Molecular Farming, an Effective System for the Production of Immunogenic Crimean-Congo Hemorrhagic Fever Virus Glycoprotein

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Abstract

The main aim of this study was to obtain the Crimean-Congo hemorrhagic fever virus (CCHFV) glycoprotein, through either stable transgenic plants or using a transient expression system, and determine the yield, quality and finally the immunogenicity of the plant-made CCHFV glycoprotein in a mouse model. We designed and synthesized a codon-optimized G1/G2 gene from the G1 and G2 parts of the CCHFV glycoprotein by bioinformatic analysis. The synthetic construct was cloned into a plant expression vector and tobacco plants were both transiently and stably transformed. The transgenic plantlets or tobacco-derived hairy roots confirmed by PCR and Southern blot analyses. The intact 98 kDa G1/G2 glycoprotein was produced by a transient expression system at as much as 3.3 mg/kg fresh weight. The recombinant G1/G2 protein was analyzed in stable lines by G1/G2 ELISA and Western blot. The yield in the transgenic hairy root line was significantly higher than that in transgenic tobacco lines. Finally, the immunogenicity of the plant-made G1/G2 glycoprotein was evaluated by its subcutaneous administration in mice when compared with positive and negative controls. This plant-purified G1/G2 protein produced a high titer of anti-CCHFV glycoprotein IgG antibodies in mice.

Keywords: Molecular farming, transgenic plant, CCHFV

Introduction

Plant-based expression systems have been shown as economical for large-scale production of pharmaceuticals, industrial and therapeutic enzymes and antibodies and vaccine antigens (Mason, Warzecha *et al.* 2002; Streatfield 2007). Unlike bacteria, they are capable of eukaryotic post-translational modifications, most importantly glycosylation (Karnoup, Turkelson *et al.* 2005). This can be obtained without the hyperglycosylation that is often observed with certain yeast systems (Streatfield 2007). Moreover, there are several additional benefits to plant systems: they are easily scalable, the recombinant protein can be transported and stored without the need to cold chain, simple processing, and the absence of contamination with human pathogens (Daniell, Streatfield *et al.* 2001; Howard 2004). Also, the use of edible plant tissues for expression and delivery of antigens offers advantages over traditional vaccine production in that the vaccination route varies from the current route.

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Although there are significant advantages, the use of stable transgenic plants for the production of a recombinant protein is generally labor intensive and time consuming (Streatfield and Howard 2003). While transient expression systems that utilize agroinfiltration have advantages in terms of rapid production of recombinant proteins in a very short time, a few transient expression technologies using genes suppressing small interfering RNA have been demonstrated to be ready for large-scale production (Giritch, Marillonnet *et al.* 2006; Villani, Morgun *et al.* 2009; Circelli, Donini *et al.* 2010; Werner, Breus *et al.* 2011). Due to the stability and high productivity of hairy root cultures, they have been investigated for their potential to produce valuable metabolites and recombinant proteins as well as for phytoremediation (Gleba, Borisjuk *et al.* 1999; Giri and Narasu 2000). Accordingly, vast numbers of recombinant proteins are now produced by hairy root cultures (Franconi, Demurtas *et al.* ; Wongsamuth and Doran 1997; Medina-Bolivar and Cramer 2004; Guillon, Tremouillaux-Guiller *et al.* 2006).

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus Nairovirus and the family Bunyaviridae, causes a zoonotic viral disease with a high mortality rate in humans (Hoogstraal 1979; Swanepoel, Gill *et al.* 1989). The virus is enveloped and possesses a tripartite, negative-sense, single-stranded RNA genome. The small (S) and the large (L) segments encode a nucleocapsid protein and an RNA-dependent RNA polymerase, respectively (Papa, Ma *et al.* 2002; Whitehouse 2004). The medium (M) segment encodes a glycoprotein precursor that that is processed to two envelope glycoproteins, G2 and G1, that bind to host cell receptors and cause the virus to enter the cell (Sanchez, Vincent *et al.* 2002;

Chinikar, Ghiasi *et al.* 2009). The virus is widely distributed across Europe, Asia, the Middle East, and Africa and is transmitted to humans through the bite of Ixodid ticks or by contact with blood or tissues from infected livestock. While a variety of livestock can become infected with CCHFV, the infections generally result in unapparent or subclinical disease (Ergonul 2006; Chinikar, Goya *et al.* 2008; Chinikar, Ghiasi *et al.* 2009). An effective and low-cost prophylactic vaccine for animals against CCHFV would decrease the rate of distribution of the virus and prevent virus reproduction in the animal and consequent transmission to humans. Expression of the entire M segment of the CCHFV glycoprotein in mammalian systems has been used to study its cellular localization, characterization, and processing and has revealed how the precursor glycoprotein becomes the mature CCHFV glycoprotein (Sanchez, Vincent *et al.* 2002; Bertolotti-Ciarlet, Smith *et al.* 2005; Altamura, Bertolotti-Ciarlet *et al.* 2007; Bergeron, Vincent *et al.* 2007; Erickson, Deyde *et al.* 2007). Monoclonal antibodies specific to the G1 and G2 portions of the CCHFV glycoprotein revealed neutralizing epitopes in both. These epitopes are conserved across the viral strains studied, regardless of significant antigenic differences between the M-segment groups (Ahmed, McFalls *et al.* 2005). In addition, a CCHFV DNA encoding the glycoprotein was shown to elicit neutralizing antibodies in some of the immunized mice (Spik, Shurtleff *et al.* 2006). Although previous studies have expressed the complete M segment in mammalian cells, there is no report on the production of the CCHFV glycoprotein in plants as an alternative eukaryotic system. Nonetheless, there is a CCHF vaccine for human injection which contains inactivated CCHF virus particles (Papa, Papadimitriou *et al.* 2011). However, the most important

vaccination approach against CCHFV is to design an efficient vaccine to control the virus propagation in the livestock which play an important role in the transmission cycle of the CCHFV. Also, development of a safe and cheap vaccine which can be easily delivered through plants within the livestock the feed would be more likely accepted by endemic countries. Here, we engineered and synthesizeda plant-optimized gene cassette containing the G2 and G1 portions of the CCHFV glycoprotein. The amino acid sequence of this synthetic gene was analyzed by bioinformatic tools for probable parameters including surface accessibility, antigenicity, and N-glycosylation sites. The gene cassette was cloned into a plant expression vector. Transient expression of the construct produced a high yield of the G1/G2 glycoprotein. Both stable transgenic lines, including tobacco plant and tobacco-originated hairy roots, were also able to constitutively produce the G1/G2 glycoprotein. The immunogenicity of the produced G1/G2 glycoprotein was confirmed in mice by subcutaneous administration.

Materials and Methods
Codon optimization and DNA constructs

The sequence of the Iranian strain (DQ446215) of the CCHF virus glycoprotein was retrieved from the GenBank data base and analyzed by DNAstar software (DNASTAR, Inc.). The base composition of the CCHFV glycoprotein and its modified genes were also analyzed by the software. The codon usage data of tobacco plants (Nicotianatabacum) and the base composition were obtained from the CUTG (Codon Usage Tabulated from GenBank) website (http://www. kazusa. or. jp/codon/). The codon adaptation index (CAI) has been used as a parameter to estimate the degree of

codon optimization of an entire gene (Serke, Schwarz et al. 1991). The values of all transgenes were calculated using the CAI Calculator (http://www. evolvingcode. net/codon/CAI Calculator. php) with tobacco codon usage values from CUTG as the codon usage template.

A gene encoding the CCHFV G1/G2 glycoprotein was designed and optimized for plant expression. The Kozak sequence and the nucleotide sequence of an endoplasmic reticulum retention signal, KDEL, were added to the beginning and the end of the synthetic gene, respectively. Four repeats of an alpha helix making linker, EAAAK, were introduced between the G1 and G2 sequences (Wriggers, Chakravarty et al. 2005) and a six repeat array of histidine (6-His tag) was added to both side of the gene. The construct was synthesized (Shine gene, Shanghai, China) and confirmed by restriction enzyme analysis and whole gene sequencing (accession number: HM537014). The synthetic gene was cloned into the plant expression vector, pBI121 (Novagene), at the Cfr9I and SacI restriction sites.

Stable transformation of tobacco plants with the G1/G2 glycoprotein

Seeds of tobacco plant (Nicotianatabacum L., cultivar; Xanthi) were sterilized and germinated on MS medium with 30 g/L sucrose which solidified with 8 g/L agar. The recombinant plasmid (pBI121-G1/G2) was introduced into the Agrobacteriumtumefaciens strain LBA4404 by the freeze and thaw method (Topping 1998). Recombinant A. tumefaciens was grown overnight at 28 °C in LB medium supplemented with 50 μg/ml kanamycin. The culture was centrifuged at 5, 000 rpm for 5 min at 4 °C and the cells were suspended in MS medium (pH 5.2) with 0.05 mMacetosyringone (3′, 5′-dimethoxy-4′-hydr - oxyacetophenone). Sterile leaf disks about 1 cm2 were excised and infected

by recombinant Agrobacterium. Two days later the leaf disks were transferred to co-cultivation medium supplemented with 200 mg/L cefotaxime and 50 mg/L kanamycin. The selection was continued for 14 days at 22 °C on a photoperiode (light-dark, L/D) cycle of 16 hours light, 8 h dark (LD 16:8). An additional selection for 14 days with 100 mg/L kanamycin was performed for the explants. The selected explants were transferred to solidified root inducing medium (MS basal salts with 15 g/L sucrose, 0.2 mg/L IBA, and 200 mg/L cefotaxime) (Carrer, Hockenberry *et al.* 1993). The rooted shoots were transferred to soil and grown under greenhouse conditions (LD 16:8 at 20 °C).

Transient expression of the G1/G2 glycoprotein in tobacco leaf

Recombinant A. tumefaciens were grown overnight at 28 °C in LB medium supplemented with 50 μg/ml kanamycin. A 50 μl aliquot of the overnight culture was used to inoculate 10 ml of LB medium supplemented with 10 mM MES buffer (pH 5.7), 50 μg/ml kanamycin, and 150 μMacetosyringone. The pre-cultures were grown overnight at 28 °C in a shaker (150 rpm). Cells were harvested by centrifugation andresuspended to a final concentration corresponding to an optical density (OD) of 1.0 at 600 nm in a solution containing 10 mM MgCl2, 10 mM MES (pH 5.7), and 450 μMacetosyringone. Cultures were incubated at room temperature for 3 hrs before infiltration. Leaves from 2–4 weeks old Nicotianatabacum L., cultivar; Xanthi plants were submerged in the bacterial suspension and subjected to a vacuum of 290 kPa for 10–20 min, with occasional agitation to release trapped air bubbles. The vacuum was released rapidlyand the leaves were placed upside down in Petri dishes containing wet filter paper. The Petri dishes were kept at 22 °C under 16 h of fluorescent light per day

for 5 days (Voinnet, Rivas *et al.* 2003; Lee and Yang 2006).

Production of the G1/G2 glycoprotein by tobacco-derived hairy root

The induction of hairy roots was stimulated by inoculation with recombinant A. rhizogenes (ATCC 15834) harboring the pBI121-G1/G2 plasmid. All preparations for hairy root induction from leaf disks of tobacco plants were the same as described previously. Two to three weeks following infection, hairy roots emerged from the infection sites. Single transformed roots were excised after one subculture and maintained separately as independent transformants. Axenic root cultures were derived and grown at 22 °C under LD 16:8 and transferred to fresh MS solid medium every 5–6 weeks.

Transgene analyses

For PCR analysis, genomic DNA was isolated from both non-transgenic and transgenic tobacco leaf tissues and hairy roots using the Plant Mini Kit (Intron, South Korea). The concentration of genomic DNA was measured at 260 nm in a UV spectrophotometer. The presence of the g1/g2 gene in transgenic plant genomic DNA was determined by PCR analysis using gene specific primers, JG1NosF 5′-CTTTGCTTTTACATTGTGGAGAGGG-3′ and JG1NosR 5′-GCGTATT AAATGTA TAATTGCGGGACT-3′, that amplify 462 bp around the attachment region between the G1 gene and Nos terminator sequences.

For Southern blot analysis, genomic DNA was extracted from plant leaves as previously described (S. L. Dellaporta 1983). Fifty micrograms of genomic DNA from wild-type and transgenic lines were digested with BglII to determine the transgene copy number. Digested DNA was electrophoresed on a 0.8% agarose gel and

blotted on a Hybond N membrane (Roche, Germany) following a standard procedure (J. Sambrook 2001). A DIG-labeled probe (602 bp) was generated with the PCR DIG Labeling Mix (Roche) using the g1/g2 gene as a template and specific primers, JG2G1F 5′-GGCATTCCTCTTTTGGTTCAGTTTC 3′ and JG2G1R 5′- TTGA TTCAGACCTTC CAGACACGA-3′. Hybridization was done using the PCR DIG Detection kit following the supplier's instructions (Roche).

Total soluble protein extraction from transgenic lines

Approximately 40 g and 5 g of tobacco leaves were used for purification of the recombinant G1/G2 glycoprotein from transiently- and stably-expressing lines, respectively. In brief, the tobacco leaves and hairy roots were ground to a fine powder in liquid nitrogen with a mortar and pestle. Total soluble protein (TSP) was extracted using 0.1 ml of protein extraction solution (Intron, South Korea) per 1 gram of leaf or root material. Cell debris was removed by two rounds of centrifugation at 20, 000 g for 30 min at 4 °C and the supernatant was used for expression analyses and protein purification by affinity chromatography.

Purification of the G1/G2 glycoprotein

The extracted TSP was filtered through Whatman 3M paper and loaded onto a 12 cm column packed with 3 ml Ni-NTA resin (Qiagen, Germany) pre-equilibrated using buffer A (50 mMTris-HCl, 300 mMNaCl, 20 mM imidazole, and 3 mM protease inhibitor PMSF, pH 8.0). The column was then washed with 100 ml buffer B (50 mMTris-HCl, 300 mMNaCl, 50 mM imidazole, and 3 mM PMSF, pH 8.0) to remove unbound proteins. The recombinant G1/G2 glycoprotein was eluted with increasing concentrations of imidazole (150, 200, and 250 mM) in 5 ml buffer C (50

mMTris-HCl, pH 8.0, 300 mMNaCl, and 3 mM PMSF). After measuring its concentration by the Bradford protein assay, the purified G1/G2 glycoprotein was subjected to Western blot and ELISA analyses.

G1/G2 Glycoprotein analysis by ELISA

The ELISA plate (Nunc, Denmark) was coated with 10 μg of TSP in coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH9.8) and incubated for 2 hrs at 37 °C. The plate was washed three times with PBST (PBS with 0.05% Tween 20) and blocked with 3% (w/v) skim milk in PBST for 1 h at 37 °C. After another three washes, human anti-serum taken from a healed CCHF patient (Provided by the National Reference Laboratory of Arbovirusesand Viral Hemorrhagic Fevers, Pasteur Institute of Iran) at a dilution of 1:500 was added to each well and incubated for 2 hrs at 37 °C. After standard washes, a 1:4, 000 dilution of anti-human IgG conjugated to peroxidase (Sigma, USA) was added to the plate without wash and the peroxidase substrate OPD was added for detection (Sigma, USA). The reaction was stopped after 5 min with 1N H_2SO_4 and the optical density was read at 492 nm. To calculate the relative amount of the G1/G2 glycoprotein in the plant material, the OD of the wild-type plant sample was deducted from the OD of the transgenic plant and was then estimated based on a standard curve taken by ELISA using 0.2–1.9 μg of the plant-purified G1/G2 glycoprotein. All samples were analyzed twice and an ANOVA test was carried out using SPSS version 12. In all ELISA tests, wild type tobacco plant, and CCHF vaccine containing the inactivated viral particles and or the plant-purified G1/G2 glycoprotein was used as negative and positive controls, respectively.

Transgenic plant analysis by Western blot

One hundred micrograms of TSP per sample was mixed with loading buffer (300 mMTris-HCl, pH 6.8, 600 mMdithiothreitol, 12% SDS, 0.6% bromophenol blue, and 60% glycerol), boiled for 10 min, placed on ice, and then separated on a 12% SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Roche, Germany) using a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, USA) in transfer buffer (50 mMTris, 40 mM glycine, 0.04% SDS, 20% methanol, pH 8.3). The membrane was blocked with 4% skim milk in PBST for 2 h at room temperature. After a brief wash in PBST, the membrane was incubated with the human anti-serum at a dilution 1:250 in 1% in PBST for 1 h at 37 °C. After three 10 min washes, the membrane was incubated with horseradish peroxidase conjugated to goat polyclonal IgG antibody against human (Sigma, USA) at a dilution of 1:4, 000 in PBST. The membrane was washed three times with PBST and HRP staining solution was applied (Sigma, USA). The chromogenic reaction was stopped by rinsing the membrane in distilled water. In all immunoblotting tests, wild type tobacco plant, and CCHF vaccine containing the inactivated viral particles and or the plant-purified G1/G2 glycoprotein was used as negative and positive controls, respectively.

Mice immunization and antibody measurement

Thirty female BALB/C mice, aged 6 to 8 weeks were purchased from the animal breeding department, Pasteur Institute of Iran. . The test group (n=10) was subcutaneously immunized with 5, 10, 15, or 20 μg of the purified G1/G2 glycoprotein along with complete Freund's adjuvant (Sigma, USA) in the first administration and with incomplete Freund's adjuvant (Sigma,

USA) in the rest periods at two weeks intervals. The positive control vaccinated group (n=10) was subcutaneously injected with 4 doses of the CCHF vaccine, an inactivated form of the CCHF virus approved for human vaccination (BulBio Company, Bulgaria), at two-week intervals. The negative control group (n=10) received no treatment. Sera were collected before injection, after the second injection, and 2 weeks after the final immunization. The G1/G2-specific antibodies were analyzed by ELISA as described previously (Amani, Salmanian *et al.* 2010). The purified transiently expressed G1/G2 glycoprotein and CCHF vaccine were used, respectively, as the solid phase coating antigen for ELISA analysis and as the positive control groups.

Data analysis

All data were processed and analyzed by SPSS version 13.0 Data Editor (SPSS Inc., Chicago, IL, USA). Groups were considered to be different if $p < 0.05$ and significantly different if $p \leq 0.01$.

Results
Construction of a codon-optimized G1/G2 glycoprotein gene

Like the other members of the family Bunyaviridae, the glycoprotein of the CCHF virus is synthesized as a polyprotein precursor (Haferkamp, Fernando *et al.* 2005) that undergoes proteolytic cleavage to yield the mature glycoprotein (Vincent, Sanchez *et al.* 2003), namely G1 and G2. For this reason, the open reading frames encoding the G1 (284 aa) and G2 (554 aa) portions of the CCHFV glycoprotein were selected. An expression cassette was designed containing, from N-terminus to C-terminus, EcoRI and Cfr9I restriction sites, the Kozak sequence specific to plants, a 6xHis tag, the G2 portion of the CCHFV

glycoprotein, an alpha helix-making sequence with four repeats of EAAAK for discrete folding of the G1 and G2 glycoprotein portions, the G1 portion of the glycoprotein, a 6xHis tag, the signal KDEL for efficient accumulation of the recombinant protein in the endoplasmic reticulum (ER), a stop codon (TGA), and SacI and XhoI restriction sites (Fig. 1). The cassette was expected to produce an 876 aa protein of approximately 98 kDa. In this case, the native gene contained rare codons in tandem that could reduce the efficiency of

translation or even disengage the translational machinery. The codon usage bias in Nicotianatabacum was addressed by increasing the Codon Adaptation Index (CAI) to 0.9. The GC content (averaging up to 41.9%) and unfavorable peaks were optimized to prolong the half-life of the mRNA. The stem-loop structures, which impact ribosomal binding and the stability of the mRNA, were broken. In addition, the optimization process successfully modified the negative cis-acting sites and repeat sequences.
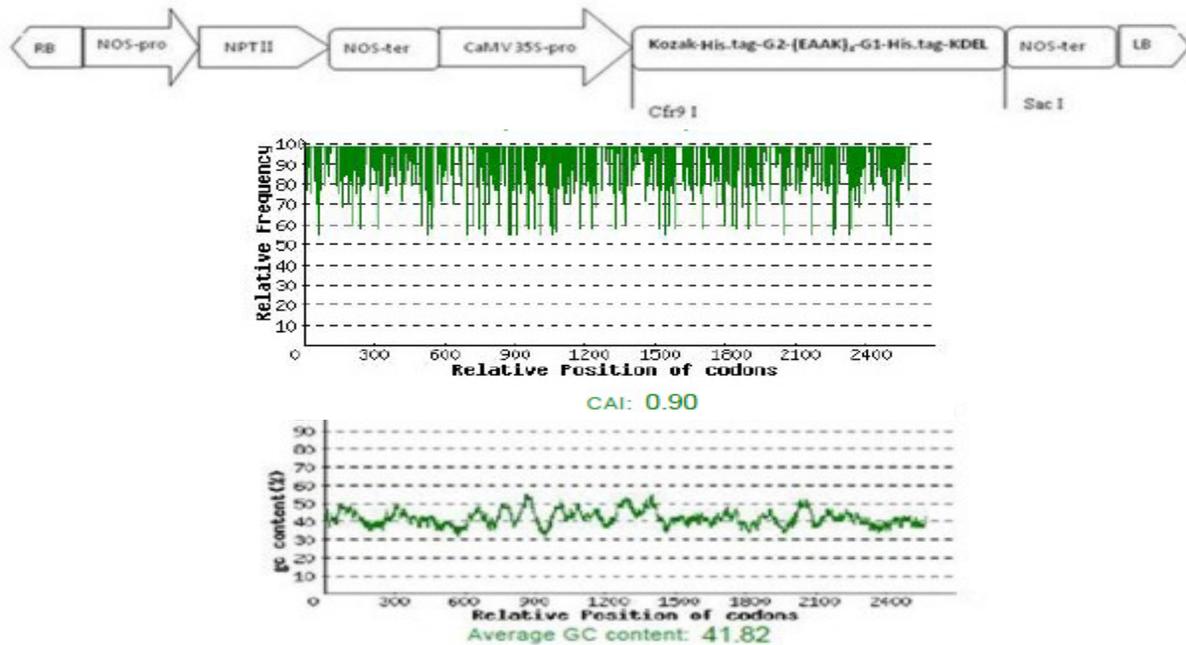
Figure 1. Schematic representation of the pBI121-G1/G2 construct. The construct contains thesynthetic G1/G2 glycoprotein gene, with the coding sequences for the immunogenic G1 and G2 portions of the CCHFV glycoprotein, under the control of a CaMV35 promoter. The Kozak sequence and KDEL signal are located at the 5・ and 3・ ends, respectively. The restriction enzyme sites of Cfr9I and SacI were inserted for cloning.

The G1/G2 glycoprotein can be transiently expressed in tobacco plants

Vacuum agroinfiltration was used to transiently express the construct harboring the G1/G2 glycoprotein in plants. A recombinant Agrobacterium strain carrying this construct and another agrobacterium with the GUS gene (positive control for agroinfiltration) were used to agroinfiltrate leaves of the Nicotiantabaccum. After two

passages through Ni-NTA affinity chromatography columns the purity of the recombinant G1/G2 was greater than 95% and a sufficient quantity was obtained (3.3 mg/kg agroinfiltrated leaves). The purity of the G1/G2 glycoprotein was tested by 12% SDS-PAGE, Western blot, and ELISA. As shown in Fig 2a, one sharp band of about 98 kDa, two bands around 65 kDa and 63 kDa, and two other weak bands of approximately

35kDa and 33kDa were observed by SDS-PAGE of the purified protein. Western blot analysis of the purified samples using anti-CCHF antibodies confirmed the intact expression of the 98kDa G1/G2 glycoprotein and two other specific bands of about 65 kDa and 63 kDa, consistent with the SDS-PAGE data (Fig. 2b). However, no bands were observed at 33 kDa or 35 kDa, suggesting that the lower molecular weight bands may correspond to minimal partial degradation products of the mature protein. The antigenicity of the purified G1/G2 glycoprotein was examined by specific ELISA (Fig. 2c), confirming its presence in the purified fraction.
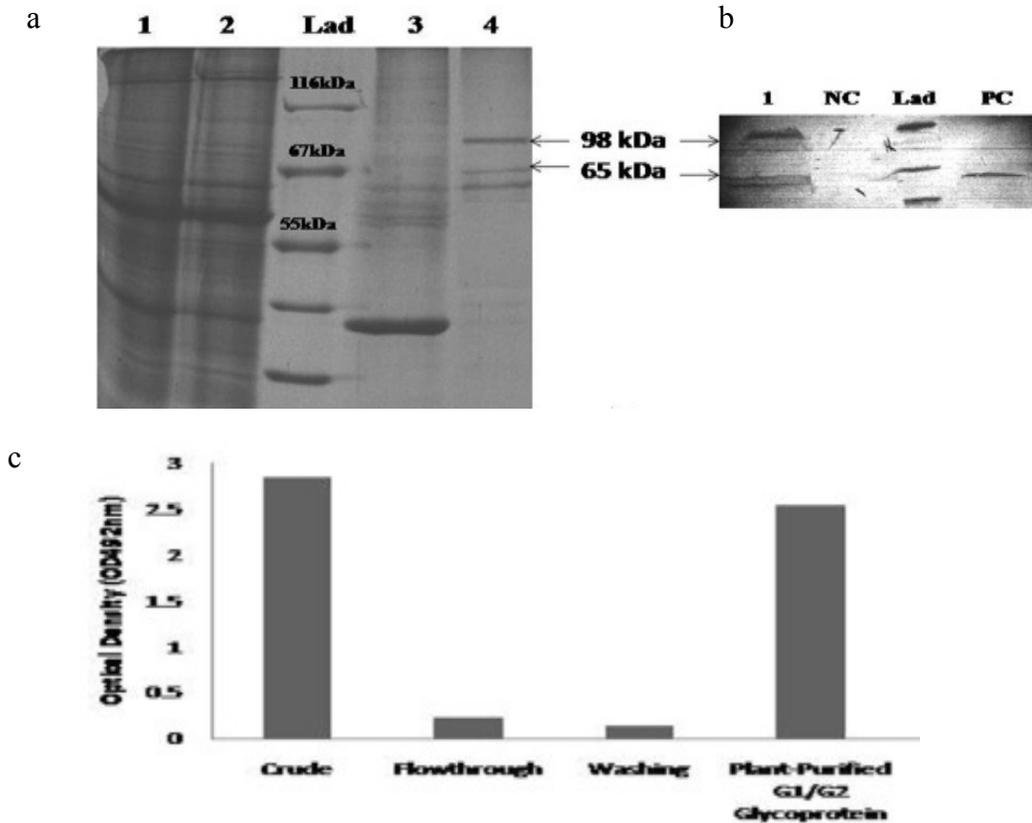
Figure 2. G1/G2 glycoprotein expression analysis of agroinfiltrated tobacco leaves. The total soluble protein (TSP) of infiltrated leaves was extracted and G1/G2 was purified through affinity chromatography. The purified fractions were visualized by (a) 12% SDS-PAGE; 1: crude TSP, 2: flow through, 3: washes, 4: plant-purified G1/G2, Lad: protein ladder. The presence of the G1/G2 glycoprotein was confirmed in the purified fraction by (b) Western blot; PC: positive control includes the CCHF vaccine containing the inactivated viral particles, NC: negative control includes non-agrofiltrated leaves, and 1: plant-purified G1/G2 glycoprotein and (c) IgG specific ELISA using human anti-serum.

The G1/G2 glycoprotein is stably expressed in tobacco plants and hairy roots

Genomic DNA from the leaves of each 2-month-old transgenic plant was isolated for PCR analysis. Most of the transgenic tobacco plants (7 lines) and tobacco-derived transgenic hairy roots (4 lines) had the 462 bp PCR product (Fig. 3). The PCR products were confirmed by sequencing. All kanamycin-selected tobacco plants and tobacco-derived hairy roots were assayed for the transgene of the G1/G2 glycoprotein gene by Southern blotting with a gene-

specific probe. One to three copies of the gene were found in the transgenic tobacco plant lines, including T1 to T7, and in the

hairy root lines, H1 to H4, but not in the untransformed tobacco plant (Fig. 3).
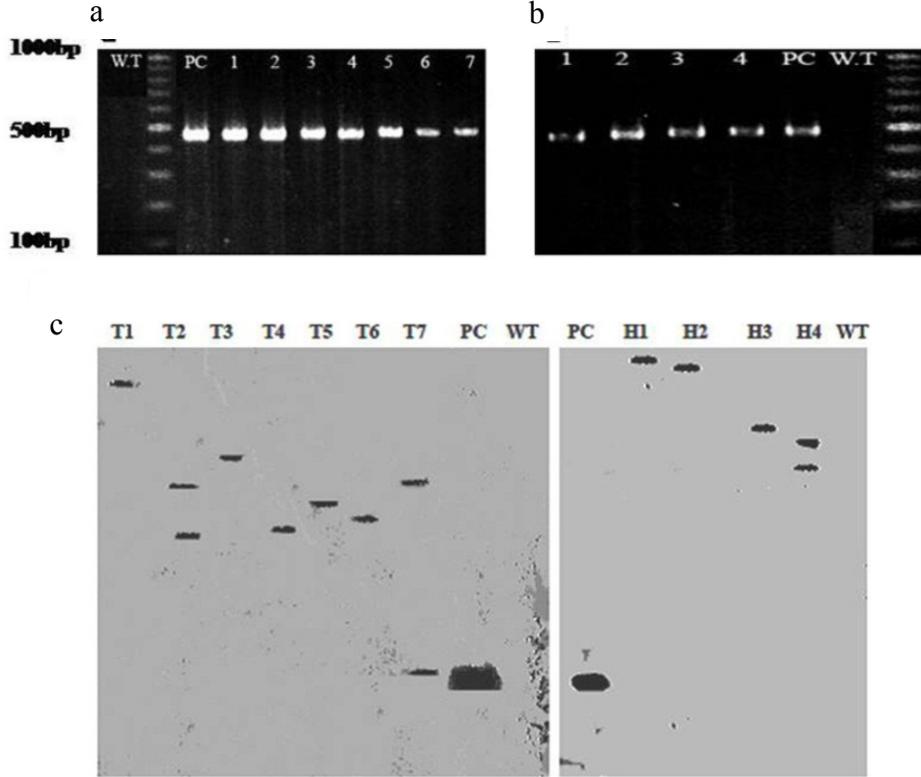
Figure 3. g1/g2 transgene analysis. Genomic DNA was extracted from transgenic plants. The presence of the g1/g2 gene was confirmed in the 7 lines (1-7) of tobacco plants (a) and 4 (1-4) lines of hairy roots (b) using gene specific primers that amplified a 462 bp fragment. (c) Also, 25 µg of DNA from each line was tested by Southern blot with a 602 bp gene-specific probe (T1-T7 for tobacco lines and H1-H4 for hairy root lines). P. C: positive control includes the construct harboring the g1/g2 gene, W. T: wild-type genome as a negative control, and a 100bp DNA ladder.

Total soluble protein (TSP) was extracted from selected transgenic leaves (7 lines) and hairy roots (4 lines) of tobacco plants to examinethe expression of the G1/G2 glycoprotein. Western blot showed the presence of the recombinant G1/G2 glycoprotein (98 kDa) in three lines of tobacco plants, namely T2, T5 and T6, and one line of tobacco-derived hairy roots, namely H1, whereas no G1/G2 protein was detected in the untransformed plant (Fig. 4a). A lower weight band was also observed, indicating degradation of the intact protein either during purification or sub-organelle accumulation in the ER. A standard curve was calculated by specific ELISA using

concentrations of the G1/G2 protein within the linear range of 0.2–1.9 µg with 0.1 µg increments. The yield of the recombinant protein was 0.96, 1.2, 1.4, and 1.8 µg/g of fresh leaf or hairy root weight for T6, T5, T2, and H1, respectively. The G1/G2 glycoprotein made up 0.45% of the TSP of H1 and 0.28%, 0.31%, and 0.34% of the TSP of the transgenic tobacco leaves in lines T6, T5, and T2, respectively (Fig . 4b, c).

Discussion

For more than two decades, plants have been considered for the production of heterologous proteins. While biofactories

have enormous advantages over established production technologies for the large scale expression of recombinant proteins, several issues remain to be addressed in terms of improving yield and quality (Fischer, Stoger *et al.* 2004; Daniell, Singh *et al.* 2009). Codon optimization of the gene of interest according to plant codon usage has increased the quality and quantity of recombinant proteins (Maclean, Koekemoer *et al.* 2007; Daniell, Ruiz *et al.* 2009). Here we optimized the G1/G2 glycoprotein gene and added two 6-histidine tags at both the C-terminus and N-terminus of the gene to facilitate the purification of the protein from plant tissues. In addition, we tried to ensure that the two portions of the CCHFV glycoprotein folded separately by inserting an alpha helix-making sequence of four repeats of the amino acid array EAAAK. However, further examination by circular dichroism (CD) and mass spectrometery is necessary to confirm their separate folding. The ER retention signal KDEL in the C-terminus allowed the G1/G2 glycoprotein to accumulate in the ER and prevented further plant-specific O-glycosylations, which could be allergenic in a vertebrate immune system (Himly, Jahn-Schmid *et al.* 2003; Leonard, Petersen *et al.* 2005). Because of this consideration, the N-terminus of the CCHFV M segment gene was not considered in this construct because of massive number of O-glycosylation sites. The presence of a slightly larger band than expected size could possibly be an N-glycosylated form of the G1/G2 glycoprotein. Thus far, adding ER retention signals and conformation facilitating sequences have resulted in more reliable outcomes of recombinant protein production in plants (Laguia-Becher, Martin *et al.* ; Suo, Chen *et al.* 2006; Daniell, Ruiz *et al.* 2009; Fujiyama, Misaki *et al.* 2009). Our Western blot results from both transiently and stably expressing lines revealed a band of approximately 98 kDa, corresponding to the G1/G2 glycoprotein. In spite of the absence of proteolytic sites in the peptide sequence, extra bands were seen at about 63 kDa and 66 kDa, the former of which would be consistent with the size of the G1 portion of the glycoprotein. The existence of these extra bands could be the result of protein degradation during protein purification or the presence of a new proteolytic site in the optimized sequence of the G1/G2 glycoprotein, which had been reported for other antigens (Mason, Ball *et al.* 1996; Webster, Wang *et al.* 2009). Also, since our optimized G1/G2 glycoprotein gene contained a few potential N-glycosylation sites in both the G1 and G2 portions, the higher band (66 kDa) may represent N-glycosylated G1 (Cabanes-Macheteau, Fitchette-Laine *et al.* 1999; Gomez, Zoth *et al.* 2009; Amani, Mousavi *et al.* 2011).

In this study, we expressed the G1/G2 glycoprotein gene using different strategies. Initially, we evaluated the efficacy of the synthetic gene by transient expression. After optimization, we were able to obtain a remarkable yield of 3.3 mg/kg fresh weight, which is much greater others have obtained (Santi, Batchelor *et al.* 2008). The yield may have increased had we used gene silencing suppressor genes such as P19 (Lombardi, Circelli *et al.* 2009; Saxena, Hsieh *et al.* 2011). The presence of the histidine tags in the protein assists in purifying the intact protein (98 kDa). . The 63 kDa and 65 kDa bands may be explained by positional effects of the histidine tags, or protein degradation, as extra bands have been reported while using a C-terminal histidine tag fused to a Plasmodium antigen (PyMSP4/5) gene (Webster, Wang *et al.* 2009).

Previous studies of other recombinant proteins expressed in stable transgenic tobacco plants have yields ranging from 0.019–0.31% of TSP (Kim, Kim *et al.* ;

Ashraf, Singh *et al.* 2005; Zhou, Badillo-Corona *et al.* 2008), whereas our recombinant protein was expressed in the transgenic tobacco lines at 0.28–0.34% of TSP. We achieved a yield in hairy roots of 0.45% of TSP, which was much higher than the previously found yield of mouse inteleukin-12 by hairy roots (Liu, Dolan *et al.* 2008; Pizzuti and Daroda 2008). Taken together, while stable transgenic tobacco is more suitable for oral delivery of antigens, both transient expression and hairy root expression may be more appropriate for production of recombinant antigens in terms of yield and time. These systems can be considered for high production to obtain

recombinant antigen for serological and immunological applications as well as for manufacturing, rather using new approaches like gene silencing suppressors (Circelli, Donini *et al.* 2010). In addition, both systems, particularly hairy root through suspension culture could be easily scaled up as already reported (Srivastava and Srivastava 2007; De Guzman, Walmsley *et al.* 2011). But, the authors strongly suggest designing a secretory vector for recombinant protein production through hairy root that leads to excrete in the culture medium in order to facilitate the purification of the protein.
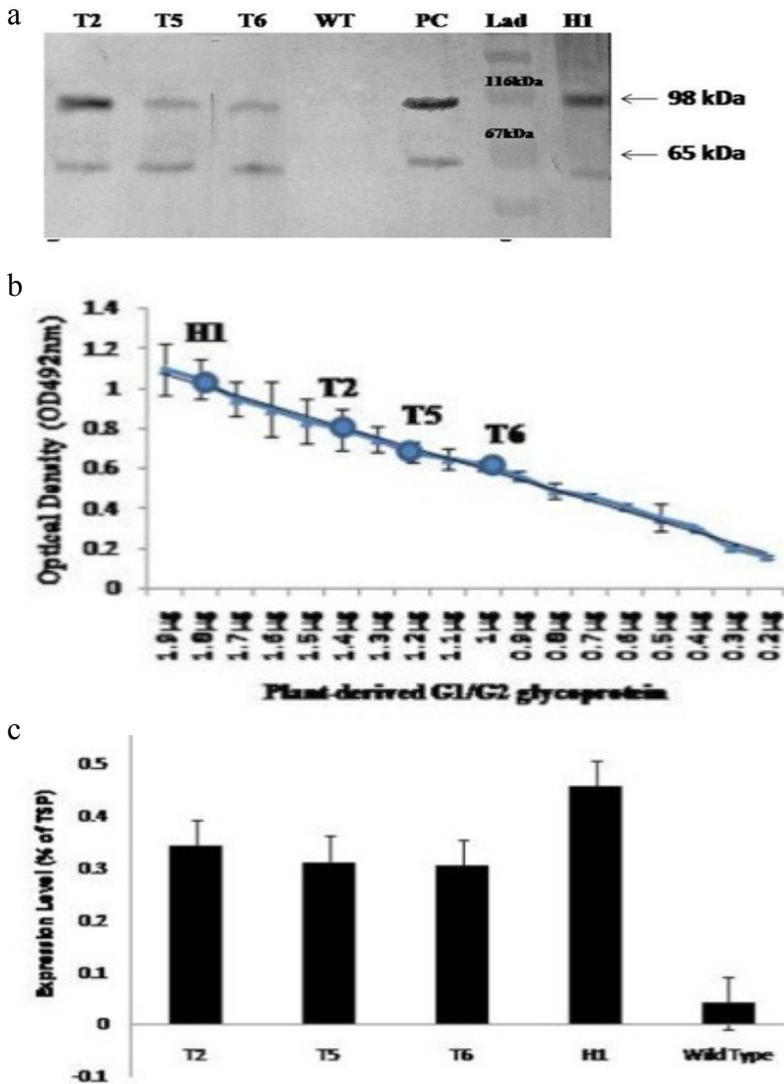
Figure 4. Measurement of the G1/G2 glycoprotein in stable transgenic lines. In the quantitative analysis of G1/G2, the amount of wild-type was deducted from the transgenic lines, and then estimated by the corresponding standard curve.
(a) Western blot analysis of transgenic tobacco lines T2, T5, and T6, and a hairy root line (H1) and Lad: protein ladder, PC: positive control includes the plant-purified G1/G2 glycoprotein, and WT: wild-type tobacco plant.
(b) The yield of the G1/G2 expressed in the stable transgenic tobacco lines based on the standard curve obtained using the plant-purified G1/G2 glycoprotein.
(c) The estimated percentage of the expressed G1/G2 over % TSP of the transgenic lines.
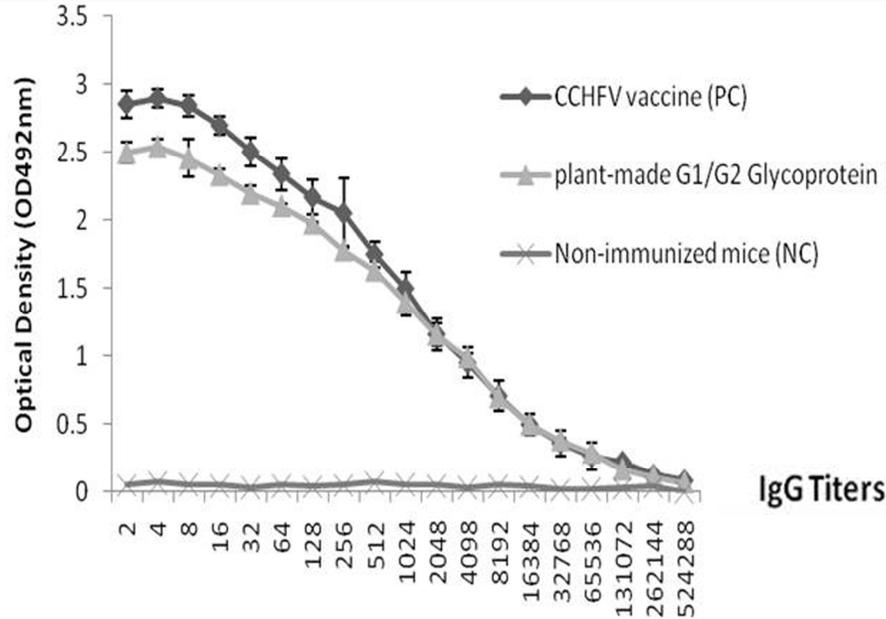
Figure 5. IgG ELISA determination of the plant-made G1/G2 glycoprotein immunogenicity in mice. Three groups were engaged in this immunization evaluation program, with a positive control group immunized with a CCHFV vaccine of inactivated virus particles, a negative control groups that was not immunized, and a test group immunized with the plant-made G1/G2 glycoprotein. All materials were subcutaneously administered.

After being purified from agroinfiltrated leaves, the G1/G2 glycoprotein was subcutaneously injected into mice at four doses. As illustrated in Fig. 5, the plant-made CCHFV glycoprotein elicited significantly high titers of anti-G1/G2 IgG antibodies by week 11 after the first boost (P≤0.01). There is no certain animal model for challenge of the CCHF disease, except the STAT1 knockout mouse that was recently studied for CCHF pathogenesis in bisafety level 4 laboratory (Bente, Alimonti *et al.* 2010). Avirus neutralization assay can be performed in high level biosafety laboratories. Our results reveal that, in contrast to other expression systems such as prokaryotic and mammalian systems, both a transient expression system and hairy root approach result in the production of the CCHFV glycoprotein on a large scale. The plant-made CCHFV glycoprotein is an efficient immunogen in eliciting neutralizing antibodies through subcutaneous injection in mice. Fusion of the glycoprotein gene to CT-B gene (Chikwamba, Cunnick *et al.* 2002; Boyaka, Ohmura *et al.* 2003), as an adjuvant, may increase the immunogenicity of the G1/G2 through oral immunization and the option should be considered for further study. Further study on the oral administration of stable transgenic plants to vertebrate hosts like livestock should be performed to determine whether an edible vaccine against CCHFV for animals is feasible.

Acknowledgments

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