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Molecular and biological studies of *Papaya ringspot virus* isolates from Brazil and Cuba

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Abstract: The coat protein genes of 21 *Papaya ringspot virus* (PRSV) isolates from Brazil and 7 isolates from Cuba were sequenced and analyzed, and the symptoms induced by the virus isolates were evaluated in papaya (*Carica papaya* L. cv. Solo) and squash (*Cucurbita pepo* L. cv. Caserta). The virus isolates were grouped in phylogenetic trees by geographic region, presenting the highest nucleotide and amino acid identities with those isolates collected in the same regions. The American and Indian isolates, from GenBank were grouped together in the tree, and the Brazilian isolates were grouped by state; the Cuban isolates from the eastern region were closer to the American isolates than to those from the central-west region. Typical mosaic symptoms, grouped according to intensity, were observed in the inoculated papaya and squash seedlings. The variability of the coat protein genes confirmed that virus control using cross-protection and transgenic plants requires the selection of region-specific virus isolates in each country.

Keywords: PRSV-P, Coat protein, Phylogeny, Papaya, Squash, Symptom intensity.

1. Introduction

Ringspot disease caused by *Papaya ringspot virus* (PRSV) is considered the major disease of papaya (*Carica papaya* L.) worldwide [1, 2]. This virus is transmitted naturally to plants of the *Cucurbitaceae* and *Caricaceae* families by many species of aphids [3, 4]. This fact, in addition to the absence of resistant varieties of papaya, makes the control of this virus and its management extremely difficult in all regions where papaya is grown.

PRSV belongs to the family *Potyviridae*, genus *Potyvirus*, and has elongated, flexuous particles measuring 760-800 x 12 nm, with positive-sense ssRNA [1]. The viral genome contains approximately 10,326 nucleotides, with an ORF encoding a polyprotein of 350 kDa, starting at positions 86/88 and ending at positions 10,118/10,120; a poly-A tail is present at the 3' terminus [5, 6]. After synthesis, the polyprotein is proteolytically cleaved into nine final products: 5'-P1 (63 K), HC-Pro (52 K), P3 (46 K), CI (72 K), K 6, VPG (21 K), NIaPro (27 K), NIb (59 K), and CP (35 K) [4, 6, 7]. PRSV isolates have been divided into two biotypes, PRSV-P and PRSV-W [1]; both are able to naturally infect plants belonging to the *Cucurbitaceae* family, though only PRSV-P can infect the *Caricaceae* family ([1, 8].

Many strategies have been adopted as control measures of PRSV; however, the results have not been satisfactory, or have been limited to local viral isolates [8]. Currently, research is focused on the demand for transgenic plants that express a PRSV gene, typically the gene encoding the coat protein (CP), and also on the selection of weak or attenuated strains that could be used in cross-protection [9]. Studies on the transformation of papaya have demonstrated that the genetic resistance to PRSV obtained through the expression of the CP gene in papaya plants depends on several factors, such as the degree of homology between the protecting virus gene and the challenging virus, a property that is considered dependent on the virus isolate [4, 10, 11]. Moreover, cross-protection with mild strains has shown limited success in protecting against severe strains, thus precluding a durable, economic, and safe control, mainly due to problems

of the protection loss from instability and a lack of specificity of mild isolates [4, 12, 13, 14, 15, 16].

There are numerous areas in Cuba with favorable conditions for papaya cultivation, and papaya crops represent 7% of the cultivated area and account for 15% to 20% of the income from fruit production in the country [17]. Currently, papaya cultivation is an alternative to the diversification of fruit crops as part of a national program for the development of the Cuban fruit agro industry, which aims to support the increasing fruit demand of its population and to expand the possibilities of the domestic market for export. In Brazil, the papaya crop is also greatly important, as evidenced by statistics that show this country as the largest producer and third largest exporter of papaya in the world. In 2011, Brazil produced more than 1.8 million tons from 35.531 hectares [18].

In both Brazil and Cuba, as also observed in the largest papaya-producing areas worldwide, PRSV-P is the main virus infecting this plant and has been a limiting factor to the establishment and development of the crop. It is very common to find papaya plants infected with this virus in commercial plantations, smallholder areas, urban household gardens, and along roadsides. These observations indicate that there are abundant sources of PRSV-P throughout the national territories, highlighting the need to develop and implement strategies for its management and control, which would reduce the harmful effects caused by this pathogen, thus increasing the income and quality of the fruits produced in the cultivation areas.

Within this context, it is necessary to know the degree of genetic variability of the isolates that occur in each specific geographic region as a basis for the development of transgenic plants and/or cross-protection programs. To assess this variability, Lima *et al.* [19] performed phylogenetic studies on the CP gene of twelve isolates of PRSV-P from different regions of Brazil, identifying a medium degree of homology of 97.3% between them. However, few studies have been performed in Cuba, with only partial CP sequences of PRSV-P available in GenBank [20, 21].

In this work, we present and discuss, for the first time, the results of sequence analyses and the comparison of the complete CP gene of seven PRSV-P isolates collected in several provinces in Cuba, the analysis of 21 PRSV-P isolates from several regions of Brazil, and the symptoms induced by the 28 isolates in papaya cv. Solo and squash cv. Caserta plants.

2. Material and Methods

2.1. Selecting a Collection and Maintenance of Isolates

Between 2010 and 2011, 28 samples were collected from papaya plants exhibiting the typical symptoms of PRSV-P in commercial plantations of ten Brazilian states and five Cuban provinces. Details of the origins of the strains and the sequences from GenBank used for comparison are shown in Table 1. The isolates were collected and mechanically inoculated onto papaya seedlings and maintained under greenhouse conditions, and the obtained infected leaves were dried and stored at -20 and -80 ºC.

Table 1. *Origin and denomination of Papaya ringspot virus (PRSV-P) isolates used in the study*

Studied Isolates (collected from Brazil and Cuba)					**Isolates from GenBank**		
Country	**Isolate**	**State/Province**	**Municipality**	**GB Access**	**Country**	**Isolate**	**GB Access**
Brazil	BrMG-1	Minas Gerais	Lavras	KC662372	Brazil	Br-PRSV-W-1	DQ374153
Brazil	BrMG-2	Minas Gerais	Lavras	KC748204	Brazil	Br-PRSV-W-C	DQ374152
Brazil	BrMG-3	Minas Gerais	Lavras	KC748205	Brazil	Brazil-CE	AF344647
Brazil	BrMG-4	Minas Gerais	Lavras	KC748206	Brazil	Brazil-PE	AF344646
Brazil	BrBA-1	Bahia	Cruz das Almas	KC748212	Brazil	Brazil-DF	AF344650
Brazil	BrBA-2	Bahia	Cruz das Almas	KC748213	Mexico	Mx-VrPro	AY231130
Brazil	BrSP-1	São Paulo	Itapira	KC748207	USA	USA-Haw-PG	EU126128
Brazil	BrSP-2	São Paulo	Itapira	KC748208	USA	USA-B9	JN132432
Brazil	BrSP-3	São Paulo	Itapira	KC748209	USA	USA-T14b	JN132470
Brazil	BrSP-4	São Paulo	Itapira	KC748210	Venezuela	Ve-Merida-6	EF189736
Brazil	BrSP-5	São Paulo	Mococa	KC748211	Venezuela	Ve-Merida-8	EF189733
Brazil	BrRJ-1	Rio de Janeiro	Rio de Janeiro	KC748214	Venezuela	Ve-Trujillo-1	EF189734
Brazil	BrPR-1	Paraná	Maringá	KC748222	Venezuela	Ve-Trujillo-5	EF189735
Brazil	BrPR-2	Paraná	Maringá	KC748223	Venezuela	Ve-Zulia-7	EF189732
Brazil	BrES-1	Espírito Santo	Linhares	KC748216	Taiwan	Ta-PRSV-SLM	DQ340771
Brazil	BrES-3	Espírito Santo	Linhares	KC748217	Taiwan	Ta-PRSV-1	X67672
Brazil	BrAM-1	Amazonas	Manaus	KC748220	Taiwan	Ta-PRSV-2	X97251
Brazil	BrMA-7	Maranhão	São Luís	KC748218	Taiwan	Ta-PRSV-SLD	DQ340769
Brazil	BrMA-8	Maranhão	São Luís	KC748219	Taiwan	Ta-PRSV-P-5-19	EU882728
Brazil	BrPA-1	Paraíba	Pombal	KC748221	Taiwan	Ta-CI	AY027810
Brazil	BrDF-1	D. Federal	DF	KC748215	China	Ch-HN-1	HQ424465
Cuba	CbMY-1	Mayabeque	Nueva Paz	KC748229	China	Ch-PRSV-P	EF183499
Cuba	CbMT-1	Matanzas	Colón	KC748227	India	In-PRSV	EU475877
Cuba	CbMT-2	Matanzas	Jagüey Grande	KC748228	India	PRSV-DEL	EF017707
Cuba	CbVC-1	Villa Clara	Santa Clara	KC748230	South Korea	SK-PRSV-W	AB369277
Cuba	CbGR-1	Granma	Jiguaní	KC748224	Thailand	Th-PRSV-P	AY162218
Cuba	CbGR-2	Granma	Jiguaní	KC748225	Thailand	Th-PRSV-W	AY010722
Cuba	CbHG-1	Holguín	Holguín	KC748226	Malaysia	Ma-PRSV	AB044342

2.2. RNA Extraction, RT-PCR, and Sequence Analysis

Total RNA was extracted from the young leaves of PRSV-infected papaya plants using the Trizol method [22]. The primers used for the amplification of a 1,238 pb fragment containing the capsid gene were designed based on the sequences available in GenBank for PRSV (http://www.ncbi.nlm.nih.gov/genbank/): PRSV9016-F (5'-CTTGARCARGCTCCATTC-3') and PRSV10253-R (5'-CTAAAAGCACGGAGG-3').

Complementary DNA (cDNA) was synthesized from the total RNA using the primer PRSV10253-R and M-MLV reverse transcriptase (Promega Corp. Madison, WI, USA). The PCR was performed with GoTaq® Flexi DNA Polymerase (Promega, Corp. Madison, WI, USA) and both primers using the following cycling conditions: 95°C for 2 minutes, followed by 35 cycles of 95°C for 45 seconds, 42°C for 1 minute, and 72ºC for 1 minute, with a final extension at 72°C for 5 minutes. The product was analyzed by electrophoresis through 0.7% agarose, purified using a purification kit (QIAGEN Sample & Assay Technologies – Venlo, The Netherlands), and sequenced by Genewiz (South Plainfield, NJ, USA).

2.3. Sequence Analysis

Based on the sequences obtained, contigs were constructed using the program DNA Baser Sequence Assembler v2 (www.dnabaser.com) and analyzed with the Basic Local Alignment Search Tool (BLAST), which is available online at the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov/).

The final sequences were analyzed with the BLAST program, and the multiple alignment was performed with the CLUSTALW program included in Molecular Evolutionary Genetics Analysis (MEGA5) [23], using, for comparison, the PRSV isolates available in GenBank (Table 1). The phylogenetic trees were constructed with the MEGA5 program using bootstrapping with 2,000 repetitions.

2.4. Survey of Symptoms

Two tests were performed to evaluate the symptoms induced by the virus isolates in papaya (*C. papaya* L. cv. Solo) and squash (*Cucurbita pepo* L. cv. Caserta) seedlings under different temperature conditions: winter (July 2010), with a minimum temperature of 4.8ºC and a maximum of 25.2°C (average 15°C), and summer (February 2012), with values of minimum and maximum temperatures of 19.8ºC and 34ºC, respectively (mean of 26.9ºC).

The studied isolates were mechanically inoculated using friction with 600 mesh silicon carbide of papaya plants (15 to 20 cm high) under greenhouse conditions. Three seedlings were used in each experiment, with three replications. Healthy plants inoculated only with phosphate buffer were used as the control. The evaluation of symptoms was performed up to 45 days after inoculation.

All the inoculated plants were maintained in a greenhouse until the end of symptom assessment, which was performed in accordance with the descriptive scale used by Rodriguez *et al.* [24] to evaluate papaya seedlings inoculated with PRSV-P.

3. Results and Discussion

Figure 1. *Amino acids sequences of different regions of CP protein of PRSV-P isolates from Brazil and Cuba showing the deletions in the BrSP-5 and BrBA-1 isolates (-), conserved DAG, WCIEN & QMKAA domains and more conserve sequences on the 3′ region than the 5′region (*). Different colors in the same column indicate different amino acids in that position. Multiple alignments were performed with the CLUSTALW program included in MEGA5.*

A total of 26 of the 28 isolates for which the PRSV CP gene was sequenced in this study produced a band with 924 nucleotides. Brazilian isolates BrBA-1 and BrSP-5 generated a 921 nucleotide band; the deletion of three nucleotides corresponds to amino acid 22 in the BrSP-5 isolate and to amino acid 18 in the BrBA-1 isolate, after the conserved DAG domain (Fig. 1). Such differences with regard to the size of the CP gene were previously reported in Brazil by Lima *et al*. [19]. These authors found 10 Brazilians isolates with a CP gene containing 924 nucleotides and 2 isolates from Bahia and Paraná with only 921 nucleotides.

Inoue-Nagata *et al.* [25] compared the genetic sequences from two strains of PRSV-W (a mild strain and a severe strain) and observed that a mild strain genome presented six additional nucleotides, located in the CP region, generating two additional amino acids (Asn and Asp). Variations in the nucleotide and amino acid lengths of PRSV CP have also been described by other authors [26, 27]. These variations are consistently found in the first 50 amino acids of the N terminus, particularly in the EK repetitive region, suggesting that this region is variable and, thus, likely to exhibit nucleotide insertions and deletions [28]. Therefore, it has been suggested that the 3' terminus of the CP gene should be used to obtain transgenic plants because this region is more conserved than the 5' region [29]. We also found a greater conservation in the 3' region (Fig. 1), confirming that this region is the most suitable for plant transformation.

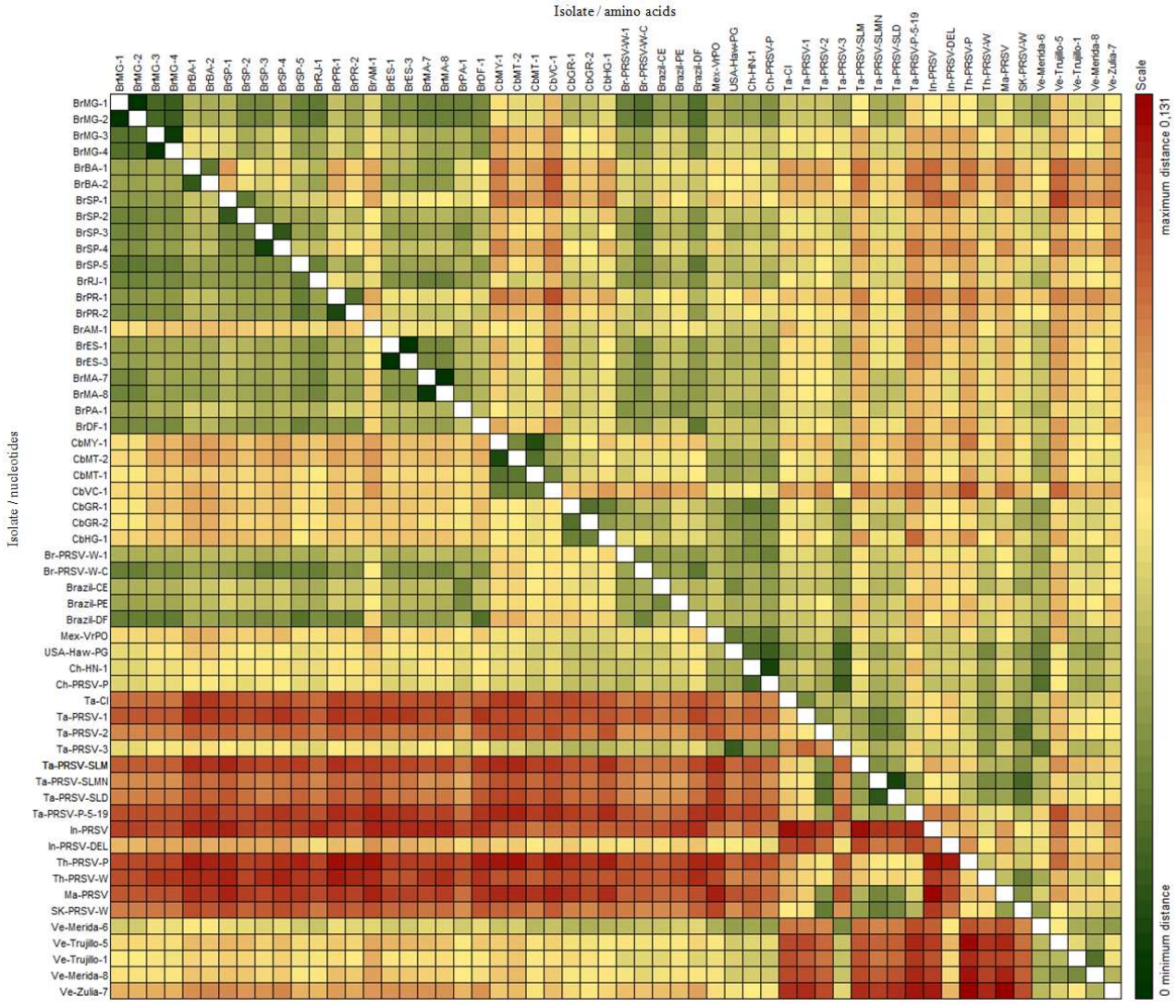

Figure 2. *Estimated genetic distance (pairwise analysis by deletion of gaps) among nucleotide sequences and among amino acid sequences of the coat protein gen of PRSV-P isolates from Brazil, Cuba and remaining isolates available in GenBank. Multiple alignments of sequences and evolutionary analyses were performed by the MEGA 5 program.*

As shown in Figure 2, the comparison using the pairwise tool of the genetic distance among the nucleotide sequences of the 28 isolates analyzed in this study and those from GenBank (n=56) indicated a range from 0 to 0.131. However, when we compared the isolates from Brazil and Cuba sequenced in this study (n=28), the genetic distance was lower, between 0 and 0.092, which is most likely because the isolates were derived from only two countries.

In this case, a zero value was observed among the isolates from the same states and municipalities in Brazil, (BrMG-1 and BrMG-2, BrES-1 and BrES-3), which shows the genetic similarity among the isolates from the same region. Moreover, the isolates that were more distant (0.092) are from the two different countries (BrAM-1 with CbMT-2 and CbVC-1). When only the Brazilian isolates were analyzed (n=21), there was a smaller distance, of up to 0.082, and the highest values (0.66 to 0.82) were found among the isolated BrMA-1, from the state of Amazonas, with the other isolates. This result is most likely due to the geographical distance between Amazonas and the other states, where the isolates were collected, because the distances varied between 0 and 0.063 among the other Brazilian isolates.

The analysis of the Cuban isolates (n=7) showed a smaller genetic distance, with values of up to 0.073, which suggests that the isolated conditions of Cuba and also its smaller territory (when compared to Brazil) allow the PRSV-P isolates to retain a higher level of conservation. In fact, the longest distance was observed between two Cuban isolates from geographic regions more distant within the archipelago (CbMT-2 and CbHG-1), whereas the lowest distance was observed in virus isolates from neighboring provinces (CbMY-1 and CBMT-1 and 2), reinforcing the hypothesis that genetic conservation is greater among isolates from nearby regions.

The identities among the gene sequences (data not shown) corroborated the results of the pairwise analyses, showing the lowest values among the isolates from America and Asia. Lower values (below 86%) were observed when the Brazilian isolates BrBA-2, BrSP-1, BrSP-4, BrPR-1, and BrMA-8 were compared with the Asian Ch-HN-1, Th-PRSV-P, and Th-PRSV-W isolates. The identities of nucleotides among the Brazilian isolates varied from 91% (among BrAM-1 and the isolates BrMA-8 and BrDF-1) to 100% (between BrMG-1 and BrMG-2 and between BrES-1 and BrES-3). Among the Cuban isolates, the identity ranged from 99% (among isolates CbMY-1 and CbMT-2) to 92% among isolates from the western region (Mayabeque, Matanzas, and Villa Clara) and the eastern region (Granma and Holguín).

Unlike the results obtained in this study, the study carried out by Lima *et al*. [19] showed higher identities between the CP sequences of PRSV isolates from different regions of Brazil, which is interesting because of the large geographical distance among the isolates evaluated by these authors. The greatest differences observed in the present study are likely due to the inclusion of an isolate from Amazonas, which was more diverse and more distant from the rest of the Brazilian isolates in both the pairwise and phylogenetic tree analyses.

When we analyzed the amino acid sequences of the studied isolates with the sequences obtained from GenBank, the conserved DAG, WCIEN, and QMKAAA domains were present (Fig. 1); these domains were also identified by other authors, who postulated that they are related to virus transmission by aphids [29, 30, 31]. Following the third amino acid after the triple block DAG domain, glutamic acid and lysine repeats known as the "EK region" [30] were observed and were more variable in the tested virus isolates.

The pairwise analysis of the amino acid sequences showed genetic distances similar to those observed for the nucleotide sequences (Fig. 2), with the lowest distance values detected among the isolates from the same states in Brazil (BrMG-1 and BrMG-2, BrES-1 and BrES-3) and the longest distances observed among the isolates from the two countries, and the highest value was between CbVC-1 and BrPR-1. In this case, the distance among all the sequences (n=56) ranged from 0 to 0.114 and among the studied isolates (n=28) were between 0 and 0.111.

Confirming the previous results, the identities between amino acids were higher than among nucleotides (data not shown). The lowest identity, 88%, occurred when all the sequences were compared (n=56) among the isolates from America and Asia. It is important to highlight the low identity (88%) that was registered between BrSP-1 and Ve-Trujillo-5, both from South America, which also showed higher distances by the pairwise method. Among the isolates sequenced in this study (n=28), the identities ranged from 89% (between CbVC-1 and BrBA-1, BrBA-2, BrSP-1, BrSP-4 and BrPR-1) to 94% (between CbGR-1 and BrMG-1, BrMG-2, BrRJ-1, BrES-1, BrES-3, BrPA-1; CbMT-1 and BrSP-2, BrMA-7, BrMA-8, BrPA-1; CbMT-2 and BrPA-1). Among the isolates from Brazil (n=21), identities varied from 90% (among the isolate from Amazonas BrAM-1, BrBA-1, BrBA-2 and BrSP-1) to 100% (among BrMG-1, and BrMG-2; BrMG-3, and BrMG-4; and also between the two isolates from Espirito Santo state and the two isolates from Maranhão state); the identities among the Cuban isolates ranged from 91% (between isolates from different regions; CbVC-1, CbGR-2, and CbHG-1) to 99% (CbMY-1 and CbMT-2).

The genetic distances among the CP amino acid sequences of the 28 isolates were slightly lower than those obtained for the nucleotide sequences, suggesting that the amino acid sequences of CP are affected by changes in the nucleotide sequences, in agreement with the results obtained by Silva-Rosales *et al.* [29]. Comparing the CP sequences of 7 Asian isolates with 3 Mexican isolates, these authors also found a higher degree of identity among nucleotides than among amino acid sequences. However, Jain *et al.* [32] detected higher degrees of identity among amino acid sequences than nucleotide sequences when comparing CP from two Indian isolates of PRSV-P, though this may have occurred because only two isolates from the same country were included.

In the phylogenetic tree (Fig. 3A), the different isolates were grouped into two clusters based on their CP nucleotide sequence: one large cluster (I) containing the isolates from America, one from Taiwan, and two from India and one minor of the Brazilian isolates (Ia). The Ia group showed a greater similarity among the isolates from the same region, except for the Amazonas isolate, which was far from all of the isolates, confirming the pairwise analyses. In the first cluster (I), we can observe the formation of another even

smaller cluster (Ib) formed by isolates from the central western region of Cuba (CbMT-2, CbVC-1, CbMY-1, and CbMT-1) (Ib1), isolates from Venezuela (Ib2), those from the eastern region of Cuba (CbGr-1, CbGR-2, and CbHG-1) (Ib3), and the isolate from Taiwan (Ta-PRSV-1) with the U.S. and Mexican (Mex-VrPo) (Ib4) isolates. Finally, the isolates from India in the first cluster were significantly more distant (In-PRSV-DEL and In-PRSV) (Ic). The remaining isolates from Asia, including China, Thailand, Taiwan, South Korea, and Malaysia, were grouped into the second largest cluster (II).

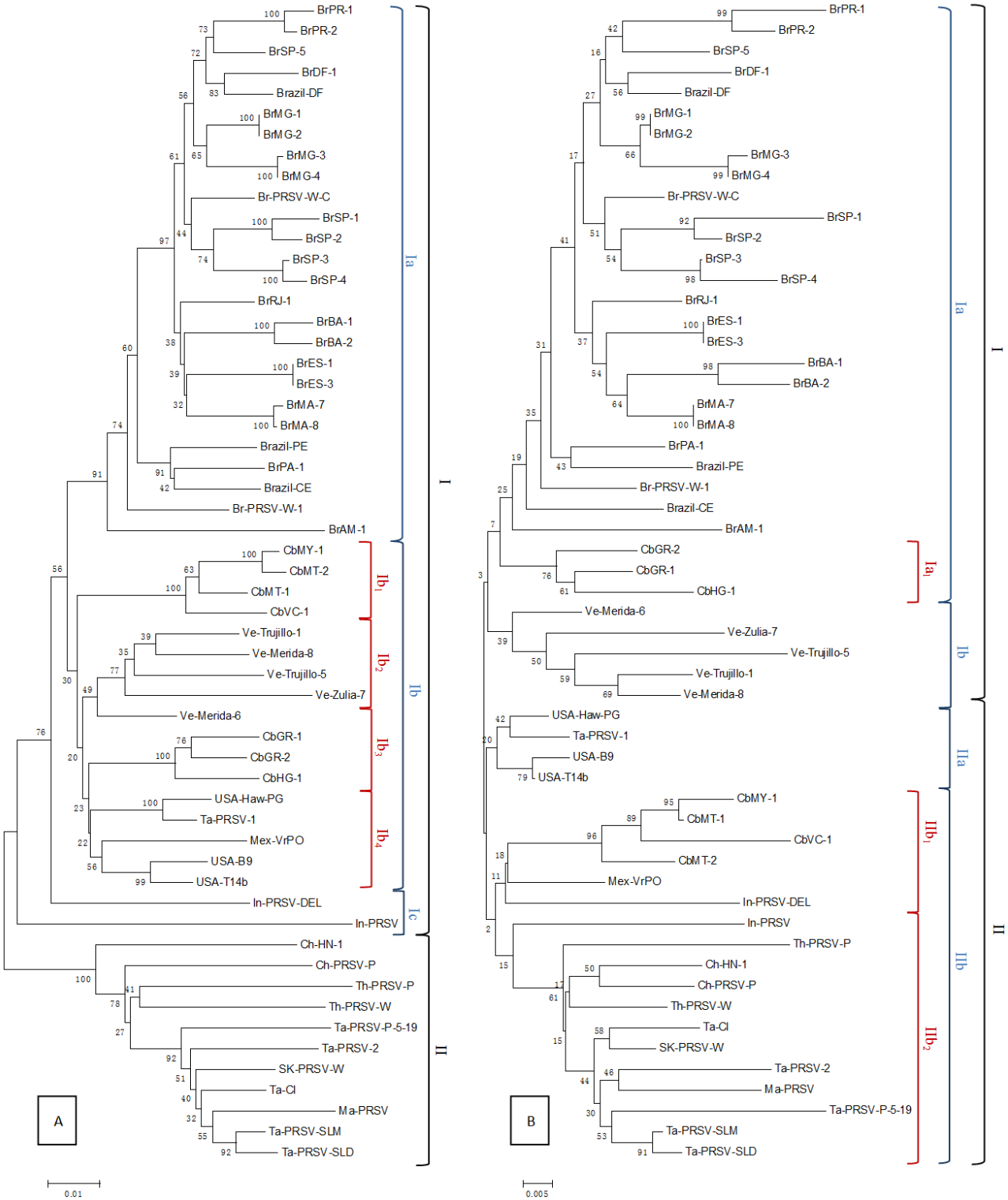

Figure 3. *Phylogenetic trees based on the nucleotide (A) and amino acids (B) sequences of the coat protein gene of PRSV isolates from Brazil, Cuba and the remaining isolates available in GenBank. Bootstrap values were obtained by MEGA5 program, using Neighbor Joining with 2.000 replicates. Equal numbers, letters and/or sub-indexes indicate common groupings.*

The phylogenetic tree based on the amino acid sequences of CP (Fig. 3B) revealed a grouping different from that observed in the tree constructed based on the nucleotide sequences. In this case, we observed two major clusters. The first cluster (I) contained the isolates from Brazil, the eastern region of Cuba (Ia), and Venezuela (Ib), which were subgrouped by country, and the Brazilian isolate from Amazon (BrAM-1) was more distant from the other isolates from Brazil, as observed in the nucleotide-based tree. The second cluster (II), containing the rest of the isolates, showed the American isolates close to each other, forming a smaller cluster (IIa), and the isolates from the western region of Cuba, Mexico (Mex-VrPo), and India (In-PRSV-DEL) (IIb1) separate from the other Asian isolates (IIb2).

Lima *et al*. [19] and Inoue-Nagata *et al*. [25] also observed that Brazilian isolates grouped into the same cluster, with few differences among them, which could be due to the use of isolates from other states that were not studied here. The results based on the CP nucleotide sequences also showed that the isolates were distributed into different groups according to the region of origin. Chin *et al*. [33] also detected a high genetic variability among the PRSV isolates from Venezuela compared to isolates from Jamaica. Such differences could be related, among other factors, to geographic isolation [34].

The phylogenetic groups obtained when the sequences studied were compared to the virus isolates available in GenBank were similar to those obtained in studies performed by Fernandez-Rodríguez *et al*. [35]. These authors observed one group containing PRSV-P isolates from Mexico, The United States, Cuba, Puerto Rico, Brazil, and Australia, as has been previously reported by several other authors, with groups of America-Australia isolates [2, 19, 25, 34, 36].

Phylogenetic relationships between the isolates from India and the isolates from other continents were also observed by some authors [28, 29, 37, 38]. These authors also noted a phylogenetic relationship among the isolates from India and America but always with a certain genetic distance among them, thus strengthening the hypothesis that PRSV may have been dispersed from India to America, via Brazil, Venezuela, or Mexico [34, 38].

The results obtained with the isolates from eastern and central-western Cuba were interesting, as they remained in separate clusters, in the trees based on both the nucleotide and on amino acid sequences. This finding is in agreement with the data obtained when Arocha *et al*. [20] analyzed the partial sequences of the CP gene of one virus isolated from the central region of Cuba, which grouped separately from the isolate collected in the eastern region. This fact might suggest different origins for these groups of isolates in Cuba.

Another fact to be noted in this study is that there were no differences in the groups containing strains PRSV-P and PRSV-W. These results were also observed by other authors when analyzing the CP sequence of isolates of these two strains from different regions of the world [25, 28, 37]. In addition to indicating possible recombination events between the two strains, the results suggested that either PRSV-P arose from PRSV-W or vice versa [34, 38].

The incubation period of PRSV-P in the papaya plants was variable among the different isolates and duration of the experiment (Table 2). In the winter, more time was required for the plants to display the first symptoms (incubation period from 10 to 38 days) when compared to the symptoms in summer (5-26 days), which suggests that summer temperatures (19.8ºC and 34ºC) favor viral multiplication and disease development. When different isolates were compared, it was observed that the BrRJ-1 and BrMA-8 isolates required a longer time period to induce symptoms under both experimental conditions, suggesting that these isolates are less aggressive than the other isolates.

Table 2. *Incubation period and intensity of symptoms induced by PRSV-P isolates on Carica papaya* L. cv. Solo *and Cucurbita pepo* L. cv. Caserta *45 and 21 days after inoculation, respectively.*

Isolate	*Carica papaya*					*Cucurbita pepo*		
	I.P. (days)		Foliar symptoms					
	Exp. 1	Exp. 2	M	B	D	S.S	I.P.	S.I
BrMG-1	15-37	9-17	++	++	+	++	9	+
BrMG-2	13-17	9-18	+++	++	-	++	9	+
BrMG-3	14-22	7-14	+++	++	++	++	9	+
BrMG-4	23-35	8-15	++	++	+	+	8	++
BrBA-1	14-17	7-10	+++	++	+++	+++	12	+
BrBA-2	11-16	6-11	+++	+++	+++	++	9	+
BrSP-1	15-27	8-23	+++	++	+	++	7	++
BrSP-2	12-35	9-15	++	+	++	++	7	++
BrSP-3	11-15	7-10	++	+	++	++	7	+++
BrSP-4	11-15	7-10	+++	+	++	++	13	++
BrSP-5	11-38	11-24	++	+	+	+	9	++
BrRJ-1	21-32	13-24	++	+	++	+	10	++
BrPR-1	12-14	7-13	++	++	++	-	11	+
BrPR-2	10-15	5-17	+++	++	+++	++	7	++
BrAM-1	13-32	7-12	++	+	+	+++	9	+
BrES-1	11-32	19-26	++	++	+	++	8	++
BrES-3	15-19	9-13	+++	++	+++	++	8	++
BrMA-7	15-30	10-21	++	+	+	+	7	+++
BrMA-8	18-20	13-23	++	+	+	+	7	++
BrPA-1	10-16	7-10	++	++	++	+	12	+
BrDF-1	11-16	8-16	+++	++	++	++	9	++
CbMY-1	11-19	12-22	++	++	+	+	12	++
CbMT-1	15-21	11-17	+	+	++	+	12	+
CbMT-2	16-31	9-15	++	++	++	++	13	+
CbVC-1	16-35	10-20	+++	++	++	+	12	+++
CbGR-1	14-20	13-21	++	+	+	+	8	++
CbGR-2	15-17	8-21	+++	++	+++	+	10	++
CbHG-1	11-22	8-14	++	++	++	+	10	++

I.P. Incubation period, Exp. experiment, M. mosaic, B. bubbles, D. distortion, S.S. stem spots, S.I. symptoms intensity

The plants inoculated with isolate BrMG-4 showed symptoms later in the winter, but the plants inoculated with BrES-1 and CbGR-1 showed symptoms later in the summer. This behavior suggests that the differential aggressiveness of these isolates could be influenced by the ambient conditions, particularly the temperature, as suggested Silva-Rosales *et al*. [29]. The same results were reported by Mangrahutia *et al*. [15], who found a maximum accumulation and

expression of viral symptoms at temperatures between 26ºC and 31ºC.

In most of the isolates, the first symptoms observed were vein clearing and mild mosaic patterning (Fig. 4A). However, the BrMG-2, BrMG-3, BrSP-1, BrSP-3, BrSP-4, BrPR-1, and BrPR-2 isolates and also induced symptoms of wrinkled leaves with vein thickening (Fig. 4B), and BrMG-3, BrMG-4, and BrRJ-1 induced oily streaks in the stem (Fig. 4C). The symptoms intensified over time. In addition to the above-described symptoms, at 45 days after inoculation, moderate or severe (Fig. 4D, E) bubbles (Fig. 4F) and leaf distortion, with strong thickening of the ribs (Fig. 4G), were also observed.

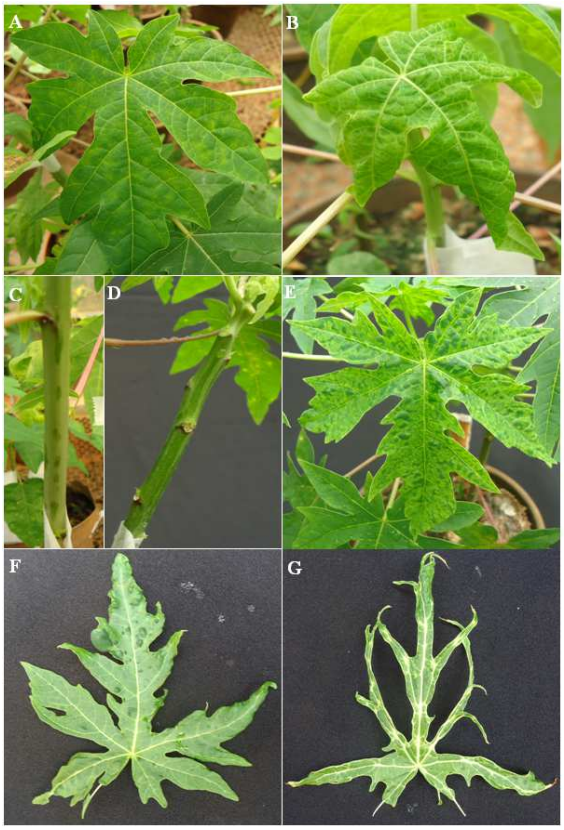

Figure 4. *Initial symptoms of vein clearing and mild mosaic (A), curling (B) and small oily streaks in the stem (C) and severe symptoms of oily streaks on the stem (D), severe mosaic (E), blistering (F) and leaf distortion (G), in plants of Carica papaya L. cv. Solo, 45 days after mechanical inoculation with PRSV-P isolates.*

The type and severity of symptoms at 45 days after inoculation varied among the isolates (Table 2): BrBA-1, BrBA-2, BrPR-2, BrES-3, and CbGR-2 induced severe symptoms; BrSP-5, BrMA-7, BrMA-8, CbMT-1, and CbGR-1 induced mild symptoms; and the remaining isolates induced moderate symptoms. In general, the less aggressive isolates induced symptoms that ranged from mild to moderate, though none induced severe symptoms, suggesting the possible existence of a relationship between symptom severity and the aggressiveness of isolates. However, it is recommended that the severity of symptoms produced by these isolates be evaluated in other cultivars of commercial interest to both countries. Rodríguez *et al*. [24] also observed variability in the intensity of symptoms in different genotypes of papaya inoculated with the same isolate of PRSV-P.

The BrMG-1 e BrMG-2, BrES-1 e BrES-3 squash plants developed chlorotic spots all over the leaf surface (Fig. 5A) and vein clearing, with larger chlorotic spots (Fig. 5D). However, not all the leaves showed these symptoms, suggesting that, although the infection is systemic, the plants demonstrated some resistance to the movement of the virus. *C. quinoa* and *C. amaranticolor* plants did not show any symptoms and were not infected by these isolates of PRSV-P. The absent of symptoms in these hosts could be caused because sometimes the latex of papaya plants inhibits the viral infection.

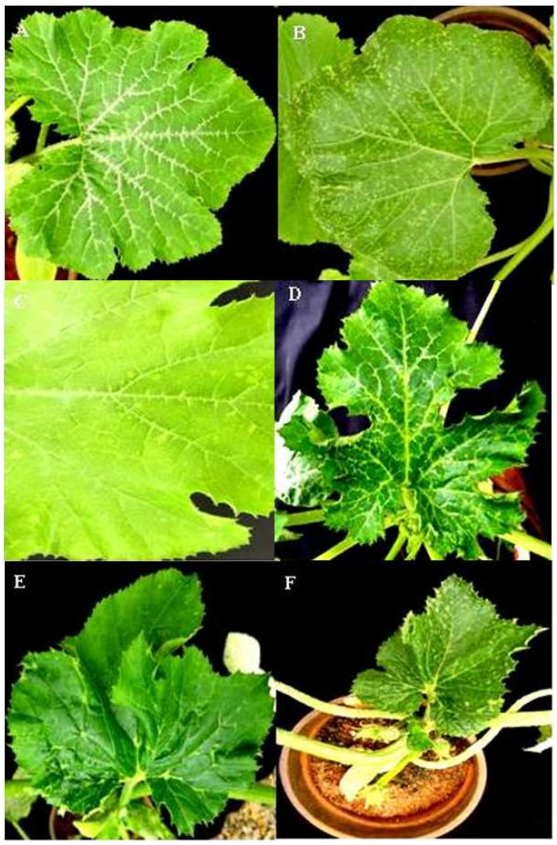

Figure 5. *Symptoms induced by PRSV-P in leaves of Cucurbita pepo L. cv. Caserta, 21 days after mechanical inoculation. Healthy plant (A), chlorotic spots (B and C), ribs yellowing (C), curling deformation (D) and plant growth reduction (F).*

The initial symptoms in the squash plants were observed at approximately seven days after inoculation. Most isolates induced symptoms of chlorotic spots, as described above, but the BrES-1, BrES-3, BrDF-1, and BrMG-4 isolates and Cuban CbVC-1 caused leaf distortion and severe curling (Fig. 5E), stunting in plant growth (Fig. 5F) and yellow vein coloration (Fig. 5D) that was also induced by BrSP-5 in the squash plants.

Silva-Rosales *et al*. [29] reported similar results, observing symptoms only in papaya and *Cucumis*

metuliferus plants, among the 13 species inoculated with PRSV-P isolates, including *C. quinoa* and *C. amaranticolor*. These results confirm that the PRSV-P isolates have a very narrow host range and a high specificity for *C. papaya* and some species of cucurbits [1, 39, 40].

The results based on the CP sequences of isolates from different regions of Brazil and Cuba and the symptoms induced in papaya and squash seedlings help to understand the genetic diversity of PRSV-P and offer valuable information for papaya ringspot management strategies, employing cross-protection and transgenic plants, in both countries. Therefore, it is recommended that biological studies should be conducted on papaya cultivars with commercial importance for both countries, before choosing the best control strategy to be employed.

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