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CONSTRUCTION AND TRANSIENT EXPRESSION OF THE CAP PROTEIN OF PORCINE CIRCOVIRUS 3 IN *Nicotiana benthamiana*

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ABSTRACT

Porcine circovirus (PCV) leads to severe illnesses in pigs, including postweaning multisystemic wasting syndrome (PMWS), reproductive impairments, and kidney disease syndrome. Among the four recognized genotypes (PCV1–PCV4), PCV3 has emerged as a globally distributed pathogen that includes Vietnam and represents a major economic burden to the global pig farming industry. Acting as a major immunogen, the Cap protein effectively induces protective immunity, highlighting its potential as a target antigen for subunit vaccines against PCV3. In this study, a plant expression vector carrying the gene encoding the Cap3 protein fused with the GCN4pII motif (Cap3-pII) was constructed and then introduced into *Agrobacterium tumefaciens*. The transformed bacteria were then infiltrated into *Nicotiana benthamiana* leaves. Western blot analysis confirmed the expression level of the Cap3-pII protein, with a yield of approximately 13 mg/kg of fresh leaf. These results provide a basis for further research on developing a subunit vaccine against PCV3 using transient expression technology in plants.

Keywords: *Agrobacterium tumefaciens,* Cap3 protein, GCN4pII motif, *Nicotiana benthamiana,* Porcine Circovirus 3, transient expression.

INTRODUCTION

Porcine circovirus (PCV) is a single-stranded DNA virus that causes severe diseases in pigs, including postweaning multisystemic wasting syndrome (PMWS), reproductive failures, and nephropathy syndrome (PDNS) (Chae, 2005; Cino-Ozuna *et al.*, 2011). PCV presents dangerous economic threats to the swine industry in several Asian countries (Maity *et al.*, 2023; Tran *et al.*, 2021). PCV belongs to the Circoviridae family and the genus circovirus, with four known types: PCV1, PCV2, PCV3, and PCV4 (Opriessnig *et al.*, 2020). Since its emergence in the United States in 2015 (Palinski *et al.*, 2017), PCV3 has spread rapidly to many countries worldwide, including Vietnam. The virus is now

recongnized as a serious swine industry pathogen (Chung *et al.*, 2021; Dinh *et al.*, 2023; Molossi *et al.*, 2022; Visuthsak *et al.*, 2021; Wen *et al.*, 2018). PCV3 has been identified in both healthy and disease swine populations. While its pathogenicity remains unclear, it is often linked to reproductive issues, PDNS, multisystemic diseases, and cardiac problems (Palinski *et al.*, 2017; Phan *et al.*, 2016). PCV3 frequently co-infects with PCV2, leading to similar symptoms (Lv *et al.*, 2023).

The genome of PCV3 comprises a circular, single-stranded DNA (ssDNA) molecule of approximately 2.0 kb. PCV3 primarily contains three open reading frames (ORFs): ORF1 and ORF2, which code for the replicase (Rep) and capsid (Cap) proteins, respectively, while the function of ORF3 remains uncertain. ORF2 encodes the Cap protein, which is 645 base pairs long and translates into 214 amino acids (Wen *et al.*, 2018). As the only structural protein that forms the virus's outer shell, the Cap protein can self-assemble into virus-like particles (VLPs) that closely resemble PCV virions. Although the genome of PCV3 has been sequenced, its homology with other porcine circoviruses is low (Guo et *al.*, 2019; Yuzhakov *et al.*, 2018). The Cap protein is vital for its antigenic properties and plays a key role in activating the immune response, making it an ideal target for developing subunit vaccines against PCV3 infection.

Plant-derived expression platforms confer multiple benefits, such as economic efficiency, enhanced biosafety owing to the minimal risk of animal pathogen contamination, and the ability to synthesize N-glycosylated recombinant proteins (Fahad *et al.*, 2015). Moreover, transient protein expression in plants requires only a biosafety level-1 laboratory and a greenhouse for plant cultivation, making this technology more accessible to a broader research community (Rybicki, 2009). Therefore, the development of Cap subunit vaccines in plants also shows promising potential. A plant-based PCV2a vaccine, commercialized by Bioapp Inc. in 2022, has demonstrated protective efficacy against four heterologous PCV2 genotypes, namely 2a, 2b, 2d, and 2e (Park *et al.*, 2023). This vaccine had a positive effect on pigs, inducing high levels of neutralizing antibody titers against PCV2d, as well as PCV2d-specific interferon-gamma-secreting cells. Currently, in Vietnam, there is only one vaccine developed against PCV-2, known as Han-Circovac, which HANVET created. To date, no commercial vaccine in Vietnam has demonstrated protection against PCV3, and there are no reports of cross-protection with existing PCV2 vaccines. This highlights the urgent need for vaccine development to prevent and control PCV3. The PCV3 Cap proteins (Cap3) have been expressed in systems such as *Escherichia coli* (Fernandes *et al.*, 2013) and insect cells (Zhang *et al.*, 2019), but they have not yet been described in plant systems, such as *N. benthamiana.*

In this study, the gene Cap3 was fused with GCN4pII, a mutant version of the "leucine zipper" from the GCN4 protein, which is a well-studied transcription factor found in *S. cerevisiae*. The modified domain can assemble from a dimer to a highly stable parallel trimeric coiled coil (Harbury et al., 1993). This is supported by evidence involving the development of the HA (hemagglutinin) subunit vaccine against the influenza virus. The recombinant trimeric sHA not only suppressed the instability of HA but also enhanced immunogenicity (Weldon et al., 2010; Phan et al., 2017). For our purposes, a plant expression vector with the Cap3-GCN4pII fusion gene (Cap3-pII)

was generated and transformed into *Agrobacterium tumefaciens*. The bacteria suspension was infiltrated into *N. benthamiana* leaves. A high expression level of Cap3-pII was observed and qualified using Western blot. This work demonstrates the potential of plant transient expression systems for the rapid development of subunit vaccine candidates against PCV3.

MATERIALS AND METHODS

Construction of a vector harboring the Cap3-pII protein

The gene encoding the PCV3 Cap3 protein was provided by the CNC Veterinary Medicine Production and Trading Joint Stock Company. This gene served as the template for PCR amplification of a truncated fragment encoding amino acids 42-213 incorporating terminal *Bam*HI and *Psp*OMI. This amplicon was subsequently ligated into the corresponding sites of cloning vector pRTRA and then transformed into *E. coli*G10. The recombinant plasmid, pRTRA-Cap3-pII, was identified in positive clones through colony PCR using the primer pair 35S-SQF (5'- CACTGACGTAAG GGATGACGC-3') and 35S-Term (5'-CTGGGAACTACTCACACA-3') and restriction digestion, with final confirmation of the insert sequence by Sanger sequencing (Ho *et al*., 2022).

Cloning of the Cap3-pII coding sequence into a plant expression vector

The pRTRA-Cap3-pII vector was treated with *Hin*dIII to collect the expression cassette with *Cap3-pII*. At the same time, the pCB301 vector was cut with *Hin*dIII and treated with shrimp alkaline phosphatase (*SAP*) for dephosphorylation. The cassette Cap3-pII was ligated with the pCB301 vector. The resulting recombinant plasmid was then transformed into *E. coli* G10 competent cells. Successful transformants were selected on Luria-Bertani (LB) agar plates with the addition of 50 mg/L kanamycin. The presence of the Cap3-pII insert in the transformants was initially confirmed via colony PCR and further verified by restriction digestion. The confirmed pCB301-Cap3-pII plasmid was efficiently delivered into the *A. tumefaciens* AGL1 cells via electroporation (2.5 kV, 25 µF and 200 Ω) in preparation for plant transformation experiments.

Agroinfiltration of Cap3-pII protein in *N. benthamiana* leaves

Transient expression of the Cap3-pII protein in *N. benthamiana* leaves was carried out following the protocol described by Ho *et al.* (2022). Two separate cultures of *A. tumefaciens* strains were established: one transformed with the pCB301-Cap3-pII recombinant vector and the other containing pIBT/Hc-Pro PVY vector (Phan *et al*., 2017) that expresses Hc-pro (Helper-componant proteinase), a known RNA silencing suppressor. Cultivation occurred in 5 mL of selective LB medium (50 mg/L carbenicillin, kanamycin, and rifamycin) at 28°C, under agitation at 120 rpm for 16 hours. The cultures were then expanded to 50 mL LB and incubated under the same conditions. Bacterial cells were harvested by centrifugation (4200 rpm, 15 min, 4°C) and resuspended in MES buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) to OD600 of 0.25. The suspension was applied to seven-week-old, hydroponically grown *N. benthamiana* plants via vacuum infiltration. The process was conducted at a pressure of 27 inches Hg (0 atm) for 90 seconds. Transformed plants were maintained in a greenhouse, and leaves were harvested 3 days post-infiltration, then kept at -80°C for further experiments.

Characterization of Cap3-pII protein expression in *N. benthamiana* leaves using SDS-PAGE and Western blot analysis.

Samples were prepared from leaf extracts in SDS sample buffer and denatured by heating at 95°C for 10 minutes before being clarified by centrifugation. The total soluble protein (TSP) concentration was determined using the Bradford method (Bradford, 1976). Thirty micrograms of TSP were separated on a 10% SDS-PAGE gel and transferred electrophoretically to a nitrocellulose membrane at 18 V overnight. To prevent nonspecific binding of the antibody, the blocking membrane steps were performed in two hours in the 5% skim milk in Phosphate-Buffered Saline (PBS). For the next hour, this membrane was immersed in the anti-His-tag antibody (1:1000 dilution) (Thermo Fisher Scientific). Before being incubated with the secondary antibody, the membrane was washed three times, for 5 minutes each, in 0.5% milk prepared in PBS. The HRP-conjugated anti-mouse IgG (Invitrogen), the secondary antibody, was used at a 1:4000 dilution for 1 hour of incubation time. Visualization of the Cap3-pII protein was achieved using a DAB-based colorimetric assay (Thermo Fisher Scientific). The reaction was performed with the addition of 0.04% H_2O_2. Images of the Western blot were acquired with an Amersham™ Imager 680 system. The protein Cap-3 concentration was quantified relative to a calibration curve generated from S1-SARS-CoV-2 protein (Sino Biological, 24–120 ng) using ImageQuant TL 8.0 software (Cytiva).

RESULTS AND DISCUSSION

Generation of a cloning vector harboring the *Cap3-pII* gene

The *Cap* gene PCR product and the pRTRA-pII vector were subjected to digestion with *Bam*HI and *Psp*OMI. The digested fragments were then purified and ligated under the catalysis of T4 ligase to construct the recombinant vector pRTRA-Cap3-pII. After transformation into *E. coli* G10, ten bacterial colonies were selected (Figure 1A) to verify the existence of the *Cap3* gene by colony PCR using the primer pair 35S-SQF and 35S-Term. Electrophoresis results of the colony-PCR products revealed that all 10 selected bacterial colonies displayed an amplicon of 0.99 kb, which corresponds to the theoretical calculation. This confirms the presence of the *Cap3* gene in all 10 colonies. Among these positive clones, two were selected and cultured for plasmid extraction and digestion using the *Bam*HI and *Psp*OMI (Figure 1B). Electrophoresis analysis of the digested plasmids showed that both displayed band patterns matching the expected sizes, with the pRTRA-pII vector backbone (~3.7 kb) and the *Cap3* gene fragment (~0.51 kb). To ensure the accuracy of the inserted *Cap3* gene fragment within the pRTRA vector, the recombinant vector was sequenced using primers located on the vector backbone. Sequence analysis also confirmed that the expected sequence was consistent with theoretical predictions. The schematic representation of the pRTRA-Cap3-pII vector is shown in Figure 1C.

Construction of a plant expression vector containing the *Cap3-pII* gene

To construct the pCB301-Cap3-pII vector, the pCB301 backbone was linearized using *Hin*dIII digestion. Simultaneously, the pRTRA-Cap3-pII plasmid was also treated with *Hin*dIII. The 1.5 kb expression cassette carrying the Cap3-pII gene was excised and purified for the ligation reaction (Figure 2A).

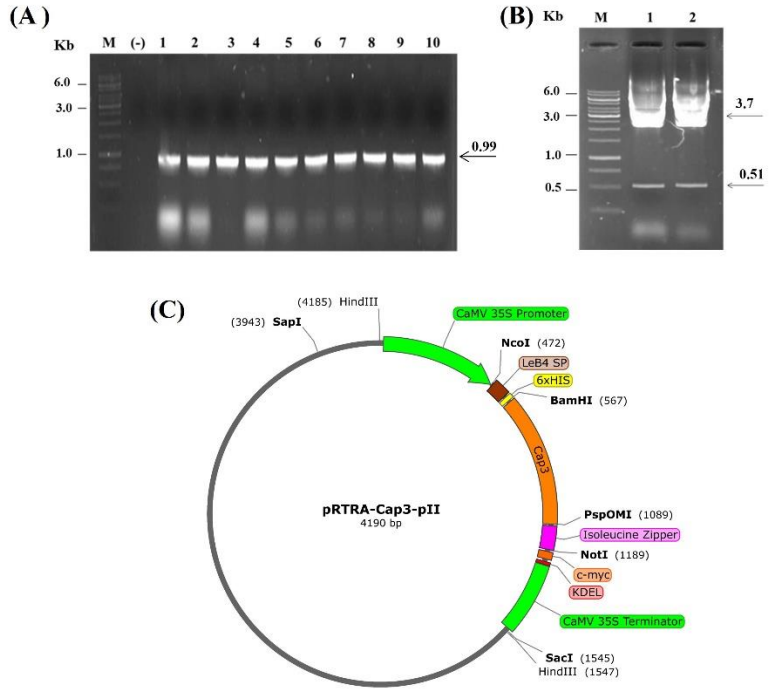

Figure 1. Construction of a vector carrying the *Cap3-pII*. (A) Results of colony PCR analysis of putative pRTRA-Cap3-pII transformants; (B) Double digestion with *Bam*HI and *Psp*OMI to confirm the integrity of the recombinant plasmid; (C) Schematic diagram depicting the structure of the pRTRA-Cap3-pII vector. CaMV 35S promoter: 35S promoter from Cauliflower mosaic virus, LeB4 SP: signal peptide, 6xHis: 6xHis-tag; KDEL: ER retention signal, isoleucine zipper: trimer motif, c-myc: cmy-tag, CaMV 35S Terminator: 35S terminator from Cauliflower mosaic virus.

Competent *E. coli* cells were transformed with the recombinant plasmid generated from the ligation reaction. Colony-PCR analysis on a 1% agarose gel revealed that six of seven tested colonies displayed distinct, bright bands corresponding to the expected theoretical size of 0.99 kb (Figure 2B). Additionally, the digestion of the pCB301-Cap3-pII plasmid with *Not*I generated bands of the expected theoretical sizes (5.6 kb and 1.49 kb). This confirms the successful construction of pCB301-Cap3-pII (Figure 2C). The pCB301-Cap3-pII vector is schematically illustrated in Figure 2D. The confirmed pCB301-Cap3-pII recombinant plasmid was delivered to competent *A. tumefaciens* AGL1 cells. To confirm successful transformation, the presence of the pCB301-Cap3-pII construct in *A. tumefaciens* AGL1 was assessed by colony PCR using 35SS-QF/35-Term-R primers. Agarose gel electrophoresis of the colony-PCR products from six bacterial colonies revealed that all six contained a DNA fragment of 0.99 kb, which matched the expected theoretical size. *A. tumefaciens* colony one was cultured and used for transient expression in *N. benthamiana* leaves.

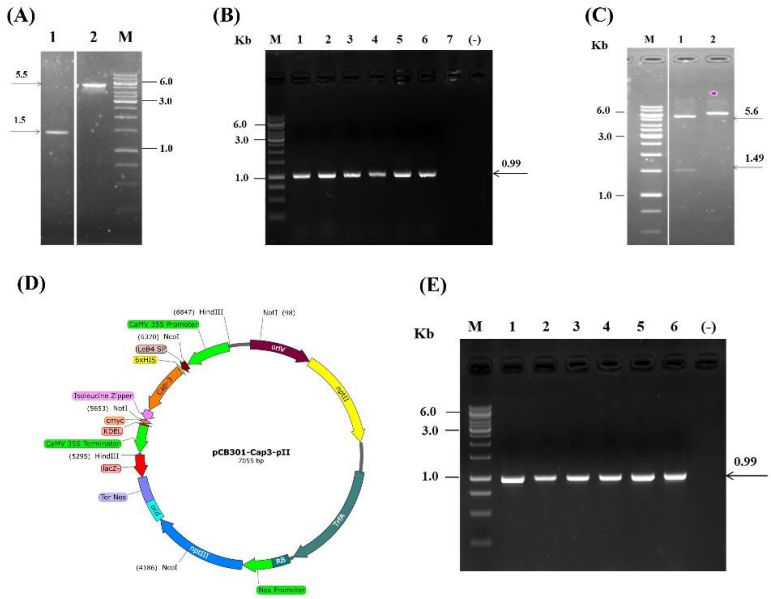

Figure 2. Construction of a plant expression vector carrying the *Cap3-pII*. (A) Digestion of pRTRA-Cap3-pII and pCB301 with *Hind*III. (B). Recombinant pCB301-Cap3-pII colony screening (1-6) by PCR using 35SS-QF/35-Term-R primers; (C) Verification of pCB301-Cap3-pII plasmids (1, 2) by *Not*I digestion; (D) Schematic representation of the pCB301-Cap3-pII vector. (E) Identification of *A. tumefaciens* clones (1-6) harboring the recombinant pCB301-Cap3-pII vector through direct PCR from colonies. (-): negative control.

Evaluation of Cap3-pII protein expression in *N. benthamiana* leaves by SDS-PAGE and Western blot analysis

Three days post-agroinfiltration, leaves were harvested to examine Cap3-pII protein expression (Figure 3A). The presence of the recombinant Cap3-pII protein was verified by Western blot analysis with an anti-6xHis tag antibody, revealing a band of molecular weight below 35 kDa. The Cap3-pII protein was successfully expressed in tobacco leaves. Its estimated theoretical molecular weight, determined using Lasergene software, is 26.93 kDa for the monomeric Cap3 protein, including the His-tag and GCN4pII sequences, but excluding the Nuclear Localization Signal region (NLS). When proteins are expressed in the plant system, they undergo a co- and post-translational modification, particularly N-Glycosylation. It is specifically linked at Asparagine residue within the consensus sequence Asp-X-Ser/Thr (where X can be any amino acid except proline) (Strasser, 2016). Using the GlycoMod program (developed by EXPASY) one N-Glycosylation site was found at the Asp 252 followed by the Gly-Ser in the sequence of Cap3-pII (https://www.expasy.org/resources/glycomod). Then, this finding may explain the observed change in its molecular weight.

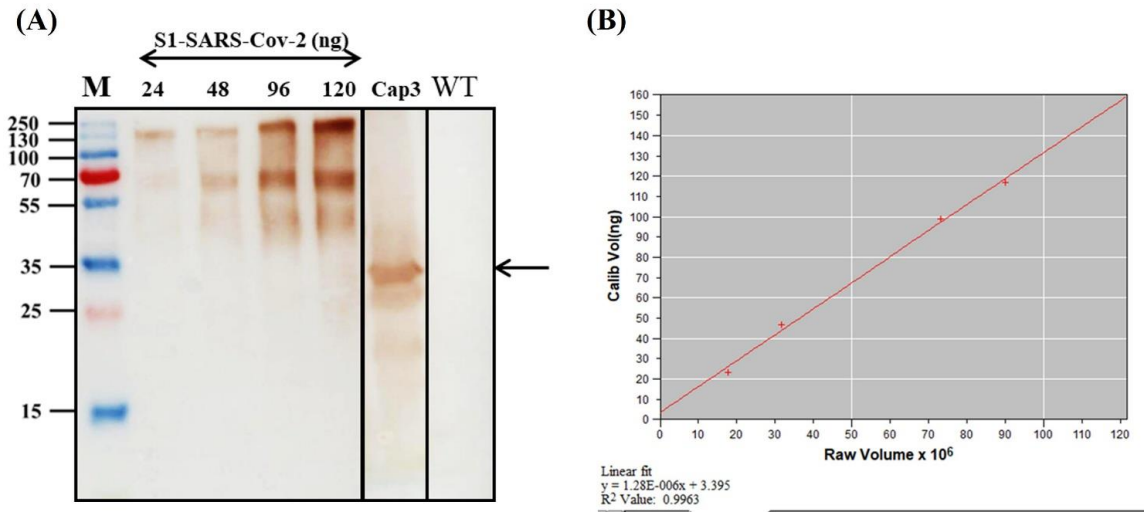

Figure 3. Assessing of recombinant Cap3-pII protein expression in *N. benthamiana*. (A) Western blot detection of the Cap3-pII protein with an anti-6x-His tag antibody. Total soluble protein (TSP, 30 µg) extracted from transformed leaves was separated on a 4–10% SDS-PAGE gel. S1-SARS-CoV-2 protein (24, 48, 96 and 120 ng) served as a reference for quantifying Cap3-pII protein in leaf extracts; (B) A standard curve for Cap3-pII protein quantification, with S1-SARS-CoV-2 protein amounts represented by red dots. WT: Wild type plant.

The accumulation of recombinant Cap3-pII protein in *N. benthamiana* was determined using the recombinant S1 protein of SARS-CoV-2. A standard curve (Figure 3B), which was established by plotting signal intensities against known concentrations of S1 protein, was used to quantify the Cap3-pII protein. It illustrates the association between the raw volume (X) and the calibrated volume (Y) of S1 protein bands. This analysis revealed that the Cap3-pII protein accumulated to approximately 13 mg/kg of fresh tissue in tobacco leaves.

Currently, there are no published reports on the expression of the PCV3 Cap protein in a plant system, specifically in *N. benthamiana*. Prior research has shown that the PCV2 Cap protein can be expressed in *N. benthamiana* and has confirmed its immunogenic properties in animal models. When compared to the accumulation of PCV2 Cap protein in a previous study (Gunter *et al.*, 2019), which was recorded at 6.5 mg per 1 kg of leaf wet weight, the Cap3-pII protein exhibited a 2-fold higher expression in *N. benthamiana* leaves. In contrast to a previous study (Park *et al.*, 2021), which reported higher yields, the quantity of the Cap3-pII protein was still comparatively lower. A PCV2a recombinant Cap subunit vaccine, produced using the *N. benthamiana* expression system, has recently become commercially available. The PCV2a Cap protein, when expressed in the form of virus-like particles (VLPs), elicited neutralizing antibodies against PCV2 in piglets (Park *et al.*, 2023). Following a co-challenge with four distinct PCV2 genotypes (2a, 2b, 2d, and 2e), vaccinated pigs exhibited a significant reduction in clinical symptoms, a decreased PCV2 viral load in the bloodstream and lymph nodes, and attenuated lymphoid lesion severity (Oh *et al.*, 2022). In a field study, a plant-based PCV2a vaccine was found to induce a robust humoral immune response, as evidenced by

the high titers of PCV2d-neutralizing antibodies observed in vaccinated pigs. Furthermore, the heightened number of PCV2d-specific interferon-γ-secreting cells was linked to a reduction in the PCV2d viral titer and the mitigation of lymphoid lesion severity (Park *et al.*, 2023). The findings collectively indicate that the plant-derived subunit vaccine has the potential to provide protection against PCV infection. The PCV2a Cap protein was expressed in the form of VLPs even though its structure had been truncated NLS (Park et al., 2021). In contrast, the VLP formation of PCV3 Cap protein was not observed without the NLS. This can be explained by the conformational change of the PCV3 NLS region, resulting in a protruding region on the VLP as opposed to the flatter region observed in PCV2. The interaction among the NLS regions of the PCV3 Cap proteins stabilizes the VLP structure. Consequently, the NLS in the PCV3 Cap protein is essential for VLP formation (Bi et al., 2020). In addition, evidence suggests that the cytotoxicity of the full-length PCV3 Cap protein in porcine cells is linked to its nuclear translocation. Truncation of the NLS in the PCV3 Cap enhanced cell viability; however, this structural modification simultaneously disables the capacity to self-assemble the VLP (Chang et al., 2023). Expression of the entire PCV3 Cap protein in the plant system yielded unsatisfactory results (data not shown). A recent study showed that incorporating the GCN4pII motif resulted in the trimerization of the avian flu hemagglutinin, which serves as the base unit of the oligomer protein. In this oligomeric form, the protein expression yield was much higher. More importantly, it elicited a better humoral immunity, as well as a higher hemagglutination inhibition titer against influenza virus (Phan et al., 2017). Similar results were also found in the trimeric COE protein, which was constructed for subunit vaccine development against porcine epidemic diarrhea virus (Ho et al., 2022). Then, removing the NLS and incorporating the GCN4pII motif led to an increased expression level of the PCV3 Cap-pII fusion protein in the plant system. Our future work aims to enhance the yield of the PCV3 Cap protein, followed by its isolation and a comprehensive evaluation of its immune-eliciting properties in relevant animal models.

CONCLUSION

This study represents the first successful transient expression of the PCV Cap3-pII protein in *N. benthamiana*, with an accumulation level of approximately 13 mg/kg fresh leaves. This work serves as a proof-of-concept for the utilization of a plant expression system to produce subunit vaccines targeting PCV in Vietnam.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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