Heterologous Expression of Hemagglutinin-Neuraminidase Protein from Newcastle Disease Virus Strain AF2240 in *Centella asiatica*

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We explored the use of the medicinally important plant *Centella asiatica* for expression of hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) strain AF2240. HN protein is the principal target for subunit vaccine development against NDV. The full-length HN gene was cloned into a plant expression construct driven by the CaMV 35S promoter and C-terminal fusion of green fluorescence protein (GFP) as reporter system. The recombinant expression construct was transformed via particle bombardment into *C. asiatica* callus. Transformants were screened using GFP and selected on MS medium supplemented with 15 mg/l hygromycin. The ~1.8 kb HN mRNA transcript was detected on the putative transformants using RT-PCR. The presence of HN protein expression was further confirmed through dot blot analysis using anti-NDV chicken serum. Here we report, for the first time, the use of a novel medicinal plant as a new platform for HN protein expression.

Key words: Antigen expression, edible vaccine, hemagglutinin-neuraminidase, medicinal plant, Newcastle disease virus.

INTRODUCTION

Newcastle disease (ND) is a highly contagious poultry disease caused by Newcastle disease virus (NDV). NDV is an enveloped virus with a negative single-stranded RNA genome encoding for at least six genes and belongs to the *Rubulavirus* genus (Paramyoxviridae family) (Murphy et al., 1995). The viral envelope is covered with two protein spikes protruding from the membrane surface known as hemagglutinin-neuraminidase (HN) and fusion (F) proteins. The HN protein plays a vital role in mediating virus attachment through binding of the sialic acid receptor on the host cells. Studies had shown that passive immunization with either polyclonal or monoclonal antibodies to the HN protein is able to confer protection in chickens against NDV challenge (Reynolds and Maraqa, 2000). Thus, the HN protein has become a major target for the development of an ND subunit vaccine. The Malaysian heat-stable viscerotropic-velogenic NDV strain AF2240 was first isolated in the 1960s; it is a highly virulent strain causing 100% mortality in susceptible chickens (Ideris et al., 1990). Tan et al. (1995) revealed that the HN protein of NDV strain AF2240 encodes 581

amino acids with a calculated *Mr* of 63.8 kDa and contains five potential asparagine-linked glycosylation sites. Previously, intensive studies on the HN protein of NDV strain AF2240 have been demonstrated using baculovirus (Ong et al., 1999) and *Escherichia coli* (Wong et al., 2009) expression systems.

Plants have emerged as viable alternatives to microbial and mammalian hosts for expression of valuable recombinant proteins (Lhernould et al., 2008). They offer several advantages, including low cost of production, lack of animal pathogenic contaminants, and ease of agricultural scale-up (Ko and Koprowski, 2005). Moreover, intact glycosylation machinery enables plants to produce various biologically active glycoproteins. Over the years, plant expression systems have proven to be a reliable and successful method for production of various essential recombinant proteins to meet the demands of the pharmacological industry (Soria-Guerra et al., 2011). In this study we explored the use of a novel medicinal plant system, *Centella asiatica*, for expressing the HN protein of NDV strain AF2240. *C. asiatica* is a promising platform for protein expression because it offers several advantages such

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as a well established regeneration system (Martin, 2004), ease of genetic transformation protocols (Krishan et al., 2008; Lai et al., 2011a), and a rapid life cycle for large-scale biomass production. Unlike tobacco, a commonly used plant biofactory, *C. asiatica* is nontoxic, edible, and of high medicinal value. It has been successfully used for the production of various essential compounds (Kim et al., 2007, 2010).

In this study we cloned and expressed the HN protein of NDV strain AF2240 using *C. asiatica* callus. The full-length HN gene was amplified and cloned into the 35SCaMV:gfp/pUC18 vector. Then the *HN* gene with C-terminal fusion of GFP was transformed and expressed in *C. asiatica* callus. The *HN* mRNA transcripts in the putative transformants were detected using RT-PCR and the presence of HN protein was confirmed through dot blot analysis using anti-NDV chicken serum. Callus was used as a target for HN protein expression because it is easy to maintain and the regeneration system is well established. Plant cell cultures have proved a useful target for downstream bioprocessing to produce various essential recombinant proteins (Millen et al., 2009).

MATERIALS AND METHODS

NDV CULTIVATION AND VIRAL RNA EXTRACTION

NDV strain AF2240 used in this study was obtained from the Faculty of Veterinary Medicine of Universiti Putra Malaysia. The NDV was cultivated in 9–10-day-old embryonated chicken eggs and purified using 30-60% (w/v) sucrose gradient centrifugation at 275,000 g for 4 h at 4°C. The resulting virus pellet was resuspended in NTE buffer (10 mM Tris, 100 mM NaCl, 1mM EDTA, pH 8.0) and used for viral RNA extraction as described previously (Yusoff et al., 1988).

CLONING OF *HN* GENE

The full-length *HN* gene was amplified using a one-step-access RT-PCR system (Promega, U.S.A.). A total of 50 μl reaction was prepared, containing 10 μl AMV/T reaction buffer, 1 μl 10 mM dNTP mixtures, 10 pmol HN gene specific forward CAGACTG-GATCCATGGACCGTGCAG TT and reverse TCATCAGGATCCTGGTTGA-CTCAACCG primers, 2 μL 25 mM $MgSO_4$, 1 μl AMV reverse transcriptase (5U/μl), 1 μl T DNA polymerase (5U/μl), 2 μl RNA sample, and nuclease-free water. The reaction conditions were 48°C for 45 min, 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 1 min, 68°C for 2 min and 68°C for 7 min. The resulting 1767 bp RT-PCR product was digested with BamH1 and ligated into similarly digested

35SCaMV:gfp/pUC18 vector to produce 35SCaMV: HN:gfp/pUC18 plasmid.

PARTICLE BOMBARDMENT TRANSFORMATION

Centella asiatica calli were induced from leaf explants in culture in vitro following a protocol described by Lai et al. (2011b). The recombinant 35SCaMV:HN:sgfp/pUC18 plasmid was bombarded into *C. asiatica* callus using an optimized protocol as described by Lai et al. (2011a). Expression of the HN protein on the transformed calli was monitored and screened using the GFP reporter by fluorescence microscopy (Nikon SMZ-1500, Japan; GFP2 filter). Images were captured through the attached camera using Nikon ACT-1 software.
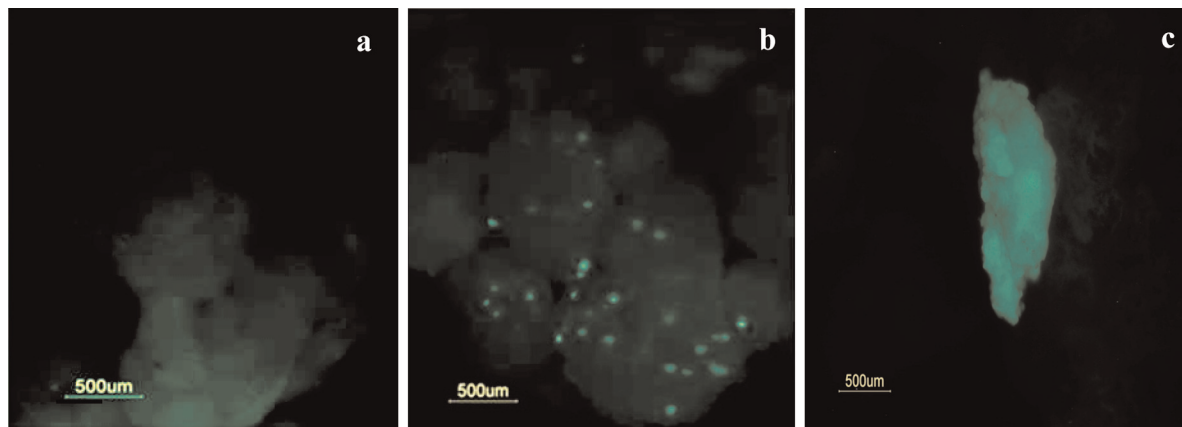
SELECTION OF PUTATIVE TRANSFORMANTS

Both hygromycin and GFP were used for selection of putative transformants. To establish the minimum fatal concentration of hygromycin for *C. asiatica* callus, calli were placed on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of hygromycin (1 to 15 mg/l at 1 mg intervals and 15 to 45 mg/l at 5 mg intervals). Data were recorded based on the percentage of surviving calli. The fatal hygromycin concentration (15 mg/l) was used to select the putative transformants under a 16 h photoperiod (25 μmol m^{-2} s^{-1}) at 24 ± 2°C.

RT-PCR ANALYSIS

Total RNA used for RT-PCR analysis was extracted from the GFP-expressing and hygromycin-resistant putative transformants. A total of 0.5 g fresh mass transformed callus was mixed with 1 ml TRIZOL®LS reagent and ground in liquid N2 until powdery with a pre-cooled mortar and pestle, after which 200 μl cold chloroform was added to the powdery mixture and it was shaken vigorously for 15 s. The mixture was left at room temperature (RT) for 2–3 min and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase containing RNA was recovered by precipitation with 500 μl chilled isopropanol. The mixture was then incubated at RT for 10 min and centrifuged at 12,000 g for 10 min at 4°C. The pellet containing RNA was washed by mixing with 1 ml chilled 70% (v/v) ethanol and centrifuged at 7500 g for 5 min at 4°C. The RNA obtained was air-dried at RT for 15 min before resuspending in 20 μl RNase-free water and treated using the DNA-free^{TM} kit (Ambion, U.S.A.). RT-PCR was performed under the above conditions using the RNA from transformed and nontransformed (negative control) calli to confirm the presence of the HN mRNA transcript.
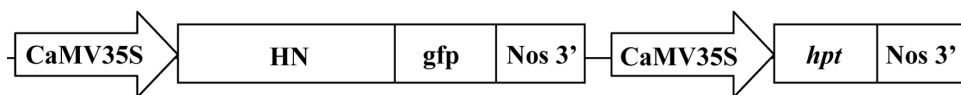
Fig. 1. Scheme of *HN* gene fusion with GFP in 35SCaMV:HN:gfp/pUC18. The HN gene is driven by CaMV 35S for expression.

Fig. 2. (**a**) Nontransformed callus (negative control), (**b**) GFP expression of calli transformed with 35SCaMV:HN:gfp/pUC18 vector, (**c**) GFP expression of hygromycin-resistant putative transformants.

DOT BLOT ANALYSIS

Total soluble proteins were extracted from the calli confirmed to contain HN mRNA using pre-chilled phosphate-buffered saline (PBS, pH 7.2) with 5 mM EDTA and 0.001% PMSF (phenyl methylsulforyl fluoride). Approximately 0.5 g fresh mass callus was ground to fine powder in a pre-cooled mortar and two volumes of ice-cold extraction PBS were added. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant obtained was used for dot blot analysis. Total soluble protein from transformed and nontransformed (negative control) calli were spotted onto a 3 mm chromatography membrane (Whatman, U.S.A.). Purified NDV strain AF2240 was used as the positive control. The dried membrane blot was blocked with 1% (w/v) skim milk in PBS, pH 7.4. Then the blot was probed with anti-NDV chicken serum at 1:5000 dilution in PBS, pH 7.4 for 1 h. The blot was washed with TPBS [PBS, pH 7.4 with 0.01% (v/v) Tween 20] 3 times, followed by incubation with alkaline phosphatase-conjugated goat anti-chicken IgG (KPL, U.S.A.) at 1:5000 dilution in PBS, pH 7.4, for 1 h at RT. Then the blot was washed again with TPBS 3 times before incubating with solution consisting of 66 µl nitro-blue tetrazolium (NBT, Sigma-Aldrich) and 33 µl bromochloroindolyl phosphate (BCIP, Sigma-Aldrich) in 10 ml alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.50) for signal development.

GENOMIC PCR ANALYSIS

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, U.S.A.) following the manufacturer's protocol. Total genomic DNA extracted was used as the template for PCR with *HN* gene-specific primers. A total 25 µl reaction containing 2.5 µl 10× reaction buffer (Takara, Japan), 2 µl 10 mM dNTP mixtures, 10 pmol gene-specific forward and reverse primers, 1 µl Ex-taq polymerase (10U/µl), 1 ul genomic DNA (~20 ng/ul) and nuclease-free water were prepared. The PCR reaction conditions were 94°C for 3 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s and 72°C for 7 min. PCR reactions using genomic DNA from nontransformed callus (negative control) and 35SCaMV:HN:gfp/ pUC18 plasmid (positive control) as templates were also performed as control.

RESULTS AND DISCUSSION

The full-length 1767 bp *HN* gene was successfully amplified by RT-PCR from the extracted viral RNA. The *HN* gene carrying the *Bam*H1 restriction site at the N- and C-terminals was ligated into the 35SCaMV:gfp/pUC18 vector producing recombinant 35SCaMV:HN:gfp/pUC18 vector (Fig. 1). The presence of the *HN* gene, its orientation, and nucleotide sequences were confirmed by DNA sequencing. The use of strong constitutive promoters such as cauli-
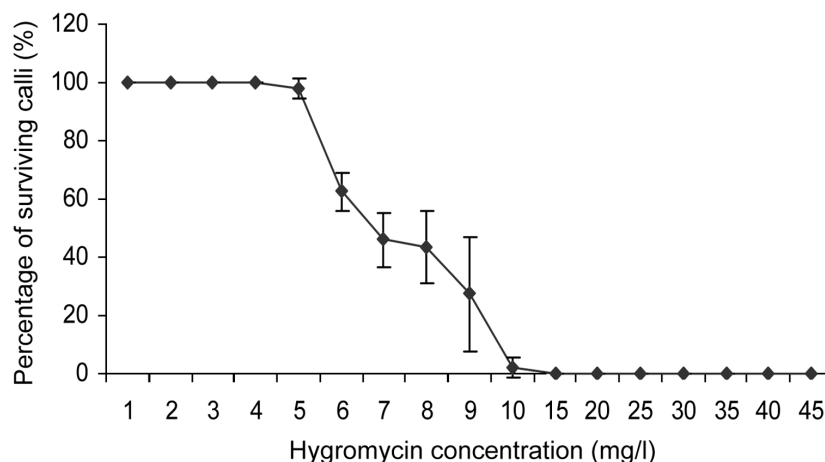
Fig. 3. Hygromycin killing level for *Centella asiatica* calli. Application of 15 mg/l was sufficient to totally inhibit callus growth in 21 days. Each point represents the mean ± SE of 3 replicates of 16 callus clumps each.

flower mosaic virus 35S RNA promoter (CaMV 35S) is needed to drive a high level of recombinant protein transcript expression in plants (Fisher et al., 2004). We also employed GFP fusion as reporter at the C-terminal of the *HN* gene to facilitate screening and identification of the transformants.

Using our previously established particle bombardment transformation protocol, 35SCaMV:HN:gfp/pUC18 vector was successfully bombarded into *C. asiatica* callus. A total of 160 clumps of calli were transformed with 35SCaMV:HN:gfp/pUC18 vector. Particle bombardment has been successfully employed to effect transient and stable transformations of plants (Gabriel and Zisheng, 2010). HN protein expression was monitored using GFP as reporter system at 60 h post-bombardment time (Fig. 2b). Using fluorescence microscopy we consistently observed a high level of GFP expression in the transformed calli. We infer that GFP cDNA and as well as HN cDNA was successfully and actively transcribed in the bombarded calli. Putative transformants detected with GFP expression by fluorescence microscopy were transferred to MS medium supplemented with 15 mg/l hygromycin (Fig. 3) for further selection. Two months after hygromycin selection, we successfully recovered 3 out of 160 T0 putative transformants with both hygromycin resistance and stable GFP expression (Fig. 2c). These 3 lines, which we named T0 lines 1, 2 and 3, were used for subsequent molecular analysis and maintained in MS medium supplemented with 15 mg/l hygromycin for future use. The use of the GFP reporter system in this study enabled us to rapidly and easily identify positive transformants with protein expression. GFP tags are commonly used for screening and confirmation of recombinant protein expression in plants (Hong and Mee, 2009). The use of GFP fusion for assessment
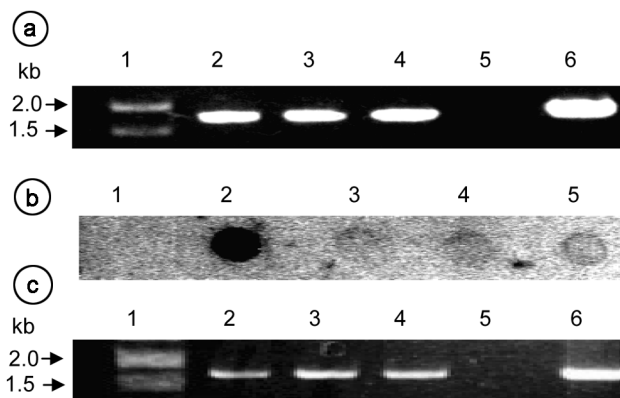
Fig. 4. (**a**) RT-PCR analysis from T0 transformants. Lane 1 – 1 kb DNA ladder; lanes 2,3,4 – T0 lines 1, 2 and 3; lane 5 -nontransformed callus (negative control); lane 6 – HN PCR product from 35SCaMV:HN:gfp/pUC18 vector (positive control), (**b**) Dot blot analysis of T0 transformants. Lane 1 – Nontransformed callus (negative control); lane 2 – Purified NDV (positive control); lanes 3,4,5 – T0 lines 1, 2 and 3, (**c**) Genomic PCR analysis from T3 transformants. Lane 1 – 1 kb DNA ladder; lanes 2,3,4 – T3 lines 1, 2 and 3; lane 5 – Nontransformed callus (negative control); lane 6 – HN PCR product from 35SCaMV:HN:gfp/pUC18 vector (positive control).

and monitoring of recombinant protein expression has also been demonstrated in numerous studies (Gabriel et al., 2006; Mohammad et al., 2009).

To verify the presence of HN mRNA transcripts in the selected lines, RT-PCR analysis was performed using the total RNA extracted from T0 lines 1, 2 and 3. All lines tested showed the presence of a ~1.8 kb band which coincides with the size of the *HN* gene (Fig. 4a). These detected HN bands indicat-

ed successful transcription of the *HN* gene in the putative transformants. To further evaluate the presence of HN protein expression, lines in which the HN mRNA transcript was detected were subjected to dot blot analysis using anti-NDV chicken serum. Dot blot analysis using the extracted total soluble protein showed a positive signal for all the tested lines, along with the purified NDV (positive control) (Fig. 4b). As expected, no signal was detected in nontransformed callus (negative control). This confirmed the presence of HN protein expression in the putative transformants. These lines were maintained and subcultured every 3 to 4 weeks in MS medium supplemented with 15 mg/l hygromycin. To further determine the stability of the *HN* transgene in the selected lines, genomic DNA PCR was performed using the total DNA extracted from T3 lines 1, 2 and 3. All tested lines showed the presence of HN bands (Fig. 4c), indicating stable integration of HN cDNA into the callus genome. Hence, the *HN* transgene is not lost in continuous subculturing and maintenance of the transgenic lines in antibiotic medium, at least through the T3 generation.

We were able to express the HN protein using the simple protocol described here. This study allowed us to assess the use of *C. asiatica* as a platform for expressing the HN protein, and confirmed its feasibility. More importantly, using the expression construct coupled with our previously established particle bombardment transformation protocol for *C. asiatica*, HN protein could be expressed to a level detectable by immunoblot assay. Our work also provides a simple, easy approach which will allow researchers to carry out immunization experiments using crude extracts of putative transformants, such as immunogen, in susceptible hosts. Since *C. asiatica* is safe to consume, it may potentially be used as a food supplement for chicken immunization against NDV. More broadly, our successful use of a novel medicinal plant system for expressing the HN protein of NDV strain AF2240 points the way toward development of *C. asiatica* as a new platform for expressing various essential therapeutic proteins.

ACKNOWLEDGMENTS

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