 (9.0) | 1821.78 (1821.64) | 54.3 |
| | G2 |  | 4.9 (5.1) | 8.4 (8.1) | 1659.66 (1659.59) | 4.4 |
| | G3 |  | 5.6 (5.8) | 8.4 (8.0) | 1659.69 (1659.59) | 23.4 |
| | G4 |  | 6.0 (6.0) | 7.0 (7.3) | 1684.71 (1684.62) | 6.8 |
| | G5 |  | 6.2 (6.3) | 7.4 (6.9) | 1654.71 (1654.61) | 2.0 |
| | G6 |  | 7.1 (7.3) | 6.5 (6.4) | 1538.66 (1538.56) | 3.6 |
| | G7 |  | 7.8 (7.9) | 7.8 (7.4) | 1700.73 (1700.61) | 5.5 |

[a] GU, glucose units

[b] Numbers in parentheses indicate the reported ODS, amide, and mass (m/z) values of PA-glycans [25]

levels of all three hCTLA4Igs rapidly declined about 10 min after dosing. The concentration declined more rapidly thereafter in the hCTLA4Ig$^P$ group than in the two other hCTLA4Ig groups. In contrast, hCTLA4Ig$^P$-Gal had an intermediate clearance rate. The half-life data for the three hCTLA4Igs are provided in Table 2. The α (distribution phase) and β (elimination phase) half-life values of the free hCTLA4Ig were higher for hCTLA4Ig$^P$-Gal than for hCTLA4Ig$^P$. Especially, the elimination half-life value of hCTLA4Ig$^P$-Gal (767.1±142.3 min) was 1.9 times longer than that of hCTLA4Ig$^P$ (414.3±89.1 min). The distribution phase involves changes of the concentration in plasma due to the movement of the drug within the body, and the

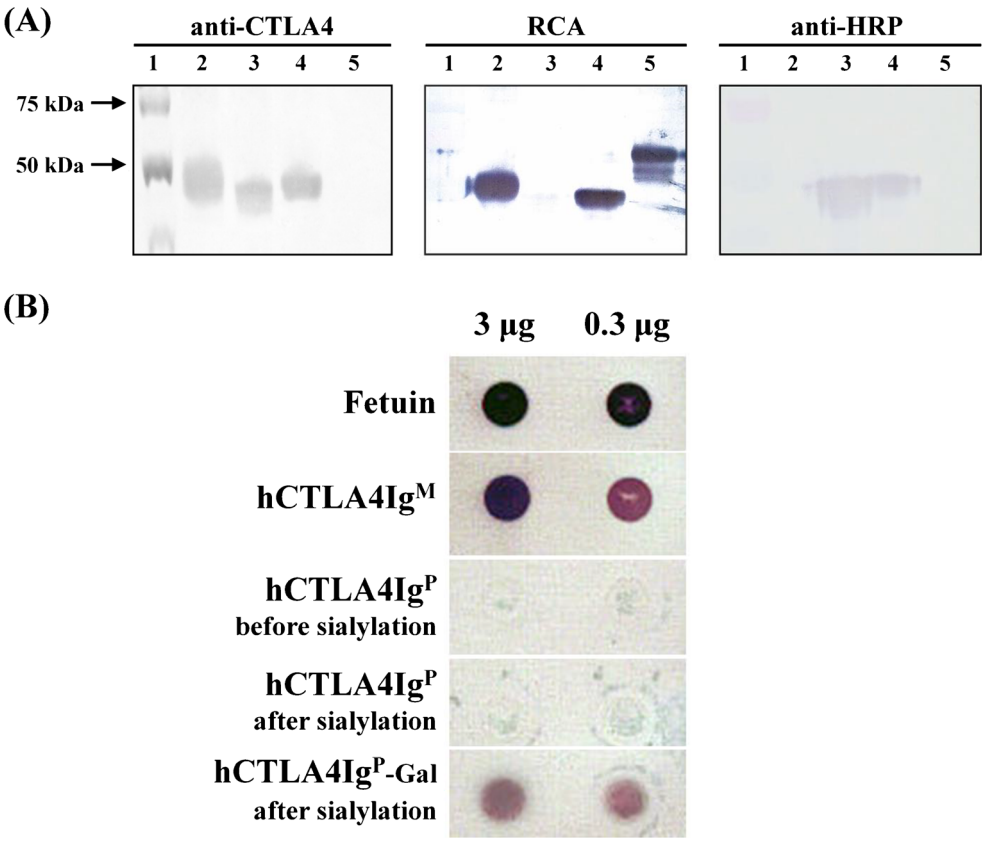



**Fig. 6** Immunoblot analyses of recombinant hCTLA4Ig from transgenic suspension-cultured rice cell lines. **a** An equal amount of purified hCTLA4Igs (1 μg) was used for each lane. The proteins were resolved by 10 % SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. The membranes were probed with mouse anti-CTLA4 antibody (*left*), biotin-labeled RCA (*center*), and rabbit anti-HRP antibody (*right*). Lane 1, protein molecular weight marker; lane 2, hCTLA4Ig$^{M}$; lane 3, hCTLA4Ig$^{P}$; lane 4, hCTLA4Ig$^{P}$-Gal; lane 5, fetuin as a glycoprotein control. **b** Samples applied to the dot blots were fetuin (a positive control), hCTLA4Ig$^{M}$ containing the sialic acid moiety, hCTLA4Ig$^{P}$ before sialylation (a negative control), hCTLA4Ig$^{P}$ after sialylation, and hCTLA4Ig$^{P}$-Gal, which was sialylated using *in vitro* sialylation

elimination phase involves changes of the concentration due to elimination from the body after reaching equilibrium.

## Discussion

In the present study, the genes encoding hGalT and hCTLA4Ig were coexpressed in rice cells to confirm whether hGalT can extend and modify the *N*-glycosylation in plants. Our results demonstrate that both genes were successfully coexpressed in the transformed rice cells, and suggest that similar to mammalian glycosyltransferase, the catalytic form of the glycosyltransferase is correctly oriented toward the luminal side of the Golgi apparatus [29]. There were no significant differences in the *in vitro* growth of rice cells expressing hCTLA4Ig$^{P}$ and those expressing hCTLA4Ig$^{P}$-Gal. These cells were maintained continuously for more than 200 weekly transfers in the laboratory (data not shown). This resistance of transgenic rice cells to changes in their *N*-glycan processing is an advantage for the production of glycan-engineered therapeutic glycoproteins.

In plant-derived antibodies with humanized glycans, Gal residues might play an important role in antibody functions

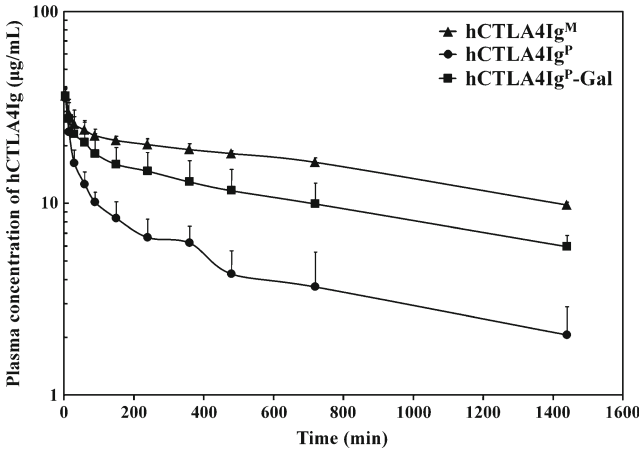



**Fig. 7** Clearance of CTLA4Ig following intravenous injection in rats. Mean serum concentrations *versus* time profiles of the three hCTLA4Igs. Rats were injected into the femoral vein with the indicated protein (2 mg/kg, six mice per group) and blood samples were taken and assayed by ELISA

**Table 2** Half-life values of the α and β phases of the recombinant hCTLA4Igs used in this study

| hCTLA4Ig | Half-life (min) | |
|---|---|---|
| | α (distribution phase) | β (elimination phase) |
| hCTLA4Ig$^{M}$ | 12.8±11.2 | 1219.6±95.9 |
| hCTLA4Ig$^{P}$ | 12.7±8.0 | 414.3±89.1 |
| hCTLA4Ig$^{P}$-Gal | 15.6±7.3 | 767.1±142.3 |

Data are mean±SD values

such as complement-dependent cytotoxicity activity [30], stability with reference to antibody folding [31], and attachment of sialic acid [31]. The glycoprotein produced in wild-type tobacco cells contains the (β1-3)-Gal; however, the hGalT produced in transgenic tobacco cells contain (β1-4)-Gal [9]. In the present study, only the (β1-4)-Gal residue was extended in hCTLA4Ig$^P$-Gal. This suggests that the human glycosyltransferase enzyme extended some complex sugar chains from nongalactose- into Gal-containing glycans [10, 32]. Our glycan analysis indicates that the hCTLA4Ig produced from rice cells coexpressing hGalT carrys both plant and mammalian glycan epitopes. The most abundant (β1-4)-Gal-extended *N*-glycan found in hCTLA4Ig$^P$-Gal was $Gal_1GlcNAcMan_5GlcNAc_2$ (peak G7, Table 1), which is consistent with the glycoproteins produced from hGalT-expressed BY2 cells [9]. This structure is rarely found in mammals and is thought to prevent the activity of α-mannosidase-II so that hGalT acts too early in the Golgi apparatus [9].

The plant-derived glycoproteins contain (β1-2)-Xyl and (α1-3)-Fuc residues in a complex-type *N*-glycan structure. It is well known that these (β1-2)-Xyl and (α1-3)-Fuc are immunogenic and allergenic for humans and other mammals [2]. To remove or reduce these unique plant *N*-glycan structure, various methods such as retention of glycoprotein in the endoplasmic reticulum, overexpression of hGalT, and production of glycoproteins from α1,3-fucosyltransferase- and β1,2-xylosyltransferase-knockout cell lines have been applied [33]. Our results confirmed that (β1-2)-Xyl and (α1-3)-Fuc residues were markedly reduced in hCTLA4Ig$^P$-Gal compared to hCTLA4Ig$^P$: glycans containing both (β1-2)-Xyl and (α1-3)-Fuc diminished to 2.0 % in total glycans of hCTLA4Ig$^P$-Gal. Another study suggested that the reductions in (β1-2)-Xyl and (α1-3)-Fuc residues are achieved by competition between hGalT and plant-specific glycosyltransferases including α1,3-fucosyltransferases and β1,2-xylosyltransferases [34]. Meanwhile, it was found that galactosylated *N*-glycans became poor acceptors for plant β1,2-xylosyltransferase and α1,3-fucosyltransferase [35]. In tobacco BY2 cells, the galactosylated glycoproteins obtained by hGalT expression did not contain any (β1-2)-Xyl residues [7]. These reductions of plant-specific glycans induced by GalT could provide a system with reduced immunogenicity when injected into humans [9].

The presence of galactosylated glycans was also demonstrated using immunoblotting with (β1-4)-Gal-specific RCA. The terminal (β1-4)-Gal residue of engineered plant glycoprotein offers a basis for the addition of sialic acid, which is an important component for absorption, serum half-life, and clearance in human glycoproteins [31]. We performed *in vitro* sialylation with hCTLA4Ig$^P$-Gal and hCTLA4Ig$^P$ using α 2, 3-sialyltransferase and found that only hCTLA4Ig$^P$-Gal can be sialylated. The expression of mammalian hGalT in plants would be a useful strategy for the humanization of plant *N*-glycans together with *in vitro* sialylation [36].

Plant-derived recombinant glycoproteins are rapidly cleared *in vivo* compared with mammalian-derived glycoproteins [37]. Our pharmacokinetic analysis shows that hCTLA4Ig$^P$ exhibits significantly lower *in vivo* stability and has low half-life value. The shortened half-life of hCTLA4Ig$^P$ may result from plant-specific *N*-glycan structures that can induce immune reactions when injected into rats [38, 39]. We found that the relatively short half-life of hCTLA4Ig$^P$ (414.3±89.1 min) was increased to 767.1±142.3 min in hCTLA4Ig$^P$-Gal. This implies that modification of *N*-glycans in plant cells through the expression of mammalian glycosylation enzymes improve the problems of plant-derived recombinant therapeutic proteins, such as the shortened *in vivo* half-life and immunogenicity [40]. However, this is still shorter than that of hCTLA4Ig$^M$ (1219.6±95.9 min), which contains sialic acid as a terminal carbohydrate in glycans. Meanwhile, high-mannose-type glycans were increased to 82.1 % of the total glycans in hCTLA4Ig$^P$-Gal in the present study. However, it has been reported that glycoprotein containing high-mannose-type glycans are rapidly cleared *in vivo* rat serum due to the mannose receptor system, and that high-mannose-type glycans are not affected by either the *in vivo* stability or half-life of a glycoprotein [41].

CTLA4Ig$^P$ exhibited high immunosuppressive activities *in vitro* that were equivalent to those of hCTLA4Ig$^M$ [27]. The human (β1-4)-Gal-extended rice strains generated in this study may thus represent a safe production system for biopharmaceuticals with human-like glycosylation patterns. Galactosylation levels have been emphasized primarily in the evaluation of humanized *N*-glycan from transgenic plants. The Gal yield in the present study (7.5 %) was lower than that found previously in a study of extended glycan produced by transgenic tobacco cells (47.3 %) [9]. The present low Gal transfer efficiency can be explained variously by, for example, steric hindrance by the antibody heavy chain [42] or differences in the incubation conditions of the cells. In addition, it is reported that change of the ammonium concentration during the culture affects the amount of Gal and GlcNAc residues in CHO cells [43]. In plants, glycosylation is also altered by supplementing glucosamine and ammonia into the culture medium [44]. Further study of the increases in galactosylated glycans is required. However, greater galactosylation of hCTLA4Ig$^P$-Gal will not necessarily increase its half-life. More important factors for increasing the half-life are probably decreasing the extent of glycans containing Xyl and Fuc as well as increasing the humanized glycans, although the galactosylation ratio will not be high. These factors should be confirmed in future studies.

In conclusion, the hCTLA4Ig$^P$-Gal fusion protein was expressed in suspension-cultured rice cells with coexpression of hGalT and hCTLA4Ig. The analysis of *N*-glycan in hCTLA4Ig$^P$-Gal indicated that the glycans containing terminal β1,4-Gal were newly expressed, and those of both

xylosylated and fucosylated glycans were decreased compared to those of hCTLA4Ig$^{P}$. The serum *in vivo* half-life of hCTLA4Ig$^{P}$-Gal was extended, and this is probably due to the newly expressed human-type glycans as well as decreased the immunogenic plant-specific glycans, in contrast to those of hCTLA4Ig$^{P}$. The present hCTLA4Ig$^{P}$-Gal is thus a newly coexpressed glycoprotein therapeutics candidate for humanized recombinant protein production in *in planta* engineering, and this is the first report of the expression, glycan analysis, and increase in the serum half-life of hCTLA4Ig$^{P}$-Gal produced from glycoengineered rice cells with humanized glycans.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with Ethical Standards** The animal experiments were performed in accordance with approval granted by the Institutional Animal Care and Use Committee at Boryung Pharmaceutical Co. Ltd.

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