Nucleotide Sequence Comparison of the Capsid Protein Gene of Severe and Protective Mild Strains of *Papaya ringspot virus**

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ABSTRACT

This study compared three mild and three severe strains of *Papaya ringspot virus* - type W (PRSV-W), based on nucleotide and amino acid sequences of the capsid protein (*CP*) gene. The *CP* nucleotide sequences of the mild strains shared 98% to 100% identity. When compared to the severe strains the identity ranged from 93% to 95%, except in the case of PRSV-W-2R, which resulted from reversion of the mild strains PRSV-W-2. The CP sequence of the reverting strain showed 100% identity with the sequence of its parental strain. An insertion of six nucleotides in the core region of the *CP* gene, which reflected the addition of two amino acids (Asn and Asp) in the deduced sequence of the protein, was found in all mild strains. These sequence comparisons were used to design strain-specific primers that were used to specifically amplify regions for either the mild or severe strains.

Additional keywords: cross protection, cucurbits, molecular virology.

RESUMO

Comparação da seqüência de nucleotídeos do gene da proteína capsidial de estirpes severas e premunizantes do *Papaya ringspot virus*

Nesse estudo foram comparadas três estirpes premunizantes e duas estirpes severas do *Papaya ringspot virus* – type W, com base na seqüência de nucleotídeos do gene da proteína capsidial. As seqüências das proteínas do capsídeo das estirpes premunizantes compartilharam identidades de 98-100%. Quando comparadas às estirpes severas, a identidade variou de 93% a 95%. PRSV-W-2R, uma estirpe severa, resultante da reversão da estirpe PRSV-W-2, mostrou 100% de identidade com a seqüência da proteína do capsídio da estirpe parental PRSV-W-2. Uma inserção de seis nucleotídeos na região conservada da proteína do capsídeo, que resultou na adição de dois aminoácidos (Asn e Asp) na seqüência deduzida da proteína foi encontrada em todas as estirpes premunizantes. A comparação destas seqüências foi utilizada para desenhar oligonucleotídeos iniciadores próprios da espécie que foram usados para amplificar essas regiões nas estirpes severas e premunizantes

Papaya ringspot virus (PRSV) is a species of the genus *Potyvirus*, family *Potyviridae*, with flexuous particles of 780 nm x 12 nm and a genome consisting of a single-stranded positive sense RNA of ~10,000 nucleotides. The nucleic acid is encapsidated by identical capsid protein (*CP*) sub-units of ~36 kDa. The PRSV isolates belong to one of two major strains, papaya (P) or watermelon (W) (Purcifull *et al*., 1984). The W strains cause systemic infection only in cucurbit species. In Brazil, PRSV-W is predominant in several states and is frequently associated with severe yield losses in different species of Cucurbitaceae (Yuki *et al*., 2000). Due to the lack of resistant cultivars of zucchini squash (*Cucurbita pepo* L.) and the low efficiency of chemical control of vectors and cultural practices for the control of the disease, studies have been carried out since 1994 to establish cross protection as an alternative disease control measure (Rezende & Pacheco, 1998). Two mild strains of the virus, PRSV-W-1 and PRSV-W-2, were selected from blisters formed on mosaic leaves of zucchini squash infected with a severe strain isolate from the county of Campinas, São Paulo (PRSV-W-C) (Rezende *et al*., 1994). A third mild strain (PRSV-W-3) was later selected from blisters on mosaic leaves of watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] plants infected with a severe strain from the county of Tupã, SP. In this study, the *CP* genes of the three mild strains and the severe strain PRSV-W-C were sequenced and compared, in order to determine if the nucleotide sequence would permit molecular differentiation of these strains, for further application in monitoring the protective strains and for studying on the mechanism(s) of cross protection. Also included in this study a severe strain collected from squash in the northeast state of Pernambuco, Brazil (PRSV-W-PE), and a reverting strain of PRSV-W-2, which induced symptoms as severe as PRSV-W-C after 17 mechanical passages to zucchini squash plants (PRSV-W-2R) (Rezende & Pacheco, 1997).

The mild and severe strains of PRSV-W were separately propagated in zucchini squash cv. Caserta under greenhouse

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conditions. The PRSV-W-2R, which was preserved for four years in dehydrated tissue at –20 °C, was also inoculated in Caserta. All strains were individually purified according to the procedure described by Shepherd & Pound (1960). Viral RNA was extracted as described by Berger & Shiel (1998), and used as a template to amplify the *CP* gene by reverse transcription and polymerase chain reaction (RT-PCR). The first strand cDNA was synthesized using Superscript II RNase H Reverse Transcriptase (Life Technologies) and a complementary 3'-end reverse primer to the *CP* gene of PRSV-P (5'-AGCTAAC CATGGGCGAGTATTCAGTTGCGC-3') (Souza Jr., 1999). The cDNA was then amplified by PCR using Platinum® *Taq* DNA Polymerase High Fidelity (Life Technology), in the presence of the 3'-end primer described above, and the 5'-end primer of the *CP* gene of PRSV-P (5'-ATCATTCCATGGGCGTGTTCCA TGAATCAA-3') (Souza Jr., 1999). The amplified PCR products were ligated into the pGem® T Vector (Promega) and cloned as described by Sambrook *et al*. (1989). The sequence of each cDNA clone was determined using the Big Dye™ Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems) or Dyenamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) in an ABI Prism 377 automatic sequencer. Homologous nucleotide sequence searches were conducted using the BLAST program (http://www.ncbi.nlm.nih.gov). Sequence alignments were performed on Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Deduced amino acid sequence was conducted using the Translate program (http://ca.expasy.org/tools/dna.html).

Eighteen clones, sequenced on both strands, were used to obtain the consensus nucleotide sequences for each of the following strains: PRSV-W-1, PRSV-W-2, PRSV-W-C and PRSV-W-PE. The consensus nucleotide sequences for strains PRSV-W-3 and PRSV-W-2R were obtained from four clones. The region covered by the amplified cDNA from all strains comprised a single open reading frame (ORF), which aligned between nucleotides 9197 and 10120 of the cDNA-derived CP sequence of PRSV, papaya strain (accession No. X67673, Yeh *et al*., 1992) (data not shown).

All studied strains revealed the presence of the DAG triplet (Atreya *et al*., 1992) in the N-terminus of the CP, which has been associated with aphid transmissibility of potyviruses (Figure 1B). Recent studies showed that mild strains PRSV-W-1 and PRSV-W-2 as well as severe strains PRSV-W-C and PRSV-W-PE were efficiently transmitted by *Myzus persicae* Sulz. and *Aphis gossypii* Glover (Giampan & Rezende, 2001). The mild strain PRSV-HA 5-1, obtained by Yeh & Gonsalves (1984) by nitrous acid treatment of the severe strain PRSV-HA, also presented the DAG triplet in the *CP*, but was not efficiently transmitted by aphids (Wang & Yeh, 1992). Aphid transmission of potyviruses is also associated with the helper component-protease (HC-Pro), which suggests that the reduction in the transmission of the mild strain PRSV-HA 5-1 might be associated with any change in this protein. The WCIEN motif present in the core region of the *CP* of potyviruses was also present in all mild and severe strains of PRSV-W analyzed (Figure 1B).

Comparison of the nucleotide sequences of the mild strains PRSV-W-1 and PRSV-W-2 indicated 100% identity. These two strains shared 98% identity with mild strain PRSV-W-3 (Table 1). This small difference might be related to the origin of these mild strains. The PRSV-W-1 and the PRSV-W-2 were obtained from blisters from different zucchini squash plants infected with severe strain PRSV-W-C (Rezende *et al*., 1994), whereas PRSV-W-3 was isolated from blisters on mosaic leaves of watermelon infected with a severe strain from Tupã, SP (unpublished data). When the mild strains PRSV-W-1 and PRSV-W-2 were compared with the severe parental strain PRSV-W-C, their identity was 95%. Wang & Yeh (1992) compared the nucleotide sequence of the *CP* gene of mild strain PRSV HA 5-1 with the severe parental strain PRSV HA and showed that they shared 99.8% identity, differing in only two nucleotide residues, which resulted in two amino acids changes. The

	9397 9404	20 30 60 70 160 170
PRSV (X67673)	.....ACTAGCCACAA---------- AACT.....	.....QSKNEAVDAGL.....NDVSTST-----KT.....VWCIENGTSPD.....
PRSV-W-C	.....ACTAGCCACAA---------- AACT.....	.....QSKNEAVDAGL.....NDVSTST-----KT.....VWCIENGTSPD.....
PRSV-W-PE	.....ACTAGCCACAA---------- AACT.....	.....QSKNEAMDAGL.....NDVSTST-----KT.....VWCIENGTSPD.....
PRSV-W-1	.....ACTAGCCAAAA**TGACAA**AACT.....	.....QSKNEAVDAGL.....NDVSTSK**ND**KT.....VWCIENGTSPD.....
PRSV-W-2	.....ACTAGCCAAAA**TGACAA**AACT.....	.....QSKNEAVDAGL.....NDVSTSK**ND**KT.....VWCIENGTSPD.....
PRSV-W-2R	.....ACTAGCCAAAA**TGACAA**AACT.....	.....QSKNEAVDAGL.....NDVSTSK**ND**KT.....VWCIENGTSPD.....
PRSV-W-3	.....ACTAGCCAAAA**TGACAA**AACT.....	.....QSKNEAVDAGL.....NDVSTSK**ND**KT.....VWCIENGTSPD.....
	A	**B**

FIG. 1 - (A) Alignment of part of the capsid protein gene of mild and severe strains of PRSV-W, comparing nucleotide positions 9398 to 9403 on the cDNA. Inserts are in bold letters and gaps are indicated by dashes (-). (B) Alignment of parts of the deduced amino acid sequence of the capsid protein of the same strains, showing the insertion of Asn (N) and Asp (D) at positions 67 and 68, respectively (inserts are in bold letters). DAG and WCIEN motifs are also shown. Accession numbers: PRSV-W-1, AY094987; PRSV-W-2, AF30088; PRSV-W-3, AY094986, PRSV-W-C, AY094985; PRSV-W-PE, AY094984; and PRSV-W-2R, AF530089.

TABLE 1 - Percentage sequence identities between the nucleotide (above the diagonal) and deduced amino acid (below the diagonal) sequences of the capsid protein of the mild strains of *Papaya ringspot virus* (PRSV), PRSV-W-1, PRSV-W-2, PRSV-W-3, reverting mild strain PRSV-W-2R and severe strains PRSV-W-C and PRSV-W-PE

Strain*	PRSV-W-1	PRSV-W-2	PRSV-W-3	PRSV-W-C	PRSV-W-PE	PRSV-W-2R
PRSV-W-1	-	100	98	96	95	100
PRSV-W-2	100	-	98	96	95	100
PRSV-W-3	99	99	-	94	96	98
PRSV-W-C	95	95	94	-	96	96
PRSV-W-PE	96	96	95	97	-	96
PRSV-W-2R	100	100	99	95	96	-

* Accession numbers: PRSV-W-1, AY094987; PRSV-W-2, AF30088; PRSV-W-3, AY094986, PRSV-W-C, AY094985; PRSV-W-PE, AY094984; and PRSV-W-2R, AF530089.

greater identity between PRSV HA 5-1 and PRSV HA might be related to the method used to obtain the mild strain. The nitrous acid treatment did not affect the *CP* gene, but might have affected one or more other genes responsible for symptom expression. A high level of identity, varying from 92 – 98%, was also found when all five strains from the present study were aligned with PRSV-W (accession numbers S89893, AF344649 and AF344648) and PRSV-P (accession numbers D00595, S46722, U14744, AF319506, AF344650 and AF 344642) isolates from different geographic origins (data not shown).

The major difference between the mild and severe strains of PRSV-W was an insertion of six nucleotide residues in the core region of the *CP* gene of the mild strains at position 9398 to 9403 (Figure 1A). This insertion was absent in the *CP* gene of all other P and W strains from the different geographic origins mentioned above, including mild strain PRSV HA 5-1 (data not shown).

The six nucleotides resulted in the addition of two amino acids (Asn and Asp) at positions 67 and 68 of the CP of the mild strains of PRSV-W (Figure 1B). This increase in the amino acid number did not permit any differentiation between the mild strains, PRSV-W-1 and PRSV-W-2, and the severe strains, PRSV-W-C and PRSV-W-PE, based on the electrophoretic mobility of the protein in SDS-PAGE and labeling with polyclonal antiserum against PRSV-W in the western blot assay. The *CP* from these strains co-migrated in the gel and showed a typical molecular weight of approximately 35 kDa (data not shown). The addition of six amino acids can be distinguished for another potyvirus protein, P1, as shown in a comparison of a mild and severe strain of *Zucchini yellow mosaic virus* (ZYMV) (Wisler *et al*., 1995).

Whether the insertion of 6 nucleotides in the core region of the *CP* gene of the mild PRSV-W strains is related to the attenuation of the symptoms induced in zucchini squash remains to be investigated. However, as the nucleotide sequence of the reverting strain PRSV-W-2R showed 100% identity with the *CP* of the parental mild strain PRSV-W-2, it is suggested that this insertion does not seem responsible for symptom attenuation. For *Tobacco vein mottling virus* (TVMV) genus *Potyviridae*, family *Potyvirus* and ZYMV, Atreya *et al*. (1992) and Gal-On (2000), respectively, reported that the HC-Pro gene of these viruses was associated with symptom attenuation, although the difference between mild and severe strains was not located at the same position of the gene in each virus. Wisler *et al*. (1995) reported that the P1 gene of ZYMV-MD contained 18 additional nucleotides (six amino acids) compared to the severe strain ZYMV-SV, but they did not carry out further experiments to determine whether this difference was related to symptom attenuation. The comparison made in the present work only reflects about one tenth of the total genome of PRSV-W; presumably, there are other differences in the remaining part of the genome of mild and severe strains of this virus. This comparison can only be done after sequencing the complete genome of these strains.

Based on the well-defined nucleotide difference between mild and severe strains of PRSV-W, two 5'-end primers were designed: one expected to anneal to cDNA from mild strains (PM; 5'-GACTAGCAAAAATGA-3') and the other expected to be specific to severe strains (PS; 5'-AACTAGCAC AAAAAC-3'). Viral RNA from mild and severe strains were extracted from infected plant tissue using Trizol® LS (Life Technologies), according to the manufacturer, and used as a template to amplify part of the *CP* gene by RT-PCR. The first strand cDNA was synthesized as before, using the reverse primer complementary to the 3'-end of the *CP* gene of PRSV-P. The cDNA was then amplified by PCR using the 3'-end primer and either the 5'-end primer PM or PS. The PCR conditions were as follows: first cycle of 30 sec at 94 °C, 30 sec at 60 °C and 1 min at 72 °C; second cycle of 30 sec at 94 °C, 30 sec at 57 °C and 1 min at 72 °C; and 29 additional cycles of 30 sec at 94 °C, 30 sec at 53 °C and 1 min at 72 °C. The amplified PCR products were analyzed on 0.8% agarose gel in the presence of ethidium bromide and photographed under UV illumination. The RT-PCR was repeated three times with different samples and the result of one test is shown in Figure 2. The *CP* gene of the mild strains PRSV-W-1, PRSV-W-2 and the reverting strain PRSV-W-2R were only amplified when the PM forward primer was used, whereas the PS primer was specific for amplification of severe strains PRSV-W-C and PRSV-W-PE. These primers will be useful for future studies on the mechanism of cross protection between strains of PRSV-W in cucurbit species.

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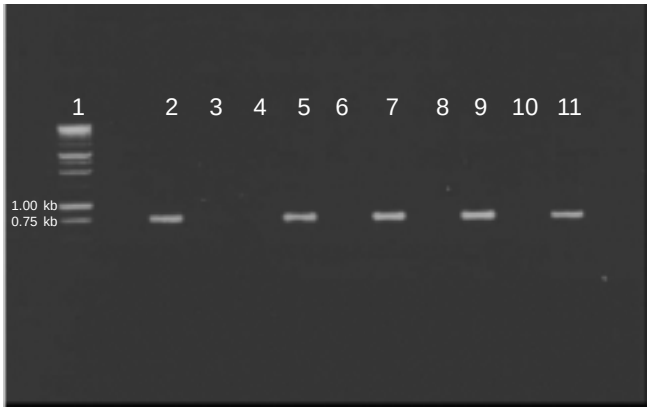

FIG. 2 - RT-PCR analysis of zucchini squash plants infected with severe strains PRSV-W-C (lanes 2 and 3) and PRSV-W-PE (lanes 4 and 5), mild strains PRSV-W-1 (lanes 6 and 7) and PRSV-W-2 (lanes 8 and 9) and reverting strain PRSV-W-2R (lanes 10 and 11). Samples on lanes 2, 5, 6, 8 and 10 were amplified with 5'-primer PS (severe strains), whereas samples on lanes 3, 4, 7, 9 and 11 were amplified with 5'-primer PM (mild strains).

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