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Short communication

Development of a pod pepper vein yellows virus-based expression vector for the production of heterologous protein or virus like particles in *Nicotiana benthamiana*

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ABSTRACT

Plant viruses are emerging as a compelling alternative system for the heterologous production of pharmaceutical proteins, offering advantages in scalability, cost-effectiveness, and biological safety over traditional expression systems. They are increasingly recognized as effective platforms for biomedical applications, frequently used in the expression of human viral proteins and the display of peptides or proteins. The pod pepper vein yellows virus (PoPeVYV), classified within the genus *Polerovirus* of the *Solemoviridae* family, can substantially increase viral titers when co-infected with pod pepper vein yellows virus-associated RNA (PoPeVYVaRNA). This mixed infection methodology facilitates the formation of rod-shaped virus-like particles (VLPs), wherein modified green fluorescent protein (mGFP) is fused to the C-terminus of the coat protein (CP) from the pepper mild mottle virus (PMMoV). Notably, the expression of hepatitis B surface antigen (HBsAg) demonstrates a marked preference for plant viruses, allowing for enhanced expression via the PoPeVYV mixed infection system in *Nicotiana benthamiana*. Consequently, the PoPeVYV-based vector presents a promising alternative for the high-level production of heterologous proteins and rod-shaped VLPs in plants.

The advancement of virus-based vectors for foreign gene expression is making a significant impact on laboratory research, therapeutic strategies, and commercial biotechnology. Plant viruses are considered nonpathogenic to animals and are generally regarded as safe for nanomedicine applications (Shahgolzari et al., 2020). Frequently employed methodologies include heterologous protein expression and cargo protein or peptide display on the virion surface, which can serve as antigens, vaccines, adjuvants, and tumor inhibitors (Cai et al., 2019; Metavarayuth et al., 2019; Pokorski et al., 2011; Shukla et al., 2017).

Recent references highlight that tobamoviruses, classified within the genus *Tobamovirus* of the *Virgaviridae* family, are one of the most extensively researched virus groups and constitute rank among the most widely utilized plant viruses. These viruses possess a positive-sense RNA genome comprising a single RNA approximately 6 kb that encodes critical components such as RNA-dependent RNA polymerase (RdRp; p126 and p183), movement protein (MP), and coat protein (CP) (Creager et al., 1999). Among various genetic engineering tools of tobamoviruses, the 30B vector, based on the tobacco mosaic virus (TMV), is noteworthy for its ability to stably express foreign proteins and is used globally (Shivprasad et al., 1999). The expression of MP and CP proteins relies on subgenomic (sg) promoters, with the distance between the sg promoters and the 3′ untranslated region (UTR) being pivotal for regulating gene expression (Man and Epel, 2004; Shivprasad et al., 1999). Notably, the full MP and CP sgRNA promoters encompass parts of RdRp-MP, and MP-CP, respectively (Grdzelishvili et al., 2000). This translational mechanism largely explains how genomic positioning affects both transcription and translation of *MP* and *CP* genes (Culver et al., 1993). As a result, the 30B vector faces challenges in expressing larger proteins, and heterologous sequences can significantly influence the expression of foreign genes.

Pod pepper vein yellows virus (PoPeVYV), a new recombinant virus and belonging to the genus *Polerovirus* within the *Solemoviridae* family (Zhao et al., 2021), comprises seven viral proteins: P0 (a silencing suppressor), P1 and the P1-P2 fusion protein (serving as RNA-dependent RNA polymerase), P3 and P5 (the major and minor capsid proteins, respectively), P3a (a non-AUG initiation protein), and P4 (movement

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protein). Our findings suggest that co-infection with PoPeVYV and pod pepper vein yellows virus-associated RNA (PoPeVYVaRNA, a Tombusvirus-like associated RNAs) can enhance the viral titers of PoPeVYV (Peng et al., 2021). Subsequent investigations will explore whether co-infection of PoPeVYV and PoPeVYVaRNA can enhance heterologous protein expression and assess whether PoPeVYV can overcome the limitations associated with tobamoviruses.

Previous study showed that at least three subgenomic RNAs of potato leafroll virus (PLRV), the reference species of *Polerovirus*, have been identified and located at positions 3536 (in the intergenic region), 5069 (in the ORF of P5), and 5347 (in the ORF of P5) nucleotides (nt), respectively (Ashoub et al., 1998; Hwang et al., 2013). To investigate the efficacy of heterologous protein expression using PoPeVYV, the modified PoPeVYV mutant vectors were constructed based on a previously developed infectious virus clone and recombination method (Zhao et al., 2021). We replaced the P3-P5 region of PoPeVYV with modified green fluorescent protein (mGFP) (Haseloff et al., 1997), resulting in a modified viral construct designated PoPeVYV$_{Del}$-mGFP (Fig. 1A, Table S1). The engineered viral vector maintained the RdRp components (P1 and P1-P2 fusion protein) and silencing suppressor (P0) from PoPeVYV, which facilitated efficient mGFP expression through viral infection. According to previous study of PLRV, the mGFP can be transcribed by promoter of P3 subgenome RNA. To evaluate the enhancement of heterologous protein expression, we performed co-infection experiments using PoPeVYV$_{Del}$-mGFP and PoPeVYVaRNA (MI), with pCB301-GUS co-infection (SI) serving as a control (Fig. 1B). These constructs were expressed into *N. benthamiana* leaves via *Agrobacterium*-mediated infiltration. At 2 days post-inoculation (dpi), mGFP expression levels were determined by examining the infiltrated leaves under UV illumination. The MI treatment exhibited higher fluorescence intensity compared to the SI treatment (Fig. 1B). The observations were continued for 6 days, both SI and MI treatments reached peak protein expression at 4 dpi. At 6 dpi, the MI treatment demonstrated a lower fluorescence quenching rate compared to the SI (Fig. 1D). The mGFP protein accumulation of inoculated leaves were also verified through Western blot analysis using anti-GFP antibodies (Fig. 1C, E). Previous studies have shown that tombusvirus-like associated RNAs (tlaRNAs), which are single-stranded positive-sense RNAs, can replicate autonomously and enhance polerovirus titers (Sanger et al., 1994). During infection, tlaRNAs generate abundant subgenomic RNAs (sgRNAs). The 3′ UTR of tlaRNAs inhibits XRN1 activity near the 5′ end of sgRNAs, suggesting that tlaRNAs not only require viruses with compatible capsids but also function as symbiotic elements that modulate host cell RNA biology (Campbell et al., 2022). This mechanism explains how the co-infection of PoPeVYV$_{Del}$-mGFP and PoPeVYVaRNA resulted in elevated PoPeVYV$_{Del}$-mGFP titers.

The surface display of cargo proteins or peptides on tobamoviruses has been accomplished through two principal strategies. The first approach involves the C-terminal fusion of cargo proteins to the CP via a flexible $(GGGGS)_3$ linker, which consists of 15 amino acids (Brown et al., 2013; Lu and Feng, 2008; Werner et al., 2006). The second method utilizes a foot-and-mouth disease virus 2A sequence inserted between the cargo protein and CP (Jiao et al., 2024; Röder et al., 2017). The assembly of tobamovirus is initiated through specific interactions between CP aggregates and the Origin of Assembly Sequence (OAS), an internal viral RNA sequence (Turner and Butler, 1986). To facilitate the assembly of rod-like particles, we engineered chimeric vectors designated as PoPeVYV$_{Del}$-CP^{G4S}-mGFP, with the CP derived from the PMMoV, a tobamovirus (Fig. 2A). A TMV OAS was inserted upstream of the 3′ untranslated region (UTR) to enhance CP aggregate recognition of the chimeric viral RNA, thereby promoting the formation of virus-like particles (VLPs) (Turner and Butler, 1986). The constructs were delivered via *Agrobacterium*-mediated infiltration, using PMMoV$_{Del}$-CP^{G4S}-mGFP as a control. Strong fluorescence was observed in leaves inoculated with either PoPeVYV$_{Del}$-CP^{G4S}-mGFP or PMMoV$_{Del}$-CP^{G4S}-mGFP at 2 dpi (Fig. 2B). Western blot analysis of total leaf extracts from tissues inoculated with PoPeVYV$_{Del}$-CP^{G4S}-mGFP or PMMoV$_{Del}$-CP^{G4S}-mGFP revealed proteins of the expected molecular mass (47.9 kDa) when probed with GFP-specific antibodies (Fig. 2C). Notably, a molecular mass corresponding to free mGFP was identified in Anti-GFP assays. The mGFP includes an N-terminal signal peptide (SP) derived from *Arabidopsis* vacuolar basic chitinase, along with a C-terminal sequence of HDEL (Haseloff et al., 1997). Free mGFP may arise through the cleavage of the fusion protein during secretion in plant cells. Transmission electron microscopy (TEM) confirmed the formation of rod-shaped particles in leaves inoculated with PoPeVYV$_{Del}$-CP^{G4S}-mGFP or PMMoV$_{Del}$-CP^{G4S}-mGFP (Fig. 2D). These also showed that the interchangeability of coat proteins among tobamoviruses does not adversely affect viral infection or particle formation (Shivprasad et al., 1999; Yu et al., 2020). Additionally, the full-length transcript RNAs of PoPeVYV$_{Del}$-CP^{G4S}-mGFP and PMMoV$_{Del}$-CP^{G4S}-mGFP measured 5290 and 7144 nucleotides, respectively. Negative-stain electron microscopy revealed that PMMoV$_{Del}$-CP^{G4S}-mGFP particles ranged in size from 260 to 319 nm, while PoPeVYV$_{Del}$-CP^{G4S}-mGFP particles varied approximately from 100 to 315 nm (Fig. 2D-E). The variation in the measured lengths of the nano-rods produced from PoPeVYV$_{Del}$-CP^{G4S}-mGFP exhibited greater diversity compared to those from PMMoV$_{Del}$-CP^{G4S}-mGFP inoculation. It suggested that tobamovirus assembly is initiated through specific interactions between CP aggregates and the OAS (Turner and Butler, 1986). Both genomic and subgenomic RNAs of PoPeVYV$_{Del}$-CP^{G4S}-mGFP contained the OAS sequences and can be recognized by CP aggregates, which facilitates nano-rod formation. This phenomenon is consistent with previously reported findings relating to the formation of nano-rods (Saunders and Lomonossoff, 2017).

To determine whether the CP^{G4S}-mGFPs in PoPeVYV$_{Del}$-CP^{G4S}-mGFP and PMMoV$_{Del}$-CP^{G4S}-mGFP were assembled into virus particles and displayed on their surfaces, the extracted virus particles were labeled with antibodies specific for either PMMoV CP or GFP using immunogold labeling, with PMMoV virions serving as a control. CP-specific labeling was observed around all three types of virus particles, with an average labeling rate ranging from 72.19 % to 80.39 % (Figure S1A-B). GFP-specific labeling was distinctly present around PoPeVYV$_{Del}$-CP^{G4S}-mGFP and PMMoV$_{Del}$-CP^{G4S}-mGFP but absent on the PMMoV virions (Figure S1B). Notably, the average labeling ratios for PoPeVYV$_{Del}$-CP^{G4S}-mGFP and PMMoV$_{Del}$-CP^{G4S}-mGFP were 57.95 % and 65.79 %, respectively, which were significantly lower than the labeling ratio for the CP (Figure S1B). Free CP was released when mGFP emerged through the cleavage of the fusion protein during secretion in plant cells (Fig. 2C). The free CPs may assemble into virus particles alongside CP^{G4S}-mGFP.

Viral nanoparticles are increasingly recognized as valuable platforms for biomedical applications, often employed for the display of peptides or proteins. However, the active replication and infection capabilities of these viruses frequently hinder their application in agriculture. Recent studies have shown that tlaRNAs can facilitate the transmission of poleroviruses by aphids and enhance the efficiency of polerovirus infections (Peng et al., 2021; Sanger et al., 1994). To investigate whether the nucleic acid of PoPeVYVaRNA was packaged into VLPs, reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the RNAs of mGFP were detected in both PoPeVYV$_{Del}$-CP^{G4S}-mGFP and PMMoV$_{Del}$-CP^{G4S}-mGFP, while PoPeVYVaRNA was not detected in any of the virus particles (Figure S1C). This suggests that the heterologous protein expression system of PoPeVYV and PoPeVYVaRNA is biosecure for use in agriculture.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is characterized by its double-stranded DNA genome of 3.2 kb (Rehermann and Nascimbeni, 2005). The infectious virion consists of hepatitis B surface antigen (HBsAg) in its lipid envelope, enclosing an inner nucleocapsid composed of hepatitis B core antigen (Gerlich and Robinson, 1980; Kim et al., 2016). Expression of HBsAg in plants for oral vaccine development represents a promising strategy to control HBV transmission. While HBsAg expression has been demonstrated in various plants and plant cells (Kumar et al., 2005a, 2005b; Lou et al., 2007; Qian

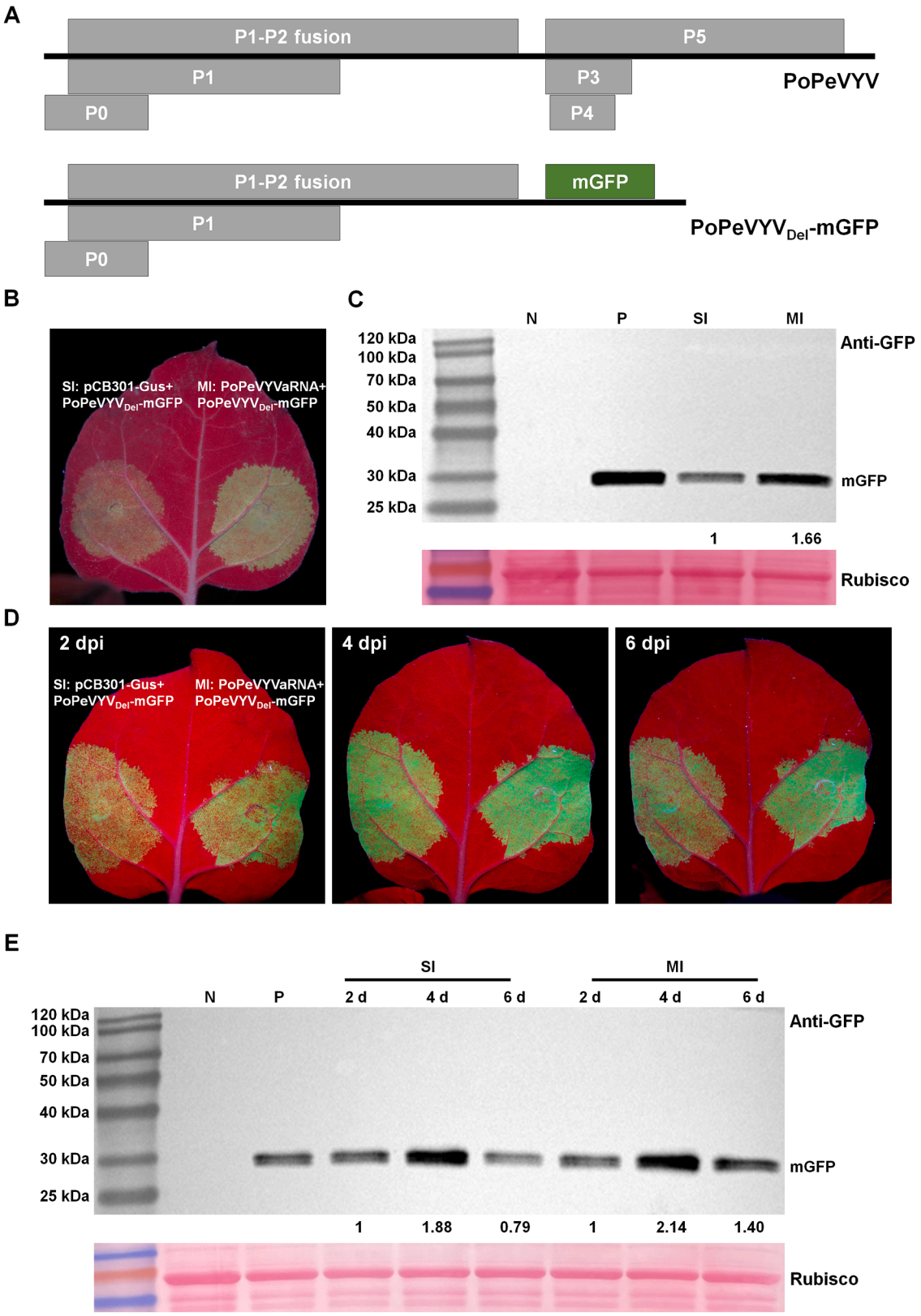
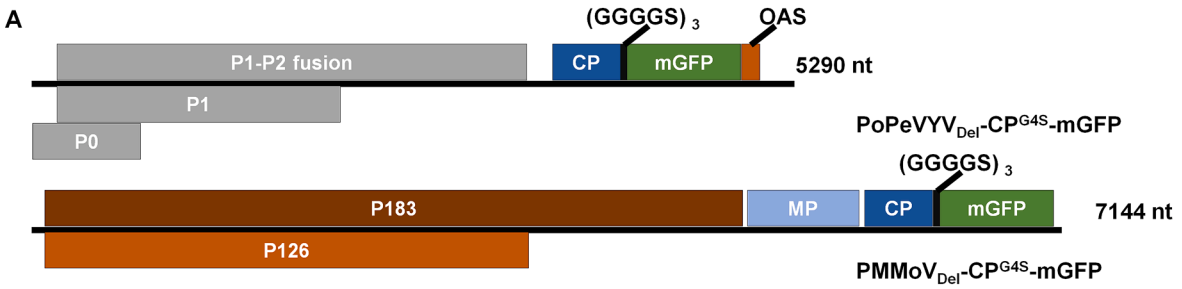
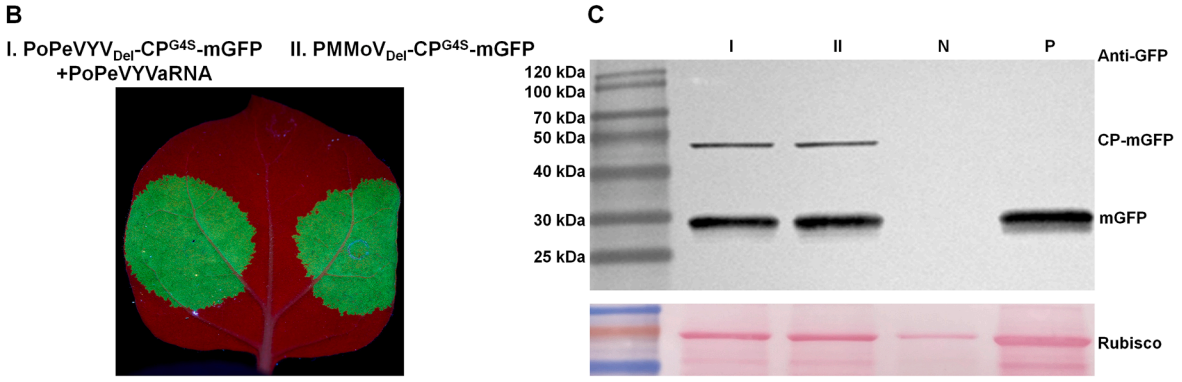
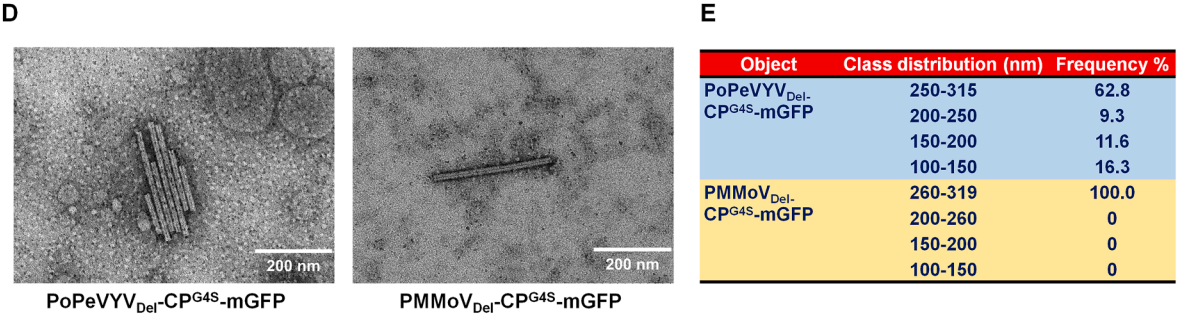


Fig. 1. mGFP expression effectivity of co-infected with PoPeVYV$_{Del}$-mGFP and PoPeVYVaRNA. (A) Schematic diagram of the PoPeVYV and PoPeVYV$_{Del}$-mGFP vectors. (B) At 2 days post inoculated, the fluorescence of PoPeVYV$_{Del}$-mGFP (SI) and co-infected with PoPeVYV$_{Del}$-mGFP and PoPeVYVaRNA (MI). (C) Western blot analysis the mGFP protein accumulations of SI and MI treatment. N: healthy plant as negative; P: transient expression of mGFP as positive. (D) The observations were continued for 6 days of the fluorescence of SI and MI treatment. (E) Western blot analysis the mGFP protein accumulations of different times. N: healthy plant as negative; P: transient expression of mGFP as positive.

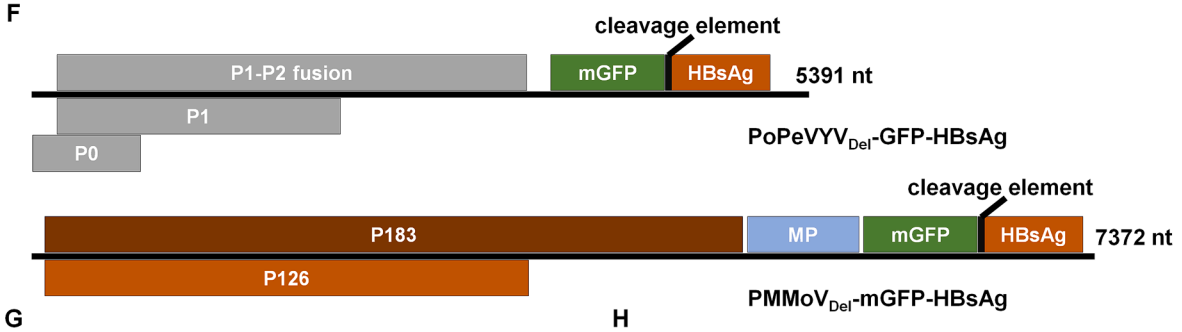






E

Object	Class distribution (nm)	Frequency %
PoPeVYV Del-CP G4S-mGFP	250-315	62.8
	200-250	9.3
	150-200	11.6
	100-150	16.3
PMMoV Del-CP G4S-mGFP	260-319	100.0
	200-260	0
	150-200	0
	100-150	0



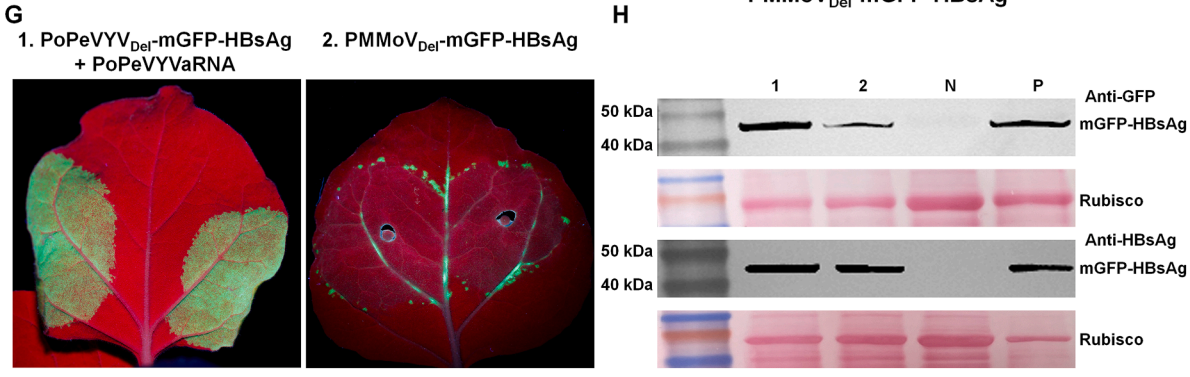


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Fig. 2. Producing of HBsAg or VLPs using PoPeVYV and PMMoV. (A) Schematic diagram of the PoPeVYV$_{Del}$-CP-mGFP and PMMoV$_{Del}$-CP-mGFP vectors. (B) At 4 days post inoculated, the fluorescence of PoPeVYV$_{Del}$-CP-mGFP and PMMoV$_{Del}$-CP-mGFP. (C) Western blot analysis the CP-mGFP fusion protein accumulations of PoPeVYV$_{Del}$-CP-mGFP or PMMoV$_{Del}$-CP-mGFP inoculated leaves. I: coinfected with PoPeVYV$_{Del}$-CP-mGFP and PoPeVYVaRNA; II: PMMoV$_{Del}$-CP-mGFP; N: healthy plant as negative; P: transient expression of mGFP or CP as positive. (D) Morphological observation of virus particles of PoPeVYV$_{Del}$-CP-mGFP in the electron microscope. Scale bar: 200 nm. (E) The statistics length of the nano-rods arising from two infiltration. (F) Schematic diagram of the PoPeVYV$_{Del}$-mGFP-HBsAg and PMMoV$_{Del}$-mGFP-HBsAg vectors. Cleavage element: $(GGGGS)_3$ and TEV cleavage site (ENLYFQG). (G) At 4 days post inoculated, the fluorescence of PoPeVYV$_{Del}$-mGFP-HBsAg and PMMoV$_{Del}$-mGFP-HBsAg. (H) Western blot analysis the mGFP or HBsAg protein accumulations of different groups. 1. Coinfected with PoPeVYV$_{Del}$-mGFP-HBsAg and PoPeVYVaRNA; 2. PMMoV$_{Del}$-mGFP-HBsAg; N: healthy plant as negative; P: transient expression of mGFP-HBsAg as positive.

et al., 2008; Streatfield, 2005), the feasibility of HBsAg expression via plant viral vectors remains to be elucidated. To investigate viral vector preferences for HBsAg expression, we constructed two chimeric virus vectors based on PoPeVYV and PMMoV. Recent studies have shown that transiently expressed HBsAg or the HBsAg fusion with GFP at the N-terminus (GFP:HBsAg) can form VLPs, whereas the HBsAg fusion with GFP at the C-terminus (HBsAg:GFP) does not, similar to yeast-derived vaccine HBsAg (Huang and Mason, 2004). To facilitate direct comparisons and ensure that the effects on immunity are assessed without interference from GFP in the future, mGFP was fused to HBsAg using a cleavage element that includes a $(GGGGS)_3$ linker and an intervening tobacco etch virus (TEV) cleavage site (ENLYFQG) (Fig. 2F). These constructs were delivered via *Agrobacterium*-mediated infiltration. At 2 dpi, leaves inoculated with PoPeVYV$_{Del}$-mGFP-HBsAg exhibited strong fluorescence, while those inoculated with PMMoV$_{Del}$-mGFP-HBsAg showed only sporadic fluorescence at 4 dpi (Fig. 2G). Western blot analysis of total protein extracts using HBsAg-specific and GFP-specific antibodies revealed bands at the expected molecular mass (Fig. 2H), with higher protein levels in PoPeVYV$_{Del}$-mGFP-HBsAg compared to PMMoV$_{Del}$-mGFP-HBsAg samples. Previous studies have demonstrated that the CP coding region of tobamoviruses influences subgenomic transcription and GFP expression from the CP subgenomic RNA promoter (Man and Epel, 2004). Inclusion of an enhancer sequence upstream of the GFP open reading frame (ORF) increased subgenomic transcription, resulting in 50-fold higher GFP accumulation compared to a minimal CP promoter where the CP ORF was replaced by GFP. Our previous work showing high protein accumulation when mGFP replaced CP supports this finding (Zheng et al., 2023). However, poor expression of the mGFP-HBsAg fusion protein in PMMoV, and successful expression of the mGFP-HBsAg fusion protein in PoPeVYV, suggests vector-dependent selectivity in plant-based HBsAg expression systems.

To investigate whether the structure and function of mGFP were affected in the mGFP-HBsAg fusion protein, we examined the subcellular localization of fluorescence using confocal microscopy. In *N.benthamiana* leaves transfected with pJP603-mGFP, fluorescence exhibited a general distribution throughout the cytoplasm and nuclear region (Figure S1D). In contrast, the fluorescence of mGFP-HBsAg was predominantly localized in punctate spots and small aggregates within the cytoplasm (Figure S1D). This observation suggests that the display of mGFP on the surface of VLPs results in the formation of punctate spots and small aggregates, which is a common phenomenon (Huang and Mason, 2004; Jiao et al., 2024).

In this study, we present the development of a heterologous protein expression system aimed at facilitating the *in vivo* production of HBsAg and the PMMoV VLPs display system in plants. This genetically tractable system enables the stable expression of HBsAg and rod-shaped VLPs, effectively overcoming the limitations associated with HBsAg protein expression when using the PMMoV vector.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Lujia Wang: Writing – original draft, Validation, Methodology, Data curation. **Ge Zhang:** Writing – original draft, Validation, Methodology, Data curation. **Shan Bu:** Methodology, Investigation. **Zina Lin:** Software, Investigation. **Jian Wu:** Writing – review & editing. **Fei Yan:** Writing – review & editing, Writing – original draft, Conceptualization. **Jiejun Peng:** Writing – review & editing, Writing – original draft, Validation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Fei Yan reports financial support was provided by the Pioneer and “Leading Goose” R&D Program of Zhejiang. Fei Yan reports financial support was provided by Chinese Agriculture Research System of MOF and MARA. Jiejun Peng has patent #202,510,269,232.5 pending to Ningbo University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199559.

Data availability

No data was used for the research described in the article.

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