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STUDY ON THE TRANSIENT EXPRESSION OF INFECTIOUS BRONCHITIS VIRUS SPIKE PROTEIN IN *NICOTIANA BENTHAMIANA* LEAVES

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SUMMARY

Infectious bronchitis virus (IBV) is considered one of the main causes of economic loss in chicken farms worldwide, especially in poultry-producing nations. This virus, a member of the *Coronaviridae* family, is classified into different genotypes based on its surface spike glycoproteins with important roles in cell attachment and immune response. Vietnam is facing both classical and new emerging IBVs such as 793B, TC07-2-like and QX-like genotypes in chickens of all ages in both small households and large farms. Compared to regular hygiene and chemical treatments, vaccination is considered the most effective tool to prevent IBV. However, due to continual genetic mutation of IBV, attenuated and inactivated vaccines show decreased effectiveness or lack of cross-protection; thus ongoing studies focus on the development of new-generation vaccines to prevent new IBV outbreaks. This paper describes our study on the expression of the antigenic region in S1 subunit of the spike in *Nicotiana benthamiana*. Two expression vectors carrying either S1 or receptor binding domain (RBD) coding gene (pCB301-S1 and pCB301-RBD) were constructed and transformed into tobacco leaves by agroinfiltration method for transient expression of the target proteins. The RBD showed a clearly higher level of expression compared to the whole S1. Purification of RBD by immobilized metal ion chromatography and size exclusion chromatography represented a mixture of monomer, dimer, and trimer glycoprotein with expected sizes in Western blot. In summary, this study demonstrated our primary success in establishing an expression model that could be used to investigate plant-based IBV recombinant vaccines.

Keywords: agroinfiltration, infectious bronchitis virus, *Nicotiana benthamiana,* receptor binding domain, S1 spike, vaccine

INTRODUCTION

Infectious bronchitis (IB) is a respiratory disease in chickens of all ages causing economic burden, especially in poultry producing nations during outbreaks. The most common symptoms of IB are coughing, watery eyes, and nasal discharge (Raj, Jones, 1997; Ganapathy, 2009). When the disease develops seriously, there are signs of injury in the kidney and reproductive tract that lead to mortality and poor egg quality (Cavanagh, Gelb, 2008; Zhong *et al.,* 2016).

The IB disease is caused by infectious bronchitis virus (IBV) which is a member of the *Coronaviridae* family (Cavanagh, Gelb, 2008). IBV was first described in the USA, then spread to all other continents (Asia, Europe, Africa, and Australia) and became a worldwide threat (Cook *et al.,* 2012). The positive-sense RNA genome of IBV encodes four structural proteins: envelope (E), membrane (M), nucleocapsid (N), and spike (S) (Perlman *et al.,* 2008). The trimer glycoprotein S on the IBV surface, which is divided into two subunits (S1 and S2), contributes to the diversity of IBV, because it contains the most variable region of the genome (Valastro *et al.,* 2016). Besides, S1 contains a receptor binding domain (RBD) that plays important roles in cell attachment and induction of neutralizing antibodies (Ignjatovic, Galli, 1994; Promkuntod *et al.,* 2014). Thus, S1 is considered as a targeted antigen in many studies on IBV classification and vaccine development (Kapczynski *et al.,* 2003; Johnson *et al.,* 2003; Zhao *et al.,* 2017).

Up to now, there are six genotypes of IBV (GI-GVI) which are sub-divided into different lineages based on the sequencing of the S1 gene (Valastro *et al.,* 2016). Among those, GI-1, GI-7, GI-13, and GI-19 have been the widespread subtypes in many countries for a long period. Several re-circulating IBV strains such as the 793B, TW and QX-like viruses become a global concern in recent years (Gao *et al.,* 2016; Tran *et al.,* 2017; Li *et al.,* 2019).

In Vietnam, although IB has affected a high number of chickens, there are only a few researches on the characterization of IBV and the development of prevention frameworks. Most of these studies focused on the detection and genotyping of IBV from chicken farms (Tran *et al.,* 2017; Le *et al.,* 2019). While vaccination was shown to be the most common method to control IB in Vietnam, most attenuated and inactivated vaccines in the market are produced from imported IBV strains such as H120 or 4/91. Due to the high rate of genetic mutation of IBV, these traditional vaccines show decreased effectiveness or lack of cross-protection (Meeusen *et al.,* 2007; Lee *et al.,* 2012; Tran *et al.,* 2017). Therefore, it is very necessary to develop new generation vaccines such as DNA or subunit vaccines to cope with the emergence of new IBV strains; however, none of these types is available to the vaccine market until now. In our previous publications, agroinfiltration in *Nicotiana benthamiana* was successfully used to express antigens of the flu H5N1 and porcine epidemic diarrhea virus (PEDV) at a high yield (Pham *et al.,* 2019; Ho *et al.,* 2022). This paper describes our study on the expression of the antigenic region in the S1 subunit from the H120 IBV commercial vaccine as the first step for establishing a model for IBV protein production in *N. benthamiana* plants. The results from this study will be a premise for further steps in IBV vaccine development.

MATERIALS AND METHODS

Materials

In this study, the commercial CEVAC® BRON vaccine was provided by Ceva-Phylaxia Veterinary Biologicals Co. Ltd. (Hungary) and CEVA Animal Health Co. Ltd. (Vietnam), and used for gene isolation. The cloning and sequencing primers were designed by Primer3 software and then synthesized by PHUSA Genomics Co. Ltd. (Vietnam).

Table 1. Primers for constructing S1 and RBD expression cassettes

No.	Primer	Sequence (5'-3')	Purpose
1	IBV_clone_F1	AGATTGAAAGCAACGCCAG	To amplify the S1 region
2	IBV_clone_R1	TTATCCATACGCGTTTGTAT	
3	IBV_seq_F1	CCTCTAATGAGACCACAGAT	To sequence the cloned gene
4	IBV_seq_R1	CTCTAGGTGACCCATCACA	
5	IBV_seq_F2	CTTTCAGTTTCAATTGCTTA	To sequence the cloned gene
6	IBV_seq_R2	ATACGAGAGCCACCGCTCTT	
7	S1_IBV_BamHI_F	AG**GGATCC**GTTTTGTATGACAGTAGT*	To construct the desired cassettes
8	S1_IBV_PspOMI_R	TCTT**GGGCCC**AAAACGACGTGTTCCA	
9	RBD_IBV_PspOMI_R	TCTT**GGGCCC**GAAATTGTGTAACGTAAA	

* Bold letter shows the sequence of restriction enzymes

The pJET1.2 vector within the CloneJET PCR Cloning Kit was purchased from Thermo Fisher Scientific (USA). The pRTRA cloning vector (Phan *et al*., 2013; Pham *et al.,* 2019) and the pCB301 shuttle vector (Xiang *et al.*, 1999) were used for constructing the expression cassettes. These vectors were kindly provided by Dr. Udo Conrad (Institute of Plant Genetics and Crop Plant Research, Germany) within the Vietnam – Germany bilateral project (N-T.07.GER.15).

The *Escherichia coli* 10G strain was used for replication of recombinant plasmids and the *Agrobacterium tumefaciens* pGV2260 strain (Vergauwe *et al*., 1996; Do *et al*., 2016; Pham *et al*., 2020) was used for plant infiltration. The *N. benthamiana* plant was cultivated in the greenhouse at the Institute of Biotechnology at the condition of 22 °C and 16 h light per day. The bacteria strains and plant seeds were provided by the Department of Plant Cell Biotechnology, Institute of Biotechnology (IBT).

The monoclonal anti-His tag antibody, horseradish peroxidase-linked sheep anti-mouse IgG antibody (GE Healthcare, UK) and DAB Substrate Kit (3,3′-diaminobenzidine tetrahydrochloride, Thermo Science Pierce, USA) were used for Western blot analysis. The SuperoseTM 6 increase 10/300GL column for protein purification was purchased from GE Healthcare (USA).

METHODS

Construction of plant expression vectors

Total RNA was isolated from the

CEVAC® BRON vaccine by Trizol (Thermo Fisher Scientific, USA) and cDNA was synthesized according to the manufacturer's instructions (Thermo Fisher Scientific, USA). The DNA fragment covering the S1 region (1880 nucleotides) was amplified by specific primers (S1_IBV_BamHI_F and S1_IBV_PspOMI_R) containing restriction sites for *Bam*HI and *Psp*OMI (Table 1). The expected PCR band in agarose electrophoresis was extracted and cloned into pJET1.2 vector. After sequencing by vector and specific primers using Sanger's method, the recombinant pJET1.2 was cut by *Bam*HI and *Psp*OMI, and cloned into pRTRA vector (Figure 1A) which contains a signal peptide from legumin (LeB4), a His tag, a trimerization GCN4-pII motif, and KDEL sequence for protein retention in endoplasmic reticulum (ER) (Phan *et al*., 2013; Pham *et al.,* 2019). The expression cassette LeB4_His_S1vac_GCN4pII_KDEL was inserted into pCB301 shuttle vector by digestion with *Hin*dIII (Xiang *et al.*, 1999; Phan *et al.,* 2016). The pCB301_S1vac vector was introduced to the *Agrobacterium* pGV2260 strain for tobacco transformation. The primer pair S1_IBV_BamHI_F and RBD_IBV_PspOMI_R (Table 1) was used for cloning the RBD region (819 nucleotides) from the pJET1.2 vector, then inserted into pCB301 vector and transformed into *Agrobacterium* pGV2260 strain using a similar protocol as described.

Agrobacterium infiltration

For the expression of recombinant proteins in tobacco, *Agrobacterium* infiltration was applied as described by Phan and Conrad (2016). Briefly, the *Agrobacteria* containing the recombinant pCB301_S1vac or pCB301_RBD and *Agrobacteria* having the plant vector for expression of HcPro to suppress gene silencing were separately cultivated in LB with 50 µg/mL kanamycin, 50 µg/mL rifampicin, and 50 µg/mL carbenicillin at 28 °C and 200 rpm. Two cultures were centrifuged at 6000 rpm for 30 min, combined in infiltration buffer (10 mM MES, 10 mM MgSO4, pH=5.6) to get an OD_{600} of 1.0, and used for *N. benthamiana* transformation. *N. benthamiana* was cultivated until four weeks old before infiltration. A vacuum for 90 s was used for every plant submerged in *Agrobacterium* culture; then these plants were put into the greenhouse at 22 °C, 16 h per day of light. The leaves were harvested on day 3 - 6 and stored at -80 °C. The wildtype leaves were also collected at the same age and used as controls.

SDS-PAGE and Western blot

Plant extracts were obtained by grinding the infiltrated leaves in liquid nitrogen, suspended in binding buffer (50 mM Na_2HPO_4, 300 mM NaCl, 100 mM Na_2SO_3, 1% Triton X100, pH 8.0), and then centrifuged at 13000 rpm for 60 min to collect the supernatant. Total protein in crude extracts was subjected to reducing SDS-PAGE (12% polyacrylamide) together with the commercial SARS-CoV2 S1 (Sino Biological, China) or our in-house p30 recombinant protein from African swine fever virus as standard protein and then transferred to PVDF membranes. The expected proteins were detected by Western blot using a monoclonal anti-His tag antibody as the primary antibody, horseradish peroxidase-linked sheep anti-mouse IgG antibody as the secondary antibody (Gahrtz, Conrad, 2009) and DAB Substrate Kit.

Protein purification by Immobilized Metal Ion Chromatography (IMAC) and Size Exclusion Chromatography (SEC)

S1 or RBD recombinant proteins were purified by IMAC described in detail by Phan *et al*. (2017). Total proteins from 110 - 140 g of leaves were mixed with Ni–NTA agarose resin overnight at 4 °C and added to a chromatography column. After washing by the wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 100 mM Na_2SO_3, 10 mM Imidazole, pH 8.0), recombinant proteins were eluted by elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 500 mM Imidazole, pH 8.0), concentrated by protein concentrators at 10 K molecular weight cutoff (MWCO) (Thermo Fisher Scientific, USA) and dialyzed against PBS (none NaCl). The IMAC-purified HA proteins were stored in 30% glycerol at -30 °C or checked by Western blot and quantified by Amersham™ Imager 680 machine and ImageQuant TL 8.0 software (Cytiva, USA).

The IMAC-purified S1 or RBD was then purified by Superose™ 6 increase 10/300GL column (GE Healthcare, USA) following the protocol as described by Phan *et al*. (2017). High molecular weight standard proteins (75 - 2,000 kDa) were used to estimate the molecular weight of the recombinant proteins. The 0.5 mL/min flow rate was used and fractions containing 500 µL were collected for Western blot detection.

RESULTS AND DISCUSSION

Isolation of S1 gene and construction of S1 (or RBD) containing vectors

In order to study the ability to produce IBV S1 or RBD protein in our plant expression system, we first amplified the 5' region covering the S1 gene from the commercial IBV vaccine by a primer pair shown in Table 1 to make it convenient for further cloning steps. A clear DNA band of approximately 1900 base pairs (bp) was obtained by agarose gel electrophoresis (Figure 1B) and successfully inserted into pJET1.2. Sequencing data showed an identity of 100% between our clone and the published H120 sequence (EU822341). It was therefore used for vector construction.

In this study, two regions of the IBV spike protein were chosen for designing the expression constructs: (1) the RBD (819 nucleotides) that is responsible for virus attachment to cell membrane, and (2) the full S1 subunit (1605 nucleotides) that contains most antigenic sites of this glycoprotein. The IBV signal peptide (SP, amino acid 1-19) was replaced by legumin signal peptide for higher expression in plant (Figure 1A). In addition, GCN4pII, KDEL, His tag and cmyc tag were already included in pRTRA cloning vector for protein trimerization, retention in ER and further purification steps (Phan *et al.,* 2014). The cassettes LeB4-His-**RBD/S1vac**-GCN4pII-cmyc-KDEL from pRTRA were successfully digested by *Hin*dIII enzyme, inserted into the pCB301 shuttle vector and confirmed by colony PCR. The digestion by *Hin*dIII showed DNA bands of expected sizes in agarose gel (~2400 bp for S1 cassette, ~1600 bp for RBD cassette, and ~5500 bp for pCB301 empty vector) (Figure 2A and 2C). The pCB301 vector containing RBD or S1 gene was transformed into the *Agrobacterium* strain pGV2260 and positive clones were selected by colony PCR (Figure 2B and 2D).

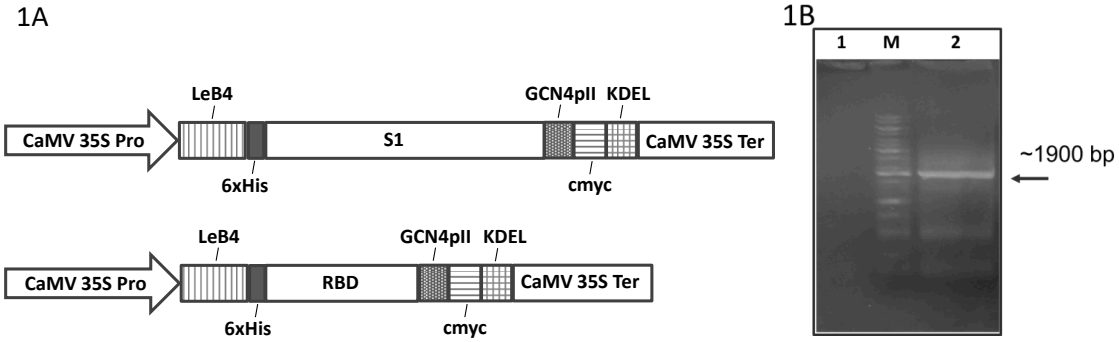

Figure 1. A. Expression cassettes of S1 and RBD in plants. CaMV 35S Pro and CaMV 35S Ter: 35S promoter and terminator from Caulifower mosaic virus (CaMV), LeB4: signal peptide from legumin, 6xHis: six-histidine tail, GCN4-pII: trimerization sequence, cmyc: cmyc tail, KDEL: signal for protein retention in ER. B. Amplification of partial IBV spike gene. (M) 1 kb DNA marker, (1) negative control, (2) PCR product from the commercial vaccine.

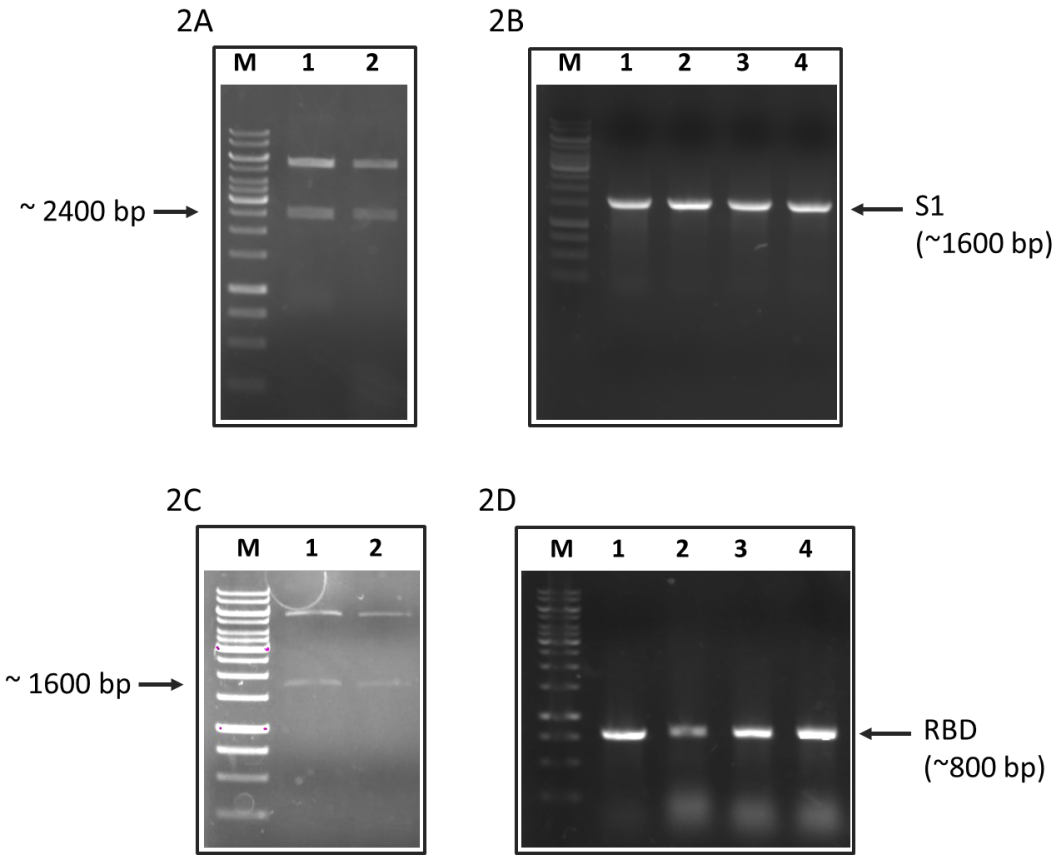

Figure 2. Construction of expression vector harboring S1 or RBD coding gene. A and C. Digestion of pCB301-S1vac and pCB301-RBD by *Hin*dIII to check the presence of expression cassettes, (M) 1 kb DNA marker, (1) and (2) clone 1 and clone 2. B and D. Selection of *A. tumefaciens* clones carrying the recombinant vector pCB301-S1vac and pCB301-RBD by colony PCR; (M) 1 kb DNA marker, (1) to (4) the selected colonies

Expression of S1 and RBD constructs in tobacco plants

After transformation of *N. benthamiana* plants with the above selected *Agrobacterium* clones carrying either S1 or RBD construct, the whole leaves were collected at days 3, 4, 5 and 6 for total protein extraction which is now called in short as S1 or RBD sample. Figure 3 showed the presence of recombinant proteins in total plant extracts detected by Western blot using His tag antibody. There was only a very faint band of about 70 kDa appearing in the wells loading with S1 samples (Figure 3A), while a strong band of approximately 40 kDa was clearly observed in RBD samples (Figure 3B), which were equal to the expected size of glycosylated S1 and RBD. This first result showed the ability of transient expression for partial IBV spike protein in tobacco leaves. Besides, day 4 post transformation was chosen for next experiments due to the most apparent band compared to other time points.

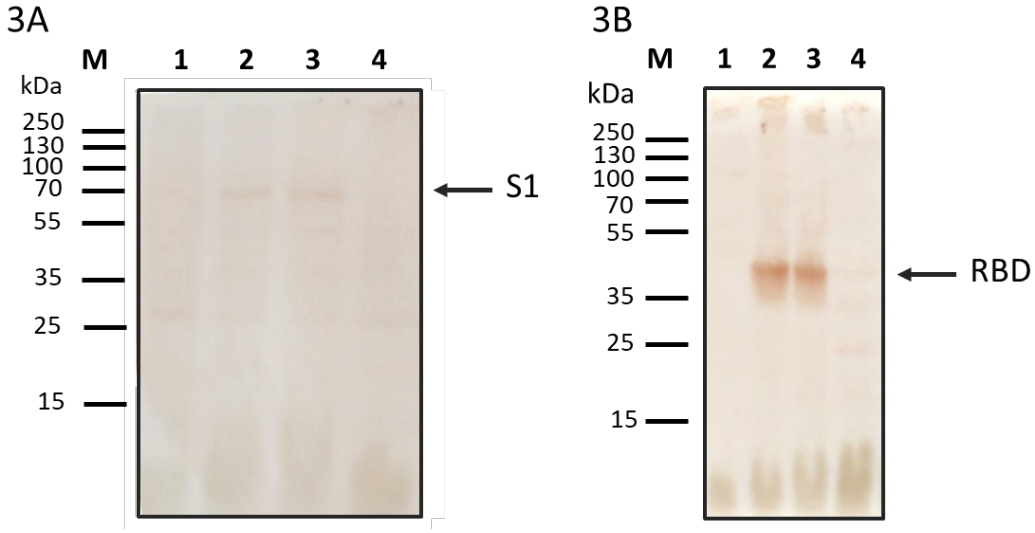

Figure 3. Expression of the recombinant S1 (A) and RBD (B) detected by Western blot using anti-His tag antibodies. (M) protein marker, (1) to (4) the extracted samples from leaves harvested on day 3,4,5 and 6 after agroinfiltration.

Purification of IBV S1 and RBD recombinant proteins

To study biological activities of the recombinant proteins, plant crude extracts collected from 140 g and 115 g of whole leaves infiltrated with RBD or S1 construct, respectively, must be purified by IMAC as described in the method part. Figure 4A showed apparent bands of RBD in contrast to the neighbouring well of the S1 sample with almost no band. This result is in agreement with the previous test showing a much lower expression of S1 compared to RBD (Figure 3A and 3B). Besides, S1 protein may be lost during the purification step and requires further optimization. Therefore, only IMAC-purified RBD protein (~1 µg/µL) was selected in this study for the next steps of SEC purification. According to the chromatogram, samples eluting in fractions 10 - 40 were analyzed by Western blot (Figure 4B). Fractions 13, 16, 19 and 25 showed three bands of approximately 40, 70 and 130 kDa which correspond to the molecular weights of a

monomer, a dimer and a trimer of RBD, but it needs to be confirmed with a crosslinking reaction using BS3 (bissulfosuccinimidyl suberate) in native SDS-PAGE. In addition, lower bands also appeared on the gel perhaps due to the degradation of RBD in reducing conditions that may lead to the decrease of protein concentration (30 ng/µL). Nevertheless, a recombinant RBD was obtained after concentrating the collective fragments and will be used for studying biological activity.

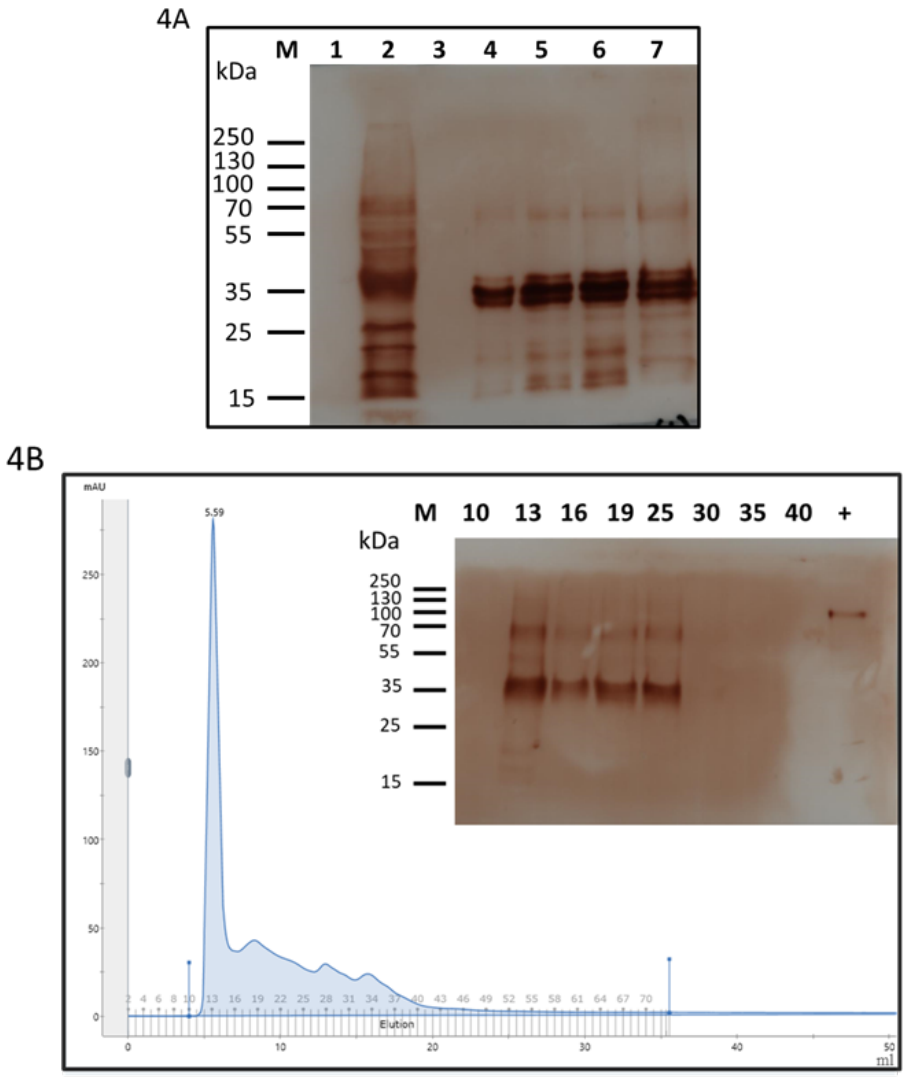

Figure 4. Purification of the recombinant proteins. A. Purification of S1 or RBD by immobilized metal ion affinity chromatography (IMAC), (M) protein marker, (1) wildtype leaf control, (2) RBD sample, (3) S1 sample, (4) to (7) p30 positive control of different volumes (5, 10, 15 and 20 µL of the 385 ng/µL p30 mixed with protein dye (3:1). B. Purification of IMAC-purified RBD protein by size exclusion chromatography (SEC) and the 10-40 SEC fractions analyzed by SDS–PAGE and Western blot using an anti–His tag antibodies, (M) protein marker, (10) to (40) number of SEC fractions, (+) SARS-CoV2 S1 positive control.

There have been studies on the expression of IBV spike protein in different systems, including bacteria, viral vectors, and transgenic potatoes (Zhou *et al.,* 2004; Cao *et al.,* 2013; Toro *et al.,* 2014). However, none of these studies were made for commercial vaccine production due to some limitations of efficacy or scaling-up (Tatsis *et al.,* 2004; Meeusen *et al.,* 2007; Ma *et al.,* 2016). Plant-derived proteins were shown to have great characteristics to be developed as vaccine candidates such as high safety, low cost and easy scale-up (Gidding *et al.,* 2001; Aswathi *et al.,* 2014). Here we used agroinfiltration to transiently express the target proteins within four days and avoid the issues of making transgenic lines. Moreover, according to our experiments (Ho *et al.,* 2022), only the RBD region was selected to get higher efficiency of expression compared to longer constructs, which was shown in this study with a concentration of 20.6 mg protein per kg of fresh leaves after IMAC purification. However, this expression yield is still lower than that of H5N1 hemagglutin and PEDV spike (Pham *et al.,* 2019; Ho *et al.,* 2022). Thus, careful considerations are needed during the purification steps before bringing our results into larger scales.

CONCLUSION

In conclusion, this study showed our first results of constructing IBV-S1 and IBV-RBD containing vectors for *in planta* expression. The IBV-RBD recombinant protein was successfully expressed and purified at an acceptable concentration. A lot of works are on-going to characterize the *in vivo* biological function of the recombinant RBD protein. In addition, other optimizing constructs are now in parallel under study for expressing IBV spike protein in *N. benthamiana* (paper in preparation). We hope that our results will contribute to the development of a new IBV vaccine for chickens.

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REFERENCES

Aswathi PB, Bhanja SK, Yadav AS, Rekha V, John JK *et al.* (2014) Plant based edible vaccines against poultry diseases: a review. *Adv Anim Vet Sci* 2 (5): 305-311.

Cao HP, Wang HN, Yang X *et al.* (2013) *Lactococcus lactis* anchoring avian infectious bronchitis virus multi-epitope peptide EpiC induced specific immune responses in chickens. *Biosci Biotechnol Biochem* 77(7): 1499-1504.

Cavanagh D, Gelb JJr (2008) "Infectious bronchitis". In *Diseases of Poultry*, Blackwell Publishing, Ames, IA, 12th edn: 117-135.

Cook JKA, Jackwood M, Jones RC (2012) "The long view: 40 years of infectious bronchitis research". *Avian Pathol* 41(3): 239-250.

Do HL, Le VS, Le TB (2016) An efficient protocol for *Agrobacterium*-mediated transformation of *Gus*/*Gusplus* gene into cassava plants (*Manihot esculenta* Crantz). *AJB* 38(4): 505-514.

Gahrtz M, Conrad U (2009) Immunomodulation of plant function by in vitro selected single-chain Fv intrabodies. *Methods Mol Biol* 483: 289-312.

Ganapathy K (2009) Diagnosis of infectious bronchitis in chickens. *In Practice* 31: 424-431.

Gao M, Wang Q, Zhao W, Chen Y, Zhang T *et al.* (2016) Serotype, antigenicity, and pathogenicity of a naturally recombinant TW I genotype infectious bronchitis coronavirus in China. *Vet Microbiol* 191: 1-8.

Giddings G, Allison G, Brokks D, Carter A (2001) Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol* 18: 1151-1155.

Ho TT, Trinh VT, Tran HX, Le PTT, Nguyen TT *et al.* (2022) The immunogenicity of plant-based COE-GCN4pII protein in pigs against the highly virulent porcine epidemic diarrhea virus strain from genotype 2. *Front Vet Sci* 9: 940395.

Ignjatovic J, Galli L (1994) The S1 glycoprotei but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. *Arch Virol* 138: 117-134.

Johnson MA, Pooley C, Ignjatovic J, Tyack SG (2003) A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. *Vaccine* 21(21-22): 2730-2736.

Kapczynski DR, Hilt DA, Shapiro D, Sellers HS, Jackwood MW (2003) Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. *Avian Dis* 47(2): 272-285.

Le TKX, Nguyen TCL, Tran NB, Doan TTH, Le TH (2019) Determining and grouping IBV in chicken in Mekong Delta. *J Vet Sci Tech* XXVI (5): 5-13.

Lee SW, Markham PF, Coppo MJ, Legione AR, Markham JF *et al*. (2012) Attenuated vaccines can recombine to form virulent field viruses. *Science* 337(6091): 88.

Li S, Du L, Xia J, Du J, You G, *et al.* (2019) Antigenic and pathogenic characteristics of QX-Type avian infectious bronchitis virus strains isolated in Southwestern China. *Viruses* 11(12): 1154.

Ma BC, Yang X, Wang HN, Cao HP, Xu PW *et al.* (2016) Characterization of a new *Lactobacillus salivarius* strain engineered to express IBV multi-epitope antigens by chromosomal integration**.** *Biosci Biotechnol Biochem* 80: 574-583.

Meeusen ENT, Walker J, Peters A, Pastoret PP, Jungersen G (2007) Current status of veterinary vaccines. *Clin Microbiol Rev* 20(3): 489-510.

Perlman S, Gallagher T, Snijder EJ (2008) *Nidoviruses*. ASM Press, Washington, DC.

Promkuntod N, van Eijndhoven REW, de Vrieze G, Gröne A, and Verheije MH (2014) Mapping of the receptorbinding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. *Virology* 448: 26-32.

Pham TV, Ho TT, Phan TH, Le TH, Pham BN *et al.* (2019) A plant-based artificial haemagglutinin (A/H5N1) strongly induced neutralizing immune responses in mice. *Appl Sci* 9(21): 4605.

Phan HT, Pohl J, Floss DM, Rabenstein F, Veits J *et al.* (2013) ELPylated haemagglutinins produced in tobacco plants induce potentially neutralizing antibodies against H5N1 viruses in mice. *Plant Biotechnol J* 11: 582-593.

Phan HT, Conrad U (2016) Plant-based vaccine antigen production. *Methods Mol Biol* 1349: 35-47.

Phan, HT, Ho, TT, Chu HH, Vu TH, Gresch U, Conrad U. (2017) Neutralizing immune responses induced by oligomeric H5N1-hemagglutinins from plants. *Vet Res* 48: 53.

Raj DG, Jones RC (1997) Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. *Avian Pathol* 26: 677-706.

Tatsis N, Ertl HCJ (2004) Adenoviruses as vaccine vectors. *Mol Ther* 10(4): 616-629.

Toro H, Zhang JF, Gallardo RA, van Santen VL, van Ginkel *et al*. (2014) S1 of distinct IBV population expressed from recombinant adenovirus confers protection against challenge. *Avian Dis* 58: 211-215.

Tran NB, Nguyen PK, Pham HD, Nguyen TCL (2017) *Can Tho Univ J of Sci* 6: 56-62.

Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW *et al*. (2016) S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infect Gene Evol* 39: 349-364.

Vergauwe A, Cammaert R, Vandenberghe D, Genetello C, Inze D *et al*. (1996) *Agrobacterium tumefaciens*-mediated transformation of *Artemisia annua* L. and regeneration of transgenic plants. *Plant Cell Rep* 15(12): 929-33.

Xiang C, Han P, Lutziger I, Wang K,Oliver D (1999) A mini binary vector series for plant transformation. *Plant Mol Biol* 40: 711.

Zhao R, Sun J, Qi T, Zhao W, Han Z et al. (2017) Recombinant Newcastle disease virus expressing the infectious bronchitis virus S1 gene protects chickens against Newcastle disease virus and infectious. *Vaccine* 35(18): 2435-2442.

Zhong Q, Hu YX, Jin JH, Zhao Y, Zhao J, Zhang GZ (2016) Pathogenicity of virulent infectious bronchitis virus isolate YN on hen ovary and oviduct. *Vet Microbiol* 193: 100-105.

Zhou JY, Cheng LQ, Zheng XJ, Wu JX, Shang SB et al. (2004) Generation of the transgenic potato expressing full-length spike protein of infectious bronchitis virus. *J Biotechnol* 111(2): 121-130.