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COMPARISON OF THE COAT PROTEIN OF A SOUTH INDIAN STRAIN OF PRSV WITH OTHER STRAINS FROM DIFFERENT GEOGRAPHICAL LOCATIONS

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SUMMARY

Papaya ring spot virus (PRSV) causes major diseases in papaya and cucurbits in the Indian sub-continent that result in significant yield losses. Molecular characterization of the coat protein (CP) gene of a South Indian strain (INP-UAS) of PRSV-P revealed an open reading frame of 849 bp that encoded the putative coat protein of 283 amino acids (GenBank Acc No. AF528190). The DAG triplet associated with aphid transmissibility and the potential protease cleavage site Q/S, located in the N-terminus of the INP-UAS CP, were conserved, as has been reported for other PRSV coat proteins. The sequence had a deletion of 24 nucleotides that corresponded to eight amino acids in the N-terminal region of the CP. A comparison of the amino acid sequence of the INP-UAS CP with those reported for other PRSV isolates showed that the N-termini were variable and suggested that the distinctiveness of INP-UAS was linked to its geographical location. Phylogenetic analysis also showed that the INP-UAS strain coat protein gene was relatively divergent from those of other PRSV-P isolates as it formed a separate and distinct group. The implications of sequence variability for the use of CP-genes in the development of transgenic plants for viral resistance are discussed.

Keywords: Coat protein, PRSV-P, Indian isolate.

INTRODUCTION

Papaya ring spot virus (PRSV), biotype P (PRSV-P) induces one of the most destructive diseases in papaya. This disease has become a major threat to papaya cultivation throughout India by rendering orchards economically unproductive. PRSV infection is reported to occur in every region where papaya is grown irrespective of the agro-climatic conditions, and the disease can result in crop losses of up to 85-90% (Lokhande *et al*., 1992; Hussain and Varma, 1994). In nature, PRSV is transmitted by numerous species of aphids in a non-persistent manner and has a limited host range that consists mainly of cucurbits and papaya. PRSV is grouped into two biotypes: Type-P (PRSV-P), which infects cucurbits and papaya, and Type-W (PRSV-W), earlier called WMV-1, which infects only cucurbits and not papaya (Purcifull *et al*., 1984). Although both types are serologically related, observations suggested that papaya is the major source for the spread of PRSV-P (Yeh *et al*., 1984). PRSV infection induces characteristic symptoms in papaya and cucurbit cultivars such as vein clearing, mottling, malformed leaves, filiformy, ring spots and streaks on fruits, stems and petioles, and stunting (Purcifull *et al*., 1984). PRSV-P is a member of genus *Potyvirus* and has flexuous filamentous particles of about 780 x 12 nm size (Gonsalves and Ishii, 1980; Rosa and Lastra, 1983). The virions contain a positive sense single stranded RNA of ~10.0 kb, encapsidated by a single species of coat protein of molecular weight of $30\text{-}36 \cdot 10^3$ kDa. The RNA is polyadenylated and has a genome-linked protein (VPg) covalently bound to the 5' end. The genome is monocistronic and is translated to give a large polyprotein that is subsequently processed into individual functional proteins (Yeh and Gonsalves, 1985).

Due to the lack of natural host resistance to PRSV-P in papaya cultivars, breeding for resistance has shown only limited success. Coat Protein-Mediated Resistance (CPMR) has been successfully used to confer resistance to a wide range of viruses including PRSV (Lomonossoff, 1995; Gonsalves, 1998). Previous studies have indicated that coat protein genes of PRSV strains are very distinct in geographic origin and in pathogenicity (Bateson *et al*., 1994; Wang *et al*., 1994). Hence, transgenic papaya with CP-genes specific to the PRSV-P strains existing in a particular region need to be developed for effective control in that region. The success of CPMR depends on the compatibility and genetic relatedness to the challenging virus of the coat protein genes expressed in the transgenic plants (Bateson *et al*., 1994; Clark *et al*., 1995; Tennant *et al*., 2001; Hema and Prasad, 2003). Therefore, in a particular region, it is essential to know the nucleotide and amino acid sequences of the PRSV coat protein (CP) gene and to determine how much this differs from those

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of other PRSV isolates. PRSV-P is prevalent in India (Mali, 1985) but the strains responsible have not been adequately characterized at the molecular level. Further, reports of molecular analysis to determine geographical specificity are limited. In the present study, we report the molecular characterization of the PRSV-P CP gene of a South Indian strain and discuss its distinctiveness from, and phylogenetic relationship with, other PRSV-P isolates reported from different geographical locations.

MATERIALS AND METHODS

Viral RNA extraction. PRSV-P-infected leaves were collected from orchards that contained naturally infected papaya plants showing symptoms typical of PRSV-P infection. The identity of the virus was confirmed by pathogenicity tests on papaya. The virus was maintained in *Carica papaya* L. var. Solo in a greenhouse. Young infected papaya leaves were collected from these plants, lyophilized for 48 h, powdered and stored at 4ºC. Viral RNA was extracted from the lyophilized leaf powder (Robertson *et al.*, 1991), resuspended in 100 ml of TE (10 mM Tris containing 1 mM EDTA, pH 8.0) and used directly as a template for cDNA synthesis.

PCR amplification and cloning of PRSV-P CP gene. Forward and reverse primers for PRSV coat protein gene corresponding to the putative 5' end, about 20 nucleotides upstream of the proteinase cleavage site, and the 3' end were designed based on the conserved regions in the PRSV-P genome reported from different isolates (Quemada *et al.*, 1990; Yeh *et al.*, 1992; Bateson *et al.*, 1994; Wang *et al.*, 1994; Jain *et al.*, 1998) with *NcoI* and *BamHI* restriction sites (forward primer, CP1-5'- CATGCCATGGTGTTTCATCAGTCCAAGAATGAAG - 3' and reverse primer, CP2-5'- CGCGGATCCTATTTAGTTGCGCATACCAGGAGAG - 3').

To synthesize the first strand cDNA, reverse primer CP-2 was annealed to the viral RNA by heating at 70°C for 5 min followed by snap chilling on ice for 2 min. First strand cDNA was synthesized using AMV reverse transcriptase (Finnzymes, Keilaranta, Finland) at 42°C for 1 h. A 900 bp fragment representing the CP gene of PRSV was amplified from 5 ml of first strand cDNA mixture using 1.5 units *Taq* polymerase with 20 pmol of CP-1 and CP-2 primers, 2 mM $MgCl_2$ and 200 mM dNTPs. Initial denaturation was done at 94°C for 3 min and the cDNA was amplified for 35 cycles by denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extending at 72°C for 1.5 min, with a final extension at 72°C for 20 min. The PCR product was analyzed by electrophoresis in 1% agarose gel (Sambrook *et al.*, 1989).

The PCR-amplified fragment was gel-eluted by using the freeze-thaw method (Ausubel *et al.*, 1989) and cloned into pTZ57R using an InstT/A PCR cloning kit (Fermentas GMBH, St. Leon-Rot, Germany) as per the manufacturer's instruction and transformed into *E. coli* strain XL1-Blue MRF' cells. The recombinant plasmids from the putative clones were isolated by the alkali lyses method (Sambrook *et al.*, 1989) and examined for the PRSV coat protein gene by digesting with *Nco*I and *Bam*HI. Clones containing the 900 bp insert were sequenced using the M13 universal primers as well as the CP-gene specific primers in both directions (forward and reverse) using ABI Prism model 377 automated sequencer.

Sequence comparison. Nucleotide sequences were aligned and translated to obtain the amino acid sequence using the BioEdit program. For pairwise multiple alignment of nucleotide and amino acid sequences and to determine the percent identity matrix, a version of Smith and Waterman (Smith and Waterman, 1981), and ClustalW algorithm (Thompson *et al.*, 1994) was implemented respectively, from BioEdit (version 5.0.6). Sequences were aligned and compared with the coat protein gene of 74 other PRSV-P isolates reported from other geographical locations. GenBank searches were done using the BLAST program (Altschul *et al.*, 1990). Cluster dendrograms were generated using Tree View in PHYLIP (Page, 1996). The gene sequence has been deposited at the NCBI GenBank under the accession number AF 528190.

RESULTS AND DISCUSSION

Cloning and sequencing of CP gene. Amplification of PRSV-P viral RNA using CP gene-specific primers by RT-PCR resulted in the production of a 900 bp fragment. The clones carrying the CP gene were confirmed as such by restriction digestion using *Nco*I and *Bam*HI and PCR amplification. These clones were, then, sequenced in both directions to generate overlapping sequences. This PRSV-P coat protein gene was referred to as INP-UAS CP and the complete sequence generated was used for further analysis. The sequence data revealed that the INP-UAS CP gene had an ORF of 849 bp, which could potentially encode a protein of 283 amino acids with an approximate molecular weight of 31000. The translated coat protein sequence indicated the presence of the conserved DAG sequence potentially associated with aphid transmissibility of many potyviruses and the protease cleavage site Q/S at the N-terminus.

Variability and phylogenetic analysis of the PRSV CP N-terminus. Amino acid sequence comparison of the INP-UAS CP with those of 74 other PRSV-P isolates reported from different geographical locations (Table 1) revealed a highly variable N-terminal region,

Table 1. PRSV isolates and sequences used in this study for comparison.

Acronym	Geographical origin	GenBank Acc. No.
INP-UAS	India	Af528190
INP-BR	India	Af120270
P-IND	India (Pune)	Af063220
US-HA	USA (Hawaii-severe)	X67672
US- HA5-1	USA (Hawaii-mild)	D00595
US-FL-H1K	USA (Florida)	Af196839
AUS-BD, AUS-BDG, AUS-1, AUS-WLGPt	Australia	U14736 - U14740
BZL-BA-CA, BZL-SP, BZL-PR, BZL-ES, BZL-PB, BZL-PE, BZL-CE, BZL-DF	Brazil	Af344641 - Af344650
MEX-VTB6, MEX-Cht11	Mexico	Aj012649, Aj012650
MEX-VPO28	Mexico	Aj012099
TWN-YK	Taiwan	X78557
PHL	Philippines	Af374863
PHL-01	Philippines	Af506902
INDONE-1, INDONE-2	Indonesia	Af374865, Af374864
PUE RICO	Puerto Rico	Af196838
CHINA	China	Af243496
JAPAN	Japan	AB044339
SRL	Srilanka	U14741
THP-BGK	Thailand	Ay010712
THP-KPS	Thailand	Af374862
THP-12- THP-14	Thailand	Af506898 – Af06900
THP-02	Thailand	Af506901
THP-01	Thailand	Af506904
THP-CMP, THP-KNKH, THP-CBRI-1, THP-CBRI-2, THP-Mild, THP-Severe, THP-CGM-1, THP-CGM-2, THP-RTCB	Thailand	Ay010713 - Ay010721
VNP-01	Vietnam	U14742
VNP-02 to VNP-29	Vietnam	Af506862 - Af506889

as previously reported for other potyviruses (Bateson *et al*., 1994; Wang *et al*., 1994; Wang and Yeh, 1997; Silva-Rosales *et al*., 2000; Hema, 2002). The core and C-terminal regions were more conserved (Fig. 1). The variability in the N-terminal region was most evident in the first 38 amino acids that contained a stretch of 'EK' (glutamic acid and lysine) repeats starting at the third amino acid after the 'DAG' aphid transmission motif (Fig. 2). The CP gene of INP-UAS had a deletion of 24 nucleotides, corresponding to eight amino acids, in this region. The other PRSV-P strain reported earlier from India (P-IND) showed a deletion of four amino acids in the N-terminal region of CP (Jain *et al*., 1998).

Comparison among the CP N-terminal regions revealed two major lineages (Fig. 3). All the American isolates (USA, Mexico, Brazil, Puerto Rico) and the Australian isolates including one of the isolates from India (P-IND) and Sri Lanka formed one lineage while the rest of the South East Asian and Western Pacific isolates (China, Indonesia, Thailand, Vietnam, Taiwan, Japan) formed another. The two strains from Southern India, INP-UAS and INP-BR did not group with either of the two lineages but formed a separate and distinct group outside the major cluster. The American and Australian isolates showed very little amino acid sequence divergence. All the four Australian isolates compared in this study showed a close clustering as previously observed (Bateson *et al*., 1994; Silva-Rosales *et al*., 2000; Bateson *et al*., 2002). Among the American isolates, the Brazilian and Mexican isolates were more diverse than were the US or Australian isolates, suggesting that they might have diverged from this population.

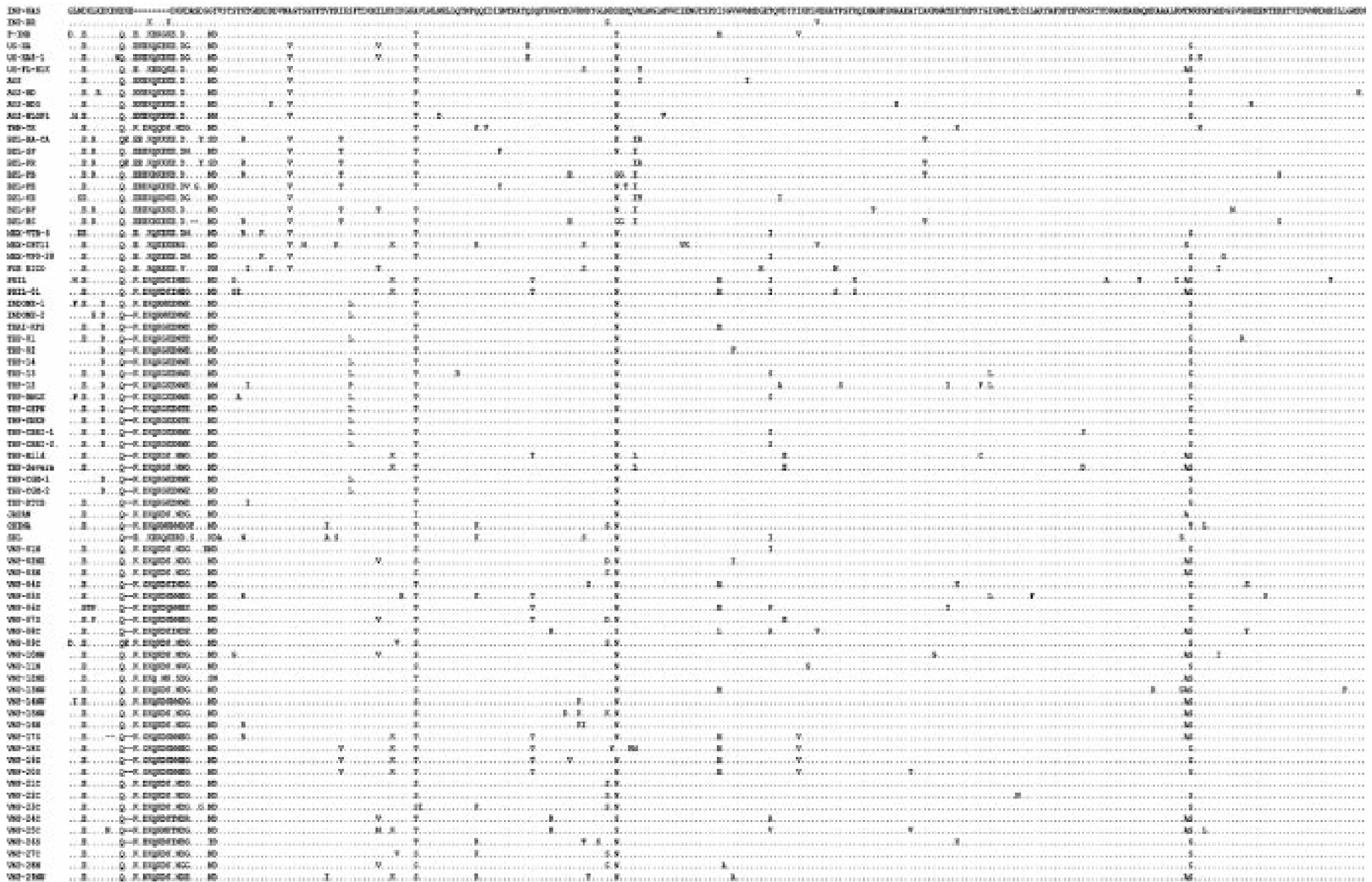

Fig. 1. Alignment of the amino acid sequences of CPs of 75 PRSV-P isolates from different geographical locations compared to INP-UAS CP. Sequences were aligned using the ClustalW algorithm from BioEdit program (version 5.0.6). The origin of the different PRSV strains is indicated in Table 1.

The Indian isolate, P-IND, also diverged from this US-Australian cluster but it did not show any particular relation to either of the two South Indian strains (INP-UAS, INP-BR) or with any of the other PRSV-P isolates from the Asian sub-continent.

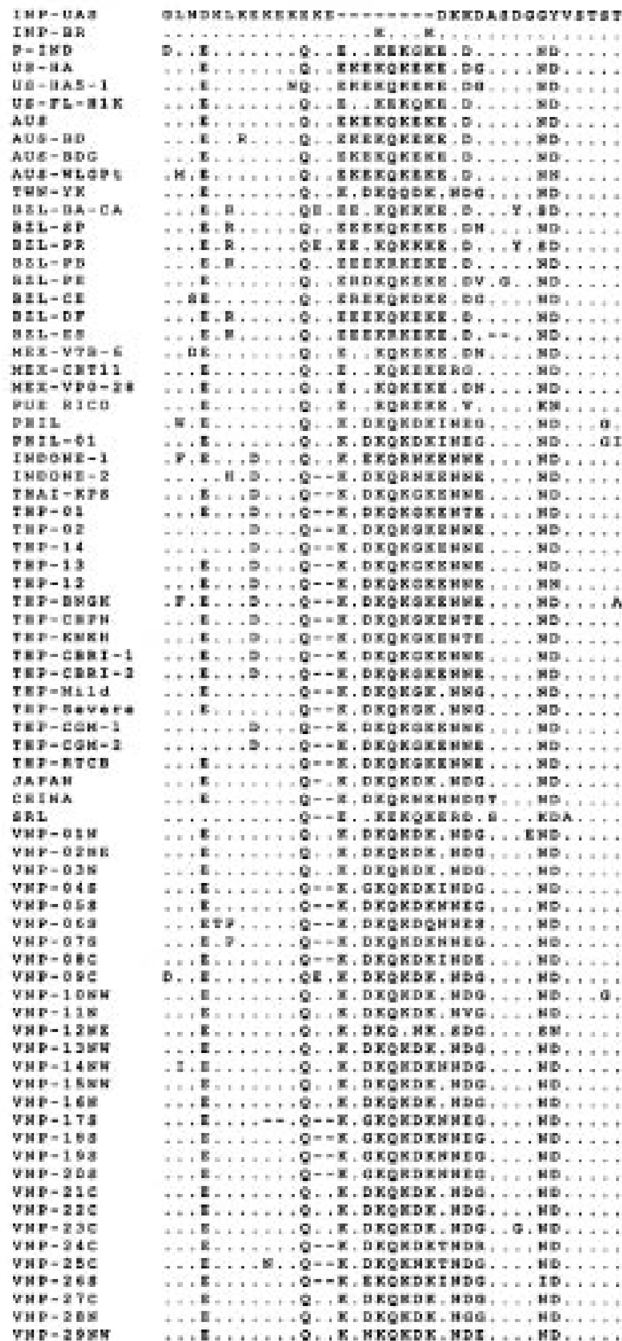

Fig. 2. Alignment of the N-terminal amino acid of the CP of PRSV-P isolates showing the variability in their sequences in comparison to INP-UAS CP. Sequences were aligned using the ClustalW algorithm from BioEdit program (version 5.0.6). The first 'G' residue in the alignment is from the 'DAG' aphid transmissibility motif. Amino acids that are the same as in INP-UAS are indicated with a '.'; deletions are indicated by '-'.

In the second lineage consisting of the South East Asian and West Pacific isolates, we observed that the PRSV-P isolates grouped mostly based on the geographical origin of the virus. There were three major sub-populations within this lineage, one consisting mainly of the Thailand isolates, and two sub-populations of Vietnam isolates. The other Asian isolates like Indonesia, Philippines, China, Japan and Taiwan clustered with any of the three sub-populations. Most of the Thai isolates clustered together showing very little sequence variation with respect to their N-terminal region. The two Indonesian isolates formed a separate cluster and seemed to diverge from the Thailand isolates.

Of the 29 Vietnam isolates compared in the present study, most showed very little divergence in the N-terminal region and clustered based on their geographical location. All the South Vietnam isolates grouped together with two strains from Philippines diverging from this group. The other sub-population showed a considerable amount of intermixing of the Central and Northern isolates. The Taiwan YK, China and Japanese isolates also clustered within this group. Given the geographical proximity between these South East Asian countries, there might well have been some local and long distance

Fig. 3. Phylogenetic tree derived from the highly variable N-terminal amino acid sequence of the CP of PRSV-P isolates from different geographical locations.

movement of the virus resulting in considerable amounts of variability in the PRSV-P population. However, it was surprising to observe that neither the Indian nor the Sri Lankan isolates showed any relatedness with the Asian isolates, despite being from the same Asian sub-continent.

Analysis of the complete and the conserved regions of CP. The CP of INP-UAS differed from those of known PRSV isolates, including that previously reported from India (P-IND), by 2-13%, both at the nucleotide and amino acid levels. The INP-UAS CP was 90-92% identical to those of American and Australian isolates, and about 87 to 92% identical to those of other Asian isolates. In terms of amino acid sequence, the INP-UAS strain showed 92 and 99% similarity with the Indian isolates, P-IND and INP-BR, respectively (data not shown). We observed that at the nucleotide level much of the variability in the CP gene was in the wobble base. This degeneracy of the third base indicated that although a higher variability is observed in the nucleotide sequence, it resulted in very few changes at the amino acid sequence thereby preserving the primary structure of the protein, which may therefore have an important functional and/or structural significance. The phylogenetic analysis revealed that the PRSV-P isolates from the Indian sub-continent are most diverse and this observation is consistent with the recent studies on molecular epidemiology of PRSV (Bateson *et al.*, 2002). The possibility of recombination between the PRSV-P populations, which could contribute significantly to sequence divergence (Garcia-Arenal *et al.*, 2001), cannot be ruled out, especially with respect to the South Indian isolates. Introduction from other areas, mutation and local and long distance movement are some of the other factors likely to contribute to the natural variation in PRSV-P populations.

Analysis of sequence divergence in the conserved region of CP, after the removal of the variable N-terminal "EK" motif, revealed an altogether different clustering pattern (Fig. 4). Except for the American and Australian isolates, which clustered into a major group, the clustering pattern varied considerably among the South East Asian isolates. The two South Indian isolates grouped together but did not cluster with any of the other isolates as observed earlier. However, the P-IND isolate from India diverged from the Vietnamese cluster, which also included the Thai isolates, suggesting that it could have originated from one of these regions. These variations in clustering pattern further emphasize the importance of the N-terminal region in determining the specificity of virus with respect to its geographical location. The significance of the N-terminal region with respect to the geographical distribution of the virus was very evident from the dendrograms derived for the isolates in the absence of the N-terminal variable region. The divergence in the amino termini of the CP can account for their differences in host specificity as reported for *Sugarcane mosaic virus* (Xiao *et al.*, 1993). It is therefore tempting to speculate that variation in the N-terminal region of the protein, which is on the outer surface of the CP (Shukla and Ward, 1989) and is close to the aphid transmission motif (Atreya *et al.*, 1990), can be correlated to the geographical distribution of PRSV.

Sequence variability has important implications for the use of CP-genes to develop transgenic plants by CPMR (Nakajima *et al.*, 1993; Taschner *et al.*, 1994; Savenkov and Valkonen, 2001). Transgenic papaya incorporating the CP gene of the PRSV mild strain (HA 5-1) of the isolate PRSV-HA, was found to impart protection specifically against infection by the severe US-isolate (HA) but not against infection with other PRSV-P isolates including Australian, Thai and Taiwan YK isolates (Chiang *et al.*, 2001). This is surprising since the Australian PRSV isolates share 96% and 98% nucleotide and amino acid sequence identity respectively in their coat proteins with the US isolates. This result suggests that sequence divergence may have a direct bearing on the management of PRSV either through cross protection or CPMR, as these forms of resistance have been

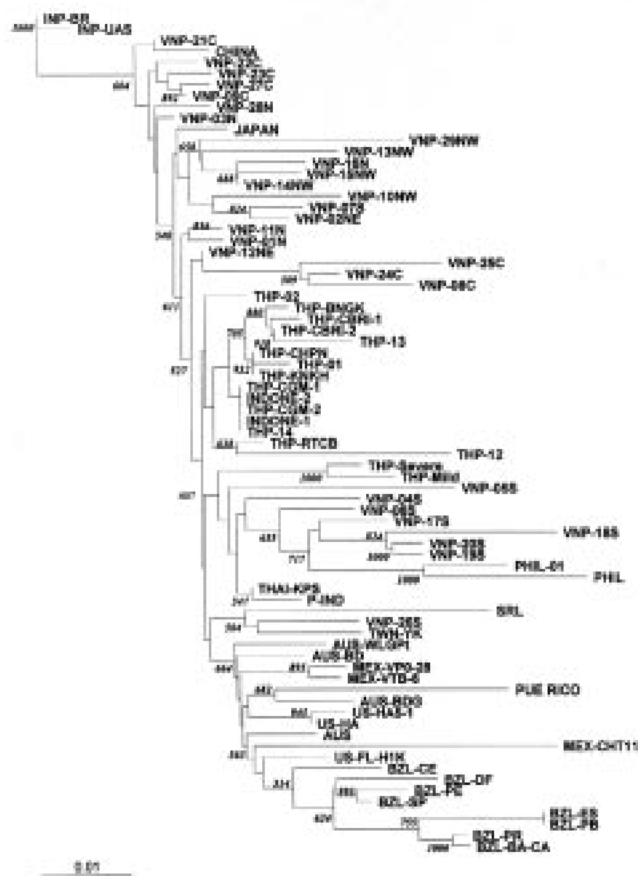

Fig. 4. Phylogenetic tree derived from the conserved region (minus the N-terminal region) of CP shows the variability in the relationship among the 75 different isolates of PRSV-P.

found to be highly sequence specific (Clark *et al*., 1995; Chiang *et al*., 2001; Tennant *et al*., 2001). Therefore the success of particular control methods may differ depending on their individual PRSV CP profiles. In India, the incidence of PRSV-P has been quite recent and coincides with the sudden increase in papaya cultivation and the introduction of new varieties from different geographical region exerting different levels of selection pressure on the virus. Availability of more sequences of PRSV-P CP from the Indian sub-continent will help a better assessment of the sequence divergence within the PRSV population. Taken together, the findings from our study emphasizes the need for developing transgenic plants with gene constructs from isolates specific to the geographical location in order to have greater compatibility and genetic relatedness between the CP-gene of the transgenic plants and the challenge virus. Currently, efforts are being made to study the divergence of PRSV CP-gene by collecting further strains from different locations in India, including the W-biotype, in order to get a better understanding of the origin of the virus, to generate constructs for engineering transgenic resistance, and to map the potential pathogenicity and host range determinants.

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