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Resistance of transgenic papaya trees to papaya ringspot in Brazil

Abstract – The objective of this work was to evaluate the resistance of transgenic papaya populations (PTPs) to *Papaya ringspot virus*-P (PRSV-P). 'Sunrise Solo' transgenic papaya plants were produced with the gene of the PRSV-P protein coat, and PRSV was mechanically inoculated in plants in greenhouse conditions. The presence of the CP/PRSV gene and homozygosis were evaluated by polymerase chain reaction (PCR). Selected plants and the 'Sunrise Solo' control were transplanted to the field for agronomic evaluations. The plants evaluated in greenhouse conditions showed resistance between 96.3 and 5.8%, without variation of symptoms. The PTPs 1/6, 1/7, 1/9, 1/10, 1/15, 2/38, 2/41, 2/56, 2/65, 3/27, 3/46, 3/48, 4/9, 4/27, 8/4, 8/23, 8/33, 18/3, 18/4, 18/8, 18/22, 18/27, 28/97, 28/104, and 28/110 showed no symptoms, they were ELISA negative, and most of them contained the CP and NPT II genes. PTPs 1/6 and 3/46 had the CP gene in homozygosis and in double insertion. PTPs 1/6/20, 1/6/59, 1/6/64, 1/6/90, 3/46/44, 3/46/52, and 18/27/97 had a well-formed fruit cluster, piriform fruit weighing approximately 500 grams, orange pulp, and less than 10% carpelloidy. PTPs 1/6/59 and 3/46/52 show resistance to PRSV, good agronomic characteristics, and the CP gene in homozygosis.

Index terms: *Carica papaya*, genetic modified organism, PRSV.

Resistência de mamoeiros transgênicos à mancha anelar no Brasil

Resumo – O objetivo deste trabalho foi avaliar a resistência de populações transgênicas de mamoeiro (PTPs) ao vírus da mancha anelar do mamoeiro [*Papaya ringspot virus*-P (PRSV-P)]. Plantas transgênicas 'Sunrise Solo' foram produzidas com o gene da proteína da capa viral PRSV-P, e o PRSV foi inoculado mecanicamente nas plantas em casa de vegetação. A presença do gene CP/PRSV e a homozigose foram avaliadas por reação em cadeia da polimerase. As plantas selecionadas e o controle 'Sunrise Solo' foram transplantados para o campo, para avaliações agronômicas. As plantas avaliadas em casa de vegetação apresentaram resistência entre 96,3 e 5,8%, sem variação nos sintomas. As PTPs 1/6, 1/7, 1/9, 1/10, 1/15, 2/38, 2/41, 2/56, 2/65, 3/27, 3/46, 3/48, 4/9, 4/27, 8/4, 23/8, 33/33, 18/3, 18/4, 18/8, 18/22, 18/27, 28/97, 28/104 e 28/110 não apresentaram sintomas, tiveram resultado negativo ao teste ELISA, e a maioria delas continha os genes CP e NPT II. As PTPs 1/6 e 3/46 apresentaram gene CP em homozigose e em dupla inserção. As PTPS 1/6/20, 1/6/59, 1/6/64, 1/6/90, 3/46/44, 3/46/52 e 18/27/97 apresentaram cacho de frutos bem formado, frutos piriformes com 500 g, polpa laranja e menos de 10% de carpeloidia. As PTPS 1/6/59 e 3/46/52 apresentam resistência ao PRSV, boas características agronômicas e o gene CP em homozigose.

Termos para indexação: *Carica papaya*, organismo geneticamente modificado, PRSV.

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Introduction

Research on papaya (*Carica papaya* L.) is essential for Brazil that is one of its main producers, with an annual production of about 1,161 thousand megagrams. Papaya tree is cultivated throughout Brazilian territory, and the states Bahia and Espírito Santo are the largest national producers (Embrapa Mandioca e Fruticultura, 2019).

Viruses are the main problems for the culture. In Brazil, papaya trees undergo incidences of viral diseases caused by *Papaya ringspot virus*-P (PRSV-P), Papaya meleira virus (PMeV) and *Papaya lethal yellowing virus* (PLYV) (Basso et al., 2016). PRSV has a positive-sense RNA; it is a species of the genus *Potyvirus*, family *Potyviridae.* The control of PRSV is difficult because it is a noncirculating virus transmitted by several species of aphids (King et al., 2012; Azad et al., 2014). Infection caused by PRSV reduces the production and quality of fruits, as they may be deformed, show rings in the bark, and reduction of their sugar content (Lima et al., 2017).

To date, no sources of resistance to PRSV have been identified in *C. papaya*; however, there are species of the papaya family with tolerance to the virus that have been used in genetic improvement programs in Thailand, Taiwan, and Hawaii (Jayavalli et al., 2011; Alviar et al., 2012; Dinesh et al., 2013). In order to obtain resistant plants to PRSV, crosses of *C. papaya* and *Vasconcellea cauliflora* were carried out (Jayavalli et al., 2011; Alviar et al., 2012; Dinesh et al., 2013). The main limiting factor for this PRSV control strategy is the long period necessary to develop these cultivars and to attain good fruit quality (Dinesh et al., 2013). Some researchers evaluated the use of cross protection, but did not obtained good results to control PRSV (Fitch, 2010; Hamim et al., 2018). Recently, the use of double strand RNA (dsRNA) has been tested to control PRSV (Vadlamudi et al., 2020). Then the main measures adopted in the Brazilian producing regions to control PRSV are escaping by the isolation of the fields and the early eradication of the infected plants (Lima et al., 2017).

The production of transgenic papaya plants resistant to PRSV is a good and durable alternative to control the virus, and it has been used and researched in several regions that produce papaya, such as Australia, Bangladesh, Brazil, China, the United States, the Philippines, India, Indonesia, Jamaica, Japan, Malaysia, Mexico, Thailand, Vietnam, and Venezuela (Souza Júnior et al., 2005; Fitch, 2010; Li et al., 2014; Lima et al., 2017; Hamim et al., 2018; Wu et al., 2018; Baranski et al., 2019).

The objective of this work was to evaluate the resistance of transgenic papaya populations (PTPs) to *Papaya ringspot virus*-P (PRSV-P) in Brazil.

Materials and Methods

The experiments were carried out from April 2003 to October 2006, in Cruz das Almas, in the state of Bahia (BA), Brazil, at the Virology Laboratory, greenhouse and experimental field of Embrapa Mandioca e Fruticultura (12º40'12"S, 39º06'07"W, at 220 m altitude). The climate in the region is Bwa type according to the Köppen-Geiger's classification, with the annual means of above 22 ºC temperature, 82% relative humidity, and 1,136 mm precipitation (D'Angiolella et al., 1998). The soil of the experimental area is classified as Latossolo Amarelo distrófico (Santos et al., 2013), with a sandy-clayey texture, which corresponds to Ferralsol (IUSS Working Group WRB, 2015).

The transgenic papaya 'Sunrise Solo' was used, which was produced in Cornell, USA, in partnership with the Cornell University. The plants were transformed by biobalistics with a cassette containing the protein coat gene of the Brazil Bahia isolate of the PRSV-P, in different translatable and untranslatable versions (Souza Júnior et al., 2005). The experiment was carried out under the permission of the Comissão Técnica de Biossegurança (CTNBio), Process 01200.004426/2000-57 and Process 01200.01214/2006-11.

Seed of 13 transgenic papaya populations (PTP) generations R-1 and R-2 were sown in greenhouse conditions, in February 2003, to attain 100 seedlings, as recommended for the crop (Sanches & Dantas, 1999). Thirty days after sowing, the plants were challenged by the mechanical inoculation of a PRSV isolate collected in the region and re-inoculated after 15 days (Souza Júnior et al., 2005). Seedlings were evaluated weekly for the presence of infection symptoms. 'Sunrise Solo' plants subjected to both inoculation and noninoculation were used as controls (Table 1). Plants were transplanted to the field in December 2003, aiming at new seed production and preliminary observations. Seed were stored in a refrigerator for 22 months, that was the waiting time for licenses to implement the field experiment (Contini et al., 2005). When they were sown, they had germinative power ranging from

0 to 56% (Table 1). Thus, it was necessary to set the field to renew the available seed. The field was set with PTP R-1 and R-2: PTP 1, PTP 2, PTP 3, PTP 4, PTP 5, PTP 6, PTP 7, PTP 8, PTP 10, PTP 17, PTP 18, and PTP 28 (Souza Júnior et al., 2005). Three plants were transplanted in each hole, and a hermaphrodite plant was maintained in the sexing. All seed produced in this field originated from self-fertilization, and seed harvested from each plant were maintained separated and received a different number. The populations were preliminary observed for the presence of PRSV symptoms and for agronomic characteristics, such as fruit size, shape, pulp color, type of fruit cluster produced and presence of fruit defects (carpelloidy and pentandry) (Sanches & Dantas, 1999; Dias et al., 2011). Samples from each PTP were evaluated by polymerase chain reaction (PCR) for the presence of CP/PRSV gene. DNA was extracted according to Doyle & Doyle's method (Almeida & Lima, 2001), and PCR was applied according to the conditions described by Souza Júnior et al. (2005).

Samples of 50 plants from transgenic papaya populations of generations R-2 and R-3 –(PTP 1/6, PTP 1/10, PTP 2/38, PTP 18/4, PTP 18/27 and PTP 28/104 – considered the most promising in the preliminary observations during multiplication in the field, were evaluated by PCR for the presence of protein coat gene (CP) of PRSV and neomycin phosphotransferase II (NPT II). The DNA extraction was made with the method proposed by Doyle & Doyle (Almeida & Lima, 2001), and PCR was applied according description by Souza Júnior et al. (2005). The CP gene segregation analysis was performed by PCR, and the results were analyzed by chi-square test (Ramalho et al., 2012).

Seedlings of the PTP R-2 and R-3 – PTP 1/6, PTP 1/10, PTP 2/38, PTP 3/46, PTP 17/92, PTP 18/4, PTP 18/22, PTP 18/27, PTP 28/75 – and the control 'Sunrise Solo' papaya (Souza Júnior et al., 2005) were produced in a greenhouse, as recommended by Sanches & Dantas (1999). One hundred plants of each population were challenged by mechanical inoculation with PRSV, as previously describe. These plants were transplanted into the experimental field in April 2005, in a randomized complete block design with three replicates per treatment, and each plot contained six plants. Plants were monitored weekly for the presence of PRSV symptoms and tested by indirect ELISA for PRSV, at the end of the experiment (Almeida & Lima, 2001). Samples were considered as testing positive for PRSV when readings showed twice the average reading of a healthy plant, in the microplate reader at 405 nm, 60 min after substrate addition to the enzyme. Each sample had two to three replicates on the plate.

Events were selected from plants without PRSV symptoms, with piriform fruit weighing approximately 500 grams, with orange pulp, well-formed fruit cluster, and showing less than 10% carpelloidy and without defects in the fruit, that are criteria adopted in the selection of papaya (Sanches & Dantas, 1999; Dias et al., 2011). From PTP 1/6/20, 1/6/59, 1/6/64, 1/6/90, 3/46/44, 3/46/52 and 18/27/97 selected in field, seedlings were produced in greenhouse and were subjected to CP gene segregation analysis by PCR, as previously described.

Table 1. Percentage of seed germination of transgenic papaya populations (PTP), generations R-1/R-2, and resistance after challenge by mechanical inoculation with *Papaya ringspot virus*-P (PRSV-P) in greenhouse, in the municipality of Cruz das Almas, in the state of Bahia, Brazil.

Population[(1)]	Germination (%)	Healthy plants (%)
PTP 1 (R-1)	13.5	96.3
PTP 2 (R-1)	52	58.2
PTP 3 (R-1)	26	78.8
PTP 4 (R-1)	15	16.7
PTP 5 (R-1)	3	80.0
PTP 6 (R-1)	4.5	22.2
PTP 7 (R-1)	29	13.8
PTP 8 (R-1)	24	23.4
PTP 9 (R-1)	0	0
PTP 10 (R-1)	34	5.8
PTP17 (R-1)	50	7
PTP 18 (R-2)	22	77.8
PTP 28 (R-1)	56	12.6

[(1)]200 seed sown per population.

Results and Discussion

In the first greenhouse evaluation of R-1/R-2 generations of PTP, the percentage of resistant plants was between 96.3% and 5.8% (Table 1). There was no variation in the symptoms that were vein clearing, leaf curl of the upper leaves, and mosaic (Figure 1). Similarly, Kung et al. (2010) observed a different percentage of transgenic 'Tainung Nº 2' resistant to PRSV, in plants containing an untranslatable construct of PRSV CP; however, in the present study, a difference in time was detected for symptom expressions. In China,

some transgenic papaya plants were infected by PRSV isolates, but they were recombinants (Wu et al., 2018).

The PTP plants 2/80, 4/30, 5/2, 6/2, 6/3, 7/21, 7/24, 7/44, 10/21, 10/25, 10/33, 17/28, 17/74, 17/92, and 28/75 transplanted in the field showed PRSV symptoms (Table 2). However, PTP 1/6, 1/7, 1/9, 1/10, 1/15, 2/38, 2/41, 2/56, 2/65, 3/27, 3/46, 3/48, 4/9, 4/27, 8/4, 8/23, 8/33, 18/3, 18/4, 18/8, 18/22, 18/27, 28/97, 28/104, and 28/110 showed no symptoms and no ELISA reaction (Tables 2 and 3). In the PCR analysis, out of 22 plants without symptoms, 20 contained the CP and NPT II genes (Figure 2), that is, in 90.9% cases showed a direct correlation between the presence of the CP gene and the resistance to PRSV. The resistance of transgenic papaya was also observed in association with trees with the presence of two or more transgene copies (Kung et al., 2010).

In the homozygosis analysis of the transgenic papaya populations selected in the field, PTP 1/6 showed the presence of transgene in all individuals, indicating the homozygous condition of the genitor, and all were resistant (Figure 3). Out of the 17 plants of the population PTP 3/46, 15 had the CP gene and showed no ringspot symptoms in the field, and two did not contain the CP gene, but showed PRSV symptoms. There was no significant difference in the segregation of the CP gene in the population PTP 3/46 (15 resistant plants, and 2 susceptible plants) which is expected when a double insertion of a gene occurs in hemizygosis (Table 4) (Ramalho et al., 2012). All individuals in PTP 17/92 showed symptoms of PRSV, and most plants from the PTP 28/75 did not contain the CP gene, thus, they were not evaluated for homozygosis. The CP gene was found to be in 15:1 segregation in PTP 1/10 and 15:3 in PTP 18/22. After the chi-square test, there was no significant difference in relation to segregation, which is indicative of two insertions in hemizygosis (Figure 3 and Table 4). PTP 2/38 and PTP 28/104 were not evaluated for homozygosis. The CP gene of Brazilian PRSV isolates has a high similarity, therefore, the production of transgenic plants for Brazilian isolates of the virus should provide a broad resistance to ringspot in Brazil (Souza Júnior et al., 2005). Some factors may influence the resistance showed by transgenic papaya trees to PRSV, such as the homology between the CP of the transgene and the CP of the present PRSV isolate, the emergence of new strains, the virus encoding of suppressors of the post-transcription silencing, plant stage, number of copies of the transgene, and environmental conditions, which may generate a synergistic or additive effect with PRSV (Kung et al., 2010; Hamim et al., 2018; Wu et al., 2018).

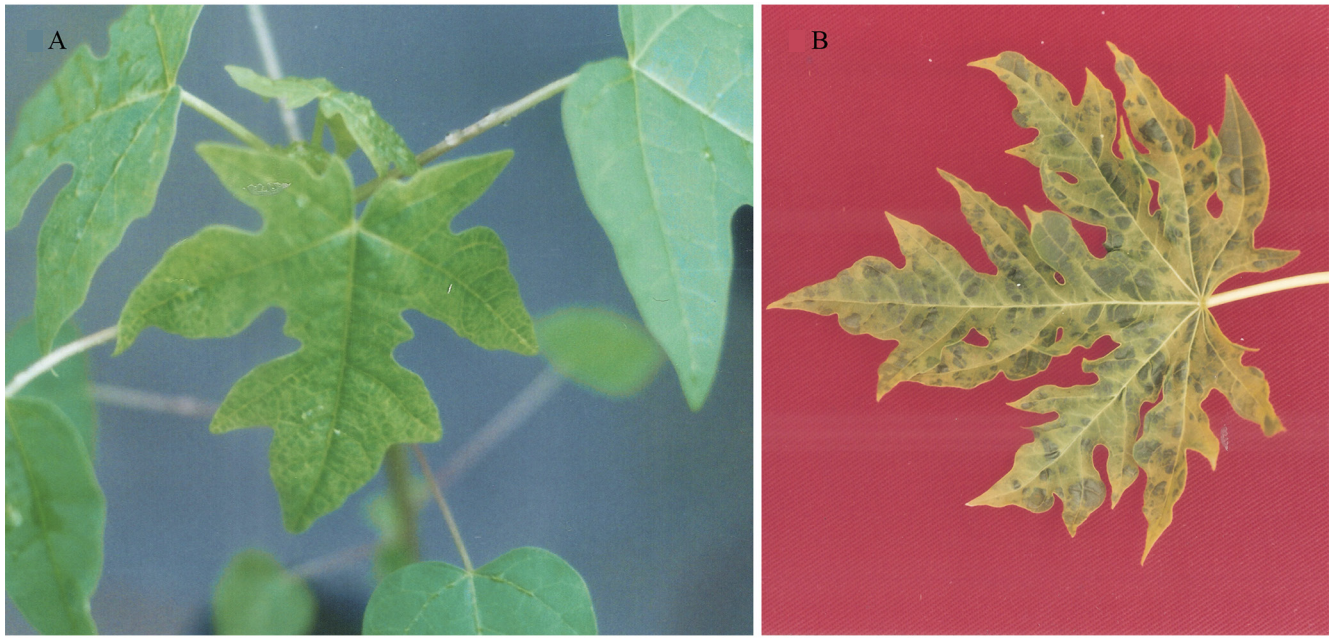

Figure 1. Symptoms showed by some plants of transgenic papaya trees challenged by mechanical inoculation with *Papaya ringspot virus* (PRSV) and kept in greenhouse: A, leaf curl and vein clearing; B, mosaic. Photos by Paulo Ernesto Meissner Filho.

Table 2. Challenge of transgenic papaya generations R-2/R-3 for *Papaya ringspot virus*-P (PRSV-P) in greenhouse and with natural exposure in the field.

Population	Healthy plants in the CV[1] (%)	Healthy plants in the field[2] (%)	Population	Healthy plants in the CV[1] (%)	Healthy plants in the field[2] (%)
PTP 1/6 (R-2)	100	100	PTP 17/92 (R-2)	58	17
PTP 1/10 (R-2)	85	88	PTP 18/4 (R-3)	86	94
PTP 2/38 (R-2)	60	0	PTP 18/22 (R-3)	87	83
PTP 2/80 (R-2)	49	NT	PTP 18/27 (R-3)	84	89
PTP 3/46 (R-2)	98	82	PTP 28/75 (R-2)	88	11
PTP 7/44 (R-2)	20	NT	PTP 28/104 (R-2)	41	NT
PTP 8/23 (R-2)	15	NT	Sunrise Solo	34	0

[1]Inoculation in 100 plants of each population. NT, not tested in the field. [2]Eighteen plants evaluated.

Table 3. ELISA evaluation of generations of transgenic papaya trees selected as resistant to *Papaya ringspot virus*-P (PRSV-P), in each transgenic population in the field.

Population	Absorbance			Absorbance average
PTP 1/6	0.078	0.030	-	0.054 (-)
PTP 1/7	0.067	0.071	-	0.069 (-)
PTP 1/9	0.000	0.000	-	0.000 (-)
PTP 1/10	0.008	0.000	-	0.004 (-)
PTP 1/15	0.067	0.071	-	0.069 (-)
PTP 2/38	NT	NT	NT	NT
PTP 2/41	0.004	0.000	-	0.002 (-)
PTP 2/56	0.044	0.000	-	0.022 (-)
PTP 2/65	NT	NT	NT	NT
PTP 3/27	NT	NT	NT	NT
PTP 3/46	0.081	0.041	-	0.061 (-)
PTP 3/48	NT	NT	NT	NT
PTP 4/9	0.078	0.005	-	0.042 (-)
PTP 4/27	0.040	0.022	-	0.031 (-)
PTP 8/4	0.043	0.030	-	0.037 (-)
PTP 8/23	0.024	0.000	-	0.012 (-)
PTP 8/33	NT	NT	NT	NT
PTP 18/3	0.010	0.015	-	0.012 (-)
PTP 18/4	0.038	0.009	-	0.024 (-)
PTP 18/8	0.023	0.000	-	0.012 (-)
PTP 18/22	0.000	0.031	-	0.016 (-)
PTP 18/27	0.000	0.045	-	0.022 (-)
PTP 28/97	0.038	0.028	-	0.033 (-)
PTP 28/104	NT	NT	NT	NT
PTP 28/110	0.042	0.011	-	0.026 (-)
PTP 1/6/20	0.121	0.102	0.083	0.102 (-)
PTP 1/6/59	0.046	0.045	0.037	0.050 (-)
PTP 1/6/64	0.170	0.169	0.187	0.175 (+)[1]
PTP 1/6/90	NT	NT	NT	NT
PTP 3/46/44	0.025	0.032	0.035	0.031 (-)
PTP 3/46/52	0.064	0.067	0.069	0.067 (-)
PTP 18/27/97	0.097	0.073	0.073	0.081 (-)
PTP 18/27/200	0.067	0.057	0.058	0.061 (-)
Healthy Sunrise Solo	0.056	0.056	0.052	0.054 (-)
Healthy Sunrise Solo (2 x)				0.108 (-)
Infected Sunrise	0.371	0.369	0.381	0.374 (+)

[1]Plants with absorbance in the ELISA show twice the value obtained in the analysis of healthy plants. NT, not tested; (-) healthy plants; (+), infected plants.

Nonetheless, in China, Kung et al. (2015) observed a breakdown of resistance to PRSV irrespectively of sequence homology by a gene silencing suppressor by more virulent strains.

The events selected in the field – PTP 1/6/20, 1/6/59, 1/6/64, 1/6/90, 3/46/44, 3/46/52 and 18/27/97 – had piriform fruit weighing approximately 500 g, with orange pulp, well-formed fruit cluster, with less than 10% of carpelloidy and without defects in the fruit, as recommended by Sanches & Dantas (1999) and Dias et al. (2011) (Figure 4). The plants showed no PRSV symptoms (Table 2), and had negative results for PRSV by indirect ELISA test (Table 3); plants 1/6/59 and 3/46/52 showed homozygosis of the CP gene (Table 4 and Figure 5). As observed in Hawaii, homozygous papaya 'SunUp' showed resistance to several PRSV

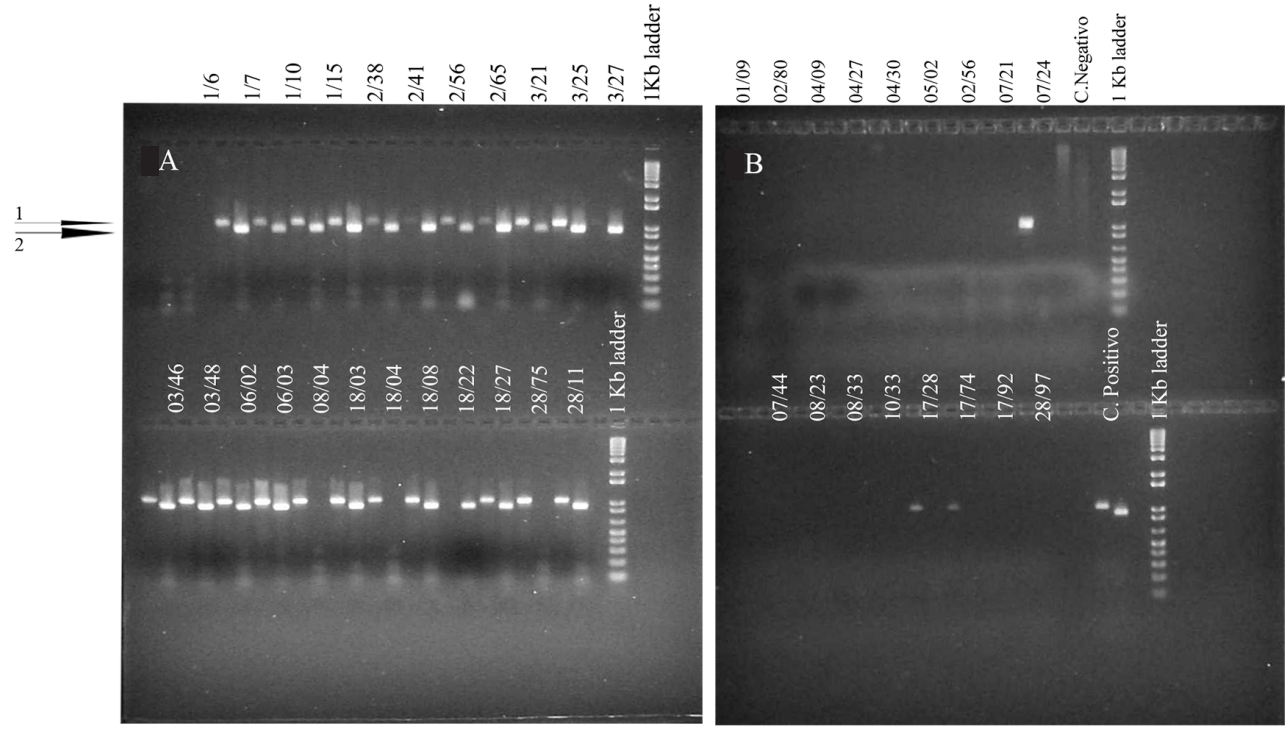

Figure 2. Gel of PCR product from transgenic and nontransgenic papaya trees using primers for the Npt II (1st band) and CP/PRSV (2nd band) genes.

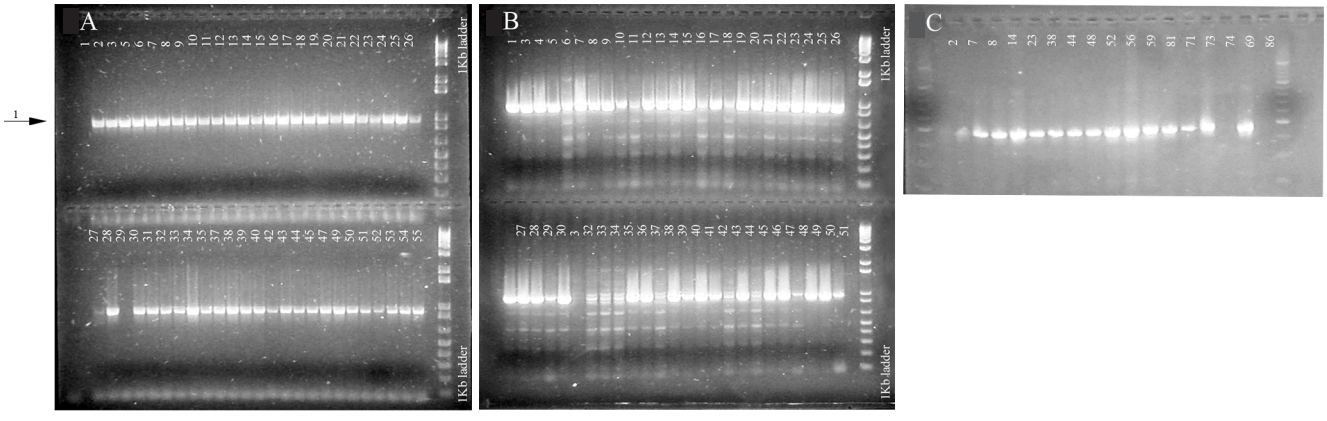

Figure 3. Gel of PCR products for the CP/PRSV of transgenic plants of different populations: A, population PTP 1/6; B, population PTP 1/10;. and C, population PTP 3/46. Molecular-weight size marker DNA Ladder 1 kb.

Table 4. Average squares of chi-square analysis of the CP/PRSV gene presence in transgenic papaya populations.

Population	GL	Band observed	Band absence	Presence of expected band	Absence ofexpected band	X^2
PTP 1/10	1	45	5	46.875	3.125	1.20^{ns}
PTP 3/46	1	15	2	15.9375	1.0625	0.88^{ns}
PTP 18/22	1	15	3	16.875	1.125	3.3^{ns}

^{ns}Nonsignificant, by X^2 test, at 5% probability.

Figure 4. General view of clusters of transgenic papaya fruit selected as elite events R-3 and R-4: A, 1/6/20; B, 1/6/59; C, 1/6/64; D, 1/6/90; E, 3/46/44; F, 3/46/52; G, 18/27/97; and H, 'Sunrise Solo' conventional. Photos by Paulo Ernesto Meissner Filho.

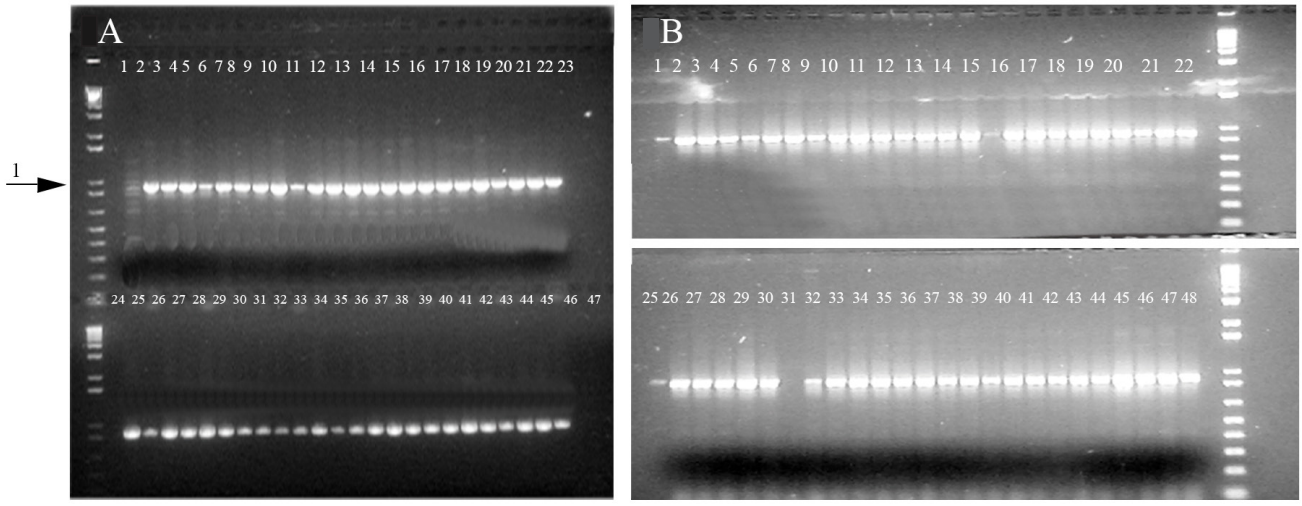

Figure 5. Homozygosis analysis of CP gene of transgenic papaya trees events in the R-3 generation by PCR: A, PTP 1/6/59; and B, PTP 3/46/52. Molecular-weight size marker DNA Ladder 1 kb.

isolates, but hemizygous 'Rainbow' did not show it (Tennant et al., 2001).

Actually, new transgenic papaya plants development in Brazil should to be resistant to both PRSV and to PMeV, the most important papaya viruses in the country.

Conclusion

The papaya populations PTP 1/6/59 and 3/46/52 show resistance to the *Papaya ringspot virus* (PRSV), as well as good agronomic characteristics, and the CP gene in homozygosis.

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