Available online at www.medicinescience.org

ORIGINAL ARTICLE

Med Science 2022;11(2):478-83

Medicine Science
International
Medical Journal

Engineering, production, and immunogenicity studies of a truncated form of rabies virus glycoprotein produced in Nicotiana benthamiana plant

Gulshan Mammadova, Irem Gurbuzaslan, Damla Yuksel, Nilufer Gun, Nedim Mutlu, Gulnara Hasanova, Tarlan Mamedov

Akdeniz University, Agricultural Faculty, Department of Agricultural Biotechnology, Antalya, Turkey

Received 21 September 2021; Accepted 07 November 2021
Available online 06.03.2022 with doi: 10.5455/medscience.2021.09.278

Abstract

Rabies is a viral disease caused by the rabies virus (Lyssavirus) that affects the central nervous system, ultimately leading to death and brain disease. Rabies usually transmitted through a bite among domestic dogs and wild carnivorous animals. The rabies virus (RABV) infects the central nervous system causing disease in the brain and death. Rabies is 100% fatal if untreated and ultimately causing more than 70,000 deaths annually worldwide, especially among children in developing countries. Rabies can be prevented by vaccination and can be cured immediately after infection. Although several human and animal vaccines against rabies are available, they are expensive, have relatively low immunogenicity and also difficult to produce. Therefore, less expensive, more immunogenic and safe rabies vaccines that can be produced in the large quantities are urgently needed. The transient plant expression system has become a more attractive and promising platform for the expression of a wide range of recombinant proteins such as vaccines, antibodies, therapeutic proteins, including mammalian complex proteins. In this study, we engineered and produced a truncated form of glycoprotein (G protein) of rabies virus in Nicotiana benthamiana (N. benthamiana) plant for the first time, using transient expression system. Plant produced RG2 protein was purified through Ni-NTA column chromatography. The purification yield of RG2 protein was ~32 mg/ kg plant leaf. The results of immunogenicity studies showed that the plant produced G-protein elicited significantly high antibody titers in mice. Plant-produced G-protein could be a cost-effective, safe and highly immunogenic rabies vaccine candidate.

Keywords: Rabies virus, G protein, subunits vaccine, plant transient expression system

Introduction

It has been well established that rabies virus (RABV), as well as other lyssaviruses, are transmitted through biting of infected animals. Rabies is a viral zoonosis, which observed nearly in 100 countries and affect many mammals, including humans [1]. Dogs, rabies are the main source of human infections (99%) and affects over 3.3 million people [2]. Rabies is 100% fatal if untreated, killing about 70,000 people each year, mainly among children in rural areas of Asian and African countries. Infection with the rabies virus leads to severe neuronal dysfunction [3,4]. Rabies can be prevented by vaccination and be cured immediately after infection. However, after clinical symptoms appear, most patients die from infection [5]. Basically, rabies prevention can be performed by two main strategies. The first strategy is the human vaccination, in which vaccine administrated before or after an exposure. The second strategy is the vaccinating dogs, which can prevent transmission of the virus to humans, since dogs are responsible for 99% of human cases (https://www.who.int/activities/human-rabies-prevention-and-management).

The rabies human diploid cell vaccine available for human use is an inactivated type vaccine that has been in use since 1967 and is an attenuated type vaccine that is derived from the Pitman-Moore virus strain L503 [6]. In addition, purified Vero cell and chicken embryo cell rabies vaccines are also now available for human use. Notably, the purified Vero cell rabies vaccine, developed by Wistar's researchers in the 1960s and 1970s uses the Wistar strain (attenuated) of the RABV. These vaccines are used for pre- and post-exposure prophylaxis and have been used more than millions of people around the world. Notable, inactivated,

***Corresponding Author:** Tarlan Mamedov, Akdeniz University, Department of Agricultural Biotechnology, Antalya, Turkey
E-mail: tmammedov@gmail.com

and attenuated vaccines have safety concerns because they are prepared from live viruses. There are other relatively safe vaccines produced with different production technologies, especially animal cells produced vaccines are available. However, vaccine production in cultured animal cells is expensive and difficult to scale-up [7,8]. A single-stranded RNA genome of rabies virus encodes five viral, structural proteins including glycoprotein (G), phosphoprotein (P), nucleoprotein (N), matrix protein (M) and RNA-dependent RNA polymerase enzyme (L) [9]. It has been demonstrated that glycoprotein G, the main antigen of RABV, can induce protective immunity [10,11]. Glycoprotein G of RABV assembles the form of homotrimers on the surface of RABV has been shown a major target for virus-neutralizing antibodies, elicits immunity response [12]. For this reason, most of the studied recombinant vaccine candidates are based on the RABV G protein. Recombinant G protein of RABV has been expressed using different protein expression systems [13-17]. It was reported that G protein was sufficient to induce high RABV-specific virus-neutralizing antibody titers in dogs, cats, and mice [18,19] even after a single vaccine dose. Therefore, G protein is a leading candidate for a new generation, protein and, subunit vaccine.

Rabies vaccines for humans and animals are currently available, enabling the effective control of the virus, however, they are expensive and some of them, especially inactivated vaccines have relatively poor immunogenicity [20,21]. In addition, high doses of antigen and multiple immunizations are needed to obtain sufficient protection. Therefore, cost effective, safer and more immunogenic rabies vaccines are urgently needed. Currently, several expression systems such as bacterial, yeast, mammalian have been developed and are used for expressing various types of recombinant protein. Transient gene expression became an alternative expression system particularly for production of difficult to express proteins [22-25]. This expression system offers several advantages over other expression platforms, for example, plants have eukaryotic post-translational modification (PTM) mechanisms and have high production capacity. In less than a week, hundreds of milligrams or grams of recombinant protein accumulate per kilogram of leaf biomass. Other advantages are lower cost, easy scalability, and safety due to the absence of endogenous human pathogens. The technology has been demonstrated to be very useful for the expression of complex proteins. Some pharmaceuticals produced in plants, such as vaccine antigens, therapeutic human proteins, and enzymes, have already reached clinical development stages. Using this technology, we have successfully produced several vaccine candidates, such as malaria [24,26], anthrax [26] and SARS-CoV-2 antigens [25] and therapeutic proteins such as human Factor IX and Furin and also bacterial and human enzymes [23,26,27] in *N. benthamiana* plant. Recently, using plant transient expression system, glycosylated (gRBD) and non-deglycosylated (dRBD) variants of Receptor Binding Domain (RBD) of SARS-CoV-2 have been successfully produced in *N. benthamiana* plant as a vaccine candidate against COVID-19, which induced high titer of RBD-specific antibodies with potent neutralizing activity against live virus, SARS-CoV-2 infection. Thus, plant transient expression platform would be also ideal for production low cost, safe, stable, and highly immunogenic vaccine against rabies. It should be noted that ERA rabies virus G protein was previously produced in transgenic plants [28,29] however, the expression level of G protein was very low. Recombinant G protein was also produced in transgenic maize [29]. After oral immunization, this plant produced recombinant rabies virus G protein could elicit a protective immune response in sheep [29]. In this study, a truncated form of the G protein (RG2) as a vaccine candidate against rabies virus was engineered and expressed in *N. benthamiana* plant using transient expression technology. We demonstrate that the RG2 antigen can be rapidly produced in *N. benthamiana* plant at a high level. Furthermore, immunogenicity studies showed that plant produced RG2 protein induced significantly high titers of antibody in mice. Thus, plant produced RG2 antigen has potential to be developed as cost-effective and safe subunit vaccine against Rabies virus.

Materials and Methods

Engineering, optimization and cloning of different variants of G protein

The G protein gene of RABV (ERA strain, GenBank: J02293.1), has been engineered, codon optimized using *N. benthamiana* codons, and two genes were de-novo synthesized at IDT (Iowa, USA) with the PR-1a signal peptide, His6 tag and ER retention peptide, KDEL at C-terminal (Figure 1). In this study, we generated two variants of G protein for expression in *N. benthamiana* plant: full length G protein, named RG1 (GenBank accession number: AAA47204.1, 20-524 AA), and truncated version, RG2 (GenBank accession number: AAA47204.1, 20-455 AA). To express G protein variants in *N. benthamiana* plants, the signal sequence (SS, amino acids 1-19) was replaced with the Nicotiana tabacum PR-1a SS (MGFVLFSQLPSFLLVSTLLLFLVISHSCRA). The schematic representations of constructs have been presented in Figure 1.

All genes were designed with Age I and Xho I restriction sites for cloning purposes. For cloning of G protein gene variants, the genes with restriction enzyme sites, Age I and Xho I, were cut with Xho I and Age I restriction enzymes and then DNA fragments run on 1% agarose gel to recovery DNA fragments (G protein genes). The two G variants were cloned into plant expression pEAQ vector [30] to generate pEAQ-RG1-His6-KDEL or pEAQ-RG2-His6-KDEL. Plasmids were then transformed into Agrobacterium strain AGL1 for infiltration into plant leaves.

Expression screening of plant-based recombinant G protein variants in N. benthamiana

To produce recombinant G protein variants (RG1 and RG2) in *N. benthamiana* , pEAQ-RG1-His6-KDEL or pEAQ-RG2-His6-KDEL plasmids harboring the RG1 and RG2 protein genes were transferred into *Agrobacterium tumefaciens (A. tumefaciens)* strain AGL1 by electroporation using Gene Pulser Xcell Total System (BioRad, USA). *A. tumefaciens* strain harboring the RG1 and RG2 genes were grown in BBL medium (5 g/L NaCl, 5 g/L yeast extract, 10 g/L soy hydrolysate and 50 mg/L kanamycin) at 28oC for overnight. Grown culture of Agrobacterium harboring RG1 and RG2 genes then introduced into *N. benthamiana* plant leaves (6-7-week-old) by manual infiltration by syringe or by vacuum infiltration using infiltration chamber to produce RG1 and RG2 proteins. Leaf samples were infiltrated with pEAQ-RG1-His6-KDEL or pEAQ-RG2-His6-KDEL plasmids, as described previously [23,24,26].

Leaf samples were harvested at 5 day of post infiltration (dpi) and then ground in a phosphate extraction buffer. Crude extract was

centrifugated at 13,000 g for 20 minute. 40 µl of clear crude extract were mixed 10 µl of 5XSDS-Sample buffer and incubated at 100°C for 5 min and then samples were run on SDS-PAGE and transferred to hydrophobic polyvinylidene fluoride (PVDF) membrane for Western blot analysis. Plant produced recombinant plant produced RG1 and RG2 proteins were visualized on the membrane; anti-His-tag antibody (cat # 652502, BioLegend) and goat anti-mouse IgG HRP (minimal x-reactivity) (cat # 405306, BioLegend, USA) were used as primary or secondary antibodies, respectively.

Purification of plant produced RG2 protein from N. benthamiana

A recombinant RG2 was purified from 25 g of *N. benthamiana* plant leaf, infiltrated with the pEAQ-RG2, by affinity Ni-column chromatography using Ni-NTA Resin (cat. no. 88221, ThermoFisher scientific) as described elsewhere [26]. The purification yield of RG2 purified protein was calculated as described previously [24].

Co-expression of RG2 with PNGase F and Endo H

To co-express RG2 with PNGase F or Endo H deglycosylating enzymes, *N. benthamiana* plant was co-infiltrated with AGL1 strain harboring pEAQ-RG2 and pGreenII-PNGase F or pGreenII-Endo H constructs [26].

Immunogenicity studies of plant produced RG2 protein in mice

Immunogenicity studies of plant produced RG2 protein was assessed in mice. Seven-week-old mice (BALB/c strain, 6 mice per group) were vaccinated intramuscularly (IM) with two doses (5 µg each dose) of plant produced RG2 protein adsorbed to 0.3% Alhydrogel at 0 and 21 days. On study days 21 and 42, serum samples were collected and then assessed by an IgG ELISA for anti-RG2 antibody responses. For ELISA, wells were coated with 200 ng of partially purified RG2 protein, or 200 ng plant produced His- tagged protein (as a negative control), recombinant PA83 of *Bacillus anthracis* in 100 mM carbonate buffer at 4oC for overnight. The next day, after blocking the wells, various serum dilutions were transferred to the wells and then plate was incubated at 37°C for 2 hours. After incubation, the plate was washed three times with 1X PBST and then anti-mouse IgG + HRP (Cat. no. MBS440122, MyBioSource, USA) was added to assess the antibody levels. After washing the plate with 1X PBST, 200 µl substrat solution, prepared using o-Phenylenediamine dihydrochloride (OPD) tablets (Cat. no. P8287, Sigma) was added to each well. The plate then was incubated in the dark, at room temperature for 30 minutes. After incubation, the plate was read at 450 nm. Mice studies were performed at Akdeniz University, Laboratory Animals Application and Research Centre. The animal protocol (protocol number: 1156/2020.07.002), we used for mice studies was approved by the Local Ethics Committee.

Results

Engineering, production, and purification of recombinant RG2 from *N. benthamiana* plants

In this study, the G protein gene was engineered and produced as full length (RG1, amino acid residues 19-524) or a truncated form (RG2, amino acid residues 19-449) in *N. benthamiana* . When full length RG1 protein was expressed in *N. benthamiana* plant no specific G protein band was observed in western blot (Figure 1, lane RG1). Therefore, we engineered a truncated form of G protein (RG2) to produce a highly soluble and immunogenic recombinant G protein in *N. benthamiana* . Western blot analysis (Figure 2) demonstrates that the RG2 protein is successfully expressed in the *N. benthamiana* plant. Plant produced RG2 protein was purified by Ni column affinity chromatography.

Figure 1. Schematic representation of the G proteins. A full length (RG1, aa 20-524 AA) and truncated (RG2, aa 20-455) G protein variants were expressed in *N. benthamiana* plant. PR-1a: *N. tabacum* PR-1a signal sequence; His6: the affinity purification His6 tag; KDEL: the ER retention signal

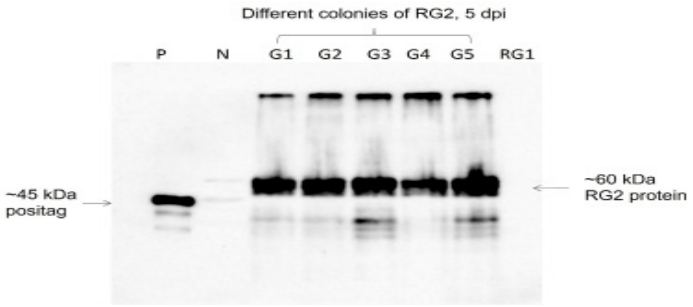

Figure 2. Western blot analysis of RG2 produced in *Nicotiana benthamiana* plants. Plants were harvested at 5 dpi (day after post infiltration). Samples were prepared as described in Materials and Methods. Lanes: N - crude extract prepared from a control, non-infiltrated plant; G1-G5 - crude extracts prepared from plants infiltrated with various colonies of Agrobacterium (AGL strain) carrying the RG2 gene. RG1- crude extract prepared from plants infiltrated with Agrobacterium (AGL strain) carrying the RG1 gene. P- 25 ng recombinant Posi-Tag Epitope Tag Protein (Positag, BioLegend, cat no: 931301) with ~45 kDa was loaded into a well as a standard protein

Figure 3 demonstrate SDS-PAGE and Western blot analysis of partially purified RG2 protein. Plant produced RG2 protein appears as a single protein with a MM of ~60 kDa (Figure 3). The purity of RG2 was about 50%. The purification yield of RG2 protein was ~32 mg/ kg plant leaf.

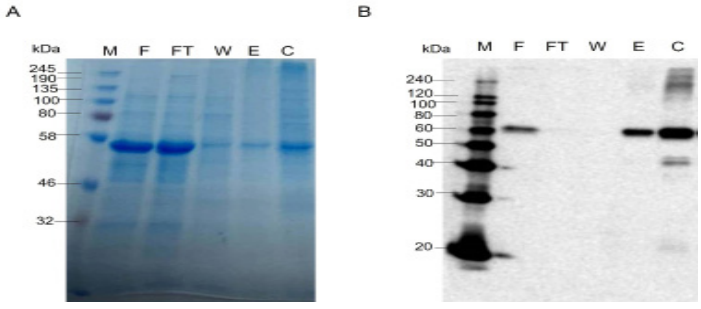

Figure 3. SDS-PAGE (A) and Western blot (B) analysis HisPur™ Ni-NTA Resin column purified; plant produced RG2 protein. A: SDS-PAGE analysis of purified, plant produced truncated RG2 protein. F: 10 µg from crude extract fraction (which was loaded into HisPur™ Ni-NTA Resin column) was loaded into gel. FT: 10 µg from flow through fraction was loaded into gel. W: 10 µg from wash fraction was loaded into gel. E: 10 µl from eluted, combined fractions was loaded into gel. C- 2 µg from concentrated eluted fractions were loaded into a gel. M: color prestained protein standard. B: SDS-PAGE analysis of purified, plant produced truncated RG2 protein. F: 200 ng from crude extract was loaded into gel. FT: flow through. W: wash. E-eluted, combined fraction. C: 200 ng from concentrated eluted fractions was loaded into gel. Plant produced RG2 protein on the membrane was detected using the purified anti-His Tag antibody (Cat. No. 652502, BioLegend). M: MagicMark XP Western Protein Standard (ThermoFisher Scientific).

In vivo co-expressing of RG2 protein with PNGase F and Endo H

RG2 protein possesses five potential N-glycosylated sites. To test if plant produced RG2 protein is glycosylated, RG2 gene was co-expressed with deglycosylating enzymes, PNGase F and Endo H [26,27]. As can be seen from Figure 4, on Western blot, there are gel shift of PNGase F (dP) or Endo H deglycosylated (dE) samples compared to glycosylated RG2 (G) suggesting deglycosylation of RG2 by co-expression with PNGase F or Endo H enzymes. These results demonstrate that produced RG2 protein is N-glycosylated when expressed in *N. benthamiana* plant.

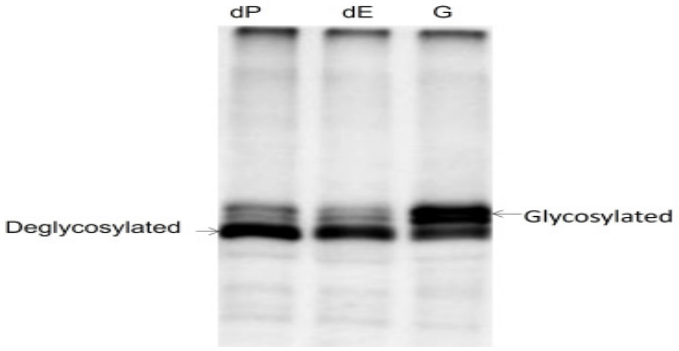

Figure 4. Co-expression of RG2 with PNGase F and Endo H. RG2 gene was *in vivo* co-expressed with bacterial PNGase F (dP) or Endo H (dE) deglycosylating enzymes as described in Materials and Method. Plant produced glycosylated (G) and deglycosylated variants (dP and dE) on the membrane were detected using the purified anti-His Tag antibody (Cat. No. 652502, BioLegend)

Immunogenicity studies of plant produced RG2 in mice

Immunogenicity studies of plant produced RG2 demonstrated that recombinant plant produced RG2 antigen was able to induce significantly higher titers of antibody in mice (Figure 5).

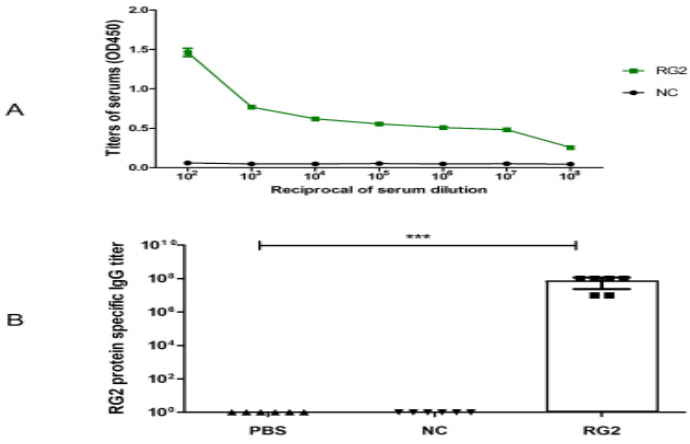

Figure 5. İmmunogenecity of plant produced RG2 antigen in mice. Mice were immunized on study days 0 and 21 IM with 5 µg (with alum) of plant produced RG2 protein. Serum samples were collected on study day 42 (post 2nd vaccination) and analyzed for anti-RG2 IgG responses by ELISA. For ELISA, 200 ng plant produced RG2 or 200 ng of plant produced recombinant PA83 of *Bacillus anthracis* (as a negative control) were coated into plate. Different dilutions of serum immunized with RG2 protein were added to test the binding affinity of plant produced RG2 and PA83 (negative control) to ant-RG2 antibody in serum immunized with RG2 protein. A: Detection of the RG2-specific IgG in different dilutions of sera from day 42. B: Detection of IgG titers in the mice serum with different dilutions specific to RG2 at day 42. Each point on the graph was derived from three replica for each dilution. One-way ANOVA test was used to compare antibody response of RG2 induced serums and PBS induced serums as control. *p<0.05;**p<0.01;***p<0.001 (n=6 mice/group)

Discussion

Rabies is a zoonotic disease that causes serious health problems worldwide and severe nerve damage in humans and animals. As mentioned above, rabies virus genome consists a single-stranded RNA encoding five proteins including G protein. G protein has been shown to play a major role of inducing virus-neutralizing antibodies and conferring protection against intracerebral challenge. RABV glycoprotein is a trimer [12] and assembles the form of homotrimers on the surface of virus or of the infected cells. G glycoprotein has been shown as a target for binding virus-neutralizing antibodies, and therefore harbors the major antigenic determinants of the virus. For this reason, most of the recombinant candidate vaccines studied so far are based on RABV-G protein. Currently, most of the vaccines prepared against RABV are produced in animal cells [31,32]. However, such vaccines have several disadvantages associated with high cost, scalability,safety and storage. Currently, several recombinant expression systems have been developed and used for expressing various types of vaccines, called a protein based, new generation subunits vaccines. Insect-cell-based systems have also been used for production of G protein of rabies virus [13,17]. Recombinant G protein produced in baculovirus expression system was shown to be immunogenic in mice. Sera of mice immunized with G protein, produced and purified from Sf9 cells had a high virus-neutralizing antibody titers. In addition, Sf9 cells produced recombinant G protein demonstrated 100% protection upon virulent intracerebral challenge [17]. The yeast expression system has been also tried to produce recombinant G protein of rabies virus, however, probably due to high-mannose glycosylation, this system was not appropriate for production of immunogenic G protein.

Plant-based transient expression platform has been demonstrated to provide safe, fast and low-cost production of variety recombinant pharmaceutical proteins in a short time. Using this expression strategy, a number of difficult to express proteins such as human Furin, Factor IX, heptamerized form of PA63 of *Bacillus anthracis* [23], full length Pfs48/45 protein of *Plasmodium falciparum* [24], human immunodeficiency virus (HIV) type 1 soluble gp140 [33], receptor binding domains (RBD) of Spike protein of SARS-CoV-2 [25,34] and other complex proteins including functional active monoclonal antibodies [22,27,33] have been successfully produced in *N. benthamiana* plant. Thus, plant transient expression strategy would be also ideal expression platform for the production low cost, safe, stable and highly immunogenic vaccine against rabies. G protein of RABV is a type-1 transmembrane protein and assembled as a trimeric spike on the surface of virus. Since G protein of RABV is known as a major antigen to induce virus neutralizing antibodies, therefore, in the present study, we have engineered and produced recombinant G protein in *N. benthamiana* plant. G protein of rabies has been produced in plants using transgenic approach [29,35-37]. However, transgenic plant approach has several concerns, associated with low target protein accumulations, long development time and also concerns with the possibility of gene transfer from transgenic plants to wild types [38]. Plant transient expression platform has several advantages over stable expression system. Recently, it was reported that rabies virus G protein produced in *N. benthamiana* by transient expression technology is an immunogenic antigen in mice [39]. In this study, full length G protein (rabies glycoprotein gene from

virus isolate SKRDG0204HC, acc. No. DQ076093) was expressed in *N. benthamiana* plant. Surprisingly, in this work, G protein was produced in *N. benthamiana* plant with native signal sequence (not plant signal sequence). Authors state that two G protein bands with molecular mass ~ 60 kDa (weak band) and ~50 kDa band (a major band) were observed on SDS-PAGE. Since in this work full length G protein was expressed in *N. benthamiana* plant, the expected size of the band should be at least~58 kDa. Based on SDS-PAGE and western blot analysis, presented in this work, two protein bands were observed is not because of N- glycosylation. The lower band was regarded as the non-glycosylated glycoprotein, and the upper as the N-glycosylated protein, please see Park et al., 2021, p. 30) as stated by authors, it is probably because of cleavage of full length G protein when expressed in *N. benthamiana* plant, as we can see that molecular mass of N-glycosylated G protein that is the major band is not higher than 50 kDa (protein band without treatment with Endo H) (Park et al. 2021, Figure 2B) [39].

For expression in plants our strategy was engineering of the RG2 sequence by fusing C-terminus with KDEL (ER retention signal) to produce of RG2 in ER, which is very well equipped with protein folding assistants and molecular chaperons for protein folding and PTMs (posttranslational modifications) of produced recombinant proteins of interest [33,40]. The other advantages of expression in ER is that ER provide high level expression of recombinant glycoproteins including complex proteins such as Pfs48/45 [24]; human Furin and Factor IX [23] with high mannose N-glycan structure, which is common in human, plants and yeast. In this study, the G protein gene (rabies glycoprotein gene from the ERA strain) was engineered and expressed as full length (RG1, amino acid residues 19-524) or a truncated form (RG2, amino acid residues 19-449) in *N. benthamiana* plant. The full-length G protein was also expressed in the *N. benthamiana* plant, but no specific protein produced as confirmed by western blot. Therefore, we engineered and expressed a truncated form of G protein (without transmembrane domain) in *N. benthamiana* plant. RG2 protein migrates as a single protein with a MM of ~60 kDa. The purification yield of RG2 was about 32 mg/kg plant leaf, which is satisfactory for commercialization. RG2 has five potential N-glycosylation sites. It was demonstrated that N-glycosylation play important role in enhanced virus production [32]. To determine the glycosylation status, RG2 was *in vivo* co-expressed with PNGase F or Endo H [26,27]. These results demonstrate that, the recombinant RG2 is glycosylated when expressed in a *N. benthamiana* plant. Immunogenicity studies have shown that the plant produced RG2 antigen can induce significantly higher antibody titers.

Thus, plant transient expression platform would be ideal expression system to produce cost-effective, safe, stable and highly immunogenic vaccine against rabies. Based on above findings plant produced RG2 could a potential vaccine candidate against rabies.

Conclusion

Our results demonstrate the feasibility of expressing truncated form of G protein in plants at high level and with high purification yield. Importantly, purified plant produced truncated form of G protein elicits significantly higher antibody titers. Thus, our findings demonstrate that plant produced truncated form of G protein could be a promising cost effective highly immunogenic and safe vaccine candidate against the rabies virus.

Conflict of interests
The authors declare that they have no competing interests.

Financial Disclosure
All authors declare no financial support.

Ethical approval
All animal experiments were carried out at Akdeniz University Experimental Animal Care Unit under permission of the Local Ethics Committee for Animal Experiments at Akdeniz University (Protocol number: 1156/2020.07.002). The animal protocols were approved by the Local Ethics Committee for Animal Experiments at Akdeniz University, for the mouse studies. All animal experiments and methods were performed in accordance with the animal experimentation guidelines and regulations approved by the Local Ethics Committee for Animal Experiments at Akdeniz University. The study was carried out in compliance with the ARRIVE guidelines.

References

1. Dietzschold B, Schnell M, Koprowski H. Pathogenesis of rabies. Curr Top Microbiol Immunol. 2005;292:45-56.
2. Knobel DL, Cleaveland S, Coleman PG, et al. Re-evaluating the burden of rabies in Africa and Asia. Bull World Health Organ. 2005;83:360-8.
3. Dhingra V, Li X, Liu Y, Fu ZF. Proteomic profiling reveals that rabies virus infection results in differential expression of host proteins involved in ion homeostasis and synaptic physiology in the central nervous system. J Neurovirol. 2007;13:107-17.
4. Gillet JP, Derer P, Tsiang H. Axonal transport of rabies virus in the central nervous system of the rat. J Neuropathol Exp Neurol. 1986;45:619-34.
5. Davis BM, Rall GF, Schnell MJ. Everything You Always Wanted to Know About Rabies Virus (But Were Afraid to Ask). Annu Rev Virol. 2015;2:451-71.
6. Fletcher MA , Hessel L, Plotkin SA. Human diploid cell strains (HDCS) viral vaccines. Developments in Biological Standardization. 1998;93:97-107.
7. Dhankhar P, Vaidya SA, Fishbien DB, Meltzer MI. Cost effectiveness of rabies post exposure prophylaxis in the United States. Vaccine. 2008;26: 4251–5.
8. Freuling C, Selhorst T, Batza HJ, Muller T. The financial challenge of keeping a large region rabies-free– the EU example. Dev Biol. 2008;131:273–282.
9. Schnell MJ, McGettigan JP, Wirblich C, Papaneri A. The cell biology of rabies virus: using stealth to reach the brain. Nat Rev Microbiol. 2010;8:51-61.
10. Foley HD, McGettigan JP, Siler CA, Dietzschold B, Schnell MJ. A recombinant rabies virus expressing vesicular stomatitis virus glycoprotein fails to protect against rabies virus infection. Proc Natl Acad Sci USA. 2000;97:14680-5.
11. Macfarlan RI, Dietzschold B, Koprowski H. Stimulation of cytotoxic T-lymphocyte responses by rabies virus glycoprotein and identification of an immunodominant domain. Mol Immunol. 1986;23:733-41.
12. Gaudin Y, Ruigrok RW, Tuffereau C, Knossow M, Flamand A. Rabies virus glycoprotein is a trimer. Virology. 1992; 187:627–32.
13. Prehaud C, Takehara K, Flamand A, Bishop DH. Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors. Virology. 1989;173:390-9.
14. Ashraf S, Singh PK, Yadav DK, et al. High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol. 2005;119:1-14.
15. Yokomizo AY, Jorge SA, Astray RM, et al. Rabies virus glycoprotein expression in Drosophila S2 cells. I. Functional recombinant protein in stable co-transfected cell line. Biotechnol J. 2007;2:102-9.
16. Huang H, Xiao S, Qin J, et al. Construction and immunogenicity of a recombinant pseudotype baculovirus expressing the glycoprotein of rabies virus in mice. Arch Virol. 2011;156:753-8.
17. Ramya R, Mohana Subramanian B, Sivakumar V, et al. Expression and solubilization of insect cell-based rabies virus glycoprotein and assessment of its immunogenicity and protective efficacy in mice. Clin Vaccine

Immunol. 2011;18:1673-9.

18. Koraka P, Bosch BJ, Cox M, et al. A recombinant rabies vaccine expressing the trimeric form of the glycoprotein confers enhanced immunogenicity and protection in outbred mice. Vaccine. 2014;32:4644-50.

19. Yang DK, Nakagawa K, Ito N, et al. A single immunization with recombinant rabies virus (ERAG3G) confers complete protection against rabies in mice, Clin Exp Vaccine Res. 2014;3:176–184.

20. McGettigan JP, David F, Figueiredo MD, et al. Safety and serological response to a matrix gene-deleted rabies virus-based vaccine vector in dogs. Vaccine. 2014;32:1716-9.

21. Huang F, Ahmad W, Duan M, et al. Efficiency of live attenuated and inactivated rabies viruses in prophylactic and post exposure vaccination against the street virus strain. Acta Virologica. 2015;59:117–24.

22. Klimyuk V, Pogue G, Herz S, e at. Production of recombinant antigens and antibodies in *Nicotiana benthamiana* using 'magnifection' technology: GMP-compliant facilities for small- and large-scale manufacturing. Curr Top Microbiol Immunol. 2014;375:127-54.

23. Mamedov T, Musayeva I, Acsora R, et al. Engineering, and production of functionally active human Furin in *N. benthamiana* plant: *In vivo* post-translational processing of target proteins by Furin in plants. PLoS One. 2019;14:e0213438.

24. Mamedov T, Cicek K, Miura K, et al. Plant produced *in vivo* deglycosylated full-length Pfs48/45 as a transmission-blocking vaccine candidate against malaria. Sci. Rep. 2019;9-9868.

25. Mamedov T, Yuksel D, Ilgin M et al. Plant-Produced Glycosylated and *In vivo* Deglycosylated Receptor Binding Domain Proteins of SARS-CoV-2 Induce Potent Neutralizing Responses in Mice. Viruses. 2021;13:1595.

26. Mamedov T, Cicek K, Gulec B, et al. *In vivo* production of non-glycosylated recombinant proteins in *Nicotiana benthamiana* plants by co-expression with Endo-β-N-acetylglucosaminidase H (Endo H) of Streptomyces plicatus. PLoS One. 2017;12:e0183589.

27. Mamedov T, Ghosh A, Jones RM, Mett V et al. Production of non-glycosylated recombinant proteins in *Nicotiana benthamiana* plants by co-expressing bacterial PNGase F. Plant Biotechnol. J. 2012;10:773-82.

28. McGarvey PB, Hammond J, Dienelt MM, et al. Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology. 1995;13:1484-7.

29. Loza-Rubio E, Rojas-Anaya E, López J, et al. Induction of a protective immune response to rabies virus in sheep after oral immunization with transgenic maize, expressing the rabies virus glycoprotein. Vaccine. 2012;30:5551-6.

30. Sainsbury F, Thuenemann EC, Lomonossoff GP. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol J. 2009;7;682–93.

31. Starodubova ES, Kuzmenko YV, Pankova EO, et al. Rabies Virus Glycoprotein with a Consensus Amino Acid Sequence and a Lysosome Targeting Signal Causes Effective Production of Antibodies in DNA-Immunized Mice. Mol Biol. 2018;52:314-317.

32. Rupprecht CE, Nagarajan T, Ert H. Current Status and Development of Vaccines and Other Biologics for Human Rabies Prevention. Expert Rev Vaccines. 2016;15:731-49.

33. Margolin E, Oh YJ, Verbeek M, et al. Co-expression of human calreticulin significantly improves the production of HIV gp140 and other viral glycoproteins in plants. Plant Biotechnol J. 2020;18;2109–117.

34. Siriwattananon K, Manopwisedjaroen S, Shanmugaraj B, et al. Plant-Produced Receptor-Binding Domain of SARS-CoV-2 Elicits Potent Neutralizing Responses in Mice and Non-human Primates. Front Plant Sci. 2021;13:682953.

35. Fleysh N, Deka D, Drath M, et al. Pathogenesis of Alfalfa mosaic virus in soybean (Glycine max) and expression of chimeric rabies peptide in virus-infected soybean plants. Phytopathology. 2001;91:941–7.

36. Roy S, Tyagi A, Tiwari S, et al. Rabies glycoprotein fused with B subunit of cholera toxin expressed in tobacco plants folds into biologically active pentameric protein. Protein Expr Purif. 2010;70:184-90.

37. Singh A, Srivastava S, Chouksey A, et al. Expression of rabies glycoprotein and ricin toxin B chain (RGP-RTB) fusion protein in tomato hairy roots: a step towards oral vaccination rabies. Mol Biotechnol. 2015;57:359–70.

38. Messeguer J. Gene flow assessment in transgenic plants. PCTOC. 2003;73:201–12.

39. Park Y, Kang H, Min K, et al. Rabies virus glycoprotein produced in *Nicotiana benthamiana* is an immunogenic antigen in mice. Czech J. Genet. Plant Breed. 2021;57:26–35.

40. Stronge VS, Saito Y, Ihara Y, Williams DB. Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates. J Biol Chem. 2001;276:39779–87.