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EVALUATION OF *PAPAYA RINGSPOT VIRUS* AS A VECTOR FOR EXPRESSION OF DENGUE E PROTEIN DOMAIN III IN *CUCURBITA PEPO* (ZUCCHINI) PLANTS

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ABSTRACT

Dengue fever is the most significant mosquito transmitted viral disease worldwide, but there is no commercially available vaccine to protect against infection. Subunit vaccines, comprising of a small antigenic region of the dengue E protein offer considerable advantages over more traditional methodologies of vaccine production, but are hampered by the requirement to produce large quantities of purified protein free from any potential pathogen or toxic agent. This study sought to determine whether Papaya ringspot virus (PRSV; Family *Potyviridae*, Genus *Potyvirus*, Species *Papaya ringspot virus*) could be engineered to accommodate the expression of a heterologous protein, specifically domain III of the DENV 2 E protein (D2EDIII), a promising subunit vaccine candidate. An infectious clone expressing DENV 2 E protein domain III was successfully constructed, and the insert showed stability over two passages in *Cucurbita pepo* (zucchini) plants. While the construct was designed to generate a discrete antigen moiety (D2EDIII) after proteolytic processing, results showed that the E protein insert was fused to the PRSV P1 protein, suggesting inefficient protease processing at the P1/ D2EDIII junction. The proof of principle results however confirm that PRSV could be used as an expression vector for heterologous protein expression in plants.

Key words: dengue; papaya ringspot virus; sub-unit vaccine; zucchini.

INTRODUCTION

Dengue virus (DENV) is a positive sense, enveloped RNA virus of the family Flaviviridae, genus Flavivirus and is the most important mosquito transmitted virus worldwide (Gubler, 1998). The virus is transmitted to humans by the bite of infected Aedes mosquitoes, and each year there are believed to be some 400 million new infections, of which some 100 million are symptomatic (Bhatt *et al*., 2013). Vaccine development for dengue has been slowed by the nature of DENV, which consists of four closely related, but antigenically distinct viruses (Gubler, 1998). A number of vaccine approaches have been investigated including live attenuated viruses, inactivated viruses, chimeric vaccines, virus-like particles, DNA vaccines and subunit vaccines all of which have their own specific advantages and disadvantages (Pulmanausahakul *et al*., 2010). Subunit vaccines aim to induce an immune response through the use of a small antigenic part of the pathogen (Moyle and Toth, 2013), and research is currently underway into subunit vaccines to protect against a wide variety of pathogens including dengue virus (Coller *et al*., 2011).

The primary drawback to subunit vaccine production is the requirement for large quantities of antigen, with the appropriate and correct glycosylation. Bacterial expression systems produce proteins without glycosylation and yeast expression systems are susceptible to the production of hyper-glycosylated antigens, while mammalian cell expression systems usually produce low yields of the antigen (Berlec and Strukelj, 2013). Plant expression systems overcome many of these problems, with the ability to be rapidly scaled in size and with an added advantage of avoidance of potentially (human) pathogenic agents being co-purified with the antigen (Yap and Smith, 2010).

Several previous studies have investigated the expression of DENV proteins in plants, and while three of these studies have established transient expression using plant virus based vectors (Saejung *et al*., 2007; Teoh *et al*., 2009; Martinez *et al*., 2010), the fourth study established expression of a consensus E protein domain III peptide in transgenic rice calli (Kim *et al*., 2013). In this study we sought to determine whether the plant virus Papaya ringspot virus (PRSV) could be employed for the transient expression of DENV E protein domain III in *Cucurbita pepo* (zucchini) plants. The zucchini plant/ PRSV system potentially has advantages over previously investigated expression systems in that as a potyvirus, PRSV equally expresses all the genes in its genome, offering the potential for the high expression of heterologous proteins and purification from a vegetable such as zucchini is a more desirable host as compared to the tobacco plant.

MATERIALS AND METHODS

Primers: The following primers were used in this study: seHC-Pro_D2D (5'-GGAACAATACAATGACG TCGC TGAGAAGTTC-3') as HC-Pro_D2D (5'-GAA CTTC

TCAGCGACGTCATTGTATTGTTCC-3') D2-R (5'-TTGCACCACAGTCAATGTCTTCAGGTTC-3') Oligo-d (5'-T TTTTTTTTTTTTTTT-3') DENV2EIII_F (5'-AAAGGAATGTCATACTCTATGTGCACAGG-3') DENV2EIII_R GAACAAGCTTTCTTAAACCAGTTGAGC-3') pR_D2EIII_F (5'-GATATACATATGCGGGACGTCCATCAT-3') pR_D2EIII_NIbR2 (5'-CTGACGTCGGATTCATGATACACCAATAGTTTCT TAAACCAGTTG-3') 5'P1HcPro (5'-CAGGAAGCAGTCGATCAACGCAGC-3') asHP-551 (5'-CGTTTGTTTCTGAAGCTCTCAACACTGCC-3') ACT-F (5'-ATCTGCTGGAAGGTGCTTAG-3') ACT-R (5'-AACGGGAAATTGTCCGTGAC-3').

Construction of *Papaya ringspot virus* (PRSV) intermediate cDNA clones: The intermediate clone, pBS-HP-EX was constructed by ligating a cDNA fragment which contained the HC-Pro gene flanked by partial P1 and P3 genes from the full-length cDNA clone of *Papaya ringspot virus* type-W Thailand isolate, pSA1164 (Yap *et al*., 2009) into the pBlueScriptKS (Stratagene) vector. PCR mutagenesis was then performed using seHC-Pro_D2D and asHC-Pro_D2D primers and pBS-HP-EX plasmid as template. The resultant construct, pBS-HP-D2D thus contained an introduced *Aat*II restriction sites at the 2nd codon of HC-Pro gene as a consequence of an introduced silent mutation (Fig. 1).

Amplification and cloning of histidine tagged dengue virus serotype 2 envelope protein domain III (His-D2EDIII) into a PRSV vector: Dengue virus serotype 2 (DENV 2) strain 16681 viral RNA was reverse-transcribed using primer D2-R to generate first strand cDNA. Using this cDNA template, the coding sequence for amino acids 295-394 of the envelope (E) protein was amplified with DENV2EIII_F and DENV2EIII_R primers. The PCR product (D2EDIII fragment) was then ligated to pRSET-B (Invitrogen) vector using blunt ended *Bam*HI (5' termini) and cohesive *Hin*dIII (3' termini) restriction sites to produce the pRSET_D2EDIII plasmid. This plasmid was then used as a template to amplify the His-D2EDIII fragment using pR_D2EIII_F and pR_D2EIII_NIbR2 primers. The PCR product which contained the D2EDIII coding sequence preceded by DNA sequence coding for 6 histidine residues and flanked by the *Aat*II restriction sites at both the 5' and 3' termini was then cloned into the corresponding *Aat*II restriction site of pBS-HP-D2D (Fig. 1) to generate pHP-D2EDIII (Fig. 2). In order to obtain the final PRSV recombinant viral vector, pVD2EDIII; the cDNA fragment flanked by the *Mlu*I restriction sites from the PRSV full length cDNA clone, pSA1164 was then exchanged with the corresponding fragment from the pHP-D2EDIII (Fig. 1). The cloning procedures using the pSA1164 vector were performed using *E. coli* STBL2 cells while the others cloning procedures were performed using *E. coli* DH5α cells. The DNA sequences of all clones constructed in this study were verified by commercial sequencing (1st BASE DNA sequencing services, Malaysia).

Introduction of recombinant viral vector into zucchini germinating seeds by particle bombardment: Hybrid squash (variety senator) *Cucurbita pepo* zucchini seeds were purchased from Seminis, USA. Seeds were soaked in tap water overnight and then placed on Petri dishes containing wet tissue paper and kept at 25°C for 3-4 days. The hard seed coats were then removed by hand and 12 seeds were arranged in circle at the center of a Petri dish layered with wet tissue papers. For each bombardment, 2µg of plasmid DNA was coated onto 600µg of 1µm in diameter gold micro-particles according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Bombardment was performed with a Bio-Rad PDS1000/He at 1350psi helium pressure from a target distance of 6cm. Bombarded seeds were transferred to soil after overnight incubation at room temperature. Plants germinated from the bombarded or control (non-bombarded) seeds were then grown in a tropical greenhouse and observed for viral symptoms development for 45 days after bombardment.

Mechanical inoculation of zucchini plants with sap cell from PRSV infected leaf: Seven day old zucchini seedlings germinated from seeds were sown in soil and grown in a tropical greenhouse for about 2 weeks until the seedlings reached the 3-4 true leaves stage. A total of 100mg of fresh or frozen PRSV infected leaf samples was ground in 150µl of inoculation buffer (0.1 M K_2HPO_4/KH_2PO_4 pH7.2 and 0.5% (w/v) Na_2SO_3) with a plastic pestle. The mixture was then centrifuged at 12000xg for 5 minutes to remove the leaf debris. The supernatant (cell sap) was then kept on ice and used for plant inoculation. For each zucchini plant, 10µl of cell sap was rubbed onto the 1st true leaf which was pre-dusted with celite. Mock inoculated plants were inoculated with 10µl of inoculation buffer. Inoculated leaves were rinsed with distilled water for about 40 – 60 seconds approximately 1 minute after inoculation to remove the excess salt from the inoculation buffer. All inoculated and non-inoculated control plants were grown in a tropical greenhouse for a further of 35 days after the inoculation to be monitored for viral infection symptom development.

Detection of viral genome by RT-PCR: Total RNA was extracted from fresh or frozen zucchini leaf with TriReagent^R (Molecular Research Center, Inc., Cincinnati, OH.) according to the manufacturer's protocol. A total of 2µg of total RNA was reverse transcribed by ImPromp-II reverse transcriptase

(Promega, Madison, WI) using a mixture of 10 pmol of Oligo-dT and 0.5µg of random hexamer (Qiagen, Germany). Subsequently, the DNA region flanking by the introduced *Aat*II site in the PRSV viral vector was amplified using 3µl of the cDNA mixture with the 5'P1HcPro and asHP-551 primers (Fig. 1). The ACT-F and ACT-R primers were used to amplify the zucchini actin gene as an internal control for the RT-PCR reaction. Band intensities were subsequently analyzed using the Scion Image software (Scion Corporation, Torrance, CA).

Detection of recombinant protein by Western blot: A total of 200mg of fresh or frozen zucchini leaf samples was ground in liquid nitrogen, and resuspended in 400µl of protein extraction buffer (3% w/v SDS, 1 mM DTT, 12.5 mM Tris-HCl, pH6.5, 10% v/v glycerol, 1% protease inhibitor cocktail (Bio Basic Inc. Catalogue No. BS386)). The homogenate was then centrifuged at 12000xg, 4°C for 15 minutes to remove the plant cell debris. The supernatant was kept in aliquots of 15µl at -20°C for SDS-PAGE and Western blot analyses. The protein homogenate was separated on 15% SDS-PAGE and electroblotted onto nitrocellulose membrane (Whatman GmbH, Germany). The membrane was first incubated with either the anti-His (GE Healthcare Catalogue No. 27-4710-01) or the Dengue Virus Type 1-4 Antibody (D1-11(3)) (Thermo Scientific Catalogue No. MA1-27093), then followed by further incubation with HRP-conjugated anti-mouse (Sigma Chemical Co., St. Louis, Mo, USA) secondary antibody. Specific binding was detected using the ECL Prime Western blot detection reagent (RPN 2232, GE Healthcare) followed by exposure to KODAK® BioMax® Maximum Resolution (MR) Autoradiography Film.

RESULTS

The recombinant PRSV viral vector, pVD2EDIII induced systemic viral infection in zucchini plants: A previously described infectious cDNA clone of the PRSV-W genome (Yap *et al*., 2009) was used to develop a recombinant viral vector, pVD2EDIII which contained the dengue virus serotype 2 E protein domain III and an added histidine tag cloned between the P1 and HC-Pro protein coding sequences. The His-D2EDIII sequence was flanked by the natural 5'- P1/HC-Pro cleavage site and an introduced 3'-7 amino acids which are recognized by the PRSV NIa proteinase (Yeh *et al*., 1992).

A total of 24 germinating zucchini seeds were bombarded with pVD2EDIII (12 plants) and the parental pSA1164 (12 plants) (Yap *et al*., 2009) viral vectors respectively. Five plants from seeds bombarded with pSA1164 viral vector developed typical ringspot and vein clearing viral symptoms on the young true leaves between 21 - 28 days post bombardment (dpb). On the other hand, one plant from seeds bombarded with pVD2EDIII viral vector developed mild ringspot and vein clearing symptoms at 35dpb. Total RNA was extracted from the symptomatic leaves harvested at 35dpb and RT-PCR was performed using the 5'P1HcPro and as-HP-551 primers. A 0.7kb and 1.1kb PCR product was observed from the RNA samples obtained from the pSA1164 and pVD2EDIII infected plants respectively (data not shown). These results confirm that pVD2EDIII recombinant viral vector is infectious as for the full-length PRSV cDNA clone, pSA1164. Therefore, the introduction of a 0.3 kb His-D2EDIII DNA fragment into the PRSV genome at the P1 and HC-Pro junction does not affect the replication or movement of the recombinant virus as the recombinant viral genome could be detected by RT-PCR in the systemic non-inoculated leaves.

pVD2EDIII viral vector induced milder viral symptoms: The symptomatic leaf samples from both pSA1164 and pVD2EDIII infected plants were used to produce cell sap for mechanical inoculation of zucchini seedlings in the first passage. Typical ringspot and vein clearing symptoms were observed around 4-12 days post inoculation (dpi) on the systemic newly developed leaves of both pSA1164 and pVD2EDIII inoculated zucchini plants. Even though no significant difference between the time on which pSA1164 and pVD2EDIII infected plants began to develop viral symptoms; the symptom severity displayed on the pVD2EDIII infected plants was much milder than that of the pSA1164 (Fig.3). Moreover, in contrast to the pSA1164 infected plants (Fig 4A, B), the viral symptoms which appeared later on the top systemic leaf (Fig. 4D) of the pVD2EDIII infected plants were less severe than that which appeared earlier on the bottom leaf (Fig. 4C).

The His-D2EDIII DNA fragment is stably maintained within the PRSV genome: pVD2EDIII second passage was performed using the cell sap prepared from either the fresh or frozen infected leaf samples from the first passage. Similar viral symptoms were observed as for the first passage. Despite the milder symptoms, RT-PCR performed using RNA samples obtained from the pVD2EDIII top leaves still showed the presence of the recombinant viral genome in these systemic infected leaves (Fig. 5) indicating that the inserted foreign DNA segment was still maintained in the recombinant viral genome. Semi-quantitative RT-PCR indicated that the amount of recombinant viral genome resulted from the pVD2EDIII infectious clone was lower as compare to that resulted from the pSA1164 infectious clone, which thus explained the milder viral symptoms observed in the corresponding infected plants (Fig. 5B, C).

Detection of His-D2EDIII protein: Total protein was extracted from respective leaf samples of pSA1164 and pVD2EDIII infected plants from the second passage and probed in a Western analysis with an anti-dengue E

protein antibody (Gromowski *et al*., 2010; Matsui *et al*., 2009). Results, Figure 6 show a clear positive signal of dengue E protein domain III in samples from plants infected with pVD2EDIII, but no signal was observed in control, pSA1164 infected samples. Surprisingly however, the signal was observed at approximately 75 kDa, instead of the expected 15 kDa. Reprobing the filter with an anti-His tag antibody showed a weak but positive signal at the same position (Figure 6).

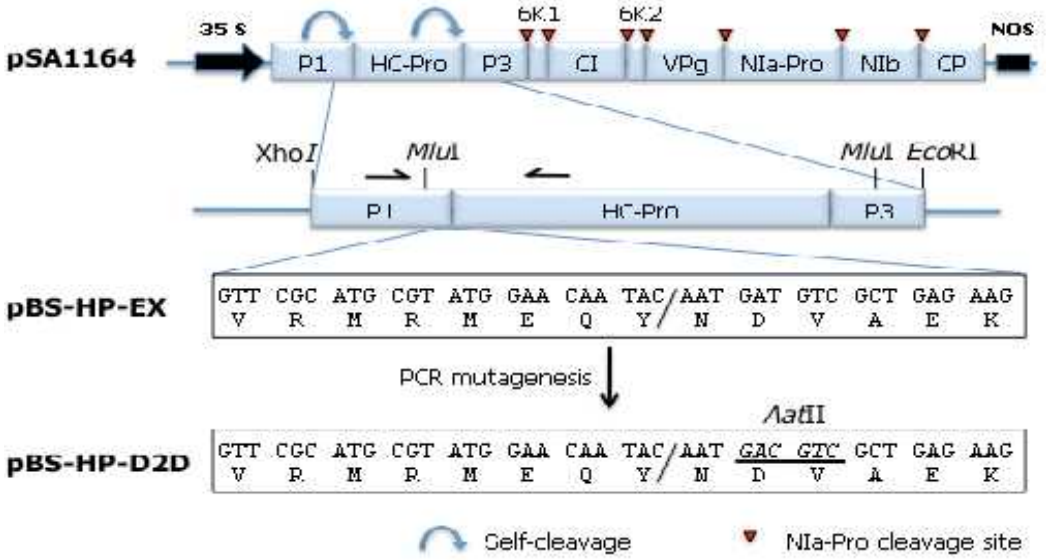

Figure 1. Papaya ringspot virus intermediate cDNA clones. The intermediate cDNA clone pBS-HP-EX was constructed by cloning the *Xho*I-*Eco*RI fragment from the PRSV full length cDNA clone, pSA1164 into the pBlueScriptKS vector. PCR mutagenesis was performed to introduce an *Aat*II restriction site into the second codon of the HC-Pro gene. The corresponding genes for the PRSV genome are indicated on the pSA1164 construct. The nucleic acid and corresponding amino acid sequences for the codons adjacent to the P1 and HC-Pro proteolytic junction in both pBS-HP-EX and pBS-HP-D2D constructs are shown in boxes. The slash represents the proteolytic site between the P1 and HC-Pro gene products. The arrows above the pBS-HP-EX construct represent the location of 5'P1HcPro (forward) and asHP-551 (reverse) primers respectively. Self cleavage and NIa-Pro cleavage sites are indicated.

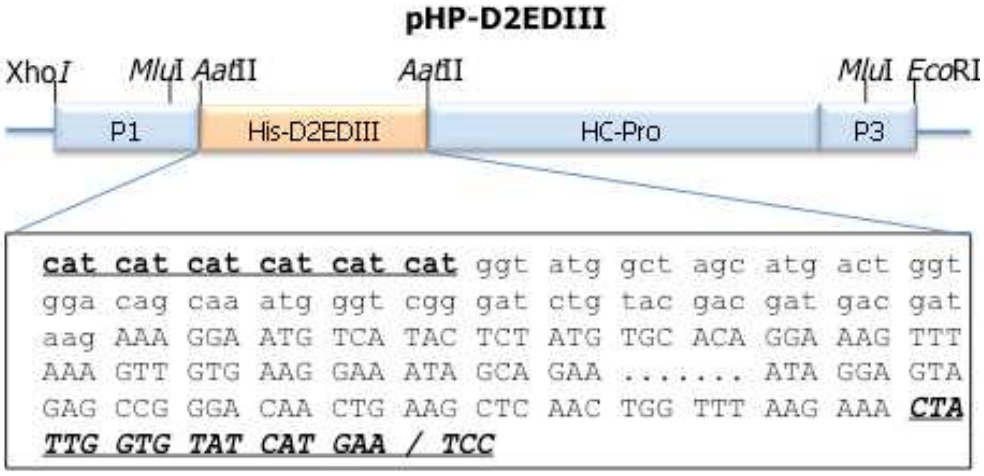

Figure 2. The pHP-D2EDIII construct. Schematic diagram and partial nucleic acid sequence of the pHP-D2EDIII construct is shown. Lower case letters represent nucleotide sequence derived from the pRSET-B plasmid, while the upper case letters represent those derived from the dengue viral cDNA. The bold, italic and underlined upper case letters represent the sequence for the PRSV NIb/CP junction which encode for the 7 amino acids which are recognized by the PRSV NIa proteinase. The slash represents the NIa proteolytic site.

Figure 3. Viral symptoms observed on pSA1164 and pVD2EDIII infected plants. Ringspot, vein clearing and mosaic symptoms were observed on the systemic leaves of pSA1164 (A, B). Only ringspot and vein clearing symptoms were observed on the systemic leaf of pVD2EDIII infected plants (C). Viral symptoms appeared on the 6dpi for both pSA1164 and pVD2EDIII plants. All the photos were taken on the 8dpi.

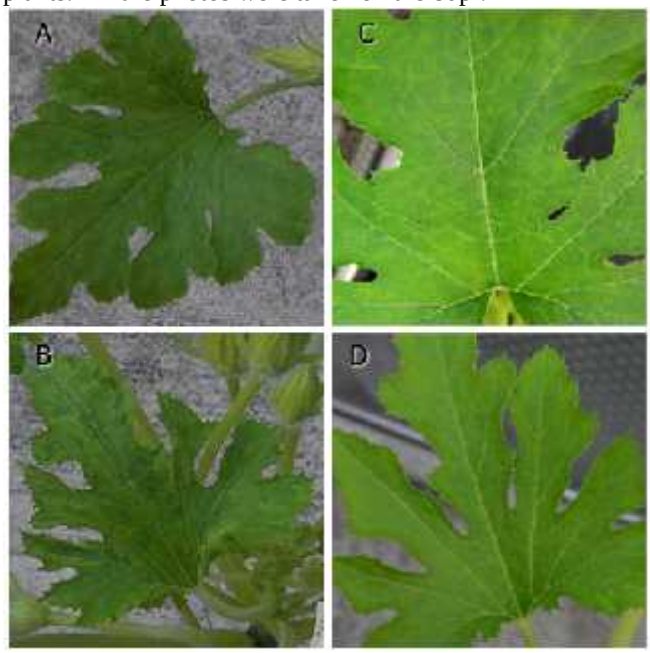

Figure 4: Progression of symptoms of pSA1164 and pVD2EDIII infected plants. Severe mosaic and leaf malformation on top leaf (B) as compared to the milder mosaic symptom on the bottom leaf (A) of a pSA1164 infected plant. Clear vein clearing symptoms on the bottom leaf (C) as compared to the mild vein clearing symptoms on the top leaf (D) of a pVD2EDIII plant. Viral symptoms appeared at 12dpi and 7dpi on the pSA1164 and pVD2EDIII infected plants respectively. All the photos were taken at 33dpi.

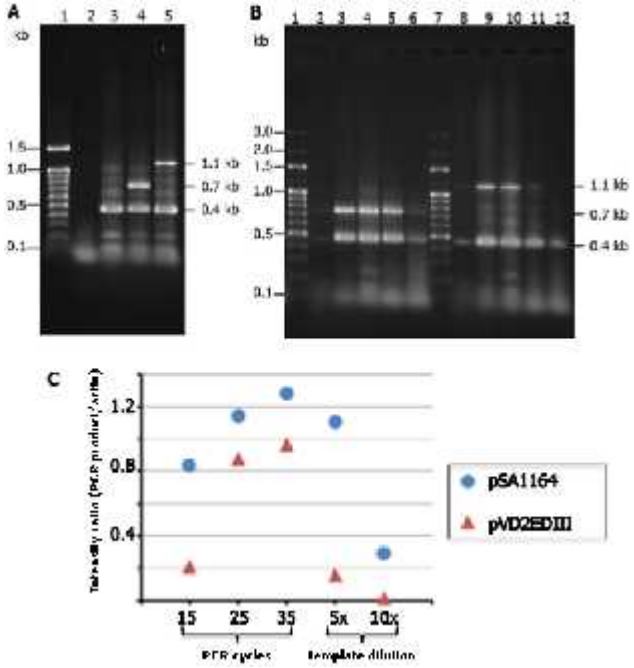

Figure 5: Detection of PRSV viral genome by RT-PCR. Total RNAs were extracted from leaf samples of zucchini plants inoculated with either inoculation buffer (mock) or cell sap of pSA1164 or pVD2EDIII infected plants (2nd passage). Multiplex RT-PCRs were performed to amplify the PRSV genome region flanking the introduced *Aat*II restriction site at the 2nd codon of HC-Pro gene and the zucchini plant actin gene. The 0.4kb PCR product represents the zucchini plant actin gene, whereas the 0.7 kb PCR product represents the product from the PRSV genome. The presence of the His-D2EDIII fragment is indicated by the additional 0.4kb in the PCR product of the PRSV genome giving a 1.1kb product. (A) Lane 1: 1.5kb-100bp DNA ladder, Lane 2 – 5: RT-PCR products from negative control, mock infection, pSA1164 and pVD2EDIII plants respectively. (B) Lane 1 and 7: 1.5kb-100bp Plus DNA ladder, Lane 2-6 and 8-12: products from pSA1164 and pVD2EDIII infected plants respectively. Lane 2-4 are products obtained from 15, 25 and 35 PCR cycles respectively. Lane 5 and 6 are products obtained from a 5x and 10x diluted cDNA template as compared to that used in lane 4. Lane 8-10 are products obtained from 15, 25 and 35 PCR cycles respectively. Lane 11 and 12 are products obtained from a 5x and 10x diluted cDNA template as compared to that used in lane 10. (C) The x-axis represents the indicated PCR reactions. The y-axis represents the intensity ratio of the PCR products from the PRSV genome over that of the zucchini plant actin gene.

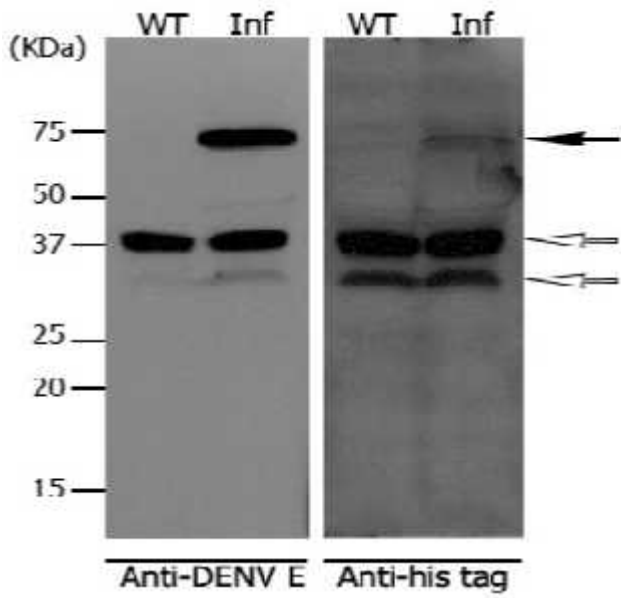

Figure 6. Western blot analysis of His-D2EDIII protein expression. Proteins were extracted from pSA1164 (WT) and pVD2EDIII infected (Inf) plants and separated by electrophoresis before transfer to a solid matrix support. Filter was probed successively with anti-DENV E (anti-DENV E) and anti-histidine tag (anti-his tag) antibodies. Membrane was stripped between probings and shown to be free of signal before the second antibody was added (data not shown). A specific signal was observed at 75kDa for both antibodies (solid arrow). Non-specific bands were also observed (open arrows).

DISCUSSION

At least four previous studies have attempted the expression of all, or a portion, of the dengue E protein in plants using a tobacco mosaic virus (TMV) based vector and mechanical inoculation of tobacco (*Nicotiana benthamiana*) leaves (Saejung *et al*., 2007), an infectious chimeric cucumber green mottle mosaic virus (CGMMV) and infection of muskmelon (*Cucumis melo*) leaves (Teoh *et al*., 2009), a combined *Agrobacterium*/plant RNA virus methodology termed "magnifection" (Martinez *et al*., 2010) and more recently the generation of transgenic rice calli (Kim *et al*., 2013). At least one of the studies (Saejung *et al*., 2007) showed that the antigen raised was antigenic and raised neutralizing antibodies in mice, while the protein produced in the transgenic rice calli was shown to possess in vitro and in vivo M cell binding activity, suggesting that this construct could raise a suitable mucosal immune response (Kim *et al*., 2013) and has potential for development as an edible vaccine.

The strategy employed in this study was most similar to the strategy of Teoh and colleagues who used CGMMV as a vehicle for expression of a truncated dengue E protein, which was fused to the terminal end of the CGMMV coat protein (Teoh *et al*., 2009). In this study, the histidine tagged dengue E protein domain III was inserted into the PRSV-W genome between the P1 and HC-Pro proteins. The insert was flanked by the natural P1/HC-Pro cleavage site and an introduced 7 amino acids which are recognized by the PRSV NIa proteinase (Yeh *et al*., 1992), that should have served to produce a free 15 kDa His-D2EDIII protein. Results of Western blotting however showed a band at approximately 75 kDa, showing that one of the two cleavage site was not cleaved, resulting in a fusion protein. The P1 protein is 63 kDa, while the HC-Pro protein is 52 k Da (Yeh *et al*., 1992; Urcuqui-Inchima *et al*., 2001). This would therefore suggest that the fusion protein of approximately 75kDa represents a fusion between P1 and His-D2EDIII and that the insert has resulted in no cleavage at the native site, rather than at the introduced NIa proteinase recognition site (Yeh *et al*., 1992).

Despite the failure to produce a correctly processed D2EDIII antigen, the insert was shown to be stable over 2 passages which contrasts sharply with the results of Teoh and colleagues (2009), who found significant instability of the recombinant insert in their chimeric CGMMV construct. Combined together our results show that PRSV is a suitable and stable vector for the expression of heterologous proteins in zucchini plants, but further research on the mechanism of proteolytic cleavage of the polyprotein is required.

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