Molecular characterization and intermolecular interaction of coat protein of *Prunus necrotic ringspot virus*: implications for virus assembly

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Abstract Coat protein (CP) and RNA3 from *Prunus necrotic ringspot virus* (PNRSV-rose), the most prevalent virus infecting rose in India, were characterized and regions in the coat protein important for self-interaction, during dimer formation were identified. The sequence analysis of CP and partial RNA 3 revealed that the rose isolate of PNRSV in India belongs to PV-32 group of PNRSV isolates. Apart from the already established group specific features of PV-32 group member's additional group-specific and host specific features were also identified. Presence of methionine at position 90 in the amino acid sequence alignment of PNRSV CP gene (belonging to PV-32 group) was identified as the specific conserved feature for the rose isolates of PNRSV. As protein–protein interaction plays a vital role in the infection process, an

attempt was made to identify the portions of PNRSV CP responsible for self-interaction using yeast two-hybrid system. It was found (after analysis of the deletion clones) that the C-terminal region of PNRSV CP (amino acids 153–226) plays a vital role in this interaction during dimer formation. N-terminal of PNRSV CP is previously known to be involved in CP-RNA interactions, but our results also suggested that N-terminal of PNRSV CP represented by amino acids 1–77 also interacts with C-terminal (amino acids 153–226) in yeast two-hybrid system, suggesting its probable involvement in the CP–CP interaction.

Keywords Rose · *Prunus necrotic ringspot virus* (PNRSV) · PV-32 · Coat protein · Interaction

Dr A. A. Zaidi—Deceased

The sequences reported in the paper were submitted to the EMBL data base with following accession numbers: PNRSV CP (AJ619958), PNRSV MP (AJ697737), PNRSV RNA 3 (AJ969095).

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Introduction

Rose is an important ornamental plant having both aesthetic as well as industrial value. In India, rose is valued for various purposes viz. worship, rose oil, rose water and in making garlands. The most onerous task in maintaining rose is preventing them from becoming overwhelmed by the attack of insects, pests and diseases. Roses are attacked by several fungi (*Verticillium albo-atrum*, *V. dahliae*, *Chondrostereum purpureum* etc.), bacteria (*Agrobacterium tumefaciens*, *A. rhizogenes* etc.), viruses (*Apple mosaic virus* (ApMV), *Arabidopsis mosaic virus* (ArMV), *Prunus necrotic ringspot virus* (PNRSV), *Strawberry latent ringspot virus* (SLRSV) etc.) and nematodes (*Meloidogyne* spp., *Pratylenchus penetrans*, *P. thornei*, *P. vulnus*, *Xiphinema diversicaudatum* etc.) [21]. Plant viruses are very important among these plant pathogens because they are easily transmitted from infected mother plant to progenies of vegetatively propagated rose. Rose mosaic disease is the

main viral disease of rose that causes severe damage to the rose plants. ApMV, ArMV, PNRSV, SLRSV and *Tobacco streak virus* (TSV) are reported to be associated with rose mosaic disease either singly or mixed. Among these viruses PNRSV was earlier reported to be more prevalent in New York than any other virus in rose [41].

Previously, growers and nurserymen growing rose crop were least concerned about the problem of virus infection in roses. When leaf symptoms appear on a plant, the affected branch is pruned off, temporarily ridding the plant of its symptoms [22]. However, rose mosaic or viruses causing rose mosaic are shown to cause flower distortion, reduced flower production, reduced flower size, reduced stem caliper at graft union, reduced vigor, early autumn leaf drop, lower bush survival rates, increased susceptibility to cold injuries and is more difficult to establish after transplantation [9–11, 31, 36–38]. The symptoms of PNRSV are highly variable among rose cultivars and are strongly influenced by weather and growing conditions [33]. Infected plants may appear to be quite healthy for much of the year, and any symptom development may be attributed to other causes, such as spray burn, nutrient deficiencies, high temperature or poor horticultural practices.

A number of viruses on rose are studied well from several parts of the world. Virus survey of rose plantations during spring season at Institute of Himalayan Bioresource Technology (IHBT) Palampur, and rose plantations in Palampur (Himachal Pradesh) revealed that PNRSV is the most prevalent virus.

The present study was attempted to characterize the PNRSV at genomic level that cause rose mosaic disease in India. Studies were also conducted to find out the portion of PNRSV coat protein (CP) responsible for interaction at the time of virus assembly.

Materials and Methods

Extraction of viral nucleic acid

The PNRSV culture was isolated from rose cultivar arjun and the pure culture of PNRSV was maintained on and purified from *Cucumis sativus*. About 1 mg purified virus suspension was taken for RNA isolation. RNA was isolated from virus using phenol:chloroform method as described [13].

Primer designing and RT-PCR

Primer pairs were designed for the amplification of PNRSV CP [CPu: 5' **AACTGCAGATGGTTTGCCGAATTTGCA** A 3' (AJ619984); CPd: 5' **GCTCTAGACTAGATCTCAAG** CAGGTC 3' (AJ619983)] and PNRSV RNA3 [RNA3u: 5' GTTGGTTGAATAGTGTTCAGTA 3'; RNA3d: 5' CGGA

GAAATTCGAGTGTGCGAACT 3' (AJ969095)] by multiple sequence alignment of available sequences for various genes of PNRSV. Alignment was carried out by MULTALIN program [12] and by CLUSTALW [18] while primers were synthesized from Microsynth (Switzerland).

RT-PCR amplification of different genes of PNRSV was standardized using RNA from purified virus preparation. For reverse transcription (RT) reaction 1–2 µg of purified RNA was mixed with 10 µl of 5 × RT buffer (reverse transcription buffer), 2.5 µl of 40 mM dNTP mix, 50 ng of downstream (reverse) primer, 20 units of RNase inhibitor and 200 units of Mu-MLV RT (GE Healthcare, USA) to a final volume of 50 µl. The reaction mixture was incubated at 37 °C for 75 min followed by 5 min incubation at 70 °C.

PCR amplification was carried out using Advantage HF-2 PCR System (BD Biosciences, USA) in 50 µl reaction containing 8 µl cDNA product, 5 µl 10× HF2 PCR buffer, 50 ng each of primers (up and down) and 5 µl of 10× dNTP mix. Respective primer pairs PNRSV CPu (AJ619984) and PNRSV CPd (AJ619983) for PNRSV CP, and PNRSV RNA3u (AJ969095) and PNRSV RNA3d (AJ969095) for PNRSV RNA 3 were utilized for PCR amplification carrying out annealing at 51 and 55 °C for CP and RNA 3, respectively for 35 cycles. Denaturation and extension were performed at 94 and 72 °C, respectively. Amplified product was electrophoresed in 1 % (w/v) TAE agarose gel and stained with ethidium bromide (0.5 µg/ml) [27]. The DNA was visualized and photographed using gel documentation system (Alpha innotech, USA). A DNA ladder was used as size standard (GE Healthcare, USA).

The amplified fragments of PNRSV CP and RNA 3 were cloned into pGEM-T easy vector system (Promega, USA). Three recombinant clones each of CP and RNA 3 were sequenced using Sanger's dideoxy chain termination method [30] in an automated sequencer (ABI Prism 310, Applied Biosystems, USA) using Big Dye Terminator Sequencing v3 sequencing kit (Applied Biosystems, USA).

The sequence was aligned with corresponding sequences of other established PNRSV sequences from the database using BLAST [2]. The program BLASTP was used to search the amino acid sequence database. Pairwise comparisons were performed by the ALIGN-2 program utilizing the DOTHELEX algorithm [35]. Multiple alignments were generated by the MULTALIN program [12]. Phylogenetic tree was constructed with the help of CLUSTALW [18] and tree was subjected to bootstrap (using 1,000 replicates) and viewed with the help of TreeView [23].

Preparation of deletion constructs of PNRSV CP

To study and identify the interacting domains of PNRSV CP, the CP gene was divided into three portions viz. N-terminal, Mid-portion and C-terminal. Primers were

designed, based on exact sequence of PNRSV CP (AJ619958) containing the restriction enzyme sites *Sma* I and *Bam* HI for the amplification of 681 bp complete CP, 228 bp N-terminal, 456 bp N+Mid-portion, 228 bp Mid-portion, 453 bp Mid+C-terminal and 225 bp C-terminal of the CP. Sequences of the different primers and various primer combinations used for the amplification of different portions of PNRSV CP have been shown in Table 1.

PNRSV CP fragments (681 bp complete, 228 bp N-terminal, 456 bp N+Mid-portion, 228 bp Mid-portion, 453 bp Mid+C-terminal and 225 bp C-terminal) were cloned into pGAD-T7 and pGBK-T7 at *Sma* I and *Bam* HI sites, so that all the fragments are present as fusion with activation and binding domains respectively. After transformation recombinant clones were sequenced for any errors that might have been introduced by Taq DNA Polymerase. Plasmid DNA was prepared from recombinant clone and used for yeast transformation.

Yeast transformation and assay

All the recombinant pGAD-T7 and pGBK-T7 plasmids were purified from 5 ml of overnight grown culture and quantified spectrophotometrically [27]. 0.1 µg Plasmid was used for yeast transformation by LiCl/PEG method as described (BD Biosciences, USA).

X-gal overlay assay, histidine dropout assay (in vivo assay) and co-immunoprecipitation (CO-IP) (in vitro assay) was performed in order to find out and confirm interactions. The protocols used for different assays were performed as described in yeast two-hybrid manual (BD Biosciences, USA).

Results

The viral nucleic acid isolated from purified virus preparation was used for the RT-PCR reaction using the viral gene specific primers. Agarose gel electrophoresis of the amplicons revealed amplification of ~680 and ~1,700 bp products corresponding to complete CP and partial RNA 3

of PNRSV, respectively. There was no amplification in healthy *C. sativus* sample used with the above RT-PCR reactions as negative control.

Three clones each were sequenced for consensus sequences and it was seen that CP gene was 681 bp in length encoding for 226 amino acids. Similarly, RNA 3 (partial sequence) revealed that it was 1,689 bp and contained two open reading frames (ORFs) of 852 (ORF 1) and 681 (ORF 2) nt separated by an intergenic region of 74 nt and 5' and 3' untranslated region (UTR) of 22 and 60 nt, respectively.

Sequence analysis of PNRSV coat protein and RNA 3

Coat protein

Multiple sequence alignment of Indian isolate of PNRSV CP with 45 other isolates of PNRSV reported from different parts of the world (Table S1) revealed that CP of the Indian isolate of PNRSV (AJ619958) contains a direct repeat of two amino acids (NRNR) after position 39, a typical characteristic feature of PV 32 group (I) of PNRSV [17].

Sequence alignment showed that the C-terminal of PNRSV CP is mostly conserved while N-terminal is most variable among all the isolates showing differences at various positions. A few changes in nucleotides that lead to change in amino acid, position 90, Indian (rose) isolate of PNRSV (AJ619958) contains methionine (M) which is in consensus with the other two rose isolates of PNRSV (DQ003584 and AY684271), while rest of the isolates contain threonine (T). It can be concluded that the presence of methionine at position 90 is a specific feature of PNRSV-rose isolates (Fig. 1).

The Indian isolate showed highest homology of 99 and 98 % at nucleotide (nt) level and 99 % homology at amino acid (aa) level with rose isolates reported from Poland (DQ003584) and China (AY684271), respectively and least homology of 88 % at nt level and 90 % at aa level with Prunus isolate of PNRSV reported from USA (L38823).

Phylogenetic tree for PNRSV CP sequences also confirmed that Indian isolate of PNRSV (rose) belongs to group I (PV 32)

Table 1 Table showing sequence of primers and primer combinations used for the amplification of various portions of PNRSV CP for CP–CP interaction studies using yeast two hybrid system

Name	Primer sequence	Region of PNRSV CP	Primer combination
P1	5' <u>TCCCCCGGG</u> GATGGTTTGCCGAATTTG 3'	Complete CP	P1 & P2
P2	5' <u>CGGGATCCC</u> TAGATCTCAAGCAGGTCC 3'	N-terminal	P1 & P3
P3	5' <u>CGGGATCCC</u> TGCGGGAGGCACATTCGGA 3'	Mid-terminal	P4 & P5
P4	5' <u>TCCCCCGGG</u> GATTCCTAAGGGTTATGT 3'	N+Mid-terminal	P1 & P5
P5	5' <u>CGGGATCCC</u> TTCTAGACAGAGCATTC 3'	C-terminal	P6 & P2
P6	5' <u>TCCCCCGGG</u> GGGGTTCTTGAAGGACCA 3'	Mid+C-terminal	P4 & P2

Bold and underlined sequence in the primers is the restriction enzymes sites

Fig. 1 Multiple sequence alignment of CP gene of Indian isolate of PNRSV (accession no. AJ619958) with 45 different PNRSV isolates reported from different hosts and causing different symptoms (Table S1) using Multalin V. 1.2.3. *Blank space showing dots* denoted the similar amino acids

of PNRSV isolates (Fig. 2). ApMV CP gene sequence (accession no. NC 003480) was taken as an out group. Recombination analysis using the PNRSV CP sequences did not reveal any possible recombination event in CP gene of Indian isolate or other isolates used in the present study.

RNA 3

PNRSV RNA 3 encodes for 283 amino acids MP and 226 amino acids CP. Coat protein is translated via subgenomic RNA 4, while MP is translated by RNA 3. The amino acid sequence of Indian isolate of PNRSV MP (accession no. AJ697737) was aligned with 14 other PNRSV MP sequences reported on different hosts.

Multiple sequence alignment revealed its high conservation in N-terminal except few changes, while the C-terminal was variable. The MP showed similarity to the level of

97–86 % at nucleotide and 98–92 % at amino acid level with MP from other isolates (data not shown) which is in accordance to previous reports [16]. The MP of Indian isolate was found to be highly similar (98 %) to PNRSV-apple isolate (accession no. Y07568). Most of the nucleotide changes are conservative and do not result in amino acid changes when translated. Some insertions and deletions in the nucleotide sequence reflected in differences in amino acid composition of the encoded protein.

Phylogenetic tree based on MP sequences also confirmed the placement of the present isolate in group I (PV 32) as it forms a cluster with the group members (ApMV MP was taken as an out group) (data not shown). No recombination events were identified in the gene.

The nucleotide sequence of RNA 3 containing CP, MP, UTR's and intergenic region (accession no. AJ969095) was also aligned with RNA 3 sequences of seven other PNRSV

Fig. 2 Phylogenetic relationship of Indian isolate of PNRSV infecting rose on the basis of coat protein (CP) gene sequence (shown by arrow). ApMV CP gene sequence was used as outgroup (accession no. NC_003480). Group designations on the right reflect major grouping identified in the analysis. Bootstrap values (% replication) are shown on the major branches. Host and isolate names are mentioned against the accession numbers (in brackets). NA Not available

isolates by multiple sequence alignment. Results showed high similarity with the corresponding RNA 3 sequences from other isolates (data not shown). Apart from the coding region present in RNA 3 the non coding region was also identified to be considerably conserved. No recombination events were identified in the gene.

PNRSV coat protein self interaction for dimer formation

Six regions of PNRSV CP were cloned so as to express fusion proteins with activation and binding domain of Gal 4. Thirty-four different combinations of various domains of the CP gene were transformed (Table S2) into yeast strain Y187 (assayed by β -galactosidase) and AH109 (assayed for β -galactosidase and histidine requirement) separately and on an average of 100 colonies per plate were obtained on minimal medium plates lacking Leucine and Tryptophan. Co-transformed colonies obtained were then used for x-gal overlay assay for β -galactosidase activity and histidine requirement.

Results of both assays showed that complete CP interacts with itself and showed very strong interaction in x-gal overlay assay (Table S2 and Fig. 3). Seventy-three amino acids (aa) of PNRSV C-terminal showed strong interactions with itself, with 76 aa of N-terminal, weak reaction with 76 aa of middle portion, 149 aa of middle+C-terminal and a very weak interaction with 226 aa of complete CP and 152 aa of N+Mid-portion (Fig. 3).

Discussion

ORF 1 of PNRSV RNA 3 encodes for 283 amino acids protein with a predicted relative molecular mass of 34 kDa that by analogy to other members of *Bromoviridae*, must correspond to the movement protein (MP) and ORF 2 encodes for 226 amino acids CP (synthesized via subgenomic RNA 4). The translation product of subgenomic RNA has previously been shown to immunoprecipitate with an antiserum against the CP in case of ApMV [24]. The sequence of this cistron and the 5' and 3' untranslated regions of RNA 4 have been characterized previously [29]. The 5' and 3' UTRs of the sequenced portion of RNA 3 were 22 and 61 nt long, respectively and the intergenic region was 74 nt long.

Presence of two amino acid insertions (NRNR) at position 39 in PNRSV CP has also been reported previously and was considered as characteristic feature of PV 32 group (Group I) of PNRSV isolates [3, 16, 17, 26, 34, 40]. The significance of this insertion to biological activity is not known, but it does serve as a group specific feature of the group I isolates that can be used to discriminate between isolates in group I and III. The nomenclature of these groups refers to that adopted in several previous reports of PNRSV sequence comparisons (PV 32-I, PV 96-II, PE 5-III) [3, 40]. PNRSV in the present study is correlated with severe symptoms on rose like oak leaf pattern and mosaic. It was demonstrated previously that the known PNRSV pathotypes fall into three overall groups.

Fig. 3 X-Gal overlay assay results of PNRSV CP interactions in various combinations. Number on the plate represents various combinations as shown in Table S2. Full length CP strongly interacts with itself in 1 [complete CP (amino acids 1–226): complete CP (amino acids 1–226)]. Positive interaction are also seen in case of a number of combinations namely 26 [Mid+C terminal (amino acids 77–226):N terminal (amino acids 1–77)], 30 [Mid+C terminal (amino acids 77–226):C terminal (amino acids 153–226)], 31 [C terminal (amino acids 153–226):complete CP (amino acids 1–226)], 32 [C terminal (amino acids 153–226):N terminal (amino acids 1–77)], 33 [C terminal (amino acids 153–226):N+Mid portion (amino acids 1–152)], 34 [C terminal (amino acids 153–226):Mid portion (amino acids 77–152)], 35 [C terminal (amino acids 153–226):Mid+C terminal (amino acids 77–226)], 36 [C terminal (amino acids 153–226):C terminal (amino acids 153–226)]. Interaction of C-terminal with itself (36) and Mid+C terminal (35) is quite evident from the overlay assay. Controls in the figures are shown as 37 [autoinduction control], 38 [positive control], 39 and 40 [negative controls]

Group I (PV 32) is composed predominantly of isolates reported to exhibit severe symptoms, while most of the group II (PV 96) isolates exhibit mild symptoms [17]. Group III (PE 5) isolates exhibit both mild and severe symptoms [17]. The presence of methionine (M) at position 90 of PNRSV CP sequence was recognized here as a specific feature of rose isolate of PNRSV.

All group I isolates of PNRSV belongs to the rugose pathotype, similar substitutions as found in MP of Indian isolate of PNRSV were also recorded previously [16, 40]. Group II isolates have been previously characterized as mild pathotypes [16, 40]. Group III isolates were closest to an ApMV isolate (NC_003480), which was used as an outgroup in the phylogenetic analysis [40]. It has been shown that deletion of three amino acids in the C-terminus rendered this protein nonfunctional for cell-to-cell movement [39]. Difference in C-terminus sequence leads to change in the physiochemical properties of the protein and high surface probabilities [39].

Results of CP–CP interaction study showed that C-terminal plays a vital role in CP assembly in case of PNRSV, Aparicio et al. used bimolecular fluorescence complementation to study PNRSV CP dimerization. They also

found that C-terminal is required for PNRSV CP dimerization and domain located between 9 and 27 amino acids plays a critical role in dimerization [4]. During our study we also observed that N-terminal is also showing interaction with C-terminal. It indicates that N-terminal is also somehow involved in CP–CP interaction but to a minor level as compared to C-terminal, as N-terminal of PNRSV is also required for RNA binding. It may be hypothesized that N-terminal has a role during later steps in capsid assembly while for dimer formation C-terminal is the most important.

Similarly, in addition to the use of CP in virus assembly, ilarviruses and *Alfalfa mosaic virus* (AMV) shares the phenomenon of “genome activation”, i.e. they require the presence of CP to initiate infection [7, 20]. In this process, the binding of CP to specific sites near the 3′ terminus is required [43]. By electrophoretic mobility band shift experiments it has been shown that the 3′ untranslated region of AMV RNA 3 and or 4 forms two [25] or four [19] distinct complexes with the viral CP. Ribonuclease protection assays [42] and site-directed mutagenesis [19, 25] have revealed that stable hairpin structures flanked by single stranded (A/U) UGC sequences are important for CP binding. The presence of the first 25 predominantly basic amino acids of the CP is required for genome activation [7, 8]. It has also been shown that N-terminus of the CP is necessary [6, 39, 42] for genome activation of PNRSV [6]. Results obtained by Sehnke et al. [32] led them to suggest that a zinc-finger motif found in TSV CP and a similar but not identical sequence of AMV could play a role in the genome activation process. Putative zinc-finger domains have also been found in case of ApMV and PNRSV [1, 14, 15, 28]. In case of PNRSV CP 26 amino acid region located in between amino acids 25–50 from the N-terminal end represented as RNA binding domain and thus involved in genome activation [5]. Since it has been already proved that N-terminus of PNRSV CP is required for RNA binding, Present finding also showed that N-terminus might also be involved in protein–protein interaction during capsid formation whereas C-terminus is the most important for dimer formation.

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