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Transient Expression of an Immunogenic Envelope Attachment Glycoprotein of Nipah Virus in *Nicotiana benthamiana*

(Pengekspresan Protein Glikoprotein Virus Nipah secara Transien dalam *Nicotiana benthamiana*)

GAN KHAI SWAN, TAN CHON SENG, ROFINA YASMIN OTHMAN & JENNIFER ANN HARIKRISHNA*

ABSTRACT

The Nipah virus is highly virulent to swine and humans. The envelope attachment glycoprotein (G) of Nipah virus plays a key role in viral entry and induction of neutralizing antibody in mammalian hosts, thus is considered a good candidate for vaccine development. Plant transient expression systems are gaining recognition as a viable alternative for the production of vaccine antigens. In this study, we expressed the Nipah virus G protein heterologously in Nicotiana benthamiana *using an agroinfiltration approach. The highest expression of recombinant G protein in* N. benthamiana *at RNA and protein levels was detected on day 9 post-infiltration. Western blot analysis demonstrated that the purified G protein reacted specifically with rabbit anti-Nipah Virus serum, indicating its potential for vaccine use.*

Keywords: Biotechnology; KDEL; plants; tobacco PR1a; vaccine

ABSTRAK

Virus Nipah adalah virus yang sangat virulen bagi ternakan khinzir dan manusia. Pelekat sampul sel gliko protein G virus Nipah memainkan peranan penting dalam kemasukan virus dan peneutralan antibodi di dalam sel perumah mamalia. Oleh itu, ia dianggap sebagai satu calon yang baik untuk dijadikan vaksin. Memandangkan sistem asai transien dalam tumbuhan semakin diberi perhatian sebagai cara alternatif penghasilan vaksin, protein G virus Nipah telah diekspres dalam tumbuhan Nicotiana benthamiana *menerusi kaedah agroinfiltrasi di dalam kajian ini. Dalam kajian ini, pengekspresan RNA dan protein G dalam* Nicotiana benthamiana *mencapai tahap maksimum pada hari ke-9 selepas infiltrasi. Protein G yang ditulenkan menunjukkan tindak balas yang khusus dengan serum arnab anti-Nipah virus dalam analisis pedapan Western. Penemuan ini menunjukkan protein rekombinan G virus Nipah berpotensi dijadikan vaksin.*

Kata kunci: Bioteknologi; KDEL; tembakau PR1-a; tumbuhan; vaksin

INTRODUCTION

Nipah virus (NiV) belongs to the genus *Henipavirus* and is closely related to the type species, Hendra virus (Eaton et al. 2006; Mayo 2002; Wang et al. 2000). NiV is a pathologenic paramyxovirus, which has been found to be the etiologic agent for both human and swine diseases (Chua et al. 2000). It was first isolated in 1999 from pigs and adult human males affected by fever and encephalitis, some with respiratory illness, during a major outbreak in peninsular Malaysia and then Singapore (Chua et al. 1999; Paton et al. 1999). Most of the people affected in the Malaysian outbreak had a history of direct contact with live pigs and the majority pig farmers of Chinese adult male ethnic with a 40% clinical case mortality rate (Parashar et al. 2000).

The known natural reservoir hosts of NiV are pteropid fruit bats, commonly known as flying foxes, which do not exhibit any clinical disease when infected (Halpin et al. 2011). NiV can infect and cause disease in a wide range of species with very high fatality rates (Geisbert et al. 2012; Wong & Ong 2011). Fatal NiV outbreaks among people have occurred almost annually since 2001 (Broder 2012; Luby et al. 2009) most of them in India and Bangladesh. Due to poor surveillance in rural areas in Bangladesh whereNiV is endemic, the true disease burden is uncertain and likely many cases go undetected, however the estimated case-fatality rate is 67% (Herriman 2015). Unlike the Malaysian outbreak, the transmission method of NiV in Bangladesh and India was from bats to humans in the absence of an intermediate animal host via food contaminated with bat saliva (Clayton et al. 2016; Luby et al. 2012). On the other hand, human infection in the Bangladesh outbreak is associated with drinking traditional liquor made from date palm sap (Islam et al. 2016).

The NiV genome encodes two different surface glycoproteins, the receptor-binding G protein and the fusion (F) protein. The main role of the G protein is to recognize and attach the virus to receptors on host cell membranes. It also facilitates the F-mediated membrane fusion process via a mechanism of receptor-induced paramyxovirus F triggering during viral entry and cell-cell fusion as proposed by Liu et al. (2015).

Current prevention of NiV disease in endemic countries relies on behavior modifications to prevent

spillover, including farming practices which decreased livestock exposure to bats, the use of bamboo skirts to prevent date palm sap contamination and pasteurizing date palm sap. Though these approaches appear to be effective strategies of prevention, their implementation is often problematic due to cultural factors (Satterfield et al. 2016). Effective therapy and vaccine are needed to combat the threats posed by NiV. Various candidate vaccine platforms have demonstrated the feasibility of using one or both of the NiV outer-membrane proteins, the glycoprotein (G) and fusion (F) protein, as antigen(s) to stimulate a protective immune response in various preclincal animal challenge models such as hamsters (Debuysscher et al. 2014; Lo et al. 2014) cats (McEachern et al. 2008), ferrets (Mire et al. 2013; Palliser et al. 2013), African green monkeys (Prescott et al. 2015) and pigs (Weingartet et al. 2006). Little or no clinical signs of disease were observed in vaccinated animals after NiV challenge and protection against mortality was typically 100% depending on the vaccination route and dose (Broder et al. 2016). Another group of scientists, Yoneda et al. (2013) proposed a recombinant measles virus vaccine expressing NiV envelope glycoprotein as an appropriate NiV vaccine candidate for use in humans.

Plant systems have been used to produce recombinant proteins as they provide some advantages over other expression systems. Plant transient expression can be used to produce vaccines at low cost on an agricultural scale and with a low risk of contamination of the product with animal pathogens as reviewed in (Chen & Lai 2015). Furthermore, plant-based vaccines have been proposed as a new approach for oral delivery such as expression of recombinant protein in edible plants like corn and rice (Kapila et al. 1997; Nochi et al. 2007; Shoji et al. 2011). Other instances of successful expression of plant-based vaccine antigens or virus-like particles include foot and mouth disease virus structural protein VP1 expressed in transgenic alfalfa plants (Wigdorovitz et al. 1999), chimeric peptide containing antigenic determinants from rabies virus glycoprotein and nucleoprotein expressed in *Nicothiana* sp. plants (Yusibov et al. 2002), cholera toxin B subunit expressed in transgenic tobacco chloroplasts (Daniell et al. 2001), recombinant subunit hemagglutinin of influenza A virus expressed in *Nicothiana benthamiena* (Kalthoff et al. 2010; Mett et al. 2008; Shoji et al. 2011, 2008) and extracellular domain of matrix protein 2 (M2e) of influenza A virus expressed in *Nicothiana benthamiena* (Mardanova 2015).

In the current study, we investigated expression of the recombinant NiV G protein in *N. benthamiana* using a non-replicating transient expression vector. The purified product showed reactivity with the antibodies present in anti-NiV antisera, as demonstrated in Western blot analysis. This study provides an important exploration of a plant-synthesed recombinant NiV G protein as a vaccine candidate.

Materials and Methods

Plant viral vector and antiserum

CPMV-based plant viral expression vector, pEAQ-HT was a kind gift from Professor George Lomonosoff (John Innes Centre, UK). Rabbit anti-NiV serum was obtained from the Veterinary Research Laboratory (VRI), Ipoh, Malaysia.

Bacterial strains, plant materials and growth conditions

E.coli (XL1-Blue, Stratagene) and *Agrobacterium tumefaciens* strain LBA4404 were used in molecular cloning experiments and were routinely cultured at 37ºC and 28ºC, respectively, in Luria-Bertani (LB) media supplemented with appropriate antibiotics: Ampicillin (100 µg/mL), kanamycin (50 µg/mL) and rifampicin (50 µg/mL). *N. benthamiana* plants were grown in a greenhouse and maintained at 20ºC ± 2, under a 12-h light/dark cycle.

Construction of recombinant vector and transformation of agrobacterium

The NiV G gene synthesis (GenBank accession no. HM545086.1) was carried out by Genescript USA (www.genescript.com). A cDNA fragment encoding the NiV G gene was synthesized de novo as shown in Figure 1 and with the following elements: *Age*I and *Sma*I restriction sites were added at the 5' and 3' ends, respectively; the native signal region of NiVG protein was replaced with a the tobacco PR1a signal sequence of pathogenesis-related protein (90 bp) GenBank accession no. X06930); the native NiV G protein transmembrane domain (148 bp - 210 bp) was eliminated; an endoplasmic reticulum (ER) retention signal peptide (KDEL) and a cMyc-tag were added the C-terminal encoding region to facilitate target protein detection. The synthetic recombinant NiV G construct was cloned into a Cowpea mosaic virus-based plant expression binary vector, pEAQ-HT (Sainsbury et al. 2009a), using the *Age*I and *Sma*I (Promega, USA) restriction sites. The ligation generated an N-terminal hexahistidine-tag to the NiV G protein construct, for affinity purification and a stop codon to end the translation. The resulting recombinant construct (named as NiV-G/pEAQ) was then transformed into XL1-Blue *E. coli* competent cells by the heat shock method. The colonies containing the recombinant plasmid were identified and confirmed using colony PCR and by DNA sequencing using a commercial service (1st Base, Malaysia). PCR was performed in a 25 µL reaction volume containing 0.05 U of *Taq* Polymerase, 2.5 µL buffer (50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$), 100 ng template DNA, 200 µM of each dNTP and 25 nM of each primer (NiVGF 5' ACC GGT ATG GGA TTT GTT CTC 3' and NiVGR 5' CCC GGG CAG ATC TTC TTC AGA 3'). PCR was run in a programmable thermocycler (Biometra, Germany) with an initial denaturation at 94°C for 4 min followed by 20 cycles of denaturation at 94°C for 45 s, then annealing at 45°C for 45 s followed by extension at

72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR-amplified product was resolved in 1% (w/v) agarose gel by electrophoresis at 80 V and analyzed using Gene Flash Syngene Bio Imaging (TopoGEN, USA). One microliter of the purified recombinant plasmids was then introduced into chemically competent *A. tumefaciens*, LBA 4404 by transformation using a freeze-thaw method (Weigel & Glazebrook 2006).

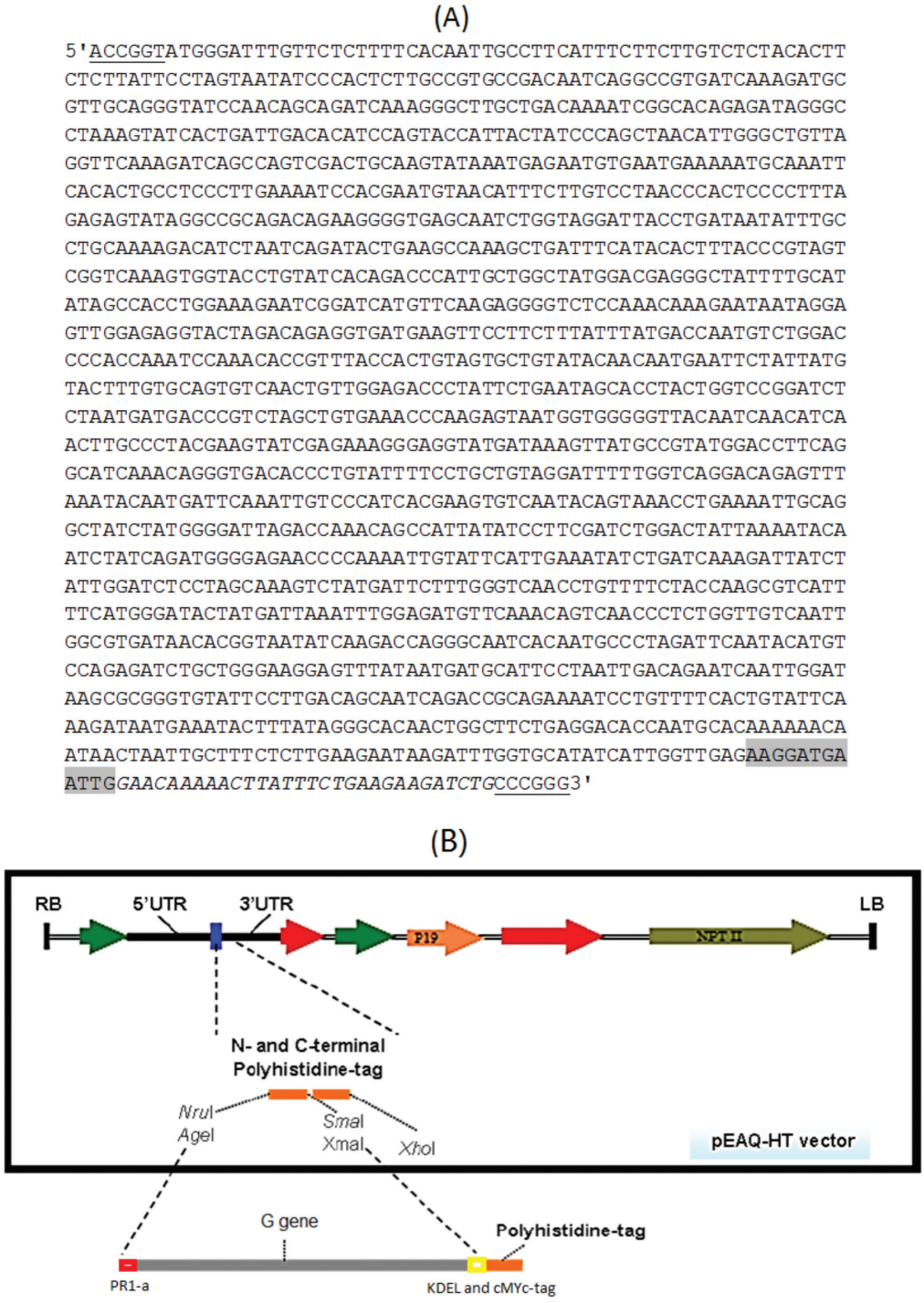

FIGURE 1. Construction of recombinant vector, NiV-G/pEAQ. (A) Sequence of the de novo synthesised cDNA fragment encoding NiV G. Restriction sites (AgeI and SmaI), are underlined; signal sequence (tobacco PR1a) shown in bold; NiV G cDNA with removal of native signal sequence and transmembrane domain are shown in normal font; KDEL sequences are shaded with grey and cMyc-tag sequences are italicised. (B) Schematic diagram of pEAQ-HT vector and insertion position of G gene in between AgeI and SmaI sites (Adapted from Sainsbury et al. 2009a)

AGROINFILTRATION OF *NICOTIANA BENTHAMIANA*

A. tumefaciens LBA4404 clones containing either empty vector pEAQ-HT or recombinant NiV-G/pEAQ were confirmed by colony PCR and thereafter named Ag/pEAQ-HT and Ag/NiVG-pEAQ, respectively. The cultures were grown to stable phase with an optical density (OD_{600}) of 1.2-1.4 in LB medium supplemented with 50 μg/mL of rifampicin and kanamycin at 28°C and shaken at 200 rpm. The cells were then collected by micro-centrifugation at 6,000 rpm for 10 min at 4°C and resuspended in infiltration buffer (10 mM 2-N-morpholinoethane sulphonic acid (MES), pH5.5, 10 mM $MgCl_2$, 0.1 mM acetosyringone) to an OD_{600} of between 0.3 and 0.4. After 2 h incubation at room temperature to induce the virulence gene, the suspensions were infiltrated into fully expanded mature leaves of 8-week old *N. benthamiana* plants using a syringe without a needle. After infiltration, the plants were grown under greenhouse conditions at 20±2ºC, under a 12-h light/dark cycle. The growth and physical appearance of the infiltrated leaves were monitored and recorded.

MRNA ASSESSMENT OF NIVG/PEAQ-INFILTRATED PLANT SAMPLES

Leaf samples from both Ag/pEAQ-HT and Ag/NiVG-pEAQ-infiltrated plants were harvested on 3, 6, 9 and 12 days post-infiltration (dpi) for total RNA extraction using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. Reverse transcription (RT) was conducted on the RNAs extracted using a Viva 2-step RT-PCR kit (Vivantis, Malaysia). The first-strand cDNA synthesis reaction mixture was prepared by adding 2 μg of total RNA, 2 μM gene-specific primer (NiVGR), 0.5 mM dNTP mixture and DEPC-treated water up to a total of 10 μL volume. Mixtures were incubated at 65°C for 5 min then placed on ice for 2 min. A 10 μL mixture of 1X RT buffer (500 mM Tris-HCl (pH 8.3 at 25°C), 750 mM KCl, 30 mM $MgCl_2$ and 100 mM DTT) and 100 U MMuLV Reverse Transcriptase was added to each tube. The mixture was incubated at 42°C for 60 min. The reaction was terminated at 85°C for 5 min and chilled on ice. PCR reactions were as described in the previous section and included a reaction with RNA without first strand cDNA synthesis as a negative control to track DNA contamination. The PCR products were then examined following electrophoresis at 80 V in 1.0% (w/v) agarose gel. RT-PCR reactions were performed in triplicate. ImageJ software was used to quantify the absolute band intensity for comparison of expression at different dpi.

PROTEIN EXTRACTION AND SDS-PAGE ELECTROPHORESIS

Proteins were extracted from the Ag/pEAQ-HT and Ag/NiVG-pEAQ infiltrated leaves at 3, 6, 9 and 12 dpi as follows: Inoculated leaf tissues were frozen in liquid nitrogen and ground to powder using mortar and pestle. Then, the samples were suspended in 2 volumes of soluble protein buffer (50 mM Tris-HCl pH 7.25, 150 mM NaCl, 2 mM DTT and 1/100 protease inhibitor cocktail) and micro-centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected and stored at 4°C. Extracted protein was quantified by spectrophotometer (ND-1000, Nanodrop) using a Bio-Rad protein assay with bovine serum albumin standards. Following this, protein was separated by electrophoresis at 120 V for 90 min in 12% SDS-PAGE.

PROTEIN PURIFICATION

A column was prepared using 5 mL of nickel–nitrilotriacetic acid (Ni–NTA) agarose (Life Technologies, USA) that was suspended thoroughly and pre-equilibrated. The supernatant was loaded onto the column. The protein-bound Ni–NTA resin was first washed with 10 mL of denaturing binding buffer (8 M urea; 20 mM $NaPO_4$, pH 7.8; 500 mM NaCl) followed by washing with 20 mL of denaturing wash buffer (8 M urea; 20 mM $NaPO_4$, pH6.0; 500 mM NaCl). The column was washed with another 20 mL of the above washing buffer at pH5. The bound recombinant protein was eluted with 10 mL denaturing elution buffer (8 M urea; 20 mM $NaPO_4$, pH4.0; 500 mM NaCl) and the sample fractions were analyzed by 12% SDS-PAGE and Western blotting (as described below). The fractions containing the purified recombinant protein were pooled and dialyzed against 6 M urea for 6 h. The dialysis was continued with the addition of 250 mL of 25 mM Tris–HCl (pH7.5) every 12 h for 36 h, then the dialysis solution was replaced with 25 mM Tris–HCl (pH7.5), 150 mM NaCl, 0.2% Triton X-100 and 5 mM β-mercaptoethanol and dialysed for another 12 h. The concentration of the recombinant G protein pooled from the eluted fractions containing the purified recombinant protein was quantified with Bio-Rad protein assay reagent (Bio-Rad, USA) using bovine serum albumin as a standard.

WESTERN BLOTTING ANALYSIS

After protein sample separation on 12% SDS-PAGE gel, the gel was either stained with Coomassie Brilliant blue or transferred onto nitrocellulose membranes (Invitrogen, USA) by electroblotting. Membranes were blocked with 5% (w/v) non-fat dried milk in 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.05 % (v/v) Tween-20) to prevent nonspecific binding of antibodies and then probed with primary antibody for 2 h at room temperature. Then, each membrane was washed three times with 1X PBS buffer and incubated with secondary antibody for 1.5 h at room temperature. The following antibody combinations were used: Murine anti-cMyc antibody (1/500 dilution) as the primary antibody and alkaline phosphatase conjugated rabbit anti-mouse IgG (1/5000 dilution) as the secondary antibody; primary rabbit polyclonal IgG antibody against NiV (1:10,000 dilution) generously provided by the Veterinary Research Laboratory (VRI), Ipoh, Malaysia and alkaline phosphatase conjugated rabbit anti-rabbit IgG as the secondary antibody (Zymed, USA) (1: 10,000 dilution). After washing three

times in 1X PBS buffer, the membranes blotted with G-protein were incubated with 3 mL of 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) colour development substrate solution (Sigma-Aldrich) until signal was observed. Western blot was performed in triplicate. ImageJ software was used to quantify the absolute band intensity for comparison of expression at different dpi.

Results

CONSTRUCTION AND TRANSFORMATION OF NiV-G/pEAQ

The NiV G gene was inserted successfully into pEAQ-HT vector via *AgeI* and *SmaI* restriction sites. Following the gene cloning, the recombinant vector was characterised and identified by PCR and restriction endonuclease digestion. The digestion of the vector with *AgeI* and *XhoI* yielded fragments of approximately 10 kbp and 1.65 kbp as expected for pEAQ-HT vector and NiV G fragments, respectively, while digestion of the vector with *SmaI* yielded a band of about 12 Kbp, the expected size for the linearized NiVG/pEAQ (Figure 2A). The resulting recombinant vector was introduced into *A. tumefaciens*, LBA 4404 by transformation and the recombinant transformants were selected using kanamycin (100 µg/mL) on LB agar plates and confirmed by colony PCR (Figure 2B).

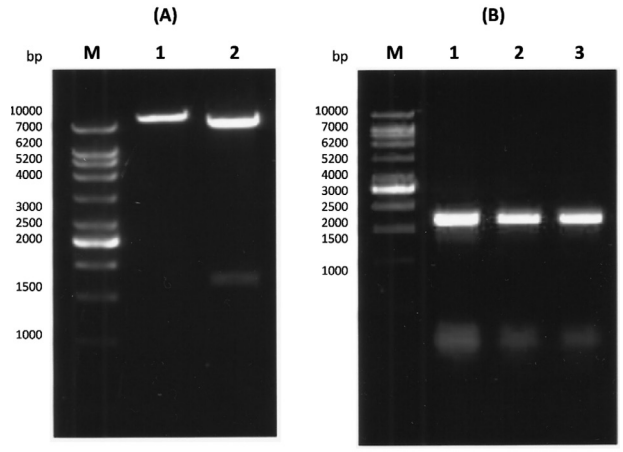

FIGURE 2. Restriction digestion profile and colony PCR of recombinant NiVG/pEAQ

(A) Restriction digestion profile of recombinant NiVG/pEAQ. Lane M: Vivantis 1kb ladder; Lane 1: NiVG/pEAQ digested with SmaI; Lane 2: NiVG/pEAQ digested with AgeI and SmaI. (B) Lanes 1-3: Colony PCR of recombinant NiVG/pEAQ. Lane M: Vivntis 1kb ladder

TRANSIENT EXPRESSION, PURIFICATION AND QUANTIFICATION OF RECOMBINANT PROTEIN

When infiltration was conducted with Ag/pEAQ-HT, the plant leaves showed the infiltrated zones bleached gradually (Figure 3). RT-PCR amplification of RNA samples extracted from Ag/NiVG-pEAQ-infiltrated leaves at 3, 6, 9 and 12 dpi using G-specific primers (NiVGF and NiVGR) produced a distinct band of approximately 1.65 kbp, indicating transcription of *G* gene sequences in the plants (Figure 4). Transcripts were detected 3 days after inoculation and reached maximum expression at 9 dpi.

After total protein extraction and Western blotting analysis, the soluble fraction of protein was specifically recognised by anti-cMyc antibody. Bands of approximately 60 kDa corresponding to the predicted size for the recombinant G protein were observed on Western blots of the protein fractions from infiltrated leaves at 3, 6, 9 and 12 dpi (Figure 5). The strongest band was observed from the protein fraction of infiltrated leaves at 9 dpi which showed the maximum expression of recombinant NiV G protein. Moreover, the recombinant NiV G protein showed reactivity with rabbit anti-NiV serum. Based on the expression results from RT-PCR (Figure 4) and Western blotting (Figure 5), infiltrated plants at 9 dpi showed the highest expression and so plants at 9 dpi were harvested for NiG protein quantification. The recombinant protein was purified using Ni-charged IMAC resin and each 1 mL elution fraction was collected and analysed by SDS-PAGE (Figure 6). Purified protein was observed in elution fractions 2 to 6 and those fractions were pooled, dialysed and the protein concentration determined, giving a maximum estimated yield of 0.1 mg recombinant NiV G-protein per gram of fresh weight of leaves.

Discussion

Outbreaks of NiV with human fatalities have occurred almost annually since 2001 in India and Bangladesh (Broder 2012; Clayton et al. 2016; Herriman 2005; Luby et al. 2009). Hence, it is imperative to find effective measures to avert future NiV pandemics. Vaccine development has

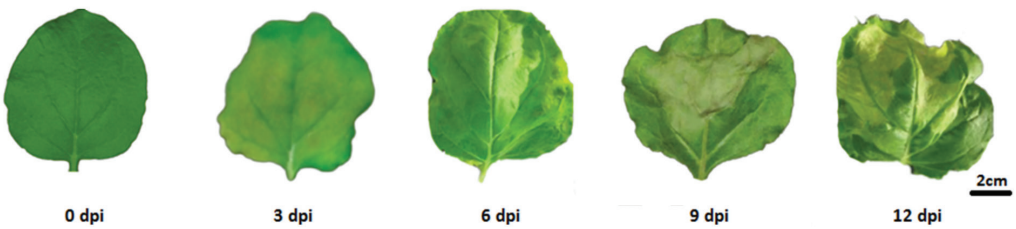

FIGURE 3. Physical appearances of *N. benthamiana* leaves infiltrated with Ag/NiVG-pEAQ cultures

Each leaf was infiltrated with OD600 0.3-0.4 of Ag/NiVG-pEAQ cultures. Representitve leaves shown at 0, 3, 6, 9 and 12 dpi.

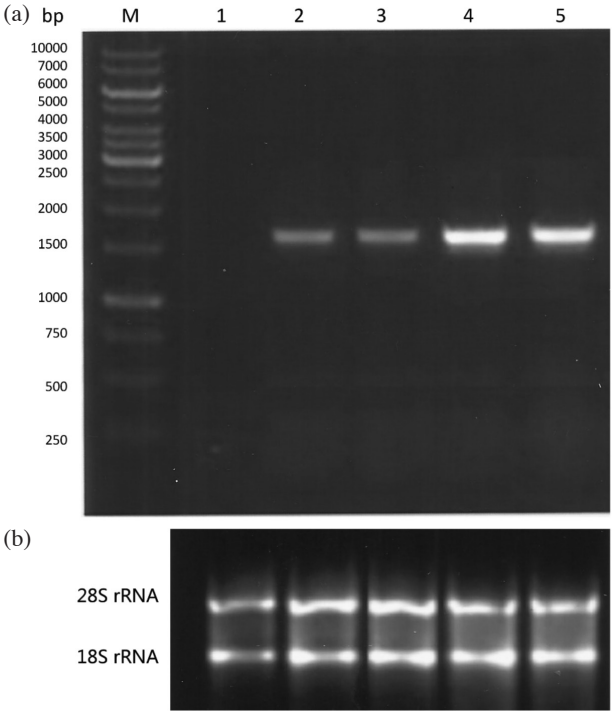

FIGURE 4. RT-PCR amplification of RNA extracted from *N. benthamiana* leaves infiltrated with Ag/NiVG-pEAQ

(a) Upper panel: Lane M: FermentasGeneRuler™ 1 kb ladder; lane 1: cDNA of N. benthamiana leaves infiltrated with Ag/pEAQ-HT harvested at 9 dpi as a negative control; lanes 2-5: cDNA of Ag/NiVG-pEAQ-infiltrated leaves harvested at 3, 6, 9 and 12 dpi, respectively. Lower panel: ethidium bromide-stained agarose gel showing 28S and 18SrRNA of the RNA samples used in RT-PCR. (b) Mean band intensity value of RT-PCR shown in (A).

become crucial to combat this disease. Some vaccines for NiV have been developed and some of the products are now in clinical trials, such as single-dose live-attenuated vesicular stomatitis virus-based vaccine (DeBuysscher et al. 2016; Prescott et al. 2015), a subunit vaccine, consisting of a recombinant soluble and oligomeric form of the G glycoprotein of Hendra virus (Bossart et al. 2005; Mason et al. 1992; Satterfield et al. 2016).

In this study, a plant-based expression system was chosen as such systems have been shown to have promise for vaccine development, for example Hepatitis B virus vaccine which is the first plant-based vaccine produced by tobacco in 1992 (Mason et al. 1992), Influenza virus vaccine (Petukhova et al. 2013), and Ebola virus vaccine (Qiu et al. 2014), both produced in *N. benthamiana*. Plant systems are favoured as they are considered safe, low-cost, rapid to upscale and less vulnerable to contamination with human or animal pathogens compared with traditional inactivated vaccines and animal or microbial cell culture–based vaccines (reviewed in Rybicki 2014). *N. benthamiana* was chosen for this study because of its status as a well-established expression host, its high biomass yields and its rapid scalability. This species is popular as a non-food, non-feed crop that reduces the risk of non-food transgenic proteins, such as those for medicinal applications, contaminating the human food chain (Fischer et al. 2004).

Various virus-based vectors have been engineered and applied successfully for recombinant protein production in plant systems, for example tobacco mosaic virus (TMV)-based (Lindbo 2007; Musiychuk et al. 2007; Roy et al. 2010). and cowpea mosaic virus (CPMV)-based vectors (Sainsbury & Lomonossoff 2008). However, none of these vectors is commercially available. In the present study, a versatile vector, pEAQ-HT, was chosen because this vector allows rapid and simple transient expression of multiple protein products from a single plasmid with a great reduction of plasmid backbone size; the P19 coding gene sequences incorporated into the T-DNA region and single step insertion of target gene sequence which eases preparation. This vector system also lacks the concerns of bio-containment as no infectious virus particles are produced (Sainsbury et al. 2009a; Sainsbury & Lomonossoff 2008). The vector pEAQ-HT is based on an adapted version of CPMV RNA2, with a hyper translatable 5'-leader sequence and modified 3'-unstranslated region (UTR) within pBINPLUS binary vector (Sainsbury & Lomonossoff 2008). This vector has been very useful for heterologous protein expression in *N. benthamiana* leaves Sainsbury et al. 2009b) and in suspension cell cultures (Sun et al. 2011).

In order to express NiV G protein in a plant-based expression system, we constructed a plant virus-based vector, NiV-G/pEAQ and expressed this transiently in *N. benthamiana* as a step towards potential vaccine development for protection against this human and animal pathogen. Several criteria were considered for high level expression of functional NiV G protein in *N. benthamiana*: the replacement of the native viral signal peptide of NiV G protein with PR1-a signal peptide, removal of the N-terminal transmembrane domain, and the addition of endoplasmic reticulum (ER) retention signal. The replacement of the native NiV G protein signal peptide with a cleavable apoplast targeting signal from tobacco pathogenesis related protein PR1-a and the removal of transmembrane domain, were to promote the target gene expression in *N. benthamiana*. This was demonstrated by Agarwal et al. (2000), where 1.5-fold higher expression was observed for human α-1-antitrypsin protein expressed using a PR1-a targeting signal as compared to other signal peptides used. This signal peptide has been regularly used for heterologous expression in *N. benthamiana* together with ER retention signal KDEL (Kanagarajan et al. 2013; Eshaghi et al 2005). As with other heterologous protein expression application, one crucial concern for vaccine production is protein solubility. In the case of NiV G protein, the presence of the transmembrane domain, the membrane anchor sequence on NiV G gene is known to interfere with solubility of the recombinant NiV G protein, so it is common practice in NiV G protein expression to remove it (Bossartet al. 2005; Eshaghi et al 2005; Liu et al. 2007). Hence, in the present study, to increase solubility, the transmembrane domain was removed.

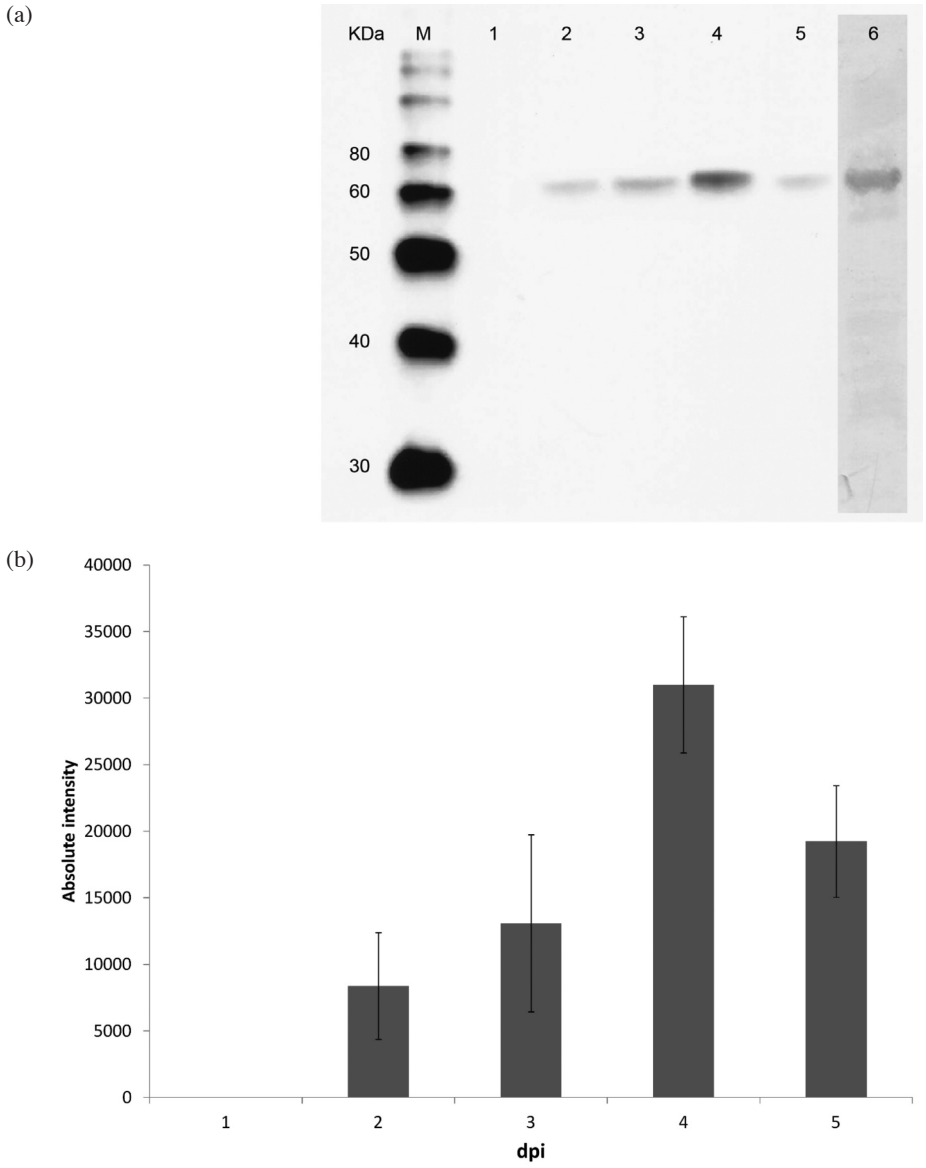

FIGURE 5. Western blot of G protein isolated from *N. benthamiana* leaves

(a) Lane M: molecular weight marker (MagicMark™ XP Western Protein Standard); Lane 1: Leaves infiltrated with Ag/pEAQ-HT (negative control) harvested at 9 dpi and probed with anti-cMyc antibody; Lanes 2-5: Leaves infiltrated with Ag/NiVG-pEAQ at 3, 6, 9, and 12 dpi respectively, probed with anti-cMyc antibody; Lane 6, Nickel column-purified G protein extracted at 9 dpi and probed with anti-NiV antibody. (b) Mean band intensity value of lanes 1-5 of Western blot shown in (a). Error bars indicate standard deviation (n=3)

The addition of an endoplasmic reticulum retention signal (KDEL) in the C-terminal region can increase the protein quality and stability as it leads to the retention of the target protein in the ER and prevents the entry of the protein into the Golgi apparatus (Schouten et al. 1996). The prevention of entry of a heterologous protein into the Golgi apparatus can reduce plant-specific glycosylation and improve recombinant protein quality (Petruccelli et al. 2006). The ER contains a relatively low amount of proteolytic enzymes and the presence of molecular chaperones favours disulphide bond formation and correct protein folding, together with an oxidizing environment (Faye et al. 2005; Nuttall et al. 2002) which can improve heterologous protein stability. The positive impacts of ER retention have been documented for various recombinant proteins production, including antibodies (Stoger et al. 2002), human interleukin-4 (Gomord et al. 2004; Ma et al. 2005), SARS corona virus S protein antigen (Pogrebnyak et al. 2005), *Aspergillus phytase* (Peng et al. 2006) and synthetic silk-like protein DR1B (Yang et al. 2005).

In this study, cMyc-tag sequences were fused to the C-terminal encoding region of the G protein cDNA to facilitate target protein detection, while a His-tag sequence was fused to the C-terminal end of G protein cDNA to facilitate the purification of recombinant proteins by Ni-NTA resin. The detection of an approximately 60 kDa protein from Ag/NiVG-pEAQ infiltrated leaves, but not in Ag/pEAQ-HT-infiltrated leaves showed that the plant-

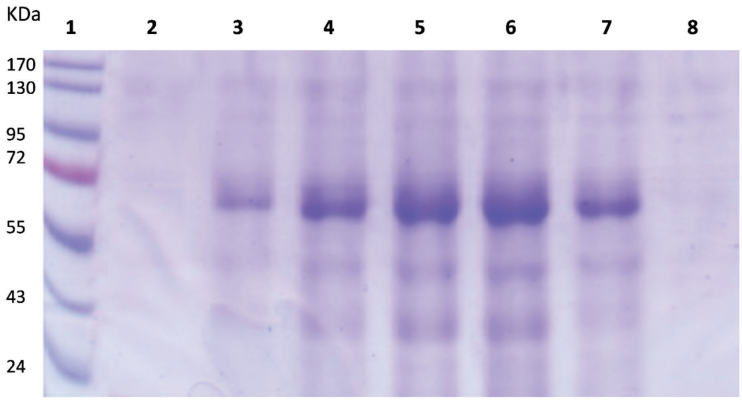

FIGURE 6. Coomassie blue-stained SDS gel image of IMAC fractions for recombinant NiV G protein

Lane 1: Protein marker. Lane 2-8: SDS-PAGE of purified NiV G protein eluted from elution fractions 1 to 7, each of 1 mL volume with 5 µL of each elution loaded into each lane. Ni-charged IMAC resin purification was done on the crude protein and similar band size was detected in elution 2 to 6 with unspecific background reduced to minimum

based Nipah virus G protein had been produced in *N. benthamiana* and could be expressed using a CPMV-based vector system. These results agree with the studies reported by Eshaghi et al. (2005) and Liu et al. (2007) and our own report (Gan et al. 2015) showing that NiV G protein expression at approximate 60 kDa in *Escherichia coli*.

RT-PCR and Western blotting analyses confirmed the transcription and translation of NiV G protein in infiltrated leaves (Figures 4, 5 and 6) with maximum levels of transcription and translation of NiV G protein in *N. benthamiana* at 9 dpi and an estimated yield of 0.1 mg recombinant NiV G-protein per gram of fresh weight of leaves. This expression profile is similar to those in other reports where maximum expression of heterologous proteins in plants was reached between 3 and 9 dpi (Pua et al. 2012; Sheludko et al. 2007; Wroblewski et al. 2005; Wydro et al. 2006). Kanagarajan et al. (2013) reported the highest relative expression of both amorpha-4,11-diene synthase and epi-cegrol synthase gene at 9 dpi in *N. benthamiana*. Wroblewski et al. (2005) have shown that the intensity of GUS staining in Arabidopsis reached the peak level within 4 to 5 dpi and decreased gradually after 5 dpi. GFP accumulation achieved maximum level at 3 dpi and remained stable up to 8 dpi in tobacco (Sheludko 2007) while Mardanova et al. (2015) observed the maximal expression of matrix protein 2 of Influenza A virus recombinant protein at 4 dpi in *N. benthamiana*. In several reports, the production of recombinant proteins in plants gradually decreased at around 9 dpi, possibly caused by RNA silencing which reduces heterologous gene expression or may induce systemic necrosis in *N. benthamiana* (Johansen & Carrington 2011; Scholthof et al. 1993; Voinnet et al. 2003; Zhang et al. 2010). However, the necrosis symptom was not observed in our study.

A specific signal was observed in Western blots probed with anti-NiV antibody confirming the specific immunoreactivity of the recombinant NiV G protein (Figure 5). This was in contrast to our previous study of Niv G protein expression in *E. coli* (Gan et al. 2015), where the 60 kDa protein reacted with anti-cMyc antibodies in Western blots but not with anti-Niv antibody, showing the plant-expressed NiV G protein to have a more specific reaction with the antibody. Based on our results, we suggest that an *N. benthamiana* transient expression system using a viral vector can be employed for large-scale production of recombinant proteins to for functional studies to elucidate their potential for various industrial and medical applications.

CONCLUSION

In summary, a modified vector containing NiV G protein sequence was constructed and has been expressed in *N. benthamiana*. The recombinant NiV G protein had reactivity to NiV antibodies, indicating its potential for vaccine use.

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Gan Khai Swan, Tan Chon Seng, Rofina Yasmin Othman & Jennifer Ann Harikrishna*
Centre for Research in Biotechnology for Agriculture
University of Malaya
50603, Kuala Lumpur
Malaysia

*Corresponding author; email: jennihari@um.edu.my

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