Protein J (2012) 31:447–456
DOI 10.1007/s10930-012-9420-y

Localization of Rabies Virus Glycoprotein into the Endoplasmic Reticulum Produces Immunoprotective Antigen

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Published online: 17 May 2012

Abstract Rabies virus surface glycoprotein (rabies G-protein) with (G+RS) and without (G−RS) endoplasmic reticulum retrieval signal was expressed and characterized in tobacco plants. Transgenically expressed rabies G-protein was estimated at 0.015–0.38 % of total leaf protein. The relative migration of the rabies G-protein on SDS-PAGE was at the position, as anticipated for the viral coat protein (~66 kDa). Immunolocalization by confocal microscopy established that immunoprotective G+RS expressed in tobacco was primarily confined to ER. G+RS showed binding to Con A lectin and was susceptible to *N*-glycosidase F activity similar to native rabies G-protein. However, the G−RS transgenically expressed in tobacco leaves was glycosylated differently and was resitant to *N*-glycosidase F. Immunological studies and Rapid Fluorescent Foci Inhibition Test (RFFIT) showed that G+RS was immunogenic and immunoprotective, whereas G−RS was moderately immunogenic but non-protective against live virus challenge. Hence, plants can express the antigenic component of rabies virus with suitable glycosylation, which is important to give protection against rabies virus infection.

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Keywords Rabies G-protein · ER retrieval signal · Glycosylation · Rabies vaccine · Plant bioreactor

Abbreviations

BSA	Bovine serum albumin
Con A	Concanavalin A
ER	Endoplasmic reticulum
G+RS	Rabies G-protein with endoplasmic retrieval signal
G−RS	Rabies G-protein without endoplasmic retrieval signal
PBS	Phosphate buffer saline
RFFIT	Rapid Fluorescent Foci Inhibition Test
VIR	Authentic rabies virus

1 Introduction

Rabies is a major zoonotic disease of all mammals causing about 55,000 annual deaths worldwide (http://www.who.int/wer) [29]. Infection progresses to disease after the rabies virus is transmitted through the bite of a rabid animal to nervous system and is inevitably fatal. Rabies glycoprotein, exposed on the surface of viral particle, is responsible for binding to cellular receptor and entry into host cells [3]. It is majorly responsible for the induction of virus neutralizing antibodies and subsequent protection after pre- and post-exposure vaccination [21]. Despite many advances in rabies vaccine technology, the incidences of rabies are high worldwide and need a safer, cheaper and effective vaccine.

Plants have emerged as promising protein production systems. Hepatitis B surface antigen was first human virus antigen that was heterologously expressed in plant that elicit immune response [14]. Majority of approved

biopharmaceutical proteins are glycoproteins and glycosylation is often essential for proper folding, stability, solubility and desired biological activity [27]. Appropriate cellular targeting of proteins in eukaryotic cells is important for the glycosylation, correct folding, assembly, post-translational modifications and stability of proteins in plant cells. Typically, *N*-glycosylation initiates into the ER following co- or post-translational transfer of a preformed dolichol phosphate-linked oligosaccharide onto the nascent polypeptide through secretory pathway. ER provides an oxidizing environment and abundance of molecular chaperones that influence folding and assembly of nascent proteins. ER residency of a protein largely depends on the presence of a unique tetra-peptide H/KDEL at C-terminus [16]. The H/KDEL-dependent retrieval mechanism has been observed in plants similar to yeast and mammalian cells [5–7]. Plants can produce proteins with complex-type *N*-linked glycans with a tri-mannosyl core substitution in the ER, similar to that of mammalian glycosylation system. Post ER processing of *N*-glycan chains in golgi apparatus is different in plants as compared to mammalian cells [12]. The tri-mannosyl core in plants is often substituted by a β 1, 2- xylose (Xyl) residue and/or α 1, 3-linked fucose (Fuc) residue at the inner core of *N*-acetyl glucosamine (GlcNAc) residue, instead of α 1, 6-linked Fuc residue, as in mammals. In the present study we have shown the successful expression of immunoprotective rabies glycoprotein in plant expression system with suitable glycosylation that can protect against rabies infection by retrieving and retaining the protein into the ER.

2 Materials and Methods

2.1 Designing, Synthesis and Vector Construction of Rabies Virus Surface Glycoprotein Gene

The strategy followed for designing, synthesis and vector construction for gene expressing rabies G-protein with ER retrieval signal (pSA5) has been described elsewhere (Fig. 1a) [1]. The rabies G-protein with ER retrieval signal was placed downstream of CaMV35S duplicated enhancer promoter. The pathogen related signal peptide was used to translocate the protein into the ER. Factor Xa peptidase site (IEGR) was included before the rabies G-protein for enzymatic removal of the His_{x6} affinity tag. The expression cassette of rabies G-protein without ER retrieval signal was constructed by deleting the SEKDEL from the gene. To achieve this, chimeric *rgp* gene, containing plasmid with SEKDEL pSA5 was used as template for amplification using 5′-CC*AAGCTT*TCTAGATAAACAATGAACT TCCTCAAGTCATTC-3′ (P1) as forward primer and

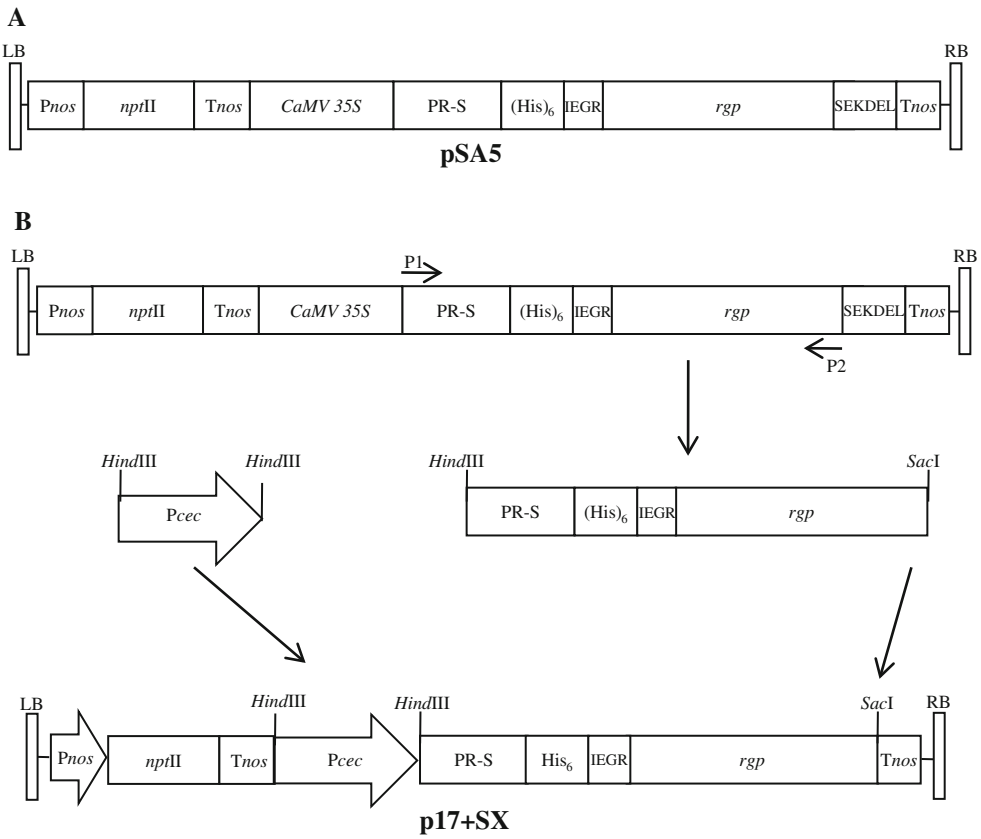

Fig. 1 Schematic diagram of coding region of G+RS (**a**) and G−RS (**b**) used for *Agrobacterium* mediated transformation to develop transgenic tobacco plants. Cloning strategy adopted for G+RS was as described by Ashraf et al. [1]. ER retrieval signal, SEKDEL, was deleted in G−RS (**b**) using primers (P1 and P2 shown by arrows). Chimeric gene (G−RS) and promoter Pcec were ligated into pBI101 binary vector at *Hin*dIII and *Sac*I restriction sites to obtain p17 + SX

5′-C*GAGCTC*TCATCACAAACGCGTCTCGCCTCC-3′ (P2) as reverse primer. This amplicon was ligated downstream of Pcec (complete expression cassette) promoter which is reported to be stronger than the CaMV35S promoter [23] and resulting expression cassette was cloned in pBI101 at *Hin*dIII and *Sac*I restriction sites to obtain binary vector construct p17 + SX (Fig. 1b).

2.2 Genetic Transformation of Tobacco and *E. coli*

Agrobacterium tumefaciens LBA4404 (pAL4404) was transformed with pSA5 (G+RS) and p17 + SX (G−RS) recombinant plasmids by electroporation and was used for transformation of tobacco (*Nicotiana tabaccum* cv. Petit Havana) using leaf disc method [11]. Kanamycin resistant T_0 plantlets expressing G+RS and G−RS were planted in soil for growth to mature vegetative stage in the green house. The plasmid pSA33 [1] was used to transform *E. coli* (BL21 DE3) to express rabies virus surface G-protein using standard methods [22].

2.3 Screening and Molecular Analyses of Transgenic Plants

The transgenic tobacco lines (T_1) were confirmed by PCR with gene specific end primers (forward primer 5′-CCAA GCTTTCTAGATAAACAATGAACTTCCTCAAGTCAT T-3′ and the reverse primer 5′-GGATATAATCTTTCCGG ACTGTGGAGTAACGGAGACCTCCCTACCGGT-3′ for G+RS and P2 as reverse primer for G−RS) using genomic DNA and cDNA as template. More than 50 individual transgenic plant lines expressing G+RS and G−RS were screened for the high level expression of rabies G-protein by sandwich enzyme-linked immunosorbent assay of total soluble protein extracted in protein extraction buffer and was probed with polyclonal horse anti-rabies antibodies (1:10,000) [1] and only the promising transgenic lines were included in subsequent experiments.

2.4 Northern Blotting

Total RNA was isolated from 100 mg fresh leaf of different transgenic lines using TRIZOL LS reagent (Invitrogen, USA) following the manufacturer's instructions. Forty µg denatured RNA was electrophoresed on 1.2 % (w/v) denaturing agarose gel containing formaldehyde. After electrophoresis, RNA was transferred onto Hybond N^+ nylon membrane following standard protocol [22]. The blot was hybridized with $\alpha[P^{32}]$ dCTP labeled *rgp* gene specific probe at 42 °C for 24 h in hybridization solution. Finally, the blot was washed with 0.1 % SDS in 0.1×SSC and was exposed to phosphorescent screen and imaged on Molecular Imager FX (Bio-Rad, USA).

2.5 Immunohistochemistry and Confocal Microscopy

Protoplasts were prepared from tobacco leaves according to [10] with minor modifications. The protoplast isolation buffer (1 % Cellulase R10; 0.25 % Macerozyme Onozuka R10; 0.4 M D-Mannitol and 10 mM 2-(*N*-morpholino)ethanesulfonic acid buffer pH 5.7) was preheated at 55 °C for 10 min and cooled to room temperature before adding 0.1 % BSA, 5 mM β-Mercaptoethanol and 30 mM $CaCl_2$. Five mm leaf pieces were submerged into protoplast isolation buffer for overnight at 28 °C. Isolated protoplasts were filtered through 70µm nylon mesh and centrifuged for 5 min at 100×*g*. The intact protoplasts were gently re-suspended in protoplast wash buffer (4 mM 2-(*N*-morpholino) ethanesulfonic acid buffer; 0.5 M Mannitol and 2 mM KCl). Isolated protoplasts were adhered on poly L-Lysine (100 µg/ml in 10 mM Tris pH 8.0) coated coverslips by incubating for 3 h at room temperature. Adhered protoplasts were washed twice with PBS, air-dried and fixed with 4 % formaldehyde for 1 h. Protoplasts were permeabilized with 0.5 % Nonidet P-40 in PBS for 10 min. Chlorophyll was leached out from the fixed protoplasts by methanol for 2 × 10 min. Air-dried protoplasts were rehydrated in PBS for 30 min. After blocking with 5 % BSA in PBS, protoplasts were incubated over-night at 4 °C with primary antibodies (monoclonal mouse anti rabies G-protein, US Biologicals, 1:10 and polyclonal rabbit anti calnexin IgG, 1:1,000 prepared in PBS-1 % BSA). Protoplasts were incubated with Alexa Fluor® 488 conjugated anti-mouse IgG (1:15) and Alexa Fluor® 594 conjugated anti-rabbit IgG (1:1,000) at 4 °C for 6 h. Protoplasts were washed with PBS for four times in between any two incubation steps. Finally, protoplasts were air dried and mounted on microscopic slides in Prolong® Gold Antifade reagent (Molecular Probes) and allowed to cure in dark at 4 °C for over-night. Flurographs were taken under Confocal Laser Scanning Microscope (Bio-Rad, USA).

2.6 Purification of Plant Expressed Rabies G-protein

Microsomes were prepared and rabies G-proteins were purified as described elsewhere [1].

2.7 Con A Affinity Chromatography of rabies G-proteins

The total protein extracted from *E. coli* (induced by 1 mM IPTG to express rabies G-protein) was solubilised in 1 M urea and dialyzed against loading buffer consisting of 20 mM Tris–Cl pH 7.5 and 0.5 M NaCl. The G+RS and

G−RS were prepared from transgenic tobacco leaves of respective transgenic plants and were partially purified on anion exchange chromatography [11]. G+RS and G−RS enriched fractions were applied on to Concanavalin A column (GE healthcare, USA) as per manufacturer's instructions. Similarly, whole cell extract of *E. coli* cells was applied on to Concanavalin A column as negative control. After washing the column, the bound glycoproteins were desorbed by 0.1 M borate buffer pH 6.5. Unbound and eluted fractions were analyzed by immunobloting to establish glycosylation state of the recombinant rabies G-proteins expressed in tobacco and *E. coli*.

2.8 *N*-glycosidase Sensitivity

Enzymatic deglycosylation of G+RS and G−RS along with authentic rabies G-protein as positive control and *E. coli* expressed rabies G-protein as negative control, was performed with *N*-glycosidase A (Roche) and *N*-glycosidase F (NEB, England). Rabies G-proteins from different sources were treated with *N*-glycosidase A for 12 h at 37 °C following manufacturer's instructions. After the incubation, immunoblotting was performed using equine anti-rabies antibodies. Similarly, rabies G-proteins from different sources were denatured with 1× glycoprotein denaturing buffer at 100 °C for 10 min. After addition of NP-40 and G7 reaction buffer, diluted *N*-glycosidase F was added and the reaction mixture was incubated overnight at 37 °C. Separation of reaction products was visualized by immunoblotting.

2.9 Immunization of BALB/c Mice

BALB/c mice were primed by injecting 25µg of purified G+RS and G−RS. Commercially available killed rabies virus vaccine containing 25µg of equivalent G protein (Rabipur, Aventis Pharma Ltd.) was used as positive control while PBS served as a negative control. Priming was performed with equal volume of Freund's complete adjuvant. Three booster doses were given on the 7th, 14th and 28th day. First two boosters were given with Freund's incomplete adjuvant while last booster was given without any adjuvant. Serum was collected from retro-orbital sinus on 35th day for anti-rabies antibody titration. Immunized mice were challenged with live challenge virus standard rabies virus. The challenge dose ($10\times LD_{50}$) was injected intra-cerebrally and mice were observed for 15 days. Experiments had three biological repetitions and values are shown as mean.

2.10 Titration of Response in Immunized Mice Blood

The presence of rabies G-protein specific antibodies in mouse sera was estimated by double sandwich enzyme-linked immunosorbent assay. The 96 well microtiter plates were coated with 100 µl/well of human anti-rabies immunoglobulin at 1:2,000 dilutions in bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. Non specific sites were blocked with 1 % BSA prepared in PBS-T and incubated at 37 °C for 2 h. The wells were filled with inactivated rabies virus, Rabipur, at 1:50 dilution in PBS-T containing 0.25 % BSA and incubated overnight at 4 °C. The wells were filled with 100 µl immunized mice serum (1:500) in PBS-T containing 0.25 % BSA and incubated overnight at 4 °C. The plates were incubated with 100 µl/well of 1:40,000 dilution of alkaline phosphatase-conjugated anti-mouse anti-IgG (Sigma, St. Louis), horseradish peroxidase conjugated anti-mouse anti-IgG1 (1:1,000) and anti-mouse anti-IgG2a (1:1,000) in PBS-T containing 0.25 % BSA at 37 °C for 2 h. Plates were washed thrice with PBS-T between each incubation step. The chromogenic reaction was allowed to take place by adding 100 µl/well of *p*-nitro phenyl phosphate for alkaline phosphatase and tetra-methylbenzidine for horseradish peroxidase conjugates and reaction was allowed for 30 min at 37 °C. The enzymatic reaction was stopped by adding 3 N NaOH or 1 N sulfuric acid (50 µl/well). Absorbance was measured at 405 nm and 450 nm, respectively.

2.11 RFFIT Test

Serum was collected on 35-day post immunization, labeled and stored at 4 °C for further analysis. The RFFIT test was performed at The Pasteur Institute of India (WHO Regional Reference Laboratory, Coonoor, India), following the procedure as described by Pandit et al. [20], including a WHO reference serum. Results are expressed in IU/ml.

3 Results

3.1 Expression of G+RS and G−RS

Transgenic tobacco plants expressing G+RS and G−RS proteins were screened for the presence of *rgp* gene. Genomic DNA isolated from the promising transgenic tobacco plants was used as template in PCR, using *rgp* gene-specific end primers. DNA fragment of the expected size of ~1.6 kb was amplified (Fig. 2a); confirming the insertion of *rgp* into the tobacco plant genome. RT-PCR using cDNA template of representative transgenic lines, amplified a fragment of ~1.6 kb (Fig. 2b) establishing full-length transcription of *rgp* gene. The full-length transcript was also noticed by northern blotting of total RNA of representative transgenic lines (Fig. 2c). Western blot analysis of the promising transgenic lines expressing G+RS and G−RS protein showed a band of ~66 kDa

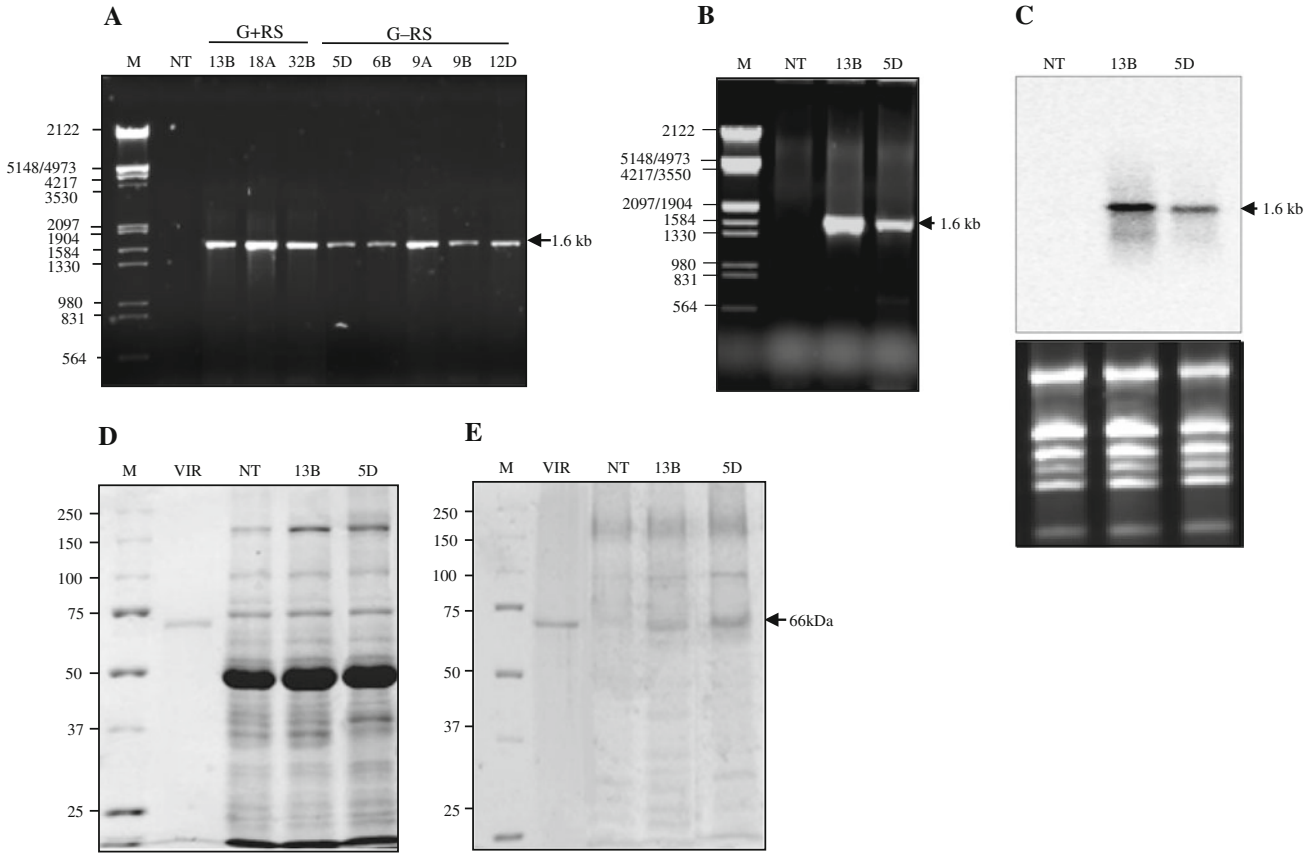

Fig. 2 Molecular analysis of transgenic tobacco plants expressing rabies G-protein: **a** Detection of *rgp* gene insert with PCR amplification of G−RS (5D, 6B, 9A, 9B, 12D) and G+RS (13B, 18A, 32B) in genomic DNA of transgenic plants, using gene specific end primers. **b** Detection of *rgp* transcripts using cDNA as template prepared from representative transgenic lines expressing G−RS (5D) and G+RS (13B), using gene specific end primers. **c** Northern blot of representative transgenic lines expressing G−RS (5D) and G+RS (13B). EtBr stained agarose gel showing loading control of total RNA (lower panel). Detection of rabies G-proteins in total protein extract from representative transgenic lines stained by Coomassie Brilliant Blue (**d**) and western blot using polyclonal anti-rabies G-protein antibody (**e**). In **d**–**e** VIR, authentic rabies G-protein; 13B, total protein from representative transgenic line expressing G+RS and 5D, total protein from representative transgenic line expressing G−RS. *M* standard molecular mass and *NT* samples from non-transformed plant

when the blot was probed with anti-rabies glycoprotein antiserum (Fig. 2e). Thus, the transgenic tobacco lines expressed full-length, glycosylated G+RS and G−RS proteins.

3.2 Immunolocalization and Confocal Microscopy

Immunolocalization was performed with isolated protoplasts from two types of transgenic and non-transgenic tobacco leaves to establish the topographic locations of rabies G-protein in cell. Calnexin, a chaperone intrinsic to endoplasmic reticulum membrane, was used as internal marker of ER. The red fluorescence emitting from anti-calnexin fluorophor indicates the topographic location of ER, whereas, the green fluorescence emitting from anti-rabies G-protein tagged fluorophor shows its localization. The protoplasts from non-transgenic tobacco emitted only red fluorescence, showing the location of calnexin into ER (Fig. 3 Top panel). The absence of green fluorescence establishes the absence of rabies G-protein in non-transgenic protoplasts. However, the protoplasts from transgenic tobacco leaf expressing rabies G-protein showed both, the green and red fluorescence from the same location (Fig. 3 Middle and lower panel); indicating that calnexin and rabies G-protein were co-localized in ER membrane. However, in the transgenic tobacco leaf expressing G−RS, the intensity of green fluorescence was remarkably low as compared to G+RS (Fig. 3 Lower panel), representing the ER localization of G−RS, similar to G+RS in ER membrane.

3.3 Glycosylation Detection in Plant Derived Rabies G-proteins

The rabies G-protein expressed in transgenic tobacco leaves and *E. coli* were analyzed for glycosylation state by observing their Con A lectin affinity. The G+RS and G−RS proteins extracted from the transgenic tobacco were

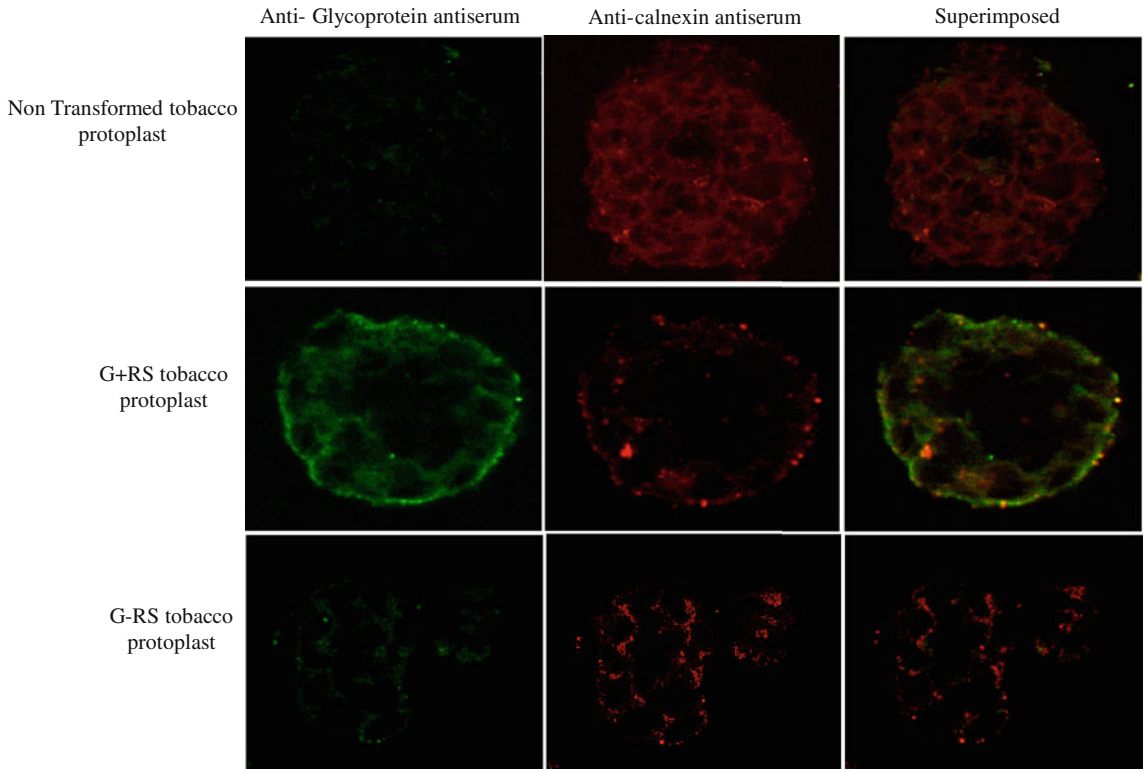

Fig. 3 Immunolocalization of rabies G-protein expressed in transgenic tobacco leaf protoplast: *Top panel* represents immunofluorescence from non-transformed protoplast; *middle panel* represents immunofluorescence from G+RS and lower panel represents immunofluorescence from G−RS. Green fluorescence indicates anti-rabies G-protein antibody labeled with Alexaflor-488 and red fluorescence indicates anti-calnexin antibody labeled with Alexaflor-520. Images were captured at 10 μm magnification

purified on Q-Sepharose fastflow matrix and immunoaffinity column. Molecular mass of the purified rabies G-proteins (Fig. 4a lane 3 G+RS and lane 5 G−RS) was similar to authentic rabies G-protein (Fig. 4a lane 2 VIR). When rabies G-protein enriched fractions obtained from the Q-Sepharose matrix were passed through Con A column and eluted with 0.1 M borate buffer, only one protein from G+RS extract (Fig. 4a lane 4) co-migrated with the authentic rabies G-protein and showed immunoreactivity with anti-rabies G-protein antibodies. While the partially purified G−RS, whose molecular mass was apparently equal to the authentic rabies G-protein was not retained on to the Con A column (Fig. 4a lane 6). The non-glycosylated *E. coli* expressed rabies G-protein whose molecular mass was ∼61 kDa (Fig. 4a lane 7), was not retained on the Con A column (Fig. 4a lane 8) as well.

3.4 Enzymatic Deglycosylation of Rabies G-proteins with *N*-glycosidase A and *N*-glycosidase F

The rabies G-proteins from authentic rabies virus, G+RS, G−RS and *E. coli*, were digested with *N*-glycosidase A (Fig. 4b lane 3, 5, 7 and 9). The molecular mass of non-glycosylated *E. coli* expressed rabies G-protein remained unchanged (Fig. 4b lane 9). However, a reduction in molecular mass was noticed in the other three cases. When the authentic rabies G-protein, G+RS, G−RS and *E. coli* expressed proteins were digested with *N*-glycosidase F, only G+RS yielded a band of increased migration (Fig. 4c lane 5) similar to authentic rabies G-protein (Fig. 4c lane 3). However, molecular mass of G−RS and non-glycosylated *E. coli* expressed rabies G-proteins remained unchanged (Fig. 4c lane 7 and 9).

3.5 Immune Response of Rabies G-proteins in Mouse Antiserum

The serological analysis of immunized BALB/c mice showed IgG response of G+RS higher than that of commercial rabies virus vaccine. However, the IgG response against G−RS was comparably lower (Fig. 5). The subisotype immunoglobulin estimation in the serum of immunized mice with G+RS and G−RS proteins showed different response. The IgG2a response was highest in the mice immunized with authentic rabies G-protein (Fig. 5 VIR); whereas it was slightly lower in the group of mice immunized with G +RS (Fig. 5 G+RS). The IgG2a response in the group of mice that received G−RS protein as immunogen was similar to the negative control (Fig. 5 G−RS).

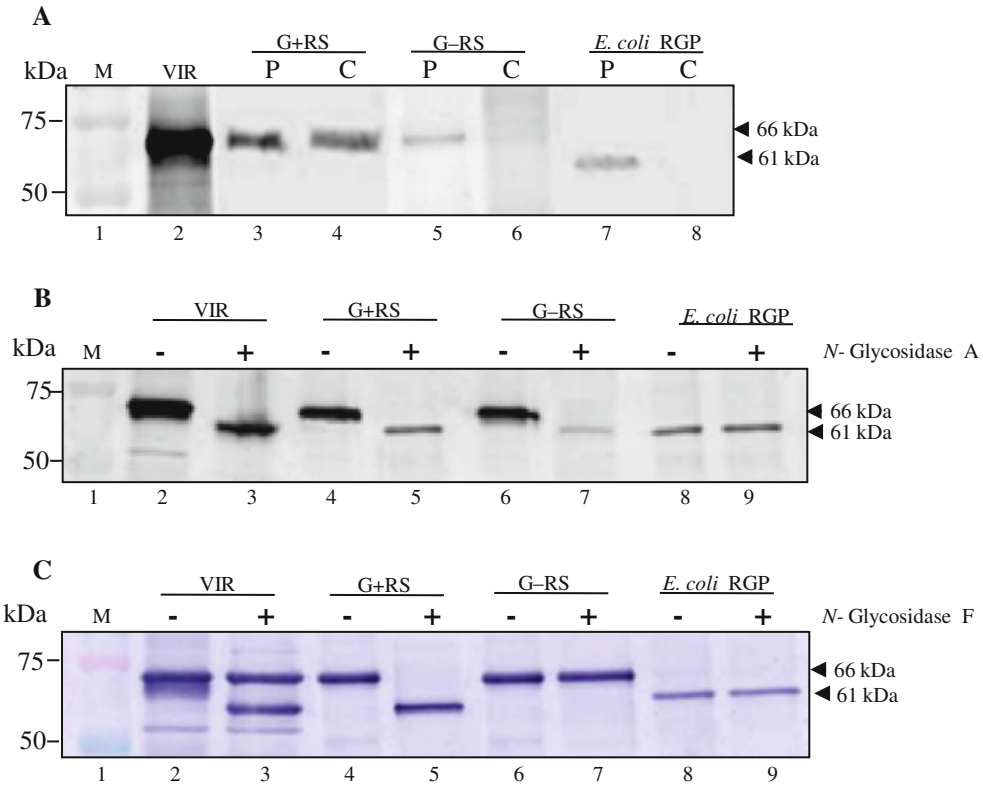

Fig. 4 Characterization of glycosylation in rabies G-protein expressed in transgenic tobacco by western blotting probed with polyclonal anti-rabies G-protein antibody. *VIR* Authentic rabies virus G-protein, *G+RS* rabies G-protein with retention signal, G−RS rabies G-protein without retention signal, *E. coli RGP* rabies G-protein expressed in *E. coli.* **a** By Con A lectin affinity: *P* purified fraction and *C* respective rabies G-protein eluted from Con A column. **b** *N*-glycosidase A and **c** *N*-glycosidase F sensitivity of respective rabies G-protein: (+) and (−) represents with and without the enzyme treated samples, respectively. *Lane M* contains molecular mass standards

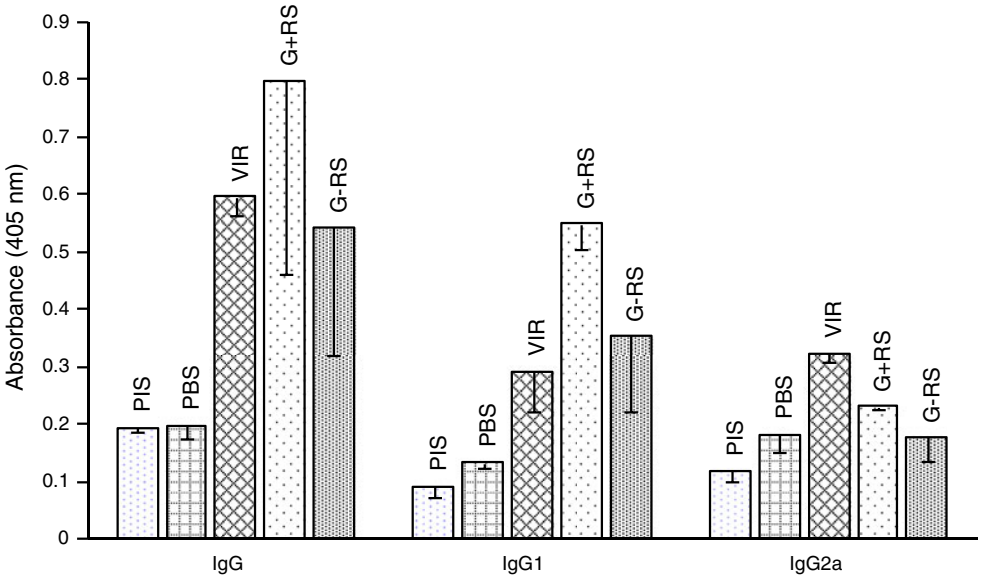

Fig. 5 Immune response against transgenically expressed rabies G-proteins in Balb/c mice serum: PIS are the levels of anti-rabies immunoglobulins (IgG, IgG1 and IgG2a) in pre-immune sera. PBS is the group of mice those received phosphate saline buffer as negative control. VIR is the group of mice those received authentic rabies G-protein as immunogen. G+RS and G−RS are the groups of mice those received rabies G-protein with and without retention signal, respectively, as immunogen

Contrary to the IgG2a response, the IgG1 response was intense in the serum of mice that received the G+RS and G−RS immunogen (Fig. 5 G+RS and G−RS). It was higher than the authentic rabies G-protein (Fig. 5 VIR). It suggests that rabies G-protein expressed in transgenic tobacco elicited higher Th2 response than Th1 response.

3.6 RFFIT Test

The post-immunization serological response following 35 days was monitored using RFFIT (virus neutralizing antibodies) tests. The estimated RFFIT titre value was ≤0.5 IU/ml in group of mice those received PBS and

G−RS along with pre-immune sera. However, the RFFIT titres value was ≥64.0 IU/ml and ≥12 IU/ml, in group of mice those received Rabipur and G+RS, respectively (Table 1).

3.7 Immunoprotection of Mice Challenged with Live Rabies Virus

After the third booster, experimental mice were challenged with live challenge virus standard rabies virus. The challenge dose of $10\times LD_{50}$ was administered intracerebrally to all groups mice and observed for 14 days for the appearance of rabies symptoms. Paralytic symptoms started appearing after 5 days of challenge, in group of mice those administered with PBS and G−RS. However, the mice those received the authentic rabies vaccine and G+RS remained healthy during the observation period. The G+RS gave complete protection, similar to the commercial authentic rabies vaccine (Fig. 6). Mice immunized with PBS and G−RS died after 14 days of challenge.

Table 1 Mean RFFIT serum titres (IU/ml, ±SD) at 35 days post-immunization

Group	Mean RFFIT titers (IU/ml, ±SD)
PIS	– (±nd)
PBS	– (±nd)
VIR	≥64.0 (±2.0)
G+RS	≥12.0 (±4.0)
G−RS	– (±nd)

– average RFFIT titer value ≤0.5 IU/ml, *nd* not detected

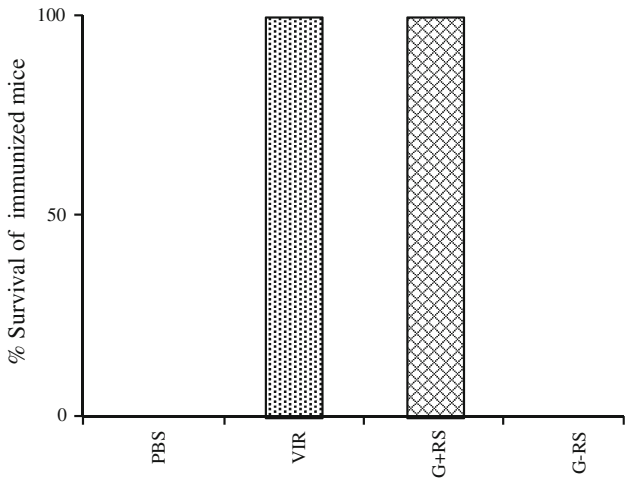

Fig. 6 Immunoprotective ability of different immunogens after intracerebral challenge of immunized mice groups (of Fig. 5) with $10\times LD_{50}$ doses of challenge virus standard and observed up to 15 days

4 Discussion

Plants have emerged as a highly versatile expression system for the production of cost-effective and contamination-safe recombinant biopharmaceutical proteins. Therefore, strategic designing of antigen coding genes for high-level expression and proper glycosylation is an important consideration. The conserved KDEL/HDEL C-terminal motif present on mature secretory proteins is recognized in golgi apparatus and is responsible for the retrieval of proteins by the transmembrane receptor, ERD2 [13]. In the present study, two variants of strategically designed *rgp* were used to study the possible role of ER retention signal, SEKDEL, on biological activity of rabies antigen due to the possible differences in glycosylation pattern. Therefore, SEKDEL was placed on the C-terminus of rabies G-protein (G+RS) for its retrieval back to ER, while it's variant was devoid of the SEKDEL motif (G−RS).

Estimation of rabies G-protein expression in transgenic tobacco lines showed that G+RS was expressed at 0.015–0.38 %, while, G−RS ranged from 0.006–0.009 % of total leaf protein. Stable expression of rabies G-protein in transgenic plants has been reported at low levels and detectable only through immunoprecipitation along with reduced molecular mass of 60–62 kDa [15]. The lowered molecular mass was attributed to differences in glycosylation, though not studied. Since the accumulation level of protein is irrespective of mRNA level [25], the increased accumulation of G+RS, unlike to that of G−RS (Fig. 3 lower panel), can be attributed to efficient retrieval of the protein into ER (Fig. 3 middle panel). Thus, addition of SEKDEL motif suggests enhanced accumulation of rabies G-protein [28]. In absence of proper targeting signals, proteins accumulated in the secretory system are secreted to apoplast where stability of protein is much lower than in the lumen of ER [4, 24]. Hence, due to the absence of SEKDEL motif or any other specific cellular localization signal in G−RS, it might have secreted into the apoplast and its subsequent degradation leading to low expression level as compared to G+RS. Thus, higher retrieval of G+RS in ER and presence of fewer proteases could lead its increased accumulation. The results from enzyme-linked immunosorbent assay (data not shown) performed with microsomal and cytosolic fractions prepared at high-speed centrifugation also suggested that G+RS mainly was confined to microsomal fraction while major part of G−RS was detected in cytosolic fraction.

The secretory systems in animals, yeast and plants share many similarities. The *N*-glycan structures of natural resident proteins of ER are devoid of golgi modifications and serve as marker of a putative recycling function [9, 17]. The differential affinity of the two plant-expressed rabies G-proteins for Con A showed the difference in their glycan

content. Con A lectin has affinity to bind with molecules containing high α-D mannopyranosyl, α-D glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups which is the feature of high mannose sugars. In the present study, G+RS was retained on the Con A matrix, suggesting the presence of biantennary high mannose type glycan moieties on it (Fig. 4a lane 4), whereas the G−RS did not show affinity and was not retained on to Con A column (Fig. 4a lane 6). It suggests that G−RS contained modified and complex type of glycans. This was supported by deglycosylation assays (Fig. 4b and c). When rabies G-proteins were treated with *N*-glycosidase A (that cleaves the *N*-glycosidic linkage irrespective of any modification of glycan side chain), the rabies G-proteins were reduced to a molecular mass similar to non-glycosylated rabies G-protein expressed in *E. coli* (Fig. 4b lane 3, 5 and 7). When rabies G-proteins were deglycosylated with *N*-glycosidase F (that cleaves between the innermost GlcNAc and asparagine residue only when the side chains of GlcNAc are not modified), G+RS was cleaved (Fig. 4c lane 5) while G−RS was resistant to *N*-glycosidase F activity (Fig. 4c lane 7). It suggests that due to lack of ER retrieval signal, SEKDEL, G−RS might travel deep up to median and *trans*-golgi apparatus, where its terminal glycans and side chains were diversely modified. G−RS might have glycoepitopes like α(1, 3)-fucose and β(1, 2)-xylose; as α(1, 3)-fucosyltransferase and β(1, 2) xylosyltransferase are found in medial cisternae of golgi apparatus. This study also established that SEKDEL motif prevented plant-specific modification of terminal glycans and side chains of GlcNAc residues of G+RS, which seems containing high mannose type oligosaccharide structure [26]. Structural analyses of plant ER resident proteins have shown that natural reticuloplasmins bear a high mannose type oligosaccharide structure, which is common to plants and mammals [4, 18, 19].

When the purified rabies G-proteins were injected into the mice to test their immunogenicity; G+RS was found more immunogenic than G−RS (Fig. 5). Titration of subisotypic antibodies in the serum of immunized mice showed that major immune response against plant derived rabies G-protein was IgG1 type (Th2) and was two fold higher for G+RS while, slightly higher in G−RS as compared to the commercial vaccine. The IgG2a response (Th1) was 1.4 fold higher in mice group immunized with authentic rabies G-protein than in G+RS group. However, there was no IgG2a response in G−RS group. Increased IgG2a response in G+RS suggest a potent induction of anti-viral effector functions through Th1 by T_C cell activation, a feature that was absent in G−RS, which proved more effective against rabies virus infection It is quite evident from RFFIT assay which shows the presence of protective level of virus neutralizing antibody in G+RS (Table 1). It explains the immunoprotective efficiency of G+RS similar to commercial vaccine against the live virus challenge (Fig. 6). However, G−RS was unable to impart comparable protection probably due to the presence of β1, 2 xylose- and/or α1, 3 fucose containing glycoepitopes, which might induce rapid immune opsonization of distinctly glycosylated rabies antigen from the blood stream, thus strongly compromising its efficacy [8]. Protection against viral infection co-relates well with development of the Th1 response [2].

Thus, the strategy to retrieve the recombinant rabies G-protein within the ER was able to produce immunoprotective rabies antigen besides enhancing its accumulation. The results also established the importance of appropriate strategies aiming plants as bioreactors for production of designer vaccines.

Acknowledgments The work was carried out at National Botanical Research Institute (NBRI), Lucknow. Council of Scientific and Industrial Research, India, is gratefully acknowledged for financial support to DKY as Senior Research Fellow at NBRI. We thank Central Drug Research Institute, Lucknow for providing confocal microscopy facility and Indian Veterinary Research Institute, Izatnagar for conducting challenge virus standard challenge of immunized mice. We thank Dr Rebecca Boston, University of North Carolina, USA, for the generous gift of anti-calnexin antibody.

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