



รายงานวิจัยฉบับสมบูรณ์

**โครงการ Function Analyses of Papaya Ringsport Virus (PRSV)
Helper Component-Proteinase (Hc-Pro)**

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ธันวาคม 2551

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Project title: Function Analyses of *Papaya ringspot virus* (PRSV) helper component-proteinase (HC-Pro)

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Abstract:

Papaya ringspot virus (PRSV) is one of the limiting factors affecting papaya production worldwide. PRSV belongs to the potyvirus genus which contains 30% of the known plant viruses. Two serological closely related strains namely type-P and W have been reported around the world. PRSV type-P infects both papaya and cucurbit, while that of W infects only cucurbit. Phylogenetic studies proposed that PRSV-P is evolved from that of type-W, while losing its pathogenicity in cucurbits. PRSV genome consists of a (+) RNA molecule of 10323 nucleotides (Thai isolates), which is first translated into a single polypeptide that further cleaved by 3 virus-encoded proteases to 10 gene products. Helper-component proteinase (HC-Pro) is encoded by the 2nd cistron of potyviral genome. It has been implicated in aphid transmission, viral movement, viral replication and suppression of host viral defense system. Independent studies from *Tobacco vein mottling virus* (TVMV) and *Tobacco etch virus* (TEV) showed contradictory results regarding the essential role of HC-Pro for the infectivity of the respective virus species. In this study, we deleted the cDNA sequences which encode for the first 54 or 100 amino acids of HC-Pro and introduced the resultant modified cDNA clones into germinating zucchini seeds through particle bombardment. Infectivity of these clones was monitored through observation of PRSV symptom development and viral genome detection by reverse-transcription polymerase chain reaction (RT-PCR). Our results indicated that as little as 54 amino acids deletion at the N-terminal of HC-Pro completely abolished the infectivity of the corresponding cDNA clone. Therefore, it is proposed that the N-terminal of HC-Pro is involved in systemic infection of PRSV in addition to its known function in aphid transmission.

Keywords: *Papaya ringspot virus* (PRSV), zucchini, cDNA infectious clone, Helper-component proteinase (HC-Pro), plant-virus interaction

Executive Summary

Rational and statement of problems:

Potyvirus infects more than 100 plant species, including food crops such as potatoes, tomatoes and legumes. *Papaya ringspot virus* (PRSV), a potyvirus which infects papaya and several cucurbit species, is one of the limiting factors for global papaya production. In Thailand, papaya is the major component of the famous Thai salad – Som Tam; and it is also widely consumed as fruits by the local people. The first outbreak of PRSV is reported in 1975 and it soon spread rapidly throughout the country. In 2003, approximately 80% of the plantation was infected, which results in a 50% reduction in production. Due to the economical importance of potyvirus, many studies have been initiated to examine the pathogenesis of various potyvirus species in different crops. However, the functional studies of PRSV genes and PRSV molecular pathogenesis in papaya and cucurbits are yet to be exploited, due to the constraint in experimental host. Current knowledge about PRSV is mainly inferred from information on other potyvirus members. This study is thus essential in order to understand the molecular pathogenesis of PRSV and its interactions with different host species. The knowledge and information gained from this study will be applied to design appropriate strategies in PRSV disease management.

The ability to cause systemic infection is one of the key determinants for disease establishment in the susceptible host. Helper-component proteinase (HC-Pro) is the gene product encoded by the second open reading frame (ORF) of potyviral genome. Analyses on HC-Pro of *Tobacco etch virus* (TEV) and *Tobacco vein mottling virus* (TVMV) have led to contradictory conclusions of its role in systemic infection of tobacco. Therefore, in this project, I will use another host-pathogen system (zucchini-PRSV) to investigate the role of HC-Pro protein in systemic infection.

Goal:

In this study, we investigate the role of *Papaya ringspot virus* (PRSV) helper component proteinase (HC-Pro) in systemic infection of zucchini plants. We examined whether HC-Pro is essential for systemic infection of PRSV and which domain is responsible for this function.

Approach:

The HC-Pro coding region within a full-length in-vivo cDNA clone of PRSV was deleted partially or fully and the infectivity of the resultant cDNA clones was accessed. The cDNA clones were introduced into germinating seeds, and the ability to induce systemic infection in the developing plants was monitored.

Table 1 – Detailed work schedule during the 2 years period.

Work description	Jul 06 – Dec 06	Jan 07 – Jun 07	Jul 07 – Dec 07	Jan 08 – Jun 08
1) Optimization of conditions to bombard zucchini seeds	→			
2) Construction of intermediate clone (i) Restriction digestion strategy (ii) PCR strategy	→ →			
3) Construction and analysis of Full length HC-Pro deletion (i) Full length deletion F1 (ii) Full length deletion F2		→ →	→ →	
4) Construction and analysis of 100 amino acids HC-Pro deletion			→	→
5) Construction and analysis of 54 amino acids HC-Pro deletion				→

Result & Conclusion:

All the cDNA clones with fully and partially deleted HC-Pro coding region were non-infectious. Therefore, it is concluded that this gene is essential for PRSV infectivity. At this point, as little as 54 amino acids deletion at the HC-Pro N-terminal was found to be deleterious for PRSV infectivity.

Function Analyses of *Papaya ringspot virus* (PRSV) helper component-proteinase (HC-Pro)

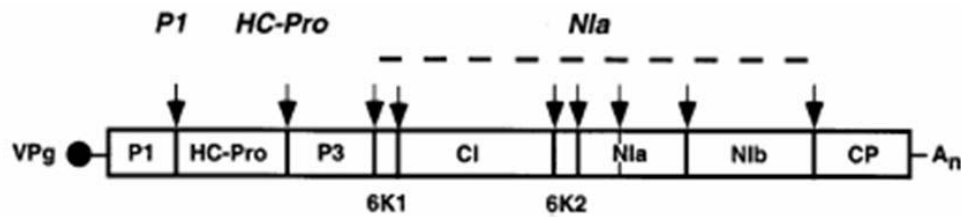
Dr. Yun-Kiam Yap

Introduction:

Potyvirus, the largest family of all known plant viruses, consists of non-enveloped, flexuous, rod-shaped virus with a dimension of 680-900nm long and 11-15nm wide. The virion consists of approximately 2000 units of single type coat protein with a single strand of positive RNA genome (Riechmann *et. al.*, 1992). *Papaya ringspot virus* (PRSV), also known as *Papaya ringspot potyvirus*, *Papaya distortion mosaic virus*, *Papaya leaf distortion virus*, *Papaw distortion ringpot virus*, *Papaw mosaic virus* and *Watermelon mosaic virus I*, is a potyvirus member which causes severe losses in papaya production worldwide. There are 2 strains of PRSV which are classified based on their host ranges. PRSV type-P infects both papaya and cucurbits species, while type-W infects only cucurbits species. PRSV type-P was first reported in *Carica papaya* (papaya), by Jensen in 1949. The symptoms on papaya hosts include mottling and malformation of leaves, ringspots and streaking on fruits, stems, and petioles. Infected papaya plants are stunt with reduced fruit production and poor food quality. PRSV type-W, formerly known as *Watermelon mosaic virus I*, was first reported by Webb in *Citrullus lanatus* (watermelon) in 1965. The symptoms on cucurbit hosts include mosaic, mottling and leaves or fruits malformation. Both strains of PRSV are transmitted by aphid vectors in a non-persistent manner, and can be mechanically inoculated onto experimental plants (Brunt *et. al.*, 1996). Independent studies on PRSV type-P and –W isolated from different regions indicated that both strains isolated from the same geographic location show higher homology at the genetic level as compared to the same strain isolated from different regions. Therefore, it is proposed that type-P from each region is evolved from that of type-W from the same region.

The first complete genome sequence of PRSV type-P was reported by Yeh *et. al.* (1992) from a Hawaii isolate. Currently, 18 full genome sequences of both PRSV strains are available in the Genbank. In Thailand, PRSV infection in papaya has been reported in many provinces including Ratchaburi, Khonkean, Saraburi, Kanjanaburi, Chiang Mai and Phuket. The complete nucleotide sequence of PRSV type-P and –W of Thailand isolates were reported in 2003 (Charoensilp *et. al.*, 2003) and 2002 (Attasart *et. al.*, 2002) respectively.

Potyviral genome consists of a +RNA which linked to the viral genome linked protein (VPg) and poly-A tail at its 5' and 3' end respectively. VPg functions as those of the cap structure of eukaryote mRNA during protein synthesis. Upon entry into the host cell, the +RNA is first translated into a single polypeptide which is then processed by 3 viral encoded protease (P1, HC-Pro and NIa) into 9 or 10 gene products as indicated in the diagram below (Urcuqui-Inchima, 2001). The respective proteolytic sites were indicated by arrows in the following diagram.



The ability to clone the cDNA of RNA viruses has opened up the opportunity to study their pathogenesis. These cDNA clone, referred as “infectious clone”, are able to produce viable virus particles upon introduction into the susceptible host plants. As a consequence, systemic infections are observed. Manipulation of these infectious clones through genetic engineering techniques enable us to study the molecular mechanisms involve in viral genome translation and replication, viral movement, symptom induction and plant – virus interaction. The first potyvirus cDNA clone was obtained in 1989 form *Tobacco vein mottling virus* (TVMV) (Domier *et. al.*, 1989), which then followed by those of *Plum pox virus* (PPV) and *Zucchini mosaic virus* (ZYMV) (Riechmann *et. al.*, 1990; Gal-On *et. al.*, 1991). The first PRSV type-P infectious clone was obtained by Chiang and Yeh (1996), from the Hawaii isolate, namely PRSV HA. In 2002, our laboratory has succeeded to clone the PRSV-W infectious clone from the Thai PRSV type-W isolate (Attasart, 2003).

Combining the cDNA mutational studies and other approaches such as protein-protein, protein-nucleic acid interaction and immuno-localization studies; many functions of the potyviral proteins were elucidated (Urcuqui-Inchima *et. al.*, 2001). However, the molecular mechanisms and the precise roles of these proteins still remained unclear. Helper component proteinase (HC-Pro) is encoded by the 2nd open reading frame of potyviral genome. PRSV HC-Pro consists of 1371 nucleotide (NCBI accession no. AY010722) that encodes for 457 amino acids (NCBI accession no. AAG47346). It is a multifunctional protein, which has been implicated in aphid transmission, viral replication and movement and suppression of host post-transcriptional gene silencing mechanism

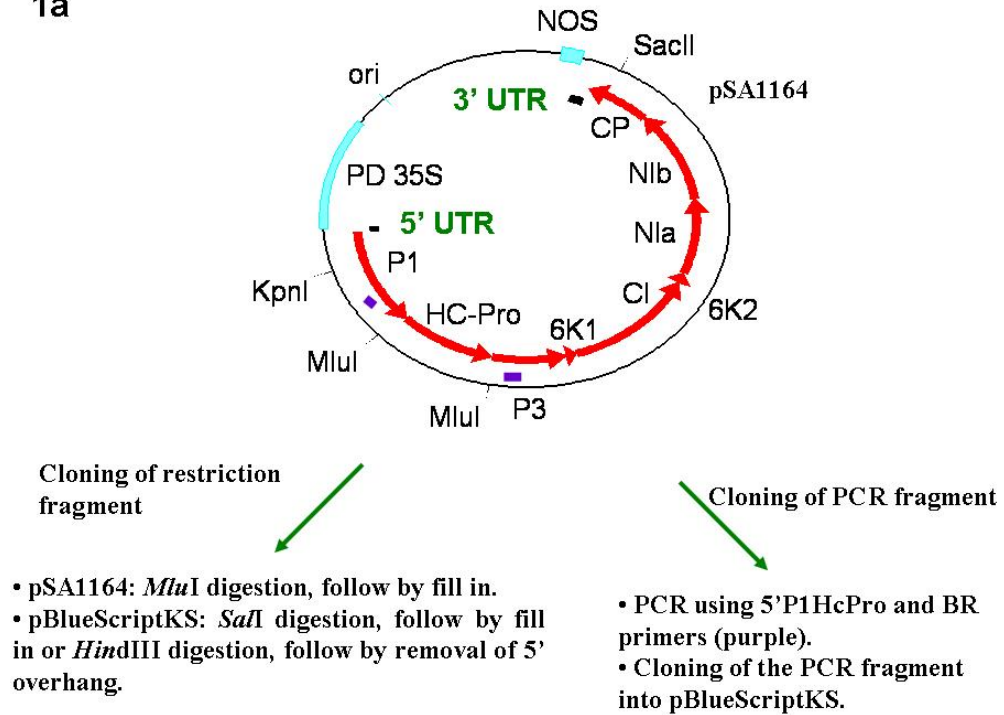
(Maia *et. al.*, 1996; Urchuqui-Ichima *et. al.*, 2001). Contradictory conclusions were reported regarding the role of HC-Pro in systemic infection of tobacco based on studies from TEV and TVMV (Atreya *et. al.*, 1992; Atreya and Pione, 1993; Dolja *et. al.*, 1993; Klein *et. al.*, 1994; Legavre *et. al.*, 1996). In the studies of TEV, viral progeny containing the N-terminal deletion of HC-Pro arise spontaneously from a GUS-recombinant virus population after being maintained in the tobacco hosts for more than a month. These results suggested that HC-Pro is not essential for viability of the virus in systemic infection of tobacco (Dolja *et. al.*, 1993). However, mutagenesis studies of TVMV indicated that N-terminal deletion abolished the infectivity of the corresponding virus. (Atreya and Pione, 1993). A current study (Stenger *et. al.*, 2005) on *Wheat streak mosaic virus* (WSMV), a tritimovirus, which is distantly related to potyvirus, reported that HC-Pro is dispensable for systemic infection in wheat. In view of all these report, I then conducted this study to investigate the role of PRSV HC-Pro for systemic infection in zucchini (a cucurbit host). In this study, we first investigate (1) whether PRSV HC-Pro is essential for systemic infection in zucchini and (2) to determine whether N-terminal deletion in HC-Pro is deleterious for PRSV infectivity in zucchini.

Methodology:

1) Construction of HC-Pro deletion cDNA clones

The PRSV type-W full-length cDNA in-vivo infectious clone (pSA1164) constructed by Dr. Attasart (2003) was used to construct all the HC-Pro deletion cDNA clones used in this study. However, the size of pSA1164 is too big (14826 bp) for manipulation. Therefore, an intermediate clone that contained only the HC-Pro cistron flanked by the 3' and 5' region of P1 and P3 cistron respectively was constructed (Fig. 1). This clone can then be used to obtain the desired HC-Pro deletions by inverse PCR strategy (Fig. 2). Subsequently, the HC-Pro cDNA fragments (with deletion) from respective modified intermediate clones (self-ligated inverse PCR products) were excised with *MluI* restriction enzyme and cloned into the similarly digested pSA1164 vector. DH5 α cells were used for transformation of plasmid with pBlueScriptKS backbone, whereas those cDNA deletion clones which contain the pSA1164 backbone were transformed into STBL2 cells.

1a



1b

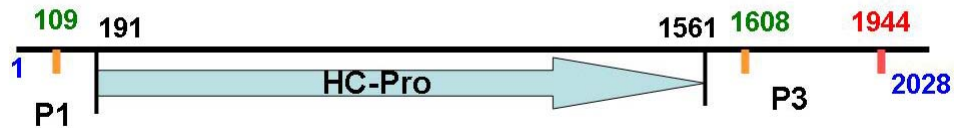


Figure 1 – Cloning strategies employed for the construction of intermediate clone.

1a – Flow chart showing the strategies used for construction of intermediate clone. pSA1164 is the full-length PRSV-W cDNA clone containing the cDNA of PRSV genome cloned within the partially duplicated 35S *Cauliflower mosaic virus* (PD 35S) promoter and Nopaline (NOS) terminator. The red arrows indicate the orientation of the respective genes as named. The 5'- and 3' un-translated region (UTR) derived from the viral genome were indicated by black boxes. The purple boxes indicate the locations of 5'P1HCPro and BR primers respectively. The unique restriction sites of *KpnI* and *SacII*, as well as *MluI* that was used for cloning the deletion cDNA clones were indicated.

1b – Schematic representation of the locations of primers, *MluI* and *EcoRI* restriction sites in relative to the cDNA sequence of the respective genes. The number 1 and 2028 represent the 1st nucleotide present in the forward (5'P1HCPro) and reverse (BR) primers (Appendix 1.1) respectively. The number 109 and 1608 represent the 1st nucleotide for the *MluI* recognition site. The number 1944 represents the 1st nucleotide for the *EcoRI* recognition site. The number 191 and 1561 represent the first and last nucleotide of the HC-Pro cDNA

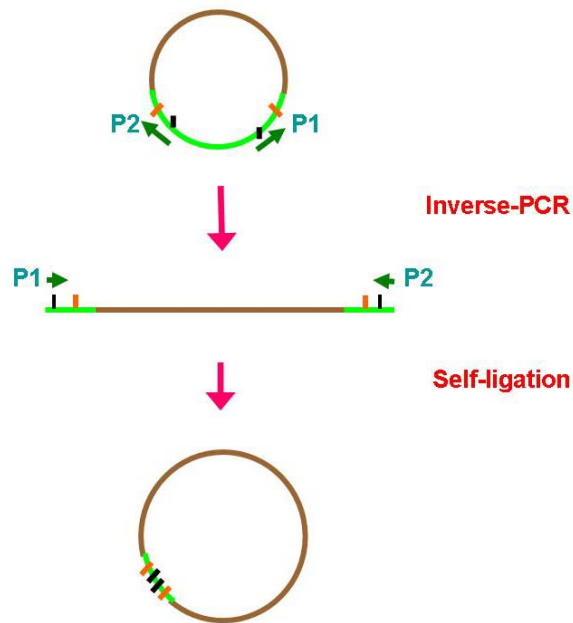


Figure 2 - Inverse-PCR strategy used to construct intermediate deletion clones which contained the desired deletion within the HC-Pro coding region.

P1 and P2 represent the primers used in the inverse PCR (Appendix 1.2, 1.3 and 1.4). Black marks indicate the border between HC-Pro and its 5' upstream (P1) and 3' downstream (P3) cistrons. Orange marks indicate the *Mlu*I restriction sites.

2) Introduction of cDNA clones into zucchini seeds.

Zucchini seeds were soaked in water for 2-4 hours to soften the seed coats. Seed coats were then removed, and seeds were placed on moisten tissues in Petri dishes kept in a paper box (to minimize water lost and provide dark condition). The box was left on shelf in the tissue culture room that is maintained at 25°C.

Bombardment is carried out three day later. Tungsten particles of 1µm diameter size were prepared and coated according to the manufacturer instruction. Seed epidermal layers were removed from the seeds just before bombardment. One percent agarose agar plate was used as a support for the seeds. The center part of the agar was removed; a piece of wet tissue is used to place 6 seeds in a circle. The seeds were then ready to be bombarded.



Figure 3 – Particle bombardment apparatus and zucchini seeds used for bombardment.

3) Plant analysis

Plants germinated from the bombarded seeds were grown under 16 hours of artificial illumination in a temperature controlled (28°C) plant growth room. Viral symptom development was observed throughout 42 days post bombardment. Leaf samples were collected at interval of 21 days and 35 days post bombardment, and total RNAs were extracted. Reverse transcriptions were then performed using oligo dT (Od_T) primer (Appendix 1.5) and the cDNAs were subjected to multiplex-PCR using primer sets (Appendix 1.6) which will amplify the coat protein gene (last cistron) of the PRSV genome, as well as the plant house keeping actin gene.

Results & Discussion

1) Optimization of conditions for bombarding zucchini seeds

The conditions of seeds used, amount of coating material and bombardment conditions were tested in order to determine the right conditions to obtain highest number of infected plants upon introduction of pSA1164 plasmids into zucchini seeds.

From the data, it was determined that 3 days old germinating seeds (with seed epidermal layer removed just before bombardment) were the best materials to be bombarded at 1350 psi from a target distance of 6 cm. Under this condition, more than 80% of seeds germinated developed PRSV viral symptom. In each bombardment, 6 zucchini seeds (Fig. 3) were bombarded with 5 µg of plasmid DNA, which were coated onto 1.5 mg of 1µm tungsten particles.

2) Construction of intermediate clone

(i) Restriction digestion strategy

As explained in the methodology session 1, a smaller intermediate clone is needed to perform the inverse PCR reaction in order to delete the HC-Pro gene. This intermediate plasmid need to contain the HC-Pro and its flanking cistrons. In addition, suitable restriction site/s (unique to pSA1164) is needed in order to clone the corresponding deleted HC-Pro fragments from the respective modified intermediate clones back into the pSA1164. Analyses of the restriction map of pSA1164 revealed that *MluI* site (Fig. 1) is a good candidate for the above purpose. However, *MluI* site is not available in the multiple cloning sites (MCS) of most of the cloning vectors. Therefore, a direct cloning using *MluI* restriction can not be used to obtain the intermediate clone. Other restriction enzyme such as combination of *EcoR1* and *XhoI* can be used to clone the HC-Pro containing fragment from the pSA1164 into the MCS of pBlueScriptKS. The resulting clone is 5.5 kb, and contained the *MluI* fragment which can then be used in the reverse cloning of deleted fragment back into pSA1164. However, the size of this intermediate clone is approximately 1kb larger than the one obtain by cloning the exact *MluI* fragment. Therefore the following digestion and modification strategies were employed to obtain the intermediate clone which contains the exact *MluI* fragment.

The pSA1164 was digested with *MluI* and filled in with T4 polymerase to produce the HC-Pro containing fragment of size approximately 1.5 kb. The pBlueScriptKS vector was digested with *SalI* and filled in with T4 polymerase. Ligation of the above fragments will result in the reconstitution of *MluI* site in the recombinant plasmid. Unfortunately, less than 30 clones were obtained from several ligation and transformation attempts. All these clones were of size smaller than the pBlueScriptKS vector.

Another approach using pBlueScriptKS vector produced from *HindIII* digestion and subsequent Mung Bean nuclease treatment was attempted twice without resulting in any positive clone. Therefore, the restriction enzyme cloning strategy was aborted after 4 months.

(ii) PCR strategy

PCR primers (Appendix 1.1) were designed and used to amplify the HC-Pro and flanking cDNA sequence, and the resultant fragment which is 2028 bp long is then digested with *EcoR1* resulting in a fragment of 1944 bp (Fig. 1b). The resultant PCR fragment is then cloned into the *SmaI-EcoRI* digested pBlueScriptKS vector. The

recombinant plasmids were screened by restriction enzyme analysis. The selected clone was subjected to sequencing, and then named as pBS1164PCR. Sequencing result from pBS1164PCR indicated that a total of 11 nucleotides mismatch (as compared with the reported NCBI record AY010722) were found within the PCR fragment (Appendix 2). The corresponding *MluI* fragment from this clone was then excised and replaced with the corresponding fragment from pSA1164. The resultant clone, pBS1164PCREx was sequenced and confirmed that those mismatched nucleotides present in pBS1164PCR were not introduced by PCR, but indeed present in the pSA1164 template. Therefore, inverse PCR was carried out as schedule using pBS1164PCREx as template.

3) Inverse PCR and construction of deletion cDNA clones

Inverse PCR was carried out as outlined in figure 2. For each set of deletion, 2 primers were designed (Appendix 1.2, 1.3 and 1.4). The PCR products for each set of inverse PCR was electrophoresed and purified from gel using a home-made silica gel and 6M NaI according to the Gene clean protocol. The purified PCR product was then self ligated using T4 ligase. Self ligated product was transformed into DH5 α and the correct clones were screened by restriction enzyme analyses and the selected clone was then subjected to sequencing. The *MluI* fragment which contained the deleted HC-Pro cDNA from each type of intermediate deletion clones was excised and cloned into similarly digested pSA1164 to obtain the respective deletion cDNA clones.

Four intermediate deletion clones were constructed. The first intermediate deletion clone, pBSHPdelF1, which used the primers set to delete almost the whole HC-Pro coding sequence retaining only 15 nucleotides at the 5' and 3' end of the gene. In this construct, 5 amino acids at the amino and carboxyl terminal of the HC-Pro will be retained to conserve the P1 and HC-Pro proteolytic recognition site between P1/HC-Pro and HC-Pro/P3 respectively. pBSHPdelF1 was then used to construct the HC-Pro full length deletion cDNA clone, pSAHPdelF1¹. Another full-length intermediate deletion clone was constructed where only 6 nucleotides at the 5' end of HC-Pro gene was retained resulting in the remaining of only 2 amino acids from the amino terminal of HC-Pro. The subsequent cDNA clone obtained was named as pSAHPdelF2².

¹ This construct was referred as pSA1164HP-del in the TRF annual meeting 2007 poster presentation.

² This construct was referred as pSA1164-HP-delF in both oral and poster presentation for TRF annual meeting 2008 and ICPP 2008 respectively.

Two other partial HC-Pro deletion cDNA clones were constructed from their respective intermediate clones. In pSAHPdelN100³, 300 nucleotides at the 5' end were deleted resulting in a deletion of 100 amino acids from the N-terminal of HC-Pro. In pSAHPdelN54⁴, 162 nucleotides at the 5' end were deleted resulting in a deletion of 54 amino acids from the N-terminal of HC-Pro. Similar to the full length deletion constructs, the partial deletion constructs retained 6 nucleotides at the 5' end of HC-Pro gene in order to conserve the corresponding P1/HC-Pro proteolytic site.

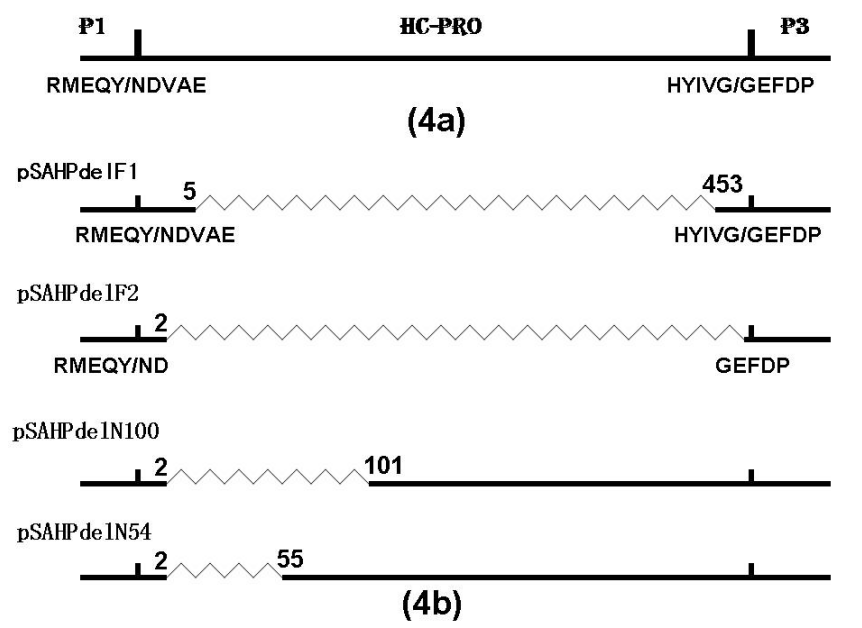


Figure 4 – Schematic representation of deletion constructs.

The figure 4a indicates the amino acids sequence for PRSV P1/HC-Pro and HC-Pro/P3 proteolytic sites. Four deletion cDNA clones were shown in figure 4b. Five amino acids (NDVAE) from the N-terminal of HC-Pro were retained in the pSAHPdelF1 construct, while the other deletion cDNA clones retained only 2 amino acids (ND) from that of HC-Pro. Five amino acids (HYIVG) from the C-terminal of HC-Pro were retained in pSAHPdelF1, while pSAHPdelF2 does not include any amino acid from the HC-Pro C-terminal. The zig-zag lines represent the corresponding deletion region present in each construct. The numbers above the zig-zag lines represent the last and first amino acids of HC-Pro that flank the respective N- and C- terminal of the corresponding deletion regions.

³ This construct was referred as pSA1164-HP-delN100 in both oral and poster presentation for TRF annual meeting 2008 and ICPP 2008 respectively.

⁴ This construct was referred as pSA1164-HP-delN54 in both oral and poster presentation for annual TRF meeting 2008 and ICPP 2008 respectively.

4) Observation of viral symptom development and detection of viral genome

Plants germinated from the seeds bombarded with the full-length cDNA clone, pSA1164 develop typical vein clearing and mosaic symptoms on the 3rd or 4th true leaves around 20 to 30 days post-bombardment (Fig. 5). However, no viral symptom was observed in plants germinated from seeds bombarded with for all the deletion clones, namely pSAHPdelF1, pSAHPdelF2, pSAHPdelN100 and pSAHPdelN54 (Fig. 6).

RT-PCR showed consistent result that no viral genome could be detected in the leaf samples collected from plants without viral symptoms (Fig. 7). Figure 7a indicated that no PCR product was amplified from C2 sample, probably due to the poor quality of RNA sample. The viral derived CP products of approximately 1 kb were observed for samples from plants 2, 3 and 4 which showed viral symptom between 25 – 35 days post-bombardment (dpb) with the pSA1164 clone. However, no viral CP product was amplified from the symptomless TE2 plant which germinated from pSA1164 bombarded seed. The 0.4 kb PCR products corresponding to the zucchini actin gene were used as the internal control to ensure that the negative results were not due to the failure of RT-PCR. Figure 7b indicated that none of those symptomless plants germinated from seeds bombarded with the fully or partially HC-Pro deleted cDNA clones contained PRSV viral genome.

The results from all the constructs were compiled in table 2. The results indicated that (1) both full-length HC-Pro deletion cDNA clones (pSAHPdelF1 & pSAHPdelF2) were not infectious (2) as little as 54 amino acids deletion at the N-terminal of HC-Pro abolishes the infectivity of the corresponding cDNA clone (pSAHPdelN54). We thus concluded that HC-Pro is essential for systemic infection in zucchini and a 54 amino acids deletion at its N-terminal is necessary for its function.

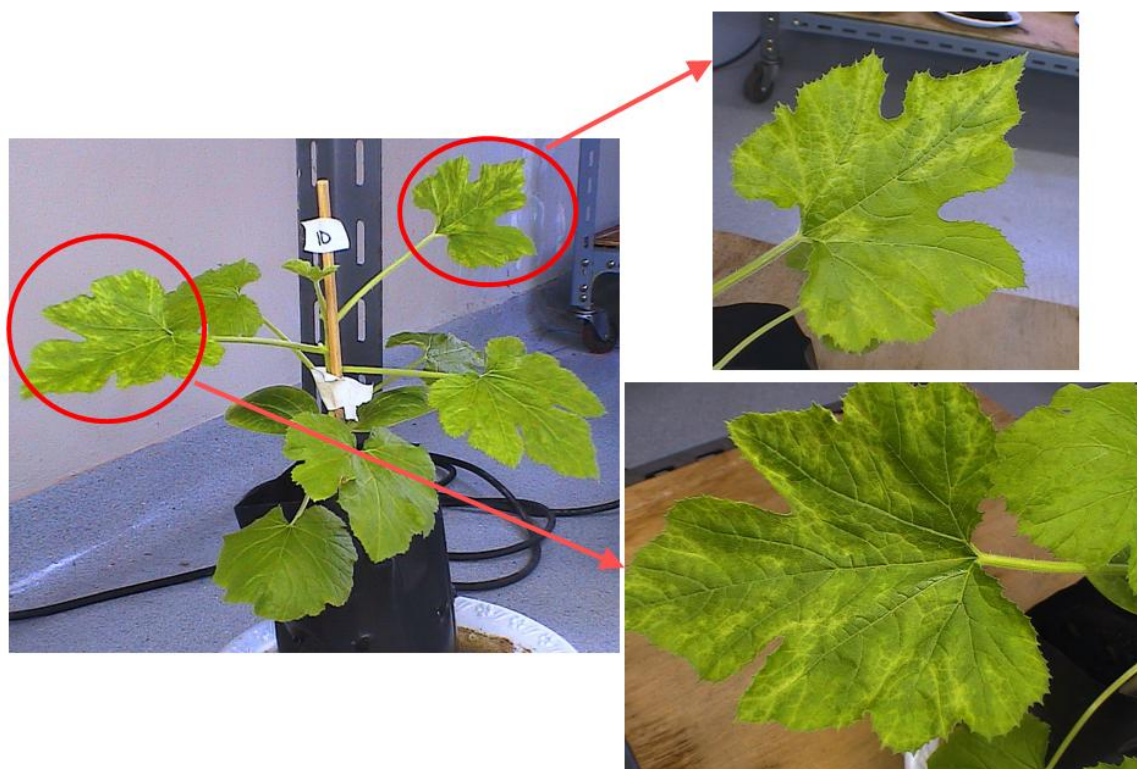


Figure 5 – Typical PRSV symptoms observed on plants germinated from seeds bombarded with pSA1164 at 30 days post-bombardment.

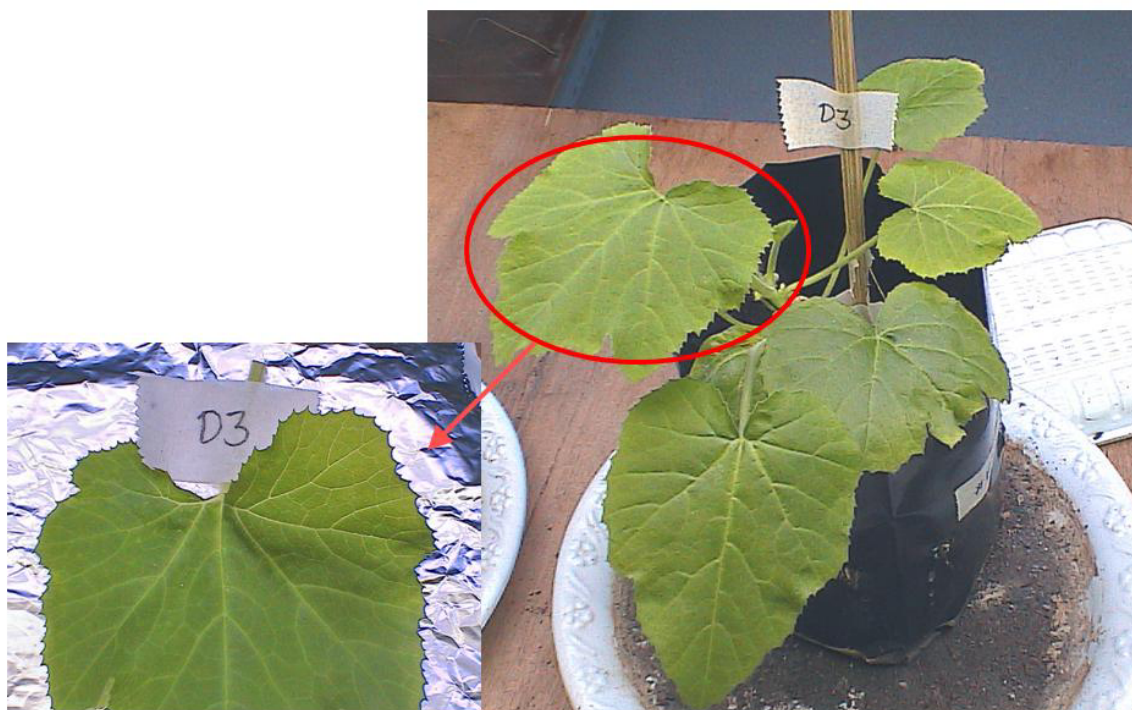


Figure 6 – Typical plant without viral symptom observed at 30 days post bombardment.

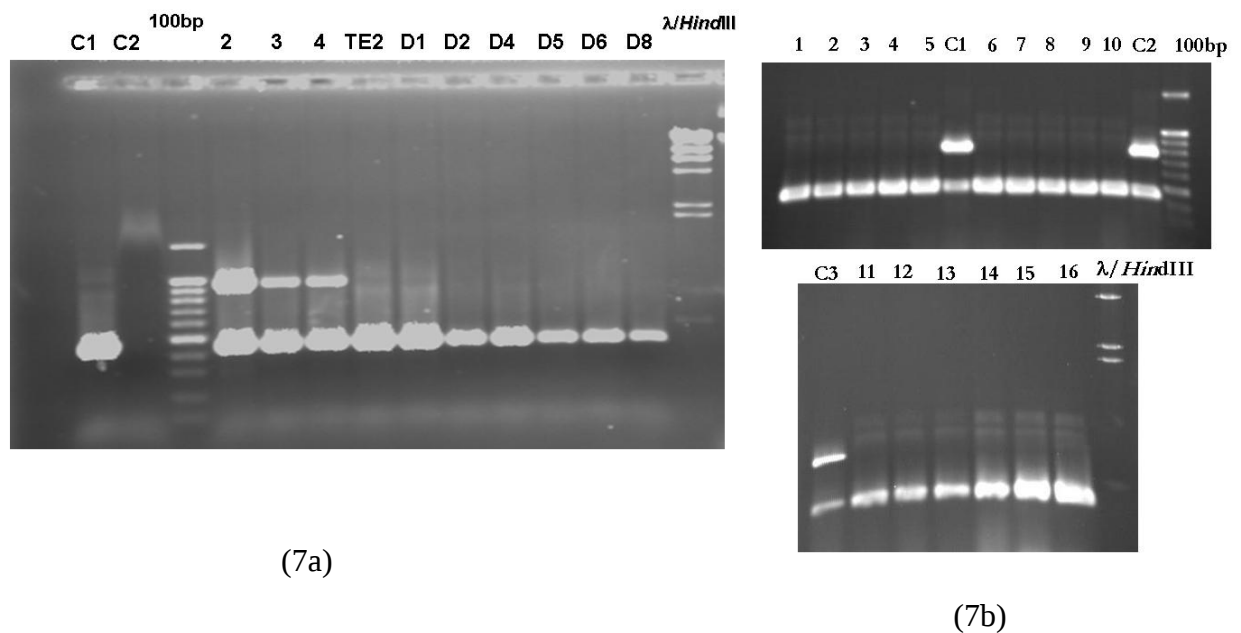


Figure 7 – Electrophoresis of RT-PCR products on 1.0% agarose gel.

RT-PCR products amplified from the total RNA extracted from respective plants germinated from seeds bombarded with different cDNA constructs were shown. A 1.0 kb product corresponds to the PRSV coat protein product indicates the presence of viral genome in the respective samples. A 0.4 kb actin product serves as an internal control for RT-PCR reaction. Figure 7a showed the RT-PCR products from leaf samples of pSAHPdelF1 plants. C1 & C2 represent plants germinated from non-bombarded seeds. The number 2, 3, 4 and TE2 represent plants germinated from seeds bombarded with pSA1164 cDNA clone. The number D1, D2, D4, D5, D6, D8 represent plants germinated from seeds bombarded with pSAHPdelF1 cDNA clone. Figure 7b showed the RT-PCR products from leaf samples of other deletion clones, namely pSAHPdelN100 and pSAHPdelN54. The number C1, C2 and C3 represent plants from seeds bombarded with pSA1164 cDNA clone. The number 1-5, 6-10 and 11-15 represent plants from seeds bombarded with pSAHPdelF2, pSAHPdelN100 and pSAHPdelN54 cDNA clone respectively.

Table 2 – Summary of bombardment experiment results for each cDNA construct.

cDNA construct	Symptom +/- total plant
pSA1164	57/82 (67%)
pSAHPdelF1	0/18
pSAHPdelF2	0/25
pSAHPdelN100	0/32
pSAHPdelN54	0/18

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Output:

Presentation at International Conference:

- 1) 3rd Asian Conference of Plant Pathology, 20th - 23rd August, 2007. Yogyakarta, Indonesia (Oral presentation)
- 2) 9th International Congress of Plant Pathology, 24th - 29th August, 2008. Torino, Italy (Poster presentation)
- 3) *Papaya ringspot virus* (PRSV) helper component proteinase (HC-Pro) is indispensable for PRSV infection. (Manuscript in preparation).

Note: The abstract/proceeding for (1) & (2) are included in the appendix.

The power point files for both oral and poster presentation of the above and those for the TRF annual meetings (2007 and 2008) are provided in the CD.

Skill development:

- 1) Throughout the project, I have developed both management and scientific skills such as proposal writing; research planning and implementation; presentation of research outputs; management of research grant and training of postgraduate students.
- 2) Part of this project was used for training a Master student in the “advance project” course conducted from 7th Jan – 29th Feb 2008.

Appendix 1

1.1– Primers used for amplifying the HC-Pro and flanking cDNA sequence.

Name	Sequences
5'P1HcPro	5' - CAGGAAGCAGTCGATCAACGCAGC
BR	5' - GCGTAGGTTTTTTCCACAGCCTCACG

1.2 - Primers used for inverse PCR of full length HC-Pro deletion

Name	Sequences
Inverse-PCR1	5' - CTCAGCGACATCATTGTATTGTTCCATAC
Inverse-PCR2	5' - CACTACATTGTTGGTGGAGAGTTTGATCC
Inv-as1	5' - ATCATTGTATTGTTCCATACGCATGCGAAC
Inv-se5	5' - GGAGAGTTTGATCCAACACTAGTTGCTTG

1.3 – Primers used for inverse PCR of 100 amino acids HC-Pro deletion

Name	Sequences
Inv-as1	5' - ATCATTGTATTGTTCCATACGCATGCGAAC
Inv-se1	5' - TTAAACAGAGTTTTTAAATGCGCGTAACATG

1.4 – Primers used for inverse PCR of 54 amino acids HC-Pro deletion

Name	Sequences
Inv-as1	5' - ATCATTGTATTGTTCCATACGCATGCGAAC
Inv-se6	5' - AACACTTGCATGAACAAGGTAAAGG

1.5 – Primers used for reverse transcription

Name	Sequences
Od_T	5' - TTTTTTTTTTTTTTTT

1.6 – Primers used for multiplex PCR

Name	Sequences
PRV-5	5' - ATCATTCATGGCTGTGGATGCTGGTTTGAATG
PRV-3	5' - GTCAAGCCATGGTTGCGCAGCCACACTGTATTCTAATG
ACT_F	5' - AACGGGAAATTGTCCGTGAC
ACT-R	5' - ATCTGCTGGAAGGTGCTTAG

Appendix 2 – ClustaW output from the comparison of PCR fragment sequence (composed) with NCBI sequence, AY010722 (Expect). The mismatched nucleotides are highlighted in bold.

composed	CAGGAAGCAGTCGATCAACGCAGCGAATCTAACACATGGTTCAAGTGGTCTCATCTTTAA	60
Expect	CAGGAAGCAGTCGATCAACGCAGCGAATCTAACACATGGTTCAAGTGGTCTCATCTTTAA	60

composed	ACCGAAGTTCGCTGACCGAACAGGGCGATA C TTTGGTGAATATTTTATCACGCGTGGACG	120
Expect	ACCGAAGTTCGCTGACCGAACAGGGCGATA T TTTGGTGAATATTTTATCACGCGTGGACG	120

composed	ATGTGAAGGAAAGCTATTTGATGGGAGATCAAAACTGGCTAAGTCGGTTCGCATGCGTAT	180
Expect	ATGTGAAGGAAAGCTATTTGATGGGAGATCAAAACTGGCTAAGTCGGTTCGCATGCGTAT	180

composed	GGAACAATACAATGATGTCGCTGAGAAGTTCTGGCTCGGTTTTAACAGGGCTTTCTTACG	240
Expect	GGAACAATACAATGATGTCGCTGAGAAGTTCTGGCTCGGTTTTAACAGGGCTTTCTTACG	240

composed	GCACAGGAAGCCGACGGATCATGTGTGCACGTCTGATATGGATGTTACCATGTGTGGTGA	300
Expect	GCACAGGAAGCCGACGGATCATGTGTGCACGTCTGATATGGATGTTACCATGTGTGGTGA	300

composed	AGTGGCGGCTCTTGCAACCATAATCCTGTTTCCATGTCATAAGATAACTTGTAACTTG	360
Expect	AGTGGCGGCTCTTGCAACCATAATCCTGTTTCCATGTCATAAGATAACTTGTAACTTG	360

composed	CATGAACAAGGTAAAGGGAAGAGTCATTGACGAGGTCGGTGAGGATCTGAATTGTGAGCT	420
Expect	CATGAACAAGGTAAAGGGAAGAGTCATTGACGAGGTCGGTGAGGATCTGAATTGTGAGCT	420

composed	CGAACGTTTGCGCGAAACCCTCTCGTCGTATGGAGGCTCATTTGGT C ATGTTTCAACATT	480
Expect	CGAACGTTTGCGCGAAACCCTCTCGTCGTATGGAGGCTCATTTGGT C ATGTTTCAACATT	480

composed	GCTTGATCAATTAACAGAGTTTTAAATGCGCGTAACATGAATGATGGAGCTTTTAAGGA	540
Expect	GCTTGATCAATTAACAGAGTTTTAAATGCGCGTAACATGAATGATGGAGCTTTTAAGGA	540

composed	GATCGCGAAGAA A ATTGATGAGAAGAAAGAAAG T CCTTGGATTCACATGACGGCCATCAA	600
Expect	GATCGCGAAGAA G ATTGATGAGAAGAAAGAAAG C CCTTGGATTCACATGACGGCCATCAA	600

composed	CAATACGCTTATTAAAGGTTTCATTAGCAACTGGCTATGAATTTGAGAGAGCGTCTGATAG	660
Expect	CAATACGCTTATTAAAGGTTTCATTAGCAACTGGCTATGAATTTGAGAGAGCGTCTGATAG	660

composed	TTTACGAGAAGTTGTAAGATGGCATCTCAAGAGAACAGAGTCAATCAAAGCTGGCAGTGT	720
Expect	TTTACGAGAAGTTGTAAGATGGCATCTCAAGAGAACAGAGTCAATCAAAGCTGGCAGTGT	720

composed	TGAGAGCTTCAGAAACAAACGTTCTGGAAAAGCTCATTTCAACCCGGCCCTCACATGTGA	780
Expect	TGAGAGCTTCAGAAACAAACGTTCTGGAAAAGCTCATTTCAACCCGGCCCTCACATGTGA	780

composed	CAATCAATTGGACAGAAATGGCAATTTCTTATGGGGTGAGAGACAATATCATGCCAAAAG	840
Expect	CAATCAATTGGACAGAAATGGCAATTTCTTATGGGGTGAGAGACAATATCATGCCAAAAG	840

composed	ATTCTTTGCTAATTACTTTGAGAAGATTGATCACAGTAAGGGTTATGAGTACTACAGTCA	900
Expect	ATTCTTTGCTAATTACTTTGAGAAGATTGATCACAGTAAGGGTTATGAGTACTACAGTCA	900

composed	ACGCCAAAACCCAAATGGAATTAGAAAGATTGCCATTGGTAATCTAATATTCTCCACAAA	960
Expect	ACGCCAAAACCCAAATGGAATTAGAAAGATTGCCATTGGTAATCTAATATTCTCCACAAA	960

composed	TTTGGAGAGGTTTCGGCAGCAAATGGTTGAACATCACATTGACCAGGGAC T AATCACTCG	1020
Expect	TTTGGAGAGGTTTCGGCAGCAAATGGTTGAACATCACATTGACCAGGGAC C AATCACTCG	1020

composed	TGAGTGTATCGCACTGCGCAATAACAATTATGT C CATGTATGTAGCTGCGTGACCTTAGA	1080
Expect	TGAGTGTATCGCACTGCGCAATAACAATTATGT T CATGTATGTAGCTGCGTGACCTTAGA	1080

composed	TGATGGAAGCTCCAGCGACGAGCGAATTGAAAAC T CCACCAAGAA C CACATTGTTCTTG	1140
Expect	TGATGGAAGCTCCAGCGACGAGCGAATTGAAAAC T CCACCAAGAA T CACATTGTTCTTG	1140

composed	TAATTCTGGTGATCCTAAGTATGTCGATTTGCCGACTCTTGAGTCTGACTCAATGTATAT	1200
Expect	TAATTCTGGTGATCCTAAGTATGTCGATTTGCCGACTCTTGAGTCTGACTCAATGTATAT	1200

composed	AGCTAAGAGAGGCTATTGCTACATGAACATCTTCCTGGCGATGCTCATAAACATACCTGA	1260
Expect	AGCTAAGAGAGGCTATTGCTACATGAACATCTTCCTGGCGATGCTCATAAACATACCTGA	1260

composed	GAATGAAGCGAAGGACTTTACAAAGAGAGTTTCGTGATCTTGAGGTTCAA A ACTTGGTGA	1320
Expect	GAATGAAGCGAAGGACTTTACAAAGAGAGTTTCGTGATCTTGAGGTTCAA A ACTTGGTGA	1320

composed	GTGGCCAACGATGTTAGATGTCGCAACATGCGCCAACCAACTGGTTATCTTT C ATCCTGA	1380
Expect	GTGGCCAACGATGTTAGATGTCGCAACATGCGCCAACCAACTGGTTATCTTT C ATCCTGA	1380

composed	TGCAGCCAATGCGGAATTGCCACGAATCTTAGTGGATCATCGACAGAAAACAATGCATGT	1440
Expect	TGCAGCCAATGCGGAATTGCCACGAATCTTAGTGGATCATCGACAGAAAACAATGCATGT	1440

composed	TATTGATTCGTTTGGATCTGTAGACTCTGGATATCACATACTGA A GCGAACACAGTGAA	1500
Expect	TATTGATTCGTTTGGATCTGTAGACTCTGGATATCACATACTGA A TGCGAACACAGTGAA	1500

composed	TCAGTTGATTCAGTTTGCCAGAGAGCCACTTGATAGTGAAATGAAGCACTACATTGTTGG	1560
Expect	TCAGTTGATTCAGTTTGCCAGAGAGCCACTTGATAGTGAAATGAAGCACTACATTGTTGG	1560

composed	TGGAGAGTTTGATCCA A CTACTAGTTGCTTGCATCAGTTGATACGCGTCATCTATAAGCC	1620
Expect	TGGAGAGTTTGATCCA A CTACTAGTTGCTTGCATCAGTTGATACGCGTCATCTATAAGCC	1620

composed	CCATGAAGCTCCGCAATCTGCT T AGAAATGAACCATATCTA A TAGTGATTGCATTGATGTC	1680
Expect	CCATGAAGCTCCGCAATCTGCT C AGAAATGAACCATATCTA G TAGTGATTGCATTGATGTC	1680

composed	GCCAAGTGACTTTTGACTTTGTTTAATAGTGCGCAATTGAGCATGCATTGAATTATTG	1740
Expect	GCCAAGTGACTTTTGACTTTGTTTAATAGTGCGCAATTGAGCATGCATTGAATTATTG	1740

composed	GATCAAAAGGGACCAAGATGTTGTTGAGGTTATTGTTTTGGTGGAGCAATTGTGTAGGAA	1800
Expect	GATCAAAAGGGACCAAGATGTTGTTGAGGTTATTGTTTTGGTGGAGCAATTGTGTAGGAA	1800

composed	AGTGACGCTTGCTAGAA C AATCTTGAGCAATTCAATGAGATTCGCCAAAATGCACGAGA	1860
Expect	AGTGACGCTTGCTAGAA T AATCTTGAGCAATTCAATGAGATTCGCCAAAATGCACGAGA	1860

composed	TATACACGAGCTAATGGATCGAAATAATAAACCTTGATTTCATACGATCGATCATTAGA	1920
Expect	TATACACGAGCTAATGGATCGAAATAATAAACCTTGATTTCATACGATCGATCATTAGA	1920

composed	GCTACTAAGTGTGTATGCGAATTC	1944
Expect	GCTACTAAGTGTGTATGCGAATTC	1944

Analyses of Papaya Ringspot Virus (PRSV) gene products

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INTRODUCTION

Papaya Ringspot Virus (PRSV), a member of the Potyvirus family which is the largest group of plant viruses infecting a vast variety of plants including many economically important crops such as potato, tomato, legumes and cucurbits. Papaya and many cucurbit plants such as cucumber, melon, watermelon, pumpkin and zucchini are commonly consumed as both vegetables and fruits in Thailand. Two types of PRSV, namely type-P and type W have been isolated from these plants. PRSV has a narrow host range and type-W only infects cucurbit plants but not papaya. PRSV genome consists of a positive RNA strand of approximately 10kb, which is translated into a single polypeptide that is cleaved by the viral encoded protease to 9-10 gene products. In this study, we have chosen to study the P3 and Hc-Pro gene products. P3 has been implicated in viral genome amplification and movement as well as viral pathogenicity determinant; however, the exact function of P3 still remained unknown. Hc-Pro is a multifunctional protein which is involved in aphid transmission, viral genome amplification and movement, and suppression of post-transcriptional gene silencing (PTGS), which is an important antiviral defense mechanism in plants.

MATERIAL & METHODS

For P3 analysis, several P3-gfp constructs (full-length and truncated) have been constructed and introduced into onion epidermal cells to examine the sub-cellular localization of the gene products. We have constructed an in-vivo PRSV type-W cDNA clone, which is able to infect zucchini by introducing the plasmid into zucchini seeds through particle bombardment. The cistron encoding for Hc-Pro gene is manipulated in order to examine the importance of Hc-Pro in PRSV-zucchini interaction. On the other hand, cistron encoding for P3 gene will also be manipulated in order to examine the link between the sub-cellular localization and function of this protein during PRSV infection.

RESULTS & DISCUSSIONS

Our results from the sub-cellular localization studies indicated that P3 protein is localized in the endoplasmic reticulum (ER) of epidermal onion cells. We have minimized the localization determination region to a 45 amino acids region containing a 19 amino acids predicted hydrophobic trans-membrane region located at the C-terminal of P3 protein. Our results suggest that the ER sub-cellular localization of the protein may play an important role in PRSV infection as the C-terminal of P3 protein have been implicated in PRSV pathogenicity in many reports. Currently, work is in progress to manipulate the above mentioned hydrophobic region in the PRSV cDNA clone. The manipulation of the Hc-Pro cistron in the cDNA infectious clone is under progress.

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Charoensilp, G., Attasart, A., Juricek, M., Panyim, S., Kertbundit, S. Sequencing and characterization of Thai Papaya Ringspot Virus isolate type P (PRSVthP). *Science Asia*. (2003) 29: 89-94

Topic area: Host-pathogen interactions

Analysis of Papaya Ringspot Virus pathogenesis

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Papaya Ringspot Virus (PRSV), a member of the *Potyviridae* family, consists of a (+) RNA genome of approximately 10kb. The genome is first translated into a single polypeptide which is then cleaved by 3 virus-encoded proteases to give 10 gene products. We have constructed a PRSV cDNA infectious clone which is able to infect zucchini and pumpkin plants through particle gun bombardment. The symptoms on both hosts are indistinguishable from those produced by PRSV virions. In this study, we investigated the role of the Hc-Pro gene in PRSV pathogenesis (infectivity and symptom severity). Hc-Pro, a multifunctional protein, has been proposed to play a role in aphid transmission, viral movement, genome amplification and suppression of host anti-viral post-transcriptional gene silencing. Various Hc-Pro regions of the full-length cDNA clone will be deleted in individual construct and these Hc-Pro mutants will be introduced into zucchini plants. Plants will be evaluated for symptom development and RT-PCR will be used to determine the presence of the PRSV genome and at the same time to semi-quantify the amount of PRSV in the plant tissues. We have tested the complete Hc-Pro deleted cDNA clone, and found it not infectious in zucchini. Therefore, Hc-Pro is indispensable for PRSV infection in zucchini. Further investigation to determine functional domains essential for zucchini infection is underway.

**Functional Analysis of Papaya Ringspot Virus (PRSV)
Helper component Proteinase (Hc-Pro)**

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Abstract

Papaya Ringspot Virus (PRSV) causes severe disease in papaya and many other cucurbits plants worldwide. PRSV is an RNA virus with a (+)-RNA genome of approximately 10kb. Its genome is first translated into a single polypeptide which is then cleaved by 3 viral encoded proteases to produce 9 gene products. Helper-component proteinase (Hc-Pro) is a multi-functional protein encoded by the 2nd open reading frame of the PRSV genome. It has been implicated to play roles in aphid transmission, protease processing of viral polypeptide and suppression of post-transcriptional gene silencing (PTGS). PTGS is one of the main antiviral defense mechanism in plants. Through evolution, many plant viruses have thus develop different type of PTGS suppressors to counter-act the antiviral system in their hosts. In this study, we aim to determine the functional domain of the Hc-Pro which is involved in PTGS suppression. This is accomplished by observing the effects of Hc-Pro gene modification on the infectivity of the modified PRSV cDNA clone on zucchini plants. First, we will investigate the effect of an Hc-Pro null mutant to determine whether this gene is essential for systemic infection. Then, we will examine the effects of different deletion mutants in the infectivity of zucchini plants. We have completed the construction of the Hc-Pro null mutant PRSV cDNA clone. This clone is then introduced into the 3 days-old germinating zucchini seeds through particle gun bombardment. The bombarded seeds were sown and plants developed from these seeds were observed for viral symptom development. Leaf samples will be collected at 30 days post-bombardment to determine the presence of PRSV virus genome by RT-PCR.

Keywords: Papaya Ringspot Virus (PRSV), Helper-Component Proteinase (Hc-Pro), post-transcriptional gene silencing (PTGS) suppressor, PRSV infectious clone, zucchini plant

Outputs

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**Function Analyses of *Papaya ringspot virus* (PRSV)
Helper Component-Proteinase (Hc-Pro)**

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Abstract

Papaya ringspot virus (PRSV) causes severe disease in papaya and many other cucurbit plants worldwide. There are 2 types of PRSV, namely type P and type W which are serologically indistinguishable. PRSV type W infects only cucurbit plants whereas that of type P has evolved to infect papaya plants while losing its pathogenicity in cucurbit plants. Both types of PRSV share high identity at the nucleic acids and protein level. The virus nucleocapsid consists of approximately 2000 units of a single type of coat protein, and a (+) RNA genome of 10323 bases. PRSV genome is first translated into a single polypeptide which is then cleaved by 3 viral encoded proteases to produce 9 gene products. Helper-component proteinase (Hc-Pro) is a multi-functional protein encoded by the 2nd open reading frame of the PRSV genome. It has been implicated to play roles in aphid transmission, protease processing of viral polypeptide and suppression of post-transcriptional gene silencing (PTGS) mechanism. PTGS is one of the main antiviral defense mechanisms in plants; through evolution, many plant viruses have thus develop different types of PTGS suppressors to counter-act the antiviral system in their hosts. To date, many of these PTGS suppressors have been identified from different viruses. However, the mechanism involved in their suppression roles is still under investigations. In this study, we made various deletions in the HC-Pro coding region of a PRSV-W full length cDNA clone, and introduced these deletion clones into zucchini seeds through particle bombardment. We then assessed the ability of these clones to induce systemic infection during the plant development process in order to reveal the functional domains of HC-Pro which are essential for infection in cucurbit plants.

Keywords: *Papaya ringspot virus*, Helper Component-Proteinase, cDNA infectious clone, pathogenesis, zucchini

Outputs

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