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Rabies virus glycoprotein produced in *Nicotiana benthamiana* is an immunogenic antigen in mice

*Youngmin Park[1], Hyangju Kang[1], Kyungmin Min[1], Nam Hyung Kim[1], Minhee Park[1], In-Ohk Ouh[2], Ha-Hyun Kim[2], Jae-Young Song[2], Dong-Kun Yang[2], Eun-Ju Sohn[1], Sangmin Lee[1]**

[1]BioApplications Inc., Pohang Techno Park Complex, Pohang, Korea
[2]Virus Disease Division, Animal and Plant Quarantine Agency, Gimcheon, Gyeongbuk, Korea

**Corresponding author: lsm1978@bioapp.co.kr*

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Abstract: Rabies remains an infectious disease among humans and animals, and requires the development of an effective vaccine essential to prevent rabies. Advances in molecular biology and biotechnology have led to the development and improvement of many rabies vaccines. Before the third-generation of the vaccine, rabies vaccines were based on the virus itself. Thus, even if effective, these vaccines may not be completely safe, resulting in a strong demand for the development of effective subunit vaccines that do not raise concerns about virus replication and infection in the host. This study investigated the ability of the glycoprotein of the rabies virus to be expressed in tobacco plants (*Nicotiana benthamiana*) and to induce an immune response in mice. Using a transient transfection, a soluble glycoprotein was successfully expressed in *N. benthamiana*. Fusing of five histidine residues at the C-terminus enabled the glycoprotein to be easily purified by affinity chromatography. The glycoprotein expressed in the plants was found to be N-glycosylated post-translationally, and the mice immunised with this glycoprotein generated neutralising antibodies against the rabies virus. These results suggest that a glycoprotein produced in the endoplasmic reticulum of *N. benthamiana* is bioactive, and might be used to generate a subunit vaccine against the rabies virus.

Keywords: glycoprotein; molecular pharming; plant-made pharmaceuticals; subunit vaccine; rabies

Abbreviations: BHK – baby hamster kidney cell; BiP – luminal binding protein; CTL – cytotoxic lymphocyte; DMEM – Dulbecco's modified Eagle medium; ER – endoplasmic reticulum; FBS – foetal bovine serum; FCA – Freund's complete adjuvant; FIA – Freund's incomplete adjuvant; HRP – horseradish-peroxidase; PVDF – polyvinylidene difluoride; SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TMB – 3,3',5,5'-tetramethylbenzidine; YEP – yeast extract peptone

Rabies is a disease caused by the bite of animals that have the rabies virus. Because house pets as well as animals living in the wild are carriers of the rabies virus, thousands of people are exposed to this virus per year, with more than 70 000 dying annually (Meslin et al. 1994; Hampson et al. 2015). Although the prevalence of rabies in pets has been sharply reduced by regular vaccination, rabies continues to be propagated by wild animals, including raccoons and badgers (Rupprecht & Smith 1994; Yousaf et al. 2012).

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The rabies virus is the most well-known pathogen of the *Rhabdoviridae* and is a *Lyssavirus*. The genomes of these viruses consist of non-segmented negative single stranded ribonucleic acids (RNAs), 11–15 kb in length and containing five genes, in the order as 3'- N (nucleoprotein) – P (phosphoprotein) – M (matrix protein) – G (glycoprotein) – L (polymerase) –5' The rabies virus possesses a bullet-shaped outer membrane, with its outer layer consisting of a lipid bilayer membrane containing a glycoprotein and a matrix protein (Coslett et al. 1980; Conzelmann et al. 1990).

Conventional vaccines were generated from viruses that had propagated in the nervous system of infected animals (first-generation) (Pasteur 1885), from viruses cultured in duck embryos (second-generation) (Culbertson et al. 1956), and from viruses cultured in cells (third-generation) (Starodubova et al. 2015; Rupprecht et al. 2016). Because they are generated from live viruses, these vaccines have safety concerns. Advances in molecular biology and biotechnology have led to efforts to overcome the safety problems of vaccines generated from live viruses, including vaccines generated from viral subunits, especially the glycoprotein and nuclear protein. Vaccines generated using a glycoprotein have been shown to prevent rabies infection by producing a neutralising antibody specific to the rabies virus, whereas vaccines generated using nuclear protein were shown to be responsible for the production of neutralising antibodies against the rabies virus, as well as other immune responses (Wiktor et al. 1973; Cox et al. 1977; Kieny et al. 1984; Tollis et al. 1991). Efforts are also actively underway to use a recombinant glycoprotein alone, or both a glycoprotein and a nuclear protein as a vaccine.

Because mammalian proteins produced in bacteria may not be folded or glycosylated as they are in mammalian cells, most vaccines to prevent the rabies virus have been produced in animal cells (Yelverton et al. 1983; Lathe et al. 1984). However, the production of vaccines in cultured animal cells is both difficult and expensive, especially due to the difficulties and costs required to expand facilities for the mass production (Dhankhar et al. 2008; Freuling et al. 2008). In addition, a rabies vaccine derived from an inactive virus and manufactured in animal cells, is both difficult to store and likely to be contaminated with a live virus from infected animals. The production of affordable vaccines in plants may be more feasible, because these vaccines are unlikely to be contaminated with a live virus from infected animals. Plant systems enable the inexpensive and simplified downstream processing and purification of expressed proteins (Streatfield 2005). In addition, these vaccines can be mass produced and stored for long periods of time (Lakshmi et al. 2013; Merlin et al. 2017; Kopertekh & Schiemann 2019).

The present study describes the high-level production of a rabies virus glycoprotein in *Nicotiana benthamiana* plants using a transient transfection. The glycoprotein produced in the plants had a high solubility, resulting in the easier purification by affinity chromatography, and it was N-glycosylated, and induced an immune response in the mice. Taken together, these results showed that the glycoprotein produced in the plants was highly immunogenic with high neutralising antibody titres against the rabies virus, suggesting its possible use as a subunit vaccine.

MATERIAL AND METHODS

Plant growth conditions. *N. benthamiana* plants were grown under a 16 : 8 h, light:dark cycle in a cultivation room maintained at 25 ± 2 °C and 50 ± 5% relative humidity.

Plasmid construction. The glycoprotein amino acid sequence was obtained from the *Rabies lyssavirus* glycoprotein (accession number AAZ29517), and the codon was optimised to express in *N. benthamiana*. The glycoprotein coding sequence (nt 1–1 575) was synthesised by GenScript Biotechnology Corp. (Nanjing, China). For cloning, the upstream sequence (5'-cgggatccaaattccccatctacacgat-3') containing a *Bam*HI restriction enzyme site and the 21 nt glycoprotein, and the downstream sequence (5'-ccgagctcatcatggtggtgatggtggtgcccccagttagggagaccca-3') containing five histidine residues, 12 nt the endoplasmic reticulum (ER) retention signal HDEL (His-Asp-Glu-Leu), and the 21 nt glycoprotein coding sequence (nt 1 551–1 572) were designed. The synthesised DNA was digested with *Bam*HI and *Sac*I, and inserted into a pCAMBIA1300 vector containing a cauliflower mosaic virus (CaMV) 35S promoter and a BiP (luminal binding protein) leader sequence (nt 1–251) from *Arabidopsis thaliana* BiP1 (BAA13947).

Transient expression of glycoprotein. The plasmid construct was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The *Agrobacterium* carrying the vector with the glycoprotein:5His and gene silencing repressor P38,

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respectively, were cultured overnight in a YEP liquid medium (LPS solution, Daejeon, Korea) to 1.0 at OD_{600}. The cells were collected by centrifugation at 3 000 × g for 10 min, and resuspended 1 : 1 (v/v) in an infiltration buffer (10 mM MES, 10 mM $MgSO_4$, 100 µM acetosyringone, pH 5.6). The cells were incubated at RT (room temperature) for 1 h in the dark. The *Agrobacterium* mixture was allowed to infiltrate into the aerial part of a six week-old *N. benthamiana* plant using a vacuum. After 4 days, 0.2 g of fresh leaf was harvested, and the plant protein was extracted with an extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100). The extract was further separated into soluble and insoluble fractions by centrifugation at 13 000 × g for 15 min.

Western blot analysis. The protein samples were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Cat. No. BC006; Biosolution, Suwon, South Korea). For the western blot analysis, the proteins were transferred to PVDF membranes (Merck Milipore Ltd., Tullagreen Carrigtwohill; Cat. No. IPVH00010). After blocking the membranes with 5% non-fat dried milk in a TBST buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) for 1 h, the membranes were incubated overnight at 4 °C with a mouse anti-His antibody (Novusbio, Centennial, USA) or an anti-rabies monoclonal antibody (In-house, clone No. 4G36) at dilutions of 1 : 1 000 in TBST containing 1% non-fat dry milk. The membranes were washed with the TBST buffer, and incubated with 1 : 5 000 diluted anti-mouse IgG HRP-conjugated (H+L) (Bethyl, Montgomery, USA) at room temperature for 1–2 h. Finally, the membranes were washed with the TBST buffer, and the positive bands were visualised by chemiluminescence.

Glycoprotein purification. Forty grams of *N. benthamiana* leaves were ground with 200 mL of a protein extract solution (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.1% Triton X-100), and followed by centrifugation. The supernatant was loaded onto a 5 mL NI^+-NTA agarose resin column (Clontech, Cat. No. 635662). The column was washed with 100 mL of a washing solution (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole), followed by the elution of the glycoprotein with an elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 300 mM imidazole). The eluted solution was replaced with phosphate-buffered saline (PBS) using a 30 kDa filter, followed by enrichment to obtain the recombinant rabies virus glycoprotein, which was analysed by SDS-PAGE and Coomassie Blue staining.

De-glycosylation of glycoprotein. One µg of the purified glycoprotein was mixed with a 10X denaturation buffer (5% SDS, 0.4 M DTT), and heated at 100 °C for 10 min. The solution was mixed with 50 U of Endo-glucosidase H or the same volume of distilled water, and incubated at 37 °C for 1 h. The protein was analysed by western blotting to determine whether the molecular weight had changed due to the removal of the N-glycans from the glycoprotein.

Mice immunisation with glycoprotein. The animal study was approved by the Institutional Animal Research Ethics Committee at the Pohang Technopark Foundation.

Nine six week-old male C57BL/6J mice were prepared. Five mice were injected subcutaneously with 10 µg of the glycoprotein in FCA at 1 week and in FIA at 3 weeks, or, as the control, four mice were injected with the same volume of PBS. At two weeks after the booster injection, the sera were collected.

In-house ELISA. The production of antibodies against glycoprotein was assessed by an in-house ELISA (enzyme-linked immunosorbent assay), in which 100 µL of a purified glycoprotein (150 ng/mL) in a carbonate-bicarbonate buffer, with a pH of 9.6, was added to each well of a 96-well plate, and the plates were incubated at 4 °C overnight. To avoid non-specific reactions, 100 µL of a blocking solution (PBS, 0.05% Tween-20, 3% skim milk) was added to each well, and the plates were incubated at 37 °C for 1 h. After being washed three times with PBST, 100 µL of the 1 : 1 000 diluted serum was added to each well, and the plates were incubated at 37 °C for 1 h. The wells were washed with PBST, and 100 µL of the HRP conjugated anti-mouse antibody, diluted to 1 : 5 000, was added to each well, followed by incubation at 37 °C for 1 h. The wells were washed again, and 100 µL of a TMB substrate was added to each well. After 10 min at room temperature, 50 µL of a stop solution (2 M H_2SO_4) was added to each well, and the absorption of each well was determined at 450 nm using a plate reader (Thermo Fisher Scientific, MULTISKAN FC, Cat. No. N07710).

Neutralising antibody titration. The sera from five mice immunised using the purified glycoprotein with FCA/FIA and from four mice immunised with PBS were used to titrate the neutralising antibodies. For each serum to be tested, 100 µL of DMEM was added to four wells per sample in a 96-well plate. Fifty microlitres of the serum was added to the first well, followed by three-fold serial dilutions in the other wells. Fifty microlitres of a standard rabies virus (CVS11),

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diluted to a concentration of 100 $TCID_{50}$ (tissue culture infective dose)/μL, was added to each well, followed by incubation at 37 °C for 1 h, followed by the addition of 50 μL of BHK/T7 cells (4 × 10^5 cells/mL) in the DMEM containing 10% FBS. To assess the infected cells by fluorescence microscopy, after the culture fluid was removed, the cells were fixed with 80% acetone, and the fluorescence signals were observed using a RABV-specific monoclonal antibody (kept in lab) and a 1 : 200 diluted FITC-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD, USA, cat. No. 172-1806). To verify the neutralisation, an OIE standard serum (Anses, Nancy, France), diluted to 0.5 international units (IU)/mL, was used as the positive control, and PBS was used as the negative control. The fluorescence signal intensity was determined using a fluorescence microscope (Nikon, ECLIPSE, TE2000-U) and quantified using ImageJ software. The antibody titres of each specimen were calculated relative to the positive control, with titres more than 0.5 IU/mL defined as positive, and titres less than 0.5 IU/mL as defined negative.

RESULTS

Rabies virus glycoprotein expression in *N. benthamiana*. To express the rabies virus glycoprotein in *N benthamiana*, its coding sequence, including its ecto- and partial transmembrane domains, was codon optimised and inserted into a plant expression vector. The preliminary results showed that the targeting of the antigen protein to the ER resulted in a high level of expression (Munro & Pelham 1987; Wandelt et al. 1992; Mason et al. 1998; Napier et al. 1998; Part et al. 2019; Stoger et al. 2000). Thus, to accumulate the target protein in the ER, the vector included a BiP leader sequence at the upstream, and an ER retention signal at the downstream of the glycoprotein coding region. To purify the glycoprotein by affinity chromatography, a tag composed of five histidine residues was fused to the C-terminus of the glycoprotein. The construct was driven by a CaMV 35S enhancer promoter (Figure 1A). To investigate whether the DNA construct was expressed in a plant, the construct was transferred to *Agrobacterium tu-*

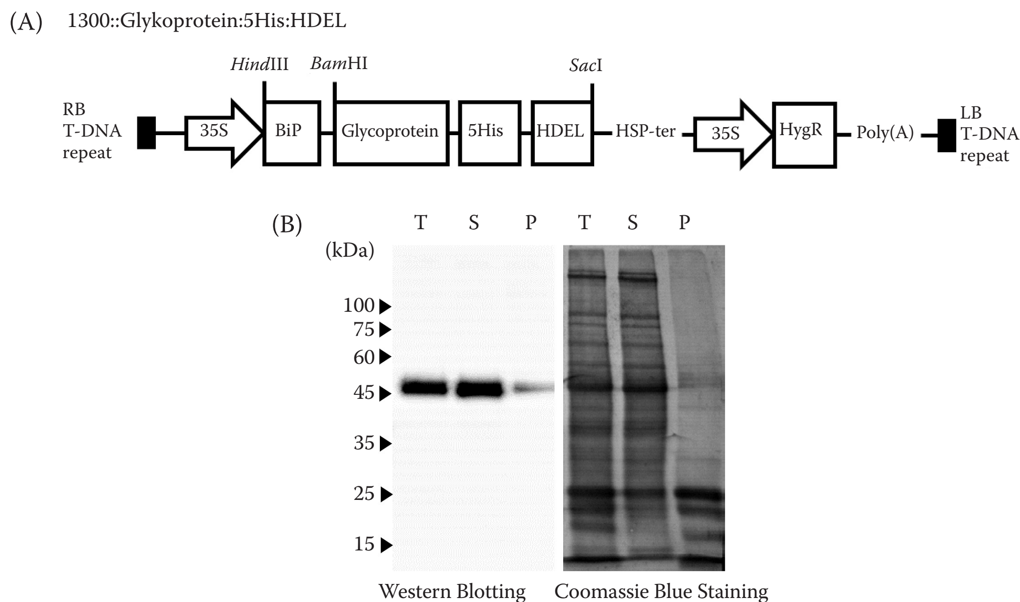

Figure 1. 1300::Glycoprotein:5His:HDEL plasmid construct, and its expression in *N. benthamiana*: (A) schematic drawing of the 1300::Glycoprotein:5His:HDEL construct. 35S, cauliflower mosaic virus 35S promoter (780 bp); BiP, ER-leader sequence (273 bp); glycoprotein, the rabies virus glycoprotein coding sequence including the ecto- and partial transmembrane domains (TM) (1 575 bp); 5His, 5 repeat histidine residues (15 bp); HDEL, ER retention signal (12 bp); HSP-ter, the heat shot protein (HSP) 18.2 terminator (250 bp); HygR, the hygromycin resistance gene; poly-A, poly A tail; (B) western blot analysis of the expression and solubility of the recombinant glycoprotein; the total protein was prepared from the *N. benthamiana* leaves, and separated into soluble and insoluble fractions by centrifugation at 20 000 × g for 15 min; after denaturation, each sample was subjected to western blotting using an anti-His antibody; the membranes were stained with Coomassie Blue to assess the protein profiles of each fraction; the molecular weights (kDa) are shown on the left; T – total extract; S – soluble fraction; P – insoluble pellet fraction

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mefaciens, followed by the transient expression in the *N. benthamiana* leaves. Four days later, the leaves were harvested, and the expression and solubility of the glycoprotein were assessed by western blotting (Figure 1B). Previously, we examined the expression levels of the target genes along with the days post-infiltration (data is not shown). Necrosis symptoms were sometimes observed as the incubation time increased and the protein levels did not notably change depending on the incubation time under our conditions. Therefore, we harvested the leaves at 4 days after infiltration. Most of the glycoprotein was detected in the soluble fraction, with only a small amount in a pellet fraction. These results indicated that the DNA construct could effectively generate a rabies virus glycoprotein in the plant cells, and this glycoprotein was highly soluble in an aqueous solution, enabling its isolation and purification.

Glycoprotein purification and de-glycosylation. The glycoprotein was subsequently isolated by Ni^{+}-NTA affinity chromatography, with an anti-His antibody used to monitor the glycoprotein throughout the purification process, including in the total plant extract (T) and in the column flow-through (FT), wash-off (W), and eluted fractions (E) (Figure 2A). Only a small amount of the glycoprotein was detected in the FT fraction, and none in the W fraction, indicating that the binding of the His-tagged glycoprotein to the nickel resin was tight. Following the elution from the column, the purified proteins were further concentrated by centrifugal filtration. The final purified proteins were seen as a doublet between 45 and 60 kDa (Figure 2B). The concentration of the purified glycoprotein measured by a Bradford assay was approximately 308 μg/mL. The assessed yield was 30 μg glycoprotein/1 g of the *N. benthamiana* leaves fresh weight (FW). The rabies virus glycoprotein has two candidate N-glycosylation sites, N_{37} and N_{319} (data not shown). The upper band was regarded as the N-glycosylated glycoprotein, and the lower as the non-glycosylated protein. The glycoprotein was likely N-glycosylated in the ER of the *N. benthamiana* leaves. The glycosylation of the rabies virus glycoprotein is essential for the generation of protective antibodies (Foley et al. 2000). To assess whether the glycoprotein was N-glycosylated, the purified protein was treated with Endo-glucosidase H (Endo H), and the product was analysed by western blotting with an anti-His antibody (Figure 2C). Endo H induced a notable band shift, indicating that the glycoprotein produced in the plants was N-glycosylated.

Glycoprotein produced in plant is immunogenic. The western blotting of the plant-produced glycoprotein with an anti-rabies monoclonal antibody showed one major band at the corresponding molecular weight, indicating that the antigenic protein produced in the plants was recognised by the rabies-

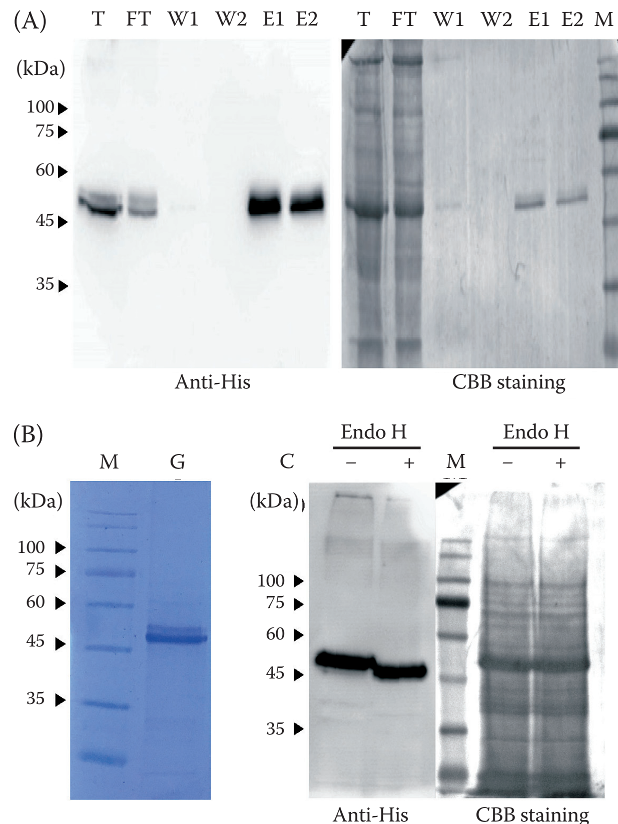

Figure 2. Purification and de-glycosylation of the glycoprotein:His recombinant protein: (A) The glycoprotein:His recombinant protein was isolated from the transiently transfected *N. benthamiana* leaves; after grinding the leaves, the total protein extract was prepared using an extraction buffer followed by binding to a Ni^{+}-NTA agarose resin; the recombinant protein was eluted from the resin; T – total fraction; FT – flow-through; W – wash-off; E – eluted fractions were analysed by western blotting with anti-His antibody; M – protein markers; (B) purified recombinant protein was concentrated by centrifugal filtration, and visualised by western blot analysis; M – protein markers; G – glycoprotein; (C) de-glycosylation of glycoprotein by treatment with endo-glucosidase H (Endo H); the purified recombinant protein was incubated in the presence or absence of 50 U Endo H, and an immunoblot analysis was performed using an anti-His antibody; the size shift of the recombinant protein was due to Endo H-mediated removal of the N-glycans

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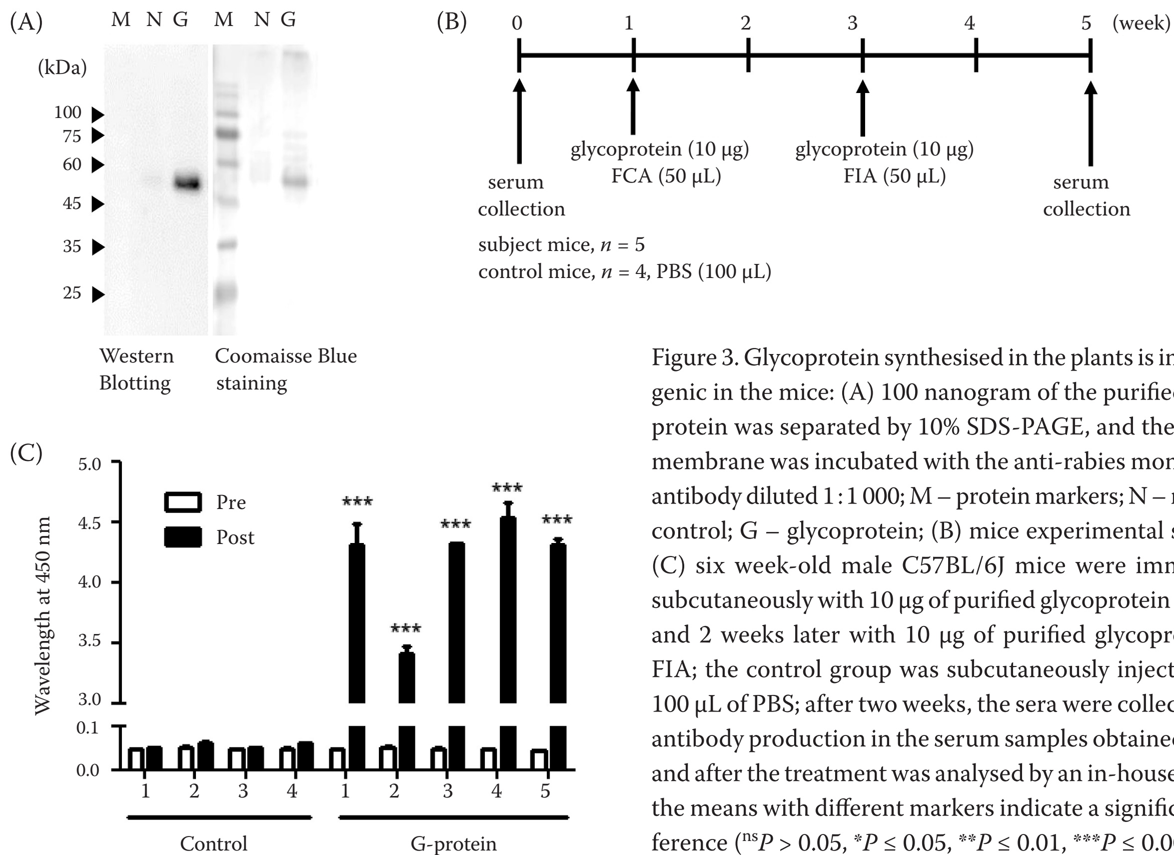

Figure 3. Glycoprotein synthesised in the plants is immunogenic in the mice: (A) 100 nanogram of the purified glycoprotein was separated by 10% SDS-PAGE, and the blotted membrane was incubated with the anti-rabies monoclonal antibody diluted 1 : 1 000; M – protein markers; N – negative control; G – glycoprotein; (B) mice experimental scheme; (C) six week-old male C57BL/6J mice were immunised subcutaneously with 10 μg of purified glycoprotein in FCA, and 2 weeks later with 10 μg of purified glycoprotein in FIA; the control group was subcutaneously injected with 100 μL of PBS; after two weeks, the sera were collected; the antibody production in the serum samples obtained before and after the treatment was analysed by an in-house ELISA; the means with different markers indicate a significant difference ($^{ns}P > 0.05$, $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$)

specific monoclonal antibody (Figure 3A). To evaluate whether this protein was immunogenic in mice, we designed an animal experiment (Figure 3B). Six week-old male C57BL/6J mice were subcutaneously injected twice with 10 μg of the purified glycoprotein in FCA at 1 week, and in FIA at 3 weeks, and the mice in the negative control group were injected with the same volume of a PBS. After two weeks, the sera were collected, and the production of specific antibodies was assessed using an in-house ELISA and a plate coated with the purified glycoprotein. The serum obtained from the mice immunised with the purified glycoprotein was highly reactive, whereas the serum obtained prior to the immunisation and the sera obtained from the control group before and after injection of the PBS showed a negative response against the specific antibodies (Figure 3C), indicating that the glycoprotein produced in the plants was immunogenic in the mice.

The ability of the sera from the mice immunised with the glycoprotein to neutralise the rabies virus was assessed by incubating these sera with BHK/T7 cells and a standard rabies virus (CVS11) for 48 h. The positive control consisted of 0.5 IU/mL of a standard positive serum from the World Organiza-

Table 1. Glycoprotein synthesised in *Nicotiana benthamiana* induces a neutralising antibody against the rabies virus

Antigen	Mouse serial No.	Neutralising test titre (IU/mL)
Glycoprotein	1	4.56
	2	1.51
	3	13.77
	4	0.87
	5	23.93
PBS con.	1	0.06
	2	0.08
	3	0.06
	4	0.07

The titres were measured as described in the materials and methods; the sera of the mice vaccinated with the purified glycoprotein or only with the phosphate-buffered saline (PBS) were incubated with a standard rabies virus (CVS11) and BHK/T7 cells at 37 °C for 48 h; the neutralising antibody response was assessed by measuring the fluorescence of the anti-rabies virus specific antibody; the OIE standard serum was diluted to 0.5 IU/mL as the positive control; the positive value was defined when the titre was more than 0.5 IU/mL; IU – international unit

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tion for Animal Health (OIE). All the sera from the mice immunised with the plant-made glycoprotein showed high neutralising antibody titres, although individual variations were observed (Table 1). Generally, the positive value was defined when the titre was more than 0.5 IU/mL (WHO 2017). These results suggested that vaccines made from glycoproteins produced in plants could provide protection against the rabies virus.

DISCUSSION

The current rabies vaccines in international markets are produced by culture in cells or eggs (Starodubova et al. 2015). These vaccines require multistep processes from culture to purification, so the processes increase the production costs. Although these vaccines are stable and immunogenic, they are difficult to store and transport, and, in some cases, have poor efficacy. Moreover, these vaccines may have adverse effects, such as allergic and encephalitic reactions. Because a glycoprotein, one of the surface proteins of the rabies viruses, could provide protection against the rabies virus by producing neutralising antibodies, efforts are being made to generate vaccines using a recombinant glycoprotein.

Recent advances in biotechnology have led to the development of several systems capable of the mass production of heterogeneous proteins. One of these systems involves the use of the protein expression systems in plants, which have been shown to generate antigenic proteins quickly, readily allowing a scale-up for mass production. Moreover, the production in plants avoids contamination with adventitious animal pathogens. Glycoproteins have been expressed in several plants, including tomatoes, tobacco, spinach, carrots, and maize (McGarvey et al. 1995; Yusibov et al. 2002; Loza-Rubio et al. 2008, 2012; Rojas-Anaya et al. 2009). However, the studies have focused on edible vaccines which are suitable for animal vaccination. Edible plants for oral delivery are a promising system because plant tissues encapsulate the antigen proteins and provide protection from digestion in the stomach. In addition, vaccination is available without needles/syringes and trained medical personnel. An edible vaccine can minimise the downstream processes, such as purification and sterilisation leading to a cost-effective vaccination. Nevertheless, several limitations, like the effect of genetically modified crops related to antibiotic resistance and allergic immune reactions (Van Ree et al. 2000) caused by plant-derived glycans have to be addressed. Above all, oral tolerance is a major hurdle to overcome in developing oral vaccines. Increasing the dose of the antigen raises the risk of inducing oral tolerance instead of the activation of immune responses (Vela Ramirez et al. 2017). Oral immunisation with only a few nano grams of plant lyophilisate following injection priming successfully boosted the production of antibodies against Hepatitis B (Pniewski et al. 2018). The oral tolerance response was distinct depending on the administration routes such as gavage and continuous feeding (Oliveira et al. 2015). Hence, it is important to establish an appropriate immunisation regime including a defined antigen dosage, vaccination schedules, injection routes and the proper formulation of vaccines.

Our study investigated the humoral response in mice to a glycoprotein expressed in *N. benthamiana* leaves. A purified rabies virus glycoprotein was injected subcutaneously into the mice, and the production of antibodies against the glycoprotein was evaluated by ELISA, and the ability to neutralise the rabies virus. The glycoprotein was specifically recognised by the antibody against the rabies virus in a western blot analysis, suggesting that the structure of the recombinant glycoprotein was similar to that of the native glycoprotein, with protein folding and assembly being similar in plant and mammalian cells. A highly homogeneous recombinant glycoprotein could be obtained by affinity chromatography, with the antibody produced against this glycoprotein in the mice having positive neutralising titres. The Endo H treatment of this purified glycoprotein confirmed that it had already been N-glycosylated in the plant. Although the glycosylation of the glycoprotein in plant may be a drawback, it did not affect the neutralising antibody response. The glycoprotein accumulation in the ER of plant cells suggests that, similar to mammalian cells, this protein may have a fewer number of mannose type glycans, with few or no plant-specific glycans like xylose. The glycosylation software predicted the glycoprotein has two candidate glycosylation sites at N_{37} and N_{319} (data not shown). Although the glycosylation of viral surface antigens often affects the protective immunity (Yelverton et al. 1983; Lathe et al. 1984), the results showed that the recombinant glycoprotein with a high number of mannose type glycans expressed in the ER of the plants was immunologically active, suggesting that this type of glycan, as well as post-translationally modified glycans, can induce immune responses.

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Ashraf et al. (2005) also produced a rabies virus glycoprotein in tobacco leaves. They accumulated the glycoproteins in the ER by fusing a PR-S signal peptide and a SEKDEL ER retention signal. They used the full length of the glycoprotein, whereas we used the ecto- and partial transmembrane domain (TM). They injected 25 μg of purified glycoprotein into mice four times (one priming and three boosters), and all the mice survived against the rabies virus challenge. On the other hand, we injected 10 μg of a purified glycoprotein into the mice two times (one priming and one booster). Although we could not perform a challenge assay, our purified glycoprotein was highly immunogenic and immune-protective in the vaccinated mice. It is possible that the difference in the two studies can be caused by the structure of the glycoprotein, because the strategy and expression system are comparable to each other with the exception of a slightly different glycoprotein coding sequence.

One of the important issues in a plant expression system is to increase the expression level of the target genes. To achieve this, many viral vectors have been developed (Shah et al. 2013). However, some of them required the co-infiltration of gene-silencing repressors. In addition, they have different expression levels in the target genes depending on the *Agrobacterium* strains such as GV3101 (Koncz & Schell 1986) and LBA4404 (Hoekema et al. 1983). Therefore, the vector system and agrobacterium strains have to be carefully considered to achieve high expressions.

The present study confirmed that a plant-produced glycoprotein is biologically active and can protect against the rabies virus. However, many issues remain to be solved in the development of a vaccine. For example, in addition to inducing neutralising antibodies, the glycoprotein induced CTL (Macfarlan et al. 1986) and T helper cell (Celis et al. 1988) responses by a $CD4^+$ T cell line, indicating a need for further investigation of cell-mediated immune responses induced by plant-derived glycoproteins. Furthermore, the glycoprotein content and vaccine efficacy should be assessed by an *in vitro* sandwich ELISA rather than by animals *in vivo* (Maas et al. 2000; Nagarajan et al. 2006; Morgeaux et al. 2017). To be recognised by the antibody, the folded form of the recombinant glycoprotein must be similar to the native form. Finally, the amounts of antigen and proper adjuvants in the vaccine formulations must be determined for vaccination of humans, pets, and wild animals.

CONCLUSION

This study showed that high levels of a soluble rabies virus glycoprotein could be produced in a plant, with a highly homogeneous protein isolated by affinity chromatography. This protein was N-glycosylated, similar to the native protein. Importantly, this study showed that (*i*) the N-glycosylated glycoprotein expressed in the plants was recognised by a specific anti-rabies-specific antibody; and (*ii*) the antibody produced in the mice injected with the plant-expressed glycoprotein could neutralise the virus. These results suggest that glycoprotein produced in the ER of plant cells is bioactive and can be used to generate a subunit vaccine against the rabies virus.

Author contributions. E.-J. Sohn and S. Lee designed and organised the overall study. Y. Park wrote the manuscript. H. Kang performed the cloning and generated constructs. K. Min carried out the protein purification and western blot analysis. N.H. Kim performed the ELISA assay and mouse vaccination. M. Park prepared the plant materials and transiently expressed the construct. I.-O. Ouh, H.-H. Kim and J.-Y. Song analysed the neutralising antibody titres against the rabies virus. D.-K. Yang provided the anti-rabies monoclonal antibody.

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