Rational design and expression of a recombinant plant rhabdovirus glycoprotein for production of immunoreactive murine anti-sera

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**Abstract**

Lettuce necrotic yellows virus (LNYV) is a plant rhabdovirus which has a type-1 transmembrane glycoprotein. Here, we describe the generation of murine anti-sera to the glycoprotein. Rational design, expression, and purification of recombinant glycoprotein, termed rLGe, was undertaken using SignalP4.1 and camSol servers to predict signal peptide cleavage and protein solubility. In order to successfully obtain expression in mammalian cells, LNYV glycoprotein native signal peptide was substituted with that of Rabies virus glycoprotein. In addition, rather than expression of the entire molecule, rLGe consisted of the LNYV glycoprotein ectodomain fused to two affinity tags to minimize the risk of protein aggregation, while facilitating detection and purification. rLGe was transiently expressed in mammalian cell culture, purified using affinity column chromatography, and used to immunize mice. Harvested anti-sera were immunoreactive and specific to the naturally occurring glycoprotein as confirmed by western blotting of plant leaf tissue infected with LNYV.

Keywords

Plant Rhabdovirus / Rabies / Glycoprotein / Signal Peptide, FLAG-tag

**Introduction**

Lettuce necrotic yellows virus (LNYV), which was first identified in Australia and New Zealand, is a negative-sense single-stranded enveloped plant rhabdovirus that infects several plant species [1-3]. The virus has an outer host-derived lipid envelope from which projects a type 1 transmembrane glycoprotein consisting of an ectodomain, a hydrophobic domain spanning the membrane, and a short cytoplasmic domain supported by the matrix protein scaffold [4]. The glycoprotein contains three N-linked glycosylation sites and disulphide bonds [5, 6]. Sequence analysis and homology-based comparisons between LNYV glycoprotein and those of other rhabdoviruses predicts that the LNYV glycoprotein contains a signal peptide of 25 amino acids at the N – terminus followed by a peptidase recognition sequence to direct the protein to the endoplasmic reticulum [7], where chaperones are thought to guide the glycoprotein folding process [8] and the sequential formation of disulphide bonds required for normal folding [9, 10].

Antibodies are a highly specific and reliable tool for detecting viral proteins [11], visualizing viral particles [12, 13] and for purification of viruses using immuno-affinity chromatography [14-16]. For LNYV, Chu *el al*. previously used anti-LNYV sera for detection of LNYV in plant hosts and insect vector [17], while Dietzgen *el al*. used monoclonal antibodies to analyze virus structural proteins [18, 19]. The objective of this study was to generate murine anti-sera to detect and purify LNYV particles, as antibodies to the virus are not available for purchase. This was part of a larger project to develop a reverse genetics system for the virus. We recently completed the first stage of this undertaking with the development of a minigenome cassette for LNYV [20].

In the studies cited previously, LNYV virus was used as immunogen: however, as this requires virus generation, followed by extensive purification of viral particles to prevent induction of antibodies to plant host components [21], we expressed recombinant LNYV glycoprotein in a mammalian cell platform for subsequent immunization of mice and generation of anti-sera. We chose the well-characterized mammalian cell HEK 293T platform to express antibody as this has previously been used successfully to express viral glycoproteins [22-24] including that from rabies virus CVS-11 [25].

In initial studies, attempts to express the entire LNYV glycoprotein with its native signal peptide were unsuccessful (data not shown). Recombinant expression of proteins with their native signal peptide substituted with expression-platform relevant equivalents has been shown to increase efficacy of signal peptide cleavage at the appropriate site and the correct processing of the protein [26]. We hypothesized that the signal peptide from Rabies virus (RABV) glycoprotein - a mammalian rhabdovirus whose glycoprotein has been successfully expressed transiently in mammalian cells [27] - might be a suitable substitute. Subsequent analysis using computational servers trained on eukaryotic sequences predicted improved cleavage of RABV signal peptide compared to that from LNYV and we therefore substituted the LNYV glycoprotein signal peptide with the RABV equivalent.

In addition, we chose to express the LNYV glycoprotein ectodomain as this was predicted to be more soluble than the entire protein, potentially minimizing the risk of protein aggregation and facilitating subsequent purification [28-30]. We also fused the ectodomain to a His6-tag and a FLAG-tag on the N – and C – terminals respectively. Dual affinity tags have been demonstrated to have a stabilizing effect on sensitive proteins and provide multiple detection and purification strategies [31, 32]. rLGe was purified using anti-FLAG column chromatography and the purified glycoprotein was used as an immunogen to elicit murine anti-sera.

# Materials and Methods

## Assessing glycoprotein signal peptide cleavage site and solubility score

SignalP 4.1 server was used to predict signal peptide score (S), the cleavage score (C) and Y-score for LNYV and RABV glycoproteins using neural networks trained on eukaryotic sequences [33, 34]. C-score and S-score are probability-based estimates derived from neural network algorithms based on experimentally determined signal peptides from both prokaryotic and eukaryotic data sets [35]. The S-score is the probability that a specific amino acid is part of the signal peptide while the C-score is the probability that the amino acid is part of the mature protein sequence. The Y-score is the combination of C-score and S-score and the cleavage site is predicted to be at the position with the highest value [36].

CamSol server [37] was used to predict the solubility of complete LNYV glycoprotein and that of the ectodomain when expressed alone. The server was also used to predict the solubility of the ectodomain following the addition of His6 and Flag-tags to the N-terminus and C-terminus respectively.

**Design and molecular cloning of recombinant LNYV glycoprotein**

The recombinant LNYV glycoprotein was designed based on the results of analyses with SignalP 4.1 and CamSol servers. The glycoprotein native signal peptide was substituted with that of RABV CVS 11 strain. The amino acid sequence of RABV signal peptide used was MVPQVLLFVLLLGFSLCFG followed by the two amino acids KF. The full 21 amino-acid sequence was synthesized as two complementary oligonucleotides strands (Sigma-Aldrich, UK). The oligonucleotides included a *Kpn*I endonuclease site at the 5’ end and a *Bsa*I site at the 3’ end (Table 1). Furthermore, LNYV glycoprotein ectodomain was used rather than complete glycoprotein (Fig. 1). The strands were annealed together at equimolar concentrations. The ectodomain of LNYV glycoprotein was PCR-amplified from LNYV cDNA (GeneArt, Germany) using the LeGp set of primers (Table 1). The primers were designed to incorporate the *Bsa*I site followed by a His6-tag at the 5’ end and the FLAG-tag codons followed by *Eco*RI site at the 3’ end of the amplicon (oligonucleotide sequences are provided in Table 1). PCR amplifications were completed using Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, UK) following the manufacturer’s protocol.

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| Primer |  | Sequence (5’ – 3’) |
| LeGp Primers Set | Forward 1 | tgagctggtctcagttccatcatcatcaccatcac |
| Forward 2 | tgagctggtctcatcatcaccatcacgtctttaaccattcggttggaccctt |
| Reverse | accattgaattctcacatatgtaatcactcgagcttgtcgtcatcgtctttgtagtccttgtgagacatgtctaagatcc |
| RABV CVS 11 leader sequence upper strand | | cctatgggtaccatggttcctcaggttcttttgtttgtactccttctggggttttcgttgtgtttcgggaagttctgagaccaatggt |
| RABV CVS 11 leader sequence lower strand | | accattggtctcagaacttcccgaaacacaacgaaaaccccagaaggagtacaaacaaaagaacctgaggaaccatggtacccatagg |

Table 1. Sequence of primers and oligonucleotides used to construct rLGe.pI.18.

RABV signal peptide and the LNYV glycoprotein ectodomain were then ligated together using the *Bsa*I-mediated Golden Gate cloning [38]. The ligation product rLGe was PCR-amplified using RABV CVS 11 leader sequence upper strand oligonucleotides as forward primer and LeGp reverse primer. rLGe PCR product was purified using QIAquick PCR Purification Kit (Qiagen, UK) then cloned into mammalian transient expression vector pI.18 [39] between *Eco*RI and *Kpn*I restriction sites yielding the rLGe.pI.18 construct which was transformed into *E. coli* strain DH5α (Invitrogen, US). The rLGe.pI.18 expression cassette was confirmed to be correct by DNA sequencing.

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Fig. 1. Schematic representing the constituent domains of (a) LNYV (b) RABV CVS 11 (c) recombinant rLGe glycoprotein. SP: signal peptide. H: His6-tag. TM D: transmembrane domain. IC D: intracellular domain. F: FLAG-tag. Amino acid sequence length of each of the domains are indicated beneath schematics (a) and (b). *Eco*RI, *Bsa*I, and *Kpn*I: restriction sites used to assemble rLGe and further cloning into pI.18 vector in schematic (c).

**Transient expression in HEK – 293T cells**

HEK-293T cells acquired from the American Type Culture Collection (US) were maintained in DMEM enriched with 15% (v/v) Fetal Bovine Sera (Sigma-Aldrich) and 1% (w/v) mixture of penicillin (Sigma-Aldrich) and streptomycin (Sigma-Aldrich) at 37°C and 5% CO2. Cultured cells were transfected with purified rLGe.pI.18 vector using FuGENE® 6 Reagent (Promega, UK). After 48 hours incubation, transfected cells were lysed using lysis buffer (50mM Tris HCl pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% (v/v) TRITON X-100), with protease inhibitor cocktail (Complete Mini, Roche, UK) and 0.02% (w/v) sodium azide (Sigma-Aldrich) at 4°C for 20 minutes on a horizontal shaker. Lysate was collected and centrifuged at 13,000 rpm, at 4°C for 10 minutes and kept at -20°C.

**Anti-Flag M2 Gel column purification**

Expressed rLGe glycoprotein was purified using an Anti-FLAG® M2 affinity gel (murine IgG1 mAb covalently attached to agarose by hydrazide linkage) (Sigma-Aldrich). A 0.5 ml gel was dispensed into a 1 cm diameter chromatography column. Supernatant from lysed cells was passed through the column at a flow rate of 1 ml per minute at 4°C overnight. The column was washed with 50 ml of TBS (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% (w/v) sodium azide), and eluted with 6 ml of 0.1 M glycine-HCl pH 3.5. The eluate was neutralized with 1 M Tris pH 8.0. Eluted rLGe glycoprotein was dialyzed overnight at 4°C against 0.01 M ammonium bicarbonate using a 12 ml Slide-A-Lyzer cassette (Thermo Scientific, UK). The dialyzed glycoprotein was vacuum-lyophilized overnight and the lyophilate resuspended in sterile PBS buffer pH 7.4 and stored at - 20°C.

**Eliciting Murine anti-sera against recombinant LNYV glycoprotein**

rLGe glycoprotein suspended in 600 µl of sterile PBS pH 7.4 was mixed with 300 µl of 4.25 mg/ml aluminium hydroxide (Sigma-Aldrich) adjuvant. The mixture was used to immunize two BALB/c mice (6–8 weeks old), with three subcutaneous injections in the base of the tail (0.55 μg rLGe in 150 μl per dose), at 10 day intervals. Two weeks after the final immunization, mice were sacrificed and sera were collected by cardiac puncture [40].

**Western blotting**

To detect rLGe glycoprotein expressed in HEK-293 T cells, samples were heated in NuPAGE® LDS Sample Buffer (Invitrogen) at 70°C for 10 minutes and loaded onto a NuPAGE® Novex® Bis-Tris 4-12% (Invitrogen) separating gel. For sample preparation under reducing conditions, 50 mM dithiothreitol (DTT) (Invitrogen) was used. Separated proteins were then blotted onto Amersham Hybond ECL Nitrocellulose Membrane (GE Healthcare, UK) using a semi-dry transfer system (Invitrogen, UK). To detect the FLAG-tag, membrane was incubated with anti-FLAG® M2−Peroxidase Clone M2 monoclonal antibody (Sigma-Aldrich). C – terminus FLAG-BAP (Bacterial Alkaline Phosphatase) fusion protein 49.1 kDa in size (Sigma-Aldrich) was used as a positive control. To detect His6-tag, membrane was incubated with monoclonal anti-polyhistidine peroxidase conjugate (clone HIS-1) (Sigma Aldrich). The positive control consisted of an irrelevant protein with an N – terminal His6-tag fusion protein (supplied by Dr G. Diogo, St George’s University of London). To detect the presence of LNYV in infected *Nicotiana glutinosa* (acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Leibniz Institute, Germany), plant leaf samples were homogenized in PBS 0.05% (v/v) Tween 20 (pH 7.4) buffer and centrifuged at 13,000 g for 20 minutes at 4℃. Supernatant treatment and preparation was as described for rLGe. Following separation and transfer, the nitrocellulose membrane was incubated with anti-sera harvested from immunized mice diluted 1:1000 in 5% (w/v) skimmed milk in PBS pH 7.4 buffer, followed by incubation with sheep anti-murine IgG HRP conjugated antibody (The Binding Site, UK). The membrane was developed by AmershamTM ECLTM Prime Western Blotting Detection Reagent (GE Healthcare UK Limited, UK) and visualized using SynGene G:Box gel imaging technology with Genesys software (Syngene, UK).

**Results and Discussion**

The highest Y-score, predicting cleavage occurrence, for LNYV glycoprotein with native signal peptide was 0.429 between amino acids at position 25 and 26 VQG|VF (Fig. 2a) which is the same as predicted previously in genomic analysis [7]. The Y-score for RABV CVS 11 strain glycoprotein with native signal peptide was 0.666 between amino acids at position 19 and 20 CFG|KF (Fig. 2b). However, when the native signal peptide of LNYV glycoprotein was substituted with that of RABV CVS 11 (including the post-cleavage site KF amino acids) the Y-score was elevated to 0.796 (Fig. 2c).



Fig. 2. SignalP 4.1 server prediction plots for the cleavage site between the signal peptides and the continuum of the glycoprotein of (a) LNYV, (b) RABV CVS 11 strain and (c) rLGe. C-score is the predicted cleavage site value, S-score is the predicted signal peptide value. Y-score is the geometric average between the C-score and S-score.

Previous studies have indicated the importance of the signal peptide in expression of viral glycoproteins. For example, analyzing signal peptide cleavage kinetics of HIV-1 envelope glycoprotein, the signal peptide was shown to retard cleavage and delay folding of the gp160 and gp120 glycoproteins when expressed in mammalian cells [41]. When the native signal was substituted with that of the human tissue plasminogen activator, the yield increased significantly. The secretion of glycoprotein was not limited by transcription, but by signal peptide sequence-dependent downstream processing [42]. This was also observed with vesicular stomatitis virus (VSV) glycoprotein expression [43]. Low efficacy signal peptide cleavage may trigger disruption of nascent protein synthesis and cause low expression levels [44-46].

CamSol server was used to analyse glycoprotein solubility. Increasing recombinant glycoprotein solubility has been demonstrated to contribute to increase in expression yield [47, 48]. The complete LNYV glycoprotein (excluding signal peptide) solubility score was 0.007 whilst that for the ectodomain was 0.342. Further modifications included the addition of His6-tag at the N-terminus and FLAG-tag at C-terminus which increased the solubility of the resulting rLGe glycoprotein to 0.543 at pH 7.

Based on the results of SignalP 4.1 and CamSol server analyses, we designed rLGe which comprised the signal peptide sequence of Rabies virus CVS 11 strain joined to the ectodomain of the LNYV glycoprotein with His6 and FLAG-tags at N and C-termini respectively.

rLGe, with a theoretical size of 56.6 kDa, could be detected in the cellular extract in the transfected cells using anti-FLAG® monoclonal antibody as shown in Fig. 3a. No bands could be detected in non-transfected cells while the positive control showed a band at approximately 50 kDa. rLGe could not be detected in the growth medium of the transfected cells (Fig. 3b) suggesting that it was not secreted out of the cell.

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Fig. 3. Western blot analysis of recombinant LNYV glycoprotein expression in HEK - 293T cells. (a) 1 µl per lane of cellular extract was run under reducing conditions on Bis-Tris 4-12% (w/v) NuPAGE gel. (b) 3 µl per lane of growth medium was run under reducing conditions on Bis-Tris 4-12% (w/v) NuPAGE gel. Detection was with anti-FLAG® M2−Peroxidase Clone M2 monoclonal antibody. (c) 1 µl per lane of cellular extract was run under reducing conditions on Bis-Tris 4-12% (w/v) NuPAGE gel. Detection was with anti-polyhistidine peroxidase conjugate (clone HIS-1) monoclonal antibody. Tr-HEK: HEK – 293 T cells transfected with rLGe.pI18 construct sample. WT-HEK: wild type HEK – 293 T cells sample. F-BAP: C – terminus FLAG-BAP fusion protein sample. Pos.H: N – terminus His6-tag protein sample.

In our design for rLGe, we removed both the cytoplasmic and transmembrane domains for expression of soluble glycoprotein. In published studies on VSV glycoprotein, efficient export from the ER required the Asp-X-Glu (where X represents any amino acid) di-acidic motif present in the cytoplasmic tail [49], the complete removal of which blocked ER exit [50]. Interestingly, in the LNYV glycoprotein, there is the Asp-X-Gln motif at the C-terminus of the cytoplasmic domain which was removed in the construction of rLGe. Whilst this is not a di-acidic motif and its function is unknown, we speculate that the cytoplasmic domain might have a role in export from the ER.

rLGe was not detected using anti-His-tag monoclonal antibody, although the positive control, an irrelevant His-tagged protein, gave an immunoreactive band at approximately 30 kDa (Fig. 3c). A marked variability in immunodetection of His-tag joined to different proteins has been previously reported [51]. A possible explanation for our inability to detect the His-tag here could be the low solubility of the rLGe N – terminus indicated by CamSol server analysis (data not shown) potentially causing histidine residues to be inaccessible to the detecting antibody.

To assess the ability of the elicited anti-sera to detect LNYV in virus-inoculated leaf samples, *Nicotiana glutinosa* infected with LNYV was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Leibniz Institute, Germany). Samples of rLGe and wild type *N. glutinosa* were also run in parallel under reducing and non-reducing conditions and immunoblotted using the harvested murine anti-sera as shown in Fig. 4. The anti-sera detected bands in the LNYV-inoculated leaf sample both in reducing and non-reducing conditions. The theoretical size of the LNYV complete glycoprotein excluding the signal peptide is 59.7 kDa. No bands could be detected in wild type *N. glutinosa*, indicating that the murine anti-sera were immunoreactive with the naturally occurring glycoprotein of the LNYV particles.

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Fig. 4. Western blotting of LNYV using anti-sera from mice immunized with rLGe. Samples were run under reducing or non-reducing conditions on Bis-Tris 4-12% (w/v) NuPAGE gel. Detection was with elicited murine anti-sera diluted 1:1000 followed by sheep anti-mouse immunoglobulin conjugated to HRP diluted 1:5000. nR-Li-Ng: non-reduced LNYV infected *N. glutinosa*. R-Li-Ng: reduced LNYV infected *N. glutinosa*. nR-rLGe: non-reduced rLGe. R-rLGe: reduced rLGe. nR-WT-Ng: non-reduced wild type *N. glutinosa*. R-WT-Ng: reduced wild type *N. glutinosa*. Asterisks indicate immunoreactive bands.

The immunoreactive band in the reduced sample from LNYV infected leaves was apparently larger than that of the non-reduced sample. This may have been due to the effect of disulphide bridges on the electrophoretic mobility of the glycoprotein as previously reported [52]. There may be differences in the exposure of hydrophobic regions of glycoproteins in reduced and non-reduced states causing anomalous binding to the detergent and a discrepancy in migration patterns [53-55].

The murine anti-sera also detected rLGe in non-reduced and reduced forms. The theoretical size of rLGe excluding the signal peptide is 56.6 kDa. The major band seen in the reduced sample was similar in size to that of the natural LNYV glycoprotein. However, a number of bands were seen in the non-reduced sample at approximately 110, 140 and 180 kDa. These may be explained by incomplete dissociation between the purified rLGe and other proteins during sample treatment or by intra-chain disulphide bond formation and generation of multimers within the cells [56]. A positive correlation between disulphide bonds formation and the growth temperature of the cell has been previously observed [57-59]. The LNYV life cycle takes place in lettuce whose optimal growth temperature is ~25°C [60] whereas HEK-293T cells were maintained at 37°C. Furthermore, the presence of non-natural disulphide bond arrangements could be explained by the rLGe potentially accumulating in the ER, resulting in aberrant bond formation [58, 61]. Similar high molecular weight bands were not observed when detecting rLGe with anti-FLAG® antibody, as samples were processed under reducing conditions.

**Conclusion**

Our study provides a model for recombinant expression of viral glycoproteins and generation of anti-sera. Expression of LNYV glycoprotein in mammalian cells, initially unsuccessful, was achieved by substituting native LNYV glycoprotein signal peptide with an equivalent from a related virus, which infects mammals. Recombinant glycoprotein was further modified to allow expression by truncation of the transmembrane and intracellular domains to increase glycoprotein solubility. The purified recombinant glycoprotein was used as an immunogen for the production of murine anti-sera which effectively detected naturally occurring LNYV glycoprotein. The anti-sera will be used in our ongoing attempts to develop a reverse genetics system for LNYV [20] allowing us to detect and purify the virus.

# GenBank Accession Numbers

Recombinant LNYV glycoprotein GenBank accession no. **AJ251533.1** and RABV CVS 11 strain accession no **EU126641.1** were used in this research.

# Conflicts of interest

# The authors declare no conflict of interest.

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