Virus Genes (2007) 35:611–617
DOI 10.1007/s11262-007-0114-z

C-terminal hydrophobic region leads PRSV P3 protein to endoplasmic reticulum

Sarasate Eiamtanasate · Mila Juricek · Yun-Kiam Yap

Received: 16 January 2007 / Accepted: 7 May 2007 / Published online: 13 June 2007

Abstract P3 protein is one of the least characterized potyviral proteins in both functions and sub-cellular localization. In this study, we examined the sub-cellular localization of PRSV P3 and its intermediate, P3-6K_1 by expressing their GFP fusion proteins in onion epidermal cells. Our results showed that both P3- and P3-6K_1 GFP fusion proteins were localized at the endoplasmic reticulum. Deletion analysis indicated that C-terminal of P3 protein contained localization signal, and a 19 amino acids hydrophobic domain from this region was able to target the GFP fusion protein to endoplasmic reticulum. C-terminal of P3 proteins has been suggested to be involved in both viability and pathogenicity of the potyvirus. Therefore, our result suggests that localization of P3 protein at endoplasmic reticulum is essential for functionality of P3 protein.

Keywords Potyvirus · P3 · Localization · Hydrophobic · Endoplasmic Reticulum · PRSV

Introduction

Papaya Ringspot Virus (PRSV) causes severe disease in papaya and cucurbits plants worldwide. PRSV is a member of Potyvirus, the largest group of plant viruses which contain an approximate 10 kb ss(+)RNA genome with 5′ VPg and 3′ Poly-A tail. The genome is translated into a single polyprotein, which is processed into 9–10 functional proteins by three viral encoded proteases [1–4].

P3 protein is one of the least characterized proteins of potyvirus due to its high sequence variability among species, the lack of structural or functional motif, the toxicity for expression in *E.coli* and difficulty in refolding. Nevertheless, P3 protein was suggested to play a role in viral replication [5–7), pathogenicity determination [8–10), host resistance [10–12), and viral movement [5). However, the localization and functions of P3 protein still remained unclear. Previous P3 protein localization studies using immunogold labeling indicated that Tobacco Vein Mottling Virus (TVMV) P3 protein was associated with viral CI protein in cytoplasm of infected cells [13), whereas Tobacco Etch Virus (TEV) P3 protein was localized inside the nucleus and associated with viral NIb and NIa proteins [14). These contradictory results might arise from different localization between functional and excessive non-functional proteins [15).

The 6K_1 protein is another least characterized non-structural protein, which is encoded by the cistron downstream of P3. The existence of this protein is first predicted from the potyviral genome sequence based on the presence of the NIa proteinase site between P3 and 6K_1 proteins [16). Analysis from Plum Pox Virus (PPV), Potato Virus A (PVA), TVMV, and TEV indicated that the P3-6K_1 junction is partially processed [2, 3, 16, 17) and this processing is not essential for the viability of PPV [18). The presence of 6K_1 mature protein was recently confirmed in plant cells infected with PPV [19). Therefore, it was suggested that cleavage between P3 and 6K_1 proteins to release two free proteins is a key step for regulating P3 function by differential localization of P3 and P3-6K_1 proteins [18).

S. Eiamtanasate · M. Juricek · Y.-K. Yap (✉)
Institute of Molecular Biology and Genetics, Mahidol University, Salaya campus, 25/25 Phuttamonthon 4 Road, Salaya, Phuttamonthon, Nakhon Pathom 73170, Thailand
e-mail: fryky@mahidol.ac.th

Present Address:
M. Juricek
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Pernikářce 15, Prague 6 160 00, Czech Republic

In this study, we examined the sub-cellular localization of PRSV P3 protein and P3-6K$_1$ intermediate by expressing their GFP fusion proteins in onion epidermal cells. Our results indicated that both fusion proteins were localized at the endoplasmic reticulum of transformed onion epidermal cells. We also showed that a short-hydrophobic region between amino acids 252–270 of P3 protein acts as an internal endoplasmic reticulum localizing signal for the P3 protein. The importance of the C-terminal of P3 protein has been reported by different research groups; thus, our sub-cellular localization results suggested that endoplasmic reticulum localization is essential for functionality of P3 protein.

Materials and methods

Constructions of expression vectors

All gfp and dsRed2 fusion constructs were obtained by overlapping PCR [20) using *Pfu* DNA polymerase (Promega). Sequences of primers were indicated in Table 1. PCR products were purified from agarose gel using silica beads [21).

PRSV sequences were amplified using a PRSV-w cDNA clone [22) as template and the gfp sequence was amplified from the *mgfp4* gene [23). The overlapping PCR products were then cloned into pSA1032 plant expression vector or its derivative. pSA1032 contains a multiple cloning sites flanked between the 35SCaMV promoter and the poly-A signal. All gfp fusion constructs were indicated in Fig. 1.

GFP construct contained the PCR amplified *mgfp4* gene [23) cloned into the pSA1032 derived plasmid. ER-targeted dsRed2 construct contained the *dsRed2* gene fused with the ER-localizing signal (*MVKTNLFLFFIFSLLLSLSSAD*) from *Arabidopsis thaliana* basic chitinase gene [23) and the ''KDEL'' retention signal at its amino and carboxyl terminus respectively. The *dsRed2* gene was amplified from pDsRed2 plasmid (Clontech). The sequence of ''KDEL'' ER retention signal was incorporated in the reverse primer for *dsRed2* gene amplification (Table 1). The PCR product of *ER-dsRed2* was then cloned into pSA1032 derived plasmid. ER-GFP is expressed from the pKar6-gfp plasmid (constructed by Dr. Robert Blanvillain[1]) which contained the *gfp* gene fused with the same ER-localizing and retention signals as the ER-targeted dsRed2 construct described above.

Onion epidermal cells bombardments

Onion (*Allium Cepa*) from local market was surface-sterilized by 70% ethanol. The epidermal layer from an onion

[1] Current affiliation: Department of Plant & Microbial Biology, University of California, Berkley, USA.

Table 1 Sequences of oligonucleotide primers used for amplification of *gfp*-fusion gene and ER-targeted *dsRed2* gene, respectively. (Underlined and double underlined sequences represent the *gfp* and *dsRed2* gene priming sequences for overlapping PCR, respectively; italic sequences represent the ''**KDEL**'', ER-retention signal)

Gene	Forward	Reverse
Full-length P3	GTGGAGAGTTTGATCCAACTACTAG	*GTTCTTCTCCTTTACTCATGGATCCGCCTTGATGAATGACTGGTAT*
Full-length P3 with 6K1	GTGGAGAGTTTGATCCAACTACTAG	*GTTCTTCTCCTTTACTCATGGATCCGCCCTGGTGATAAACATTTGTGTTGC*
5′P3 (P3 amino acid 1–179)	GTGGAGAGTTTGATCCAACTACTAG	*GTTCTTCTCCTTTACTCATGGATCCGCTGAATGCAAAGTACCTTAAGTGC*
3′P3 with 6K1 (P3 amino acid 173–345 with full-length 6K$_1$)	GCACTTAAGGTACTTTGCATTC	*GTTCTTCTCCTTTACTCATGGATCCGCCCTGGTGATAAACATTTGTGTTGC*
Full-length 6K$_1$	GCAAAATCAGACAATGAAAAGAAAC	*GTTCTTCTCCTTTACTCATGGATCCGCCCTGGTGATAAACATTTGTGTTGC*
Hydrophobic region 2 (P3 amino acid 236–280)	ACGTTTATCCGGAAAAGTGCC	*CTTCTCCTTTACTCATGGATCCGCT*ATGCTCCATGGCAATCCCTTGTGC
mgfp4	GCGGATCCATGAGTAAAGGAGAAGAACTTTTCACT	GCGGATCCTTTGTATAGTTCATCCATGCCATG
dsRed2	GATCCCGGGATGGCCTCCTCCGAGAACG	CCTCTA*GAGCTCGTCCTTCCC*GGGTACCAGGAACAGGTGGTGGCGG
Arabidopsis basic chitinase (ER-targeted signal sequence)	CCGAATTCATGAAGACTAATCTTTTTCTCTTTCTCATC	*CGGAGGAGGCCATCCCGGG*ATCCTCGGCCGAGGATAATGATAGG

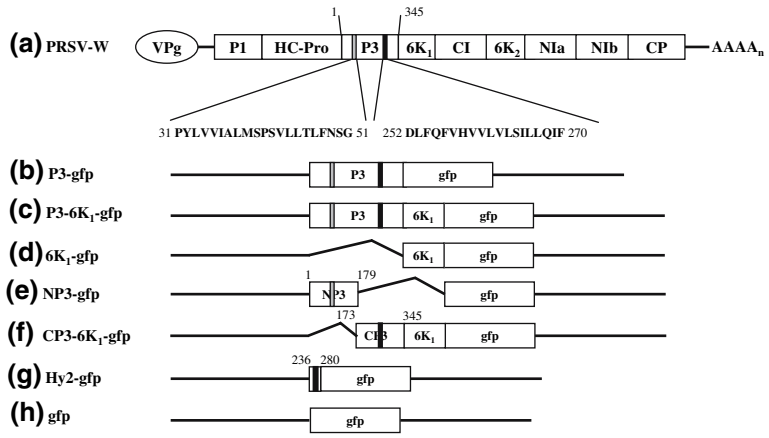

Fig. 1 Schematic representations of PRSV-W genome and GFP fusion constructs. (**a**) PRSV genome, the abbreviation of each gene products was indicated in the respective box. P3 cistron encodes for 345 amino acids and contains 2 predicted transmembrane regions as indicated by the gray and black shaded area, respectively. The amino acids sequence of the respective transmembrane region was indicated below the diagram. (**b**)–(**h**) represent different gfp fusion constructs present in each expression vector. The corresponding amino acids of the truncated P3 proteins were indicated above the P3 box in each construct

scale of approximately 2 × 2 cm size was gently peeled off and placed upside down onto 1X Murashige-Skoog (MS) media agar [24]. The samples were bombarded within 1 h.

For Co-bombardment of gfp fusion construct with ER-dsRed2 construct, 1 μg of plasmid DNA from individual gfp fusion construct was coated together with 0.5 μg of plasmid DNA from ER-dsRed2 construct onto 0.5 mg of 1 μm diameter gold microcarrier (Bio-Rad) as described in the instrument manual. DNA coated microcarriers were bombarded into onion epidermal cells using PDS-1000/He Biolistic particle delivery system (Bio-Rad) with 650 psi helium pressure from a distance of 6 cm. Bombarded onion epidermal peels were incubated overnight at 25°C in the dark.

Each bombarded onion peel was fixed onto glass slide by distilled water and observed under the Olympus BX61 fluorescent microscope. The green signals from GFP were observed under the U-MW1B2 filter set/excitation 460–490 nm/emission 510 nm, and red signals of dsRed2 protein were observed under the U-MW1G2 filter set/excitation 545–580 nm/emission 610 nm. All images were captured by the Olympus DP70 CCD camera using the manufacturer's DP Controller program (ver. 3.1.1.267). Composition images were then constructed using the above program. Adobe Photoshop program was used for photo adjustments.

Results

P3-GFP fusion protein is localized at the endoplasmic reticulum

Green fluorescence signals from the onion epidermal cell co-bombarded with P3-gfp and ER-dsRed2 constructs were found in the cytoplasm and nucleus (Fig. 2b1). The signal pattern at the nucleus resembles those of the ER-GFP protein (data not shown), which is intense around the nucleus's surface but lighter within the nucleus. This result suggested that P3-GFP protein is localized at the endoplasmic reticulum, which is in abundance on the nucleus surface. This was further confirmed by the co-localization of P3-GFP and ER-DsRed2 proteins as shown by the merging of both green and red signals within the same cell (Fig. 2b3).

P3-6K_1 intermediate product is localized at the endoplasmic reticulum

P3-6K_1 intermediate has been reported in plant cells infected by different species of potyvirus [2, 16, 17). In order to investigate the role of this intermediate during potyviral infection, we have constructed the P3-6K_1-gfp construct to study the sub-cellular localization of this fusion protein. Our result indicated that P3-6K_1 protein is localized at the endoplasmic reticulum (Fig. 2c). In contrast, 6K_1-GFP fusion protein was expressed homogenously in both cytoplasm and nucleus (Fig. 2d) as for the GFP protein (Fig. 2a). This result suggested that 6K_1 protein is not expressed in a particular organelle and the location of P3-6K_1 protein is determined by the P3 protein.

C-terminal of P3 protein contains ER localization determining sequence

Two P3 deletion gfp fusion constructs were made in order to determine the location of the ER localization determining sequence. NP3-GFP fusion protein containing P3

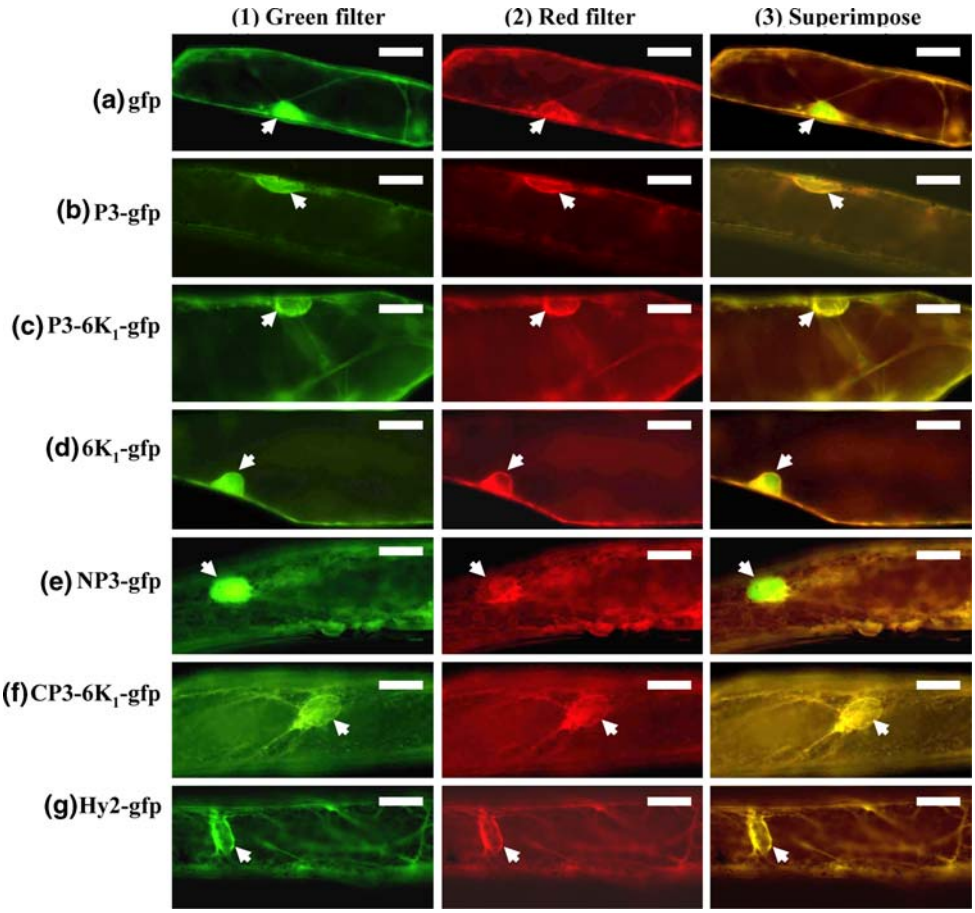

Fig. 2 Co-bombardment of onion epidermal cells. Onion epidermal cells were co-bombarded with combination of various gfp fusion constructs and ER-dsRed2 construct. The gfp construct for each co-bombardment was indicated on the left most column of the photos. Each row represents the same onion epidermal cell observed under the green filter (1st column) and the red filter (2nd column). Superimposed image are presented in the 3rd column (right most). Nucleuses are indicated with white arrows and the white bar represents 500 μm

sequence from amino acids 1–179 was found to be expressed homogenously in both cytoplasm and nucleus (Fig. 2e). In contrast, CP3-$6K_1$-GFP fusion protein containing P3 sequence from amino acids 173–345 was found to be expressed on the nucleus's surface, with similar pattern as the full length P3-GFP protein (Fig. 2f, b). These results suggested that the ER localization domain of P3 protein lies within amino acids 173–345.

A 19 amino acids hydrophobic region localized P3 protein to endoplasmic reticulum

Since there weren't any reported ER-localization signals or domains found within the P3 protein; the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html; [25, 26]) was then used to predict the presence of hydrophobic regions which might contribute to its ER localization. Two hydrophobic regions were predicted in the PRSV P3 protein; and similar hydrophobic pattern was found in the P3 proteins of another 11 potyvirus species analyzed using the same program (Fig. 3). Our result from the deletion study indicated that the ER-localization domain of P3 protein lies between amino acids 173–345, where the predicted second hydrophobic region, namely hydrophobic region 2, (amino acids 252–270) is found. In order to confirm the role of this 19 amino acids (*DLFQFVHVVLVLSILLQIF*) hydrophobic region 2 in ER localization, a Hy2-gfp fusion construct was expressed in onion cells. The result showed that Hy2-GFP proteins were co-localized with the ER-DsRed2 proteins (Fig. 2 g), which support the hypothesis that the 19 amino acids is responsible for the ER localization of P3 protein.

Discussions

The molecular mass of NP3-GFP and $6K_1$-GFP proteins are 49 kDa and 35 kDa, respectively, which enables them to diffuse passively through the nucleopore of nucleus [27]. As the consequence, these proteins are distributed homogenously in both cytoplasm and nucleus. Even though with a similar molecular weight, the CP3-$6K_1$-GFP and Hy2-GFP proteins which are 54 kDa and 32 kDa respectively are not expressed homogenously in nucleus as for NP3-GFP and $6K_1$-GFP proteins. These observations indicate that CP3-$6K_1$-GFP and Hy2-GFP proteins contain localization signal that enable them to localize specifically at the surface of nucleus.

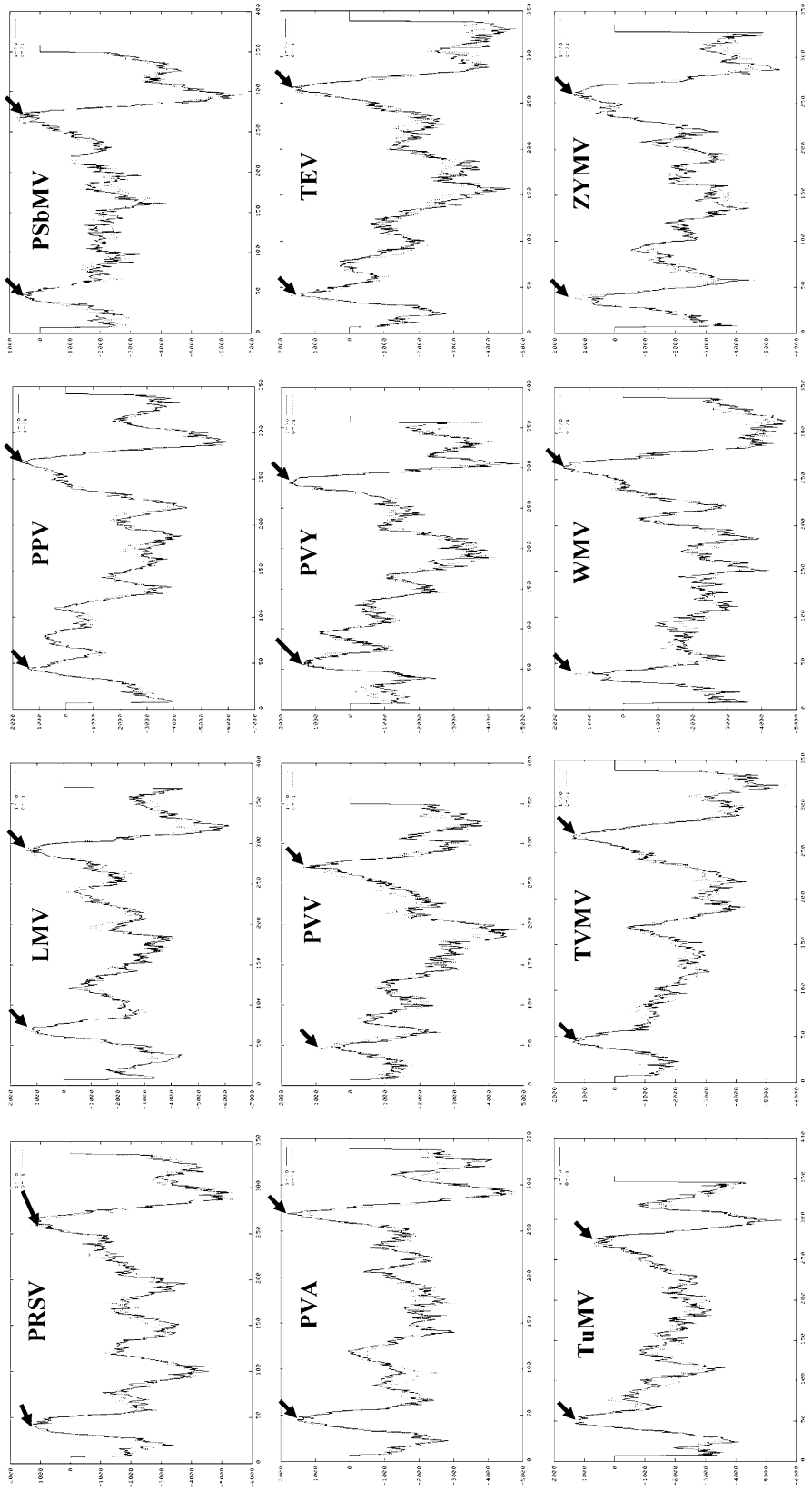

Fig. 3 Hydrophobic regions prediction by TMpred program. (http://www.ch.embnet.org/software/TMPRED_form.html). The P3 protein sequences of 12 Potyvirus species were analyzed. The predicted hydrophobic regions within each species were indicated by arrow head in the diagram. The Genebank accession number for the protein sequence of each virus is indicated in bracket as followed. PRSV (NP_734235): Papaya Ringspot Virus, LMV (NP_734155): Lettuce Mosaic Virus, PPV (NP_734341): Plum Pox Virus, PSbMV (NP_734421): Pea Seed-borne MosaicVirus, PVA (NP_734361): Potato Virus A, PVV (NP_734371): Potato virus V, PVY (NP_734251): Potato virus Y, TEV (NP_734209): Tobacco Etch Virus, TuMV (NP_734215): Turnip Mosaic Virus, TVMV (NP_734330): Tobacco Vein Mottling Virus, WMV (YP_077272): Watermelon Mosaic Virus, ZYMV (Q89330): Zucchini Yellow Mosaic Virus

In this study, P3 protein was expressed as a heterologous protein in plant cells without the presence of other PRSV gene products and the results shown that it was localized at the endoplasmic reticulum. The blast result of P3 amino acid sequence did not reveal any known ER-targeting signal. Therefore, we speculate that P3 protein localization at the ER might be attributed to the presence of hydrophobic regions within the protein [28). Using the TMpred program, we located 2 hydrophobic regions spanning the region from amino acid 31–51 and 252–270, respectively. From the deletion results, we observed that CP3-6K_1-GFP fusion protein, which contains the hydrophobic region 2 (amino acids 252–270) was localized at the ER as for the full-length P3-GFP protein. The Hy2-gfp construct further support our hypothesis that the 19 amino acids spanning from 252–270 is the ER localization determining sequence of P3 protein.

The mutation analysis of TVMV P3 protein showed that each of the two mutations in C-terminal region of TVMV P3 protein render the virus ability to replicate in tobacco cells [6). The C-terminal of TEV P3 protein was shown to contain the determinant of wilting response of Tabasco pepper [8). Similarly, this region was also reported to involve in the pathogenicity and infectivity of PPV and Turnip Mosaic Virus (TuMV), respectively [29, 30). Our localization result is consistent with the previous P3 functional studies, which suggest the importance of the C-terminal of P3 protein. Taken together, we thus propose that ER localization is important for P3 functions. Therefore, mutation to the hydrophobic region 2 within the C-terminal of P3 to disrupt its localization will enable us to further dissect the role of P3.

We found that 12 of the potyviral species that we have analyzed using TMpred program contain the same hydrophobicity pattern, even though they are varied in length and shared lower amino acids homology as compared to other potyviral gene products. This conserved pattern might implicate the importance of these hydrophobic regions in P3 protein function.

Analysis of amino acid sequences of both hydrophobic regions indicated that hydrophobic region 1 (amino acids 31–51) of the 12 species of potyvirus that we have compared shared higher similarity (87.3%, Fig. 4a) as compared to the hydrophobic region 2 (amino acids 252–270) which shows only 82.9% similarity (Fig. 4b). The presence of Proline residue in the hydrophobic region 1 (Fig. 4a) suggests that this region is unlikely to be a transmembrane region [31), and our results from the expression of NP3-gfp construct in onion cells also indicated that it is not sufficient for ER localization of P3 protein. However, we do not exclude the possibility that this hydrophobic region may serve as a transmembrane domain in addition to hydrophobic region 2. Further analysis is necessary to determine the topology of P3 protein in ER membrane and its mechanism of insertion.

P3 protein was reported to be involved in viral replication [5–7) and interacting with other viral gene products involved in replication such as CI and NIb [13, 14, 32). However, it does not bind to viral genome RNA [33). The replication complex of Potyvirus was reported to be located at endoplasmic reticulum of infected cells [34, 35). Thus, we proposed that P3 might play a role as an adaptor protein to assemble the replication components to the ER to form the replication complex. Nevertheless, ER localization of P3 protein also supports its proposed function in viral cell-to-cell movement [5) due to the inter-connection of the ER network and the plasmodesmata.

Our study found that P3 and 6K_1 proteins are localized at different sub-cellular localization. However, the P3 protein localization is not depending on the 6K_1 protein. In contrast, we found that P3 protein contains the ER localization determining sequence, which targets itself and the

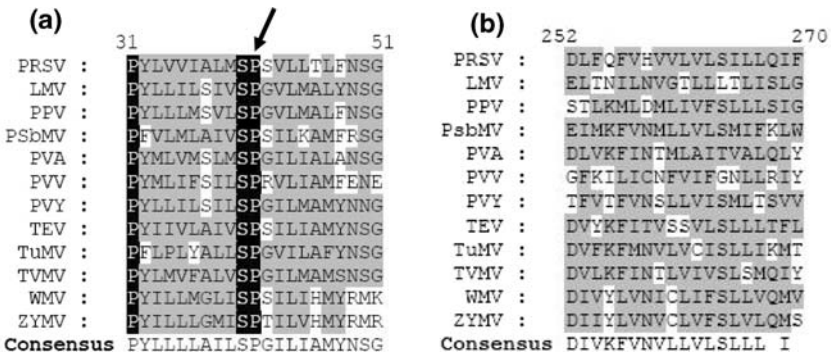

Fig. 4 Amino acid alignments of hydrophobic regions from 12 Potyvirus species. The amino acid sequences of both hydrophobic regions from 12 Potyvirus species were aligned together with the Alignx program from Vector NTI Suite 9.0.0. Identical and similar amino acids within the same row are highlighted with black and gray color, respectively. The numbers indicated on top of the sequence represent the corresponding amino acid number of PRSV P3 protein. (**a**) Hydrophobic region 1 alignment. The conserved proline amino acid in the middle of hydrophobic region 1 is indicated by arrow. (**b**) Hydrophobic region 2 alignment

P3-6K$_1$ intermediate to the endoplasmic reticulum. Therefore, we proposed that the function of P3 protein might not be regulated by the processing of the P3-6K$_1$ processing as suggested by previous report [18).

Acknowledgements We thank Dr. Kusol Pootanakit and Professor Sakol Panyim, IMBG, Mahidol University for valuable discussion and critical reading of the manuscript; Mr. Sarun Kunwittaya, NIC-FD, Mahidol University for technical advice to operate the fluorescent microscope; Dr. Scott G. Franzblau, University of Illinois and the Flanders Interuniversity Institute of Biotechnology (VIB), Ghent University, Belgium for the kind gift of pDsRed2 and pKar6-gfp plasmids respectively. S.E is the recipient of Mahidol University Medical scholar Program (PhD-MD). This study was supported by the Mahidol University Research Grant, ''RNA Interference for Plant Virus Resistance'' and TRF grant MRG4980038.

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