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Effect of a silencing suppressor gene towards the expression of VP2 protein of highly virulent infectious bursal disease virus in tobacco

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ABSTRACT

Gene silencing has been recognized as an important factor, which influence the expression of heterologous protein delivered via plant-viral based system. The application of suppressor genes that act against gene silencing mechanisms demonstrated improvement in the expression level of heterologous protein in plants. The aim of the present study was to evaluate the ability of a silencing suppressor gene to enhance the expression of VP2 protein of highly virulent infectious bursal disease virus in *Nicotiana benthamiana* plants using a plant viral-based expression system derived from potato virus X. In the present study, a silencing suppressor gene of P19 encoded by tomato bushy stunt virus was employed as a suppressor for gene silencing mechanism by co-infecting *N. benthamiana* plants with the P19 gene via agroinfiltration strategy followed by mechanical inoculation of potato virus X viral transcripts containing the cloned VP2 gene. Western blot analyses revealed that the expressed VP2 protein can be detected by 7 days post-infection in the presence of P19 gene as compared with plants without P19 gene that can only be detected at 10 days post-infection. However, there was no significant enhancement in the yield of expressed VP2 protein in the presence of P19 gene. The present findings demonstrated the employment of P19 gene and improved the expression of hvIBDV VP2 protein in *N. benthamiana* plants.

Key words: infectious bursal disease virus, VP2, protein expression, silencing suppressor gene, P19 gene, *Nicotiana benthamiana*

Introduction

The VP2 protein of highly virulent infectious bursal disease virus (hvIBDV) is the major protein that surrounds the internal components of the virus. VP2 consists of approximately 494 amino acid residues that contain the major antigenic site, which is responsible for eliciting neutralizing antibodies (Azad et al., 1987; Becht et al., 1988; Fahey et al., 1989; Reddy & Silim, 1991). VP2 possessed the antigenic determinants for serotype specificity (Azad et al., 1987; Becht et al., 1988). The ability to induce production of neutralizing antibodies makes the VP2 as a suitable target protein for development of subunit vaccine. The expression of heterologous VP2 protein has been demonstrated successful in variety of expression system including prokaryotic, eukaryotic and mammalian cells. The expression of VP2 protein in plants has become an alternative approach for more current expression systems based on animal, yeast or bacteria cell culture. The main advantages of plant system over the other systems include the low cost of production, easy to scale-up and low risk of contamination with animal pathogens (Floss et al., 2007; Ma et al., 2003). Non-food crop species like tobacco is an attractive option for recombinant protein production because it can minimize regulatory barriers by eliminating the risk of entry into the food chain.

Expression of IBDV VP2 protein in plant system has been

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reported either by employing stable transformation strategy or via plant viral-based transient expression system. In stable transformation strategy, the VP2 gene isolated from an antigenic variant E strain has been expressed in *Arabidopsis thaliana* (Wu et al., 2004), whereas the VP2 gene of a virulent IBDV strain (ZJ2000) has been expressed in rice seeds (Wu et al., 2007). Meanwhile, the expression of VP2 protein via plant viral-based epitope presentation strategy has been reported by Chen et al. (2012). The truncated VP2 protein (24.5 kDa) was expressed as chimeric viral particles (CVPs) fused to the N-terminus of the bamboo mosaic virus (BaMV) coat protein (CP). Recently, a study conducted by our group (Hasmah et al., 2013) had expressed the whole VP2 protein structure (50 kDa) in *Nicotiana tabacum* via plant viral-based transient expression system using potato virus X (PVX) as viral-based expression vector. The yield of the expressed VP2 protein demonstrated in our study was considered low, which can be enhanced if proper strategy is undertaken to improve the level of protein expression.

Post-transcriptional gene silencing (PTGS) was proposed as one of a limiting factor that influence level of protein expression in plants. Common features of PTGS in different organisms are the involvement of double-stranded (ds)RNA as initiator molecule (Fire et al., 1998; Hammond et al., 2000) and the presence of short-interfering (si)RNAs of 21 to 25 nt that are processed from dsRNA by an RNAase III-like enzyme (Bernstein et al., 2001; Elbashir et al., 2001; Hamilton & Baulcombe, 1999). The siRNAs confer sequence specificity to a nuclease that degrades any RNA-sharing sequence homology to the activating dsRNA molecules (Hammond et al., 2000). PTGS operates as an adaptive immune system targeted against viruses, and as a counter-defensive strategy, many plant viruses have evolved proteins that suppress various steps of the mechanism (Ratcliff et al., 1999; Voinnet, 2001).

Studies have shown that the expression of heterologous protein by *Agrobacterium*-mediated transient expression system can be enhanced in the presence of a suppressor of gene silencing. A viral gene encoded for P19 protein derived from tomato bushy stunt virus (TBSV) has been found as an effective gene silencing suppressor. It has been reported that the expression levels of green fluorescent protein (GFP) in the presence of P19 were substantially higher than in the absence of suppressor GFP-expressing stable transgenic lines (Voinnet et al., 2003). Similar finding was also demonstrated by Lindbo (2007) whereby the expression of GFP protein using cauliflower mosaic virus (CaMV) 35S promoter-driven TMV expression vector delivered by *Agrobacterium*-mediated transient expression system produced 10 to 25 times higher of recombinant proteins in the presence of P19 suppressor gene co-introduced via agroinfiltration strategy.

The present study was aimed to evaluate the effect of P19 silencing suppressor gene of TBSV towards the expression of hvIBDV VP2 protein via plant viral based transient expression system using potato virus X (PVX) expression vector in *N. benthamiana*. Previous studies conducted by Lindbo (2007) demonstrated that the expression of green fluorescent protein (GFP) in *N. benthamiana* was significantly increased by co-infiltrating the *A. tumefaciens*/pJL43:GFP together with *A. tumefaciens*/pJL3:p19. In this study, a modification on gene delivery strategy has been implemented by introducing the suppressor gene of P19 via agroinfiltration technique followed by mechanical inoculation of PVX viral transcripts carrying the VP2 gene in *N. benthamiana*. The efficacy of the alternative gene delivery strategy to improve the expression level of VP2 protein in tobacco was evaluated.

Materials and Methods

Virus isolation and construction of recombinant PVX:VP2 vector

The isolation of a Malaysian strain of hvIBDV and the amplification of hvIBDV VP2 gene were described in previous study (Hasmah et al., 2013). The VP2 gene was cloned onto a potato virus X (PVX) viral-based vector designated as pP2C2S, which was kindly provided by Prof. Dr. David Baulcombe from the University of Cambridge, UK. (Baulcombe et al., 1995). The VP2 gene was digested with *Eco*RV and *Sal*I restriction enzymes and subsequently cloned into the pP2C2S vector as described previously (Hasmah et al., 2013). The recombinant PVX::VP2 vector and the empty PVX vector were propagated in XL1-Blue competent cells and extracted using GeneAll Exprep™ Plasmid Quick extraction kit (GeneAll Biotechnology, Korea). The empty PVX and PVX::VP2 vectors were digested with *Sph*I restriction enzyme to produce linearized vectors for generation of *in vitro* viral transcripts.

Transformation of pJL3:P19 recombinant vector into Agrobacterium tumefaciens

The tobacco mosaic virus (TMV) recombinant binary vector, pJL3:P19 was transformed into *A. tumefaciens* strain GV3101 using the freeze-thaw method (Chen et al., 1994).

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The competent cells of *A. tumefaciens* strain GV3101 were thawed on ice and 2 µl of pJL3:P19 was added into the competent cells. The mixture was incubated on ice for 5 minutes. Then, the mixtures were transferred to liquid nitrogen and incubated for 5 minutes. The mixture was incubated for a further 5 minute at 37°C water bath. To each tube, 1 ml of LB media was added and sealed before placing on an orbital shaker for 2-4 hours at room temperature. The transformed cells were collected by spinning briefly in a microcentrifuge and then spread on LB agar plates supplemented with 50 µg/ml kanamycin and 10 µg/ml rifampicin. The LB agar plates were incubated for 2 days at 28°C.

Agroinfiltration of A. tumefaciens/ pJL3:P19 cultures

The P19 gene incorporated in tobacco mosaic virus (TMV) binary vector was first introduced into tobacco plants by infiltrating half portion of the tobacco leaves with *A. tumefaciens*/pJL3:p19. Individual colonies of *A. tumefaciens* transformed with pJL3::P19 plasmid were grown to an OD600 of 1.0 in liquid LB media supplemented with 10 mM MES pH 5.7, 50 µg/ml kanamycin, and 20 µM acetosyringone. Cells were collected by centrifugation at 4000 g for 10 minutes and resuspended in induction media containing 10 mM MES, pH 5.7 and 200 µM acetosyringone. Cells sat at room temperature in induction media for 5 hours before infiltration. Only half portion of *N. benthamiana* leaves were infiltrated with cells prepared at OD600 of 0.5 (100 µl) into the abaxial surface of leaves using a 1 ml syringe without needle. The infiltrated plants were maintained in a growth room at 23°C overnight.

Mechanical inoculation of viral transcripts and plant sap virions in tobacco plants

Two types of viral inocula in the form of capped viral transcripts and plant sap virus were prepared for mechanical inoculation of recombinant PVX::VP2 in *N. benthamiana*. The capped viral transcripts of empty PVX and recombinant PVX::VP2 were generated using Ambion mMESSAGE mMACHINE® Kits (USA) following the protocol recommended by the manufacturer. The viral sap inoculum of empty PVX and recombinant PVX::VP2 were prepared by homogenizing 0.5 g of previously infected *N. benthamiana* leaves harvested at 4 days post infection (dpi) in 2 ml of 0.1 M phosphate buffer (pH 7.0). The plants of non-infiltrated or infiltrated with *A. tumefaciens* containing pJL3:P19 were used for inoculation of the viral transcripts and plant sap virions. The remaining portion of the leaves was mechanically inoculated with the viral transcripts or sap inoculums of empty PVX vector or recombinant PVX:VP2. Approximately 0.25 µg (15-20 µl) of either empty PVX or PVX:VP2 viral transcripts were mechanically inoculated together with silicon carbide (400 mesh) on the remaining portion of the infiltrated leaves. The plant sap inoculum was diluted at 1:1 and 1:2 (v/v) in 20 mM phosphate buffer before conducting the inoculation. The co-infected plants were maintained in a growth room at 23°C and the viral infection symptoms were assessed after several days of post infection. The locally inoculated leaves were harvested at 7 dpi and 10 dpi followed by extraction of total soluble protein which analyzed by SDS-PAGE and Western blot analyses.

Antigenicity assessment of VP2 protein expression with combination treatment of viral suppressor

The *N. benthamiana* plants initially agroinfiltrated with pJL3:P19 plasmid and co-infected with PVX and PVX:VP2 either as viral transcripts or plant sap virus were harvested at 7 and 10 dpi. The leave portions, which have been infected with viral transcripts or sap inoculums, were separated from the agroinfiltrated portions during sample processing for total protein extraction. Extraction of plant total soluble protein was conducted by homogenizing the inoculated leaves in extraction buffer containing 50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 30% sucrose and 0.5% DTT in addition of 1.0 µM protease inhibitor. Approximately 10 µg of undenatured protein samples were separated on 4-10% polyacrylamide gels and stained by Coomassie blue or electrophoretically transferred into nitrocellulose membrane for Western blot analysis. The blotted membrane was incubated in blocking solution (5% milk in PBS) followed by primary antibody of chicken polyclonal anti-IBDV diluted at 1:3000 in 1X PBS containing 0.1% Tween 20. The membrane was subsequently incubated with polyclonal goat anti-chicken IgY conjugated with horseradish peroxidase (HRP) diluted at 1:8000. The membrane was developed by chemiluminescent using SuperSignal West Pico Chemiluminescent Substrate (Pierce) following the protocol recommended by the manufacturer.

Results

Simultaneous infection of A. tumefaciens/pJL3:P19 and chimeric PVX viral transcripts in tobacco plants

Tobacco plants of *N. benthamiana* were inoculated with either infectious viral transcripts or plant sap virus containing

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the cloned VP2 gene incorporated onto the PVX vector in the presence or absence of P19 suppressor gene. The plants were initially agroinfiltrated with *A. tumefaciens*/ pJL3:P19 prior mechanical inoculation with the empty PVX or recombinant PVX:VP2Mt virus. The physical appearance of *N. benthamiana* leaf infiltrated with *A. tumefaciens*/ pJL3:P19 and co-infected with the recombinant PVX:VP2 viral transcripts (A and B) as compared with mock inoculated leaf (C) is indicated in Figure 1. The physical appearance of the upper leaves of *N. benthamiana* agroinfiltrated with *A. tumefaciens*/ pJL3:P19 and co-infected with either empty PVX or PVX::VP2 viral transcripts and are shown in Figure 2. The upper leaves of *N. benthamiana* plants infected with either empty PVX (A) or PVX:VP2 (B) viral transcripts showed systemic infection whereby the young leaves developed chlorotic lesions after 10 to 15 days post infection whereas similar lesion was not developed in mock inoculated plant (C).

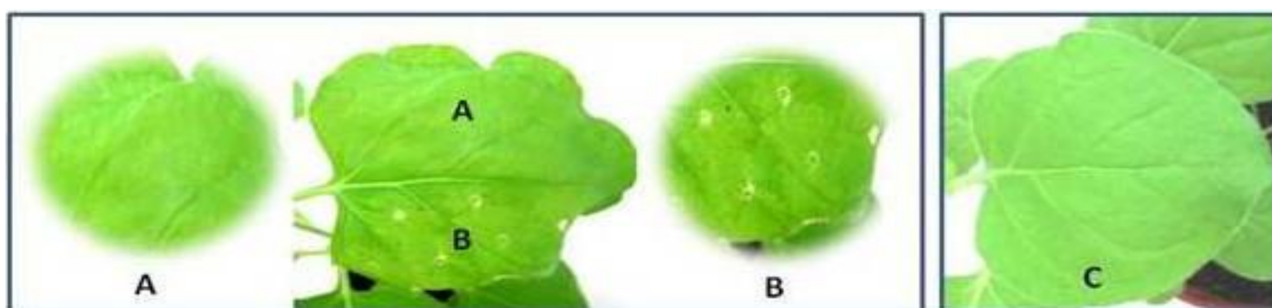

Figure 1. *The physical appearance of N. benthamiana leaf infiltrated with A. tumefaciens/pJL3:P19 and co-infected with the recombinant PVX:VP2 viral transcripts (A and B) compared to mock inoculated leaf (C). (A) Portion of leaf mechanically inoculated with PVX:VP2 viral transcripts (B) Portion of leaf agroinfiltrated with A. tumefaciens/ pJL3:P19.*

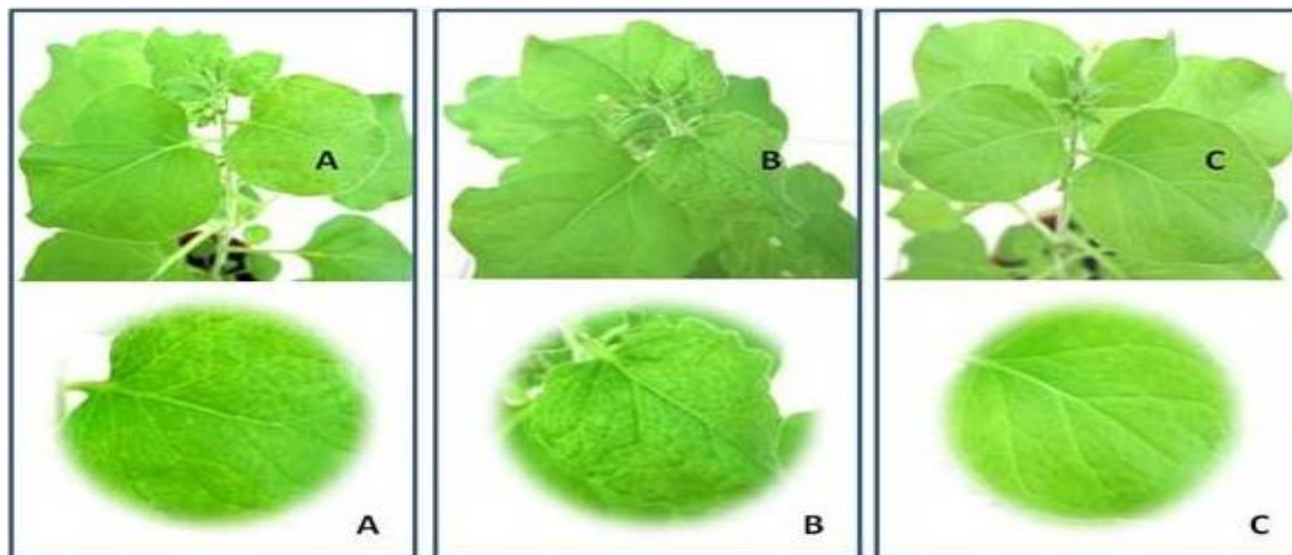

Figure 2. *The physical appearance of the upper leaves of N. benthamiana agroinfiltrated with A. tumefaciens/ pJL3:P19 and co-infected with either empty PVX (A) or PVX::VP2 viral transcripts (B) compared with mock inoculated plant (C).*

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Analysis of total soluble protein infected with viral transcripts

SDS-PAGE analysis of total soluble protein (TSP) from plants inoculated with empty PVX and PVX::VP2 viral transcripts in the presence or absence of P19 gene indicated the presence of prominent bands of approximately 50 kDa (Figure 3A). Sample from plants inoculated with PVX:VP2 revealed higher intensity of 50 kDa bands in comparison with other protein samples from non-infected and plants infected with empty PVX. Western blot analysis indicated that prominent bands of approximately 50 kDa were only detected from plants inoculated with PVX:VP2 viral transcripts (Figure 3B). In the absence of P19 gene, a prominent band was only detected at 10 dpi, whereas plants co-infected with P19 gene showed detection of prominent bands at 7 dpi and further detected at 10 dpi. Protein sample of non-infected and inoculated with empty PVX viral transcript revealed no detection of the prominent band.

Analysis of total soluble protein infected with plant sap virus

SDS-PAGE analysis of total soluble protein (TSP) indicated that all samples inoculated with plant sap virus of PVX:VP2 at dilution 1:1 (1) and 1:2 (2) in the presence or absence of P19 suppressor gene showed prominent bands of 50 kDa (Figure 4A). Meanwhile, high band intensity of 60 kDa was demonstrated from sample inoculated with PVX:VP2 at dilution 1:1 in the presence of P19 gene. A band of 50 kDa observed from non-infected protein sample was derived from the RUBISCO protein found in tobacco plants. The protein samples were further analyzed by Western blot analysis. Total protein from samples infected with PVX:VP2 at dilution 1:2 in the absence or presence of P19 gene indicated single band of approximately 50 kD (Figure 4B). On the other hand, two bands of approximately 50 kD and 60 kD were detected from sample infected with PVX:VP2 at dilution 1:1 co-infected together with P19 gene. Meanwhile, only 50 kD protein was detected from sample infected with PVX:VP2 at dilution at 1:1 in the absence of P19. Sample from non-infected leaves showed no detection of band in Western blot analysis.

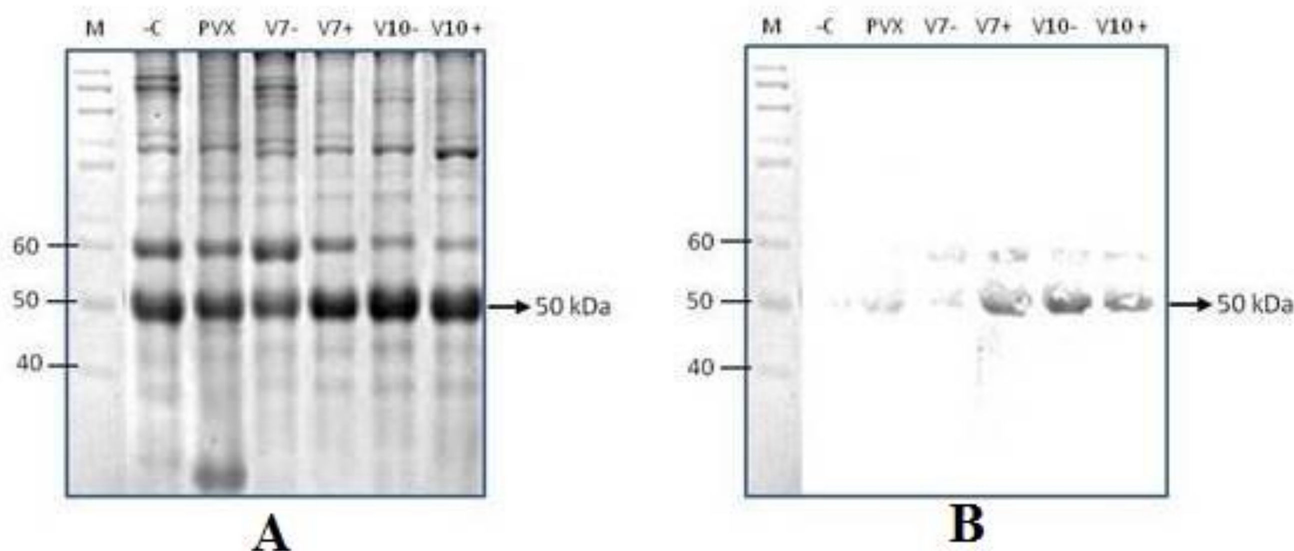

Figure 3: *SDS-PAGE (A) and Western blot (B) analyses of plant total soluble protein (TSP) of N. benthamiana from non-infected (-C) or infected with virus transcripts (0.25 µg) of empty PVX (PVX) and recombinant PVX::VP2 (V) either inoculated alone (-) or co-infected with P19 suppressor gene (+) from tomato bushy stunt virus (TBSV) harvested at 7 and 10 days post-infection (dpi).*

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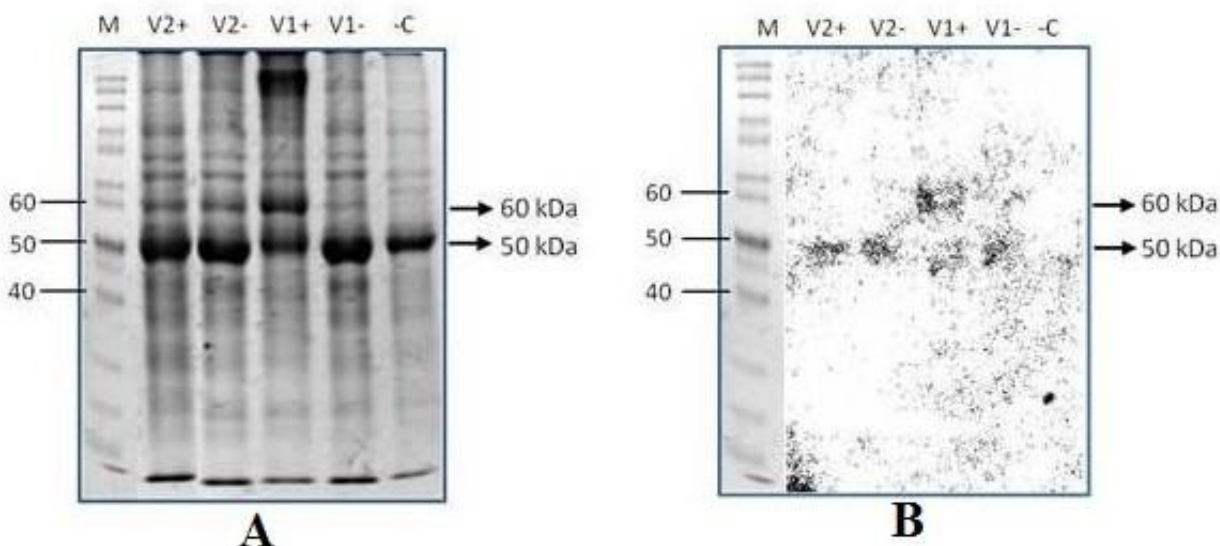

Figure 4: *SDS-PAGE (A) and Western blot (B) analyses of total soluble protein of N. benthamiana leaves infected with PVX::VP2 in plant sap inoculum at different dilutions; 1:1 (1) and 1:2 (2) in the absence of P19 gene (-) or co-infected with P19 gene (+). Lane V: Leaves infected with PVX::VP2; Lane –C: Non-infected leaves; Lane M: Page Ruler Unstained Protein Ladder.*

Discussion

In the present study, an alternative strategy has been implemented to enhance the expression of hvIBDV VP2 protein by introducing the P19 silencing suppressor gene of TBSV into tobacco leaves prior inoculation with viral inoculums carrying the VP2 gene. Modification has been made by employing a combination of two different techniques for the delivery of VP2 gene and P19 suppressor gene in *N. benthamiana*. The infectious virus either as viral transcripts or in viral sap inoculums were mechanically inoculated onto tobacco leaves initially agroinfiltrated with *A. tumefaciens*/pJL3:P19. *N. benthamiana* leaves were divided into two portions whereby one portion was agroinfiltrated with *A. tumefaciens*/ pJL3:P19 and the second portion was inoculated with the viral transcripts or sap inoculums of empty PVX or recombinant PVX::VP2 virus. The gene delivery techniques employed in this study was slightly different from the techniques reported by Lindbo (2007) whereby the *A. tumefaciens*/pJL3:P19 was mixed together with the expression vector carrying the green fluorescent protein (GFP) and delivered into *N. benthamiana* leaves simultaneously via agroinfiltration method.

The tobacco plants, which had received combination treatment of *A. tumefaciens*/pJL3:P19 agroinfiltration and chimeric viral transcripts infections, showed symptoms at inoculation sites or generated systemic infections. Observation on the morphology of *N. benthamiana* infected with empty PVX or PVX:VP2 recombinant viral transcripts in the presence of P19 gene indicated that the plants showed systemic infection characterized by chlorotic mosaic symptoms of the upper systemic leaves after 10 to 15 days post inoculation. These findings indicated that after more than 10 days of post inoculation in *N. benthamiana*, the PVX virus transcripts possessed the characteristic of an infectious virus, which is similar to wild type PVX virus. Similar characteristics of systemic infection were not detected in mock inoculated *N. benthamiana* plants. Previous study by Barajas et al. (2006) had described similar findings whereby the *N. benthamiana* plants inoculated with PVX-HCT RNA transcripts showed symptoms of systemic infections by 7 dpi followed by the appearance of severe necrosis characterized by chlorotic mosaic symptoms by 12 to 14 dpi. Based on these findings, the chimeric viral transcripts were found infectious and able to generate local and systemic symptoms. Previous studies have shown that the expression of heterologous protein in plant system was interrupted by defense mechanism strategy of post-transcriptional gene

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silencing (PTGS) that acts as an adaptive immune system targeted against plant viruses (Ratcliff et al., 1999; Voinnet, 2001). In the presence of P19 gene, the viral infection initiated by the chimeric PVX viral transcripts was suspected to become more efficient due to the activity of P19 suppressor gene that response against PTGS mechanism in plants.

Analyses on the expression profile of VP2 protein from viral transcripts either in the presence or absence of P19 gene revealed slight variations. In the absence of P19 gene, the VP2 protein was able to be expressed but require longer time to allow detection of the expressed protein by Western blot analysis. In contrast to inoculation of virus transcripts in the presence of P19 gene, the expressed VP2 protein can be detected few days earlier compared to inoculated plants in the absence of P19 gene. These findings revealed that the expression of VP2 gene in the presence of P19 gene was able to enhance the expression of the VP2 protein by reducing the time required for harvesting of the expressed protein from 10 dpi to 7 dpi. Inoculation of *N. benthamiana* with sap virus inoculum either in the absence or presence of P19 gene revealed slight variation of the protein profile. Protein sample inoculated with higher concentration of virus sap inoculum (1:1), which co-expressed together with P19 gene, showed an additional band of approximately 60 kDa. The expression of an additional band of 60 kD might be due to glycosylation process or cross-reacting plant protein (Biemelt et al., 2003). It can be speculated that the glycosylation process probably had increased the number of protein residues in VP2 protein.

The level of protein expression demonstrated in this study was found similar either in the presence or absence of P19 gene. A study conducted by Lindbo (2007) reported that the expression of GFP protein co-expressed with P19 suppressor gene delivered by agroinfiltration method was able to enhance level of protein expression between 10 to 25 times higher than the most efficient 35S promoter driven transient expression systems. The GFP protein was detected in infiltrated tissue at 6 dpi and in systemically infected plant tissue at 10 to 14 dpi. In contrast to the previous study reported by Lindbo (2007), the expression level of the VP2 protein in the presence of P19 gene was remained the same as compared with the expression of VP2 protein without P19 gene. The expressed VP2 protein was only detected from leaves locally inoculated with the viral transcripts or viral sap inoculums. Analysis of total soluble protein from the systemically infected leaves indicated that the VP2 expressed protein was not detected from the protein sample. It can be speculated that the PVX had developed characteristics of wild type virus that tend to remove the foreign gene insert via the recombination mechanism during viral infection (Chapman et al. 1992; Guo et al. 1998; Rabindran & Dawson, 2001). Previous studies also reported that PVX-based chimeric viruses tend to lose the inserted foreign sequence during infection (Chapman et al. 1992; Gonzalez-Jara et al. 2005). In RNA viruses, recombination events are frequent and likely contribute to a rapid adaptation of viruses to their hosts (Lai, 1992).

The present findings revealed that the employment of P19 gene, which act as a suppressor for gene silencing during the expression of VP2 protein via PVX viral-based vector in *N. benthamiana,* was not fully enhanced the expression level of VP2 protein. The strategy of gene delivery might influence the enhancement of protein expression level in tobacco plants. In this study, the VP2 gene was delivered mechanically in the form of infectious viral transcripts rather than introduced together with P19 gene via agroinfiltration system as described by Lindbo et al. (2007). Therefore, future studies can be focused on the construction of recombinant vector for the VP2 gene delivery, which is compatible to be expressed via agroinfiltration system.

Conclusion

The ability of P19 gene to enhance the expression level of VP2 protein in *N. benthamiana* was evaluated in this study. The VP2 protein expressed together with P19 gene showed different protein profile as compared with VP2 protein expressed alone in tobacco. These findings demonstrated that the VP2 protein was successfully expressed in *N. benthamiana* either in the presence or absence of P19 gene. The expression of VP2 protein in the presence of P19 suppressor gene demonstrated enhancement of expression level and variation in protein profile.

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