Development of Papaya Breeding Lines with Transgenic Resistance to *Papaya ringspot virus*

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ABSTRACT

Davis, M. J., and Ying, Z. 2004. Development of papaya breeding lines with transgenic resistance to *Papaya ringspot virus*. Plant Dis. 88:352-358.

Papaya (*Carica papaya*) was transformed via *Agrobacterium*-mediated transformation with four constructs containing either the unmodified or modified coat protein (CP) gene of Florida isolate H1K of *Papaya ringspot virus* (PRSV). The CP genes were in the sense orientation (S-CP), antisense orientation (AS-CP), sense orientation with a frame-shift mutation (FS-CP), or sense orientation mutated with three-in-frame stop codons (SC-CP). In all, 256 putative transgenic lines with the CP constructs were inoculated mechanically with PRSV H1K. None of the lines was immune to PRSV; however, highly resistant lines were found in each CP transgene group. For breeding purposes, 21 PRSV-resistant lines representing the four transgene constructs were selected and crossed with six papaya genotypes. The lines from the FS-CP and SC-CP transgene groups were highly fertile, but those from the S-CP and AS-CP transgene groups were practically infertile. Plants derived from 54 crosses and representing 17 transgenic lines were planted in the field. After 1 year in the field, 293 of the 1,258 the plants (23.3%) became naturally infected with PRSV; whereas, 29 of 30 of the nontransgenic control plants (96.7%) became infected. The incidence of PRSV infection varied in the R_1 progeny depending on both the transgenic line and the nontransgenic parent.

Papaya ringspot virus (PRSV) is a nonpersistent aphid-transmitted member of *Potyvirus* that causes one of the most economically important diseases of papaya. PRSV has been reported as a major limiting factor in papaya production in Hawaii (20), Florida (8), Caribbean countries (20,29), South America (19,20), Africa (21), Asia (27,34), and Australia (32). In papaya, PRSV causes mottling and distortion of leaves, ringspots on fruit, watersoaked streaks on stems and petioles, plant stunting, and drastic reductions of the fruit size and production (36). PRSV is difficult, if not impossible, to control by conventional methods (9,10,14,18,25,37). However, papaya genetically transformed with a coat protein (CP) gene of the virus has proven to be resistant to the virus (2,16,23,24). In Hawaii, where the papaya industry appeared to be doomed by PRSV, the introduction of transgenic PRSV-resistant papaya lines into commercial production has revitalized the industry (17).

PRSV is a single-stranded, positive-sense RNA virus (13,26). The RNA is encapsulated with a CP that is composed of single units with a mass of 32.6 kDa. Genetic variation exists among CP genes of PRSV strains from different geographic locations (1,2,11,33). Transgenic papaya expressing the CP gene of Hawaiian PRSV isolate HA 5-1 was shown to be highly resistant to PRSV isolates from Hawaii but more susceptible to PRSV isolates from other geographic locations (7,18,31). The degree of resistance appeared to be directly related to the extent of sequence homology between the CP transgene and the CP gene of the challenge isolate (2,31).

Because of the apparent homology dependence of PRSV CP transgene-associated resistance, we assumed that utilization of a CP gene of a local isolate might be a prerequisite to obtain effective PRSV resistance in transgenic papaya plants for the geographic region as long as genetic variation among strains in the region is not a limiting factor. To evaluate this, we examined 27 PRSV isolates from south Florida and found that they were very closely related based on nucleotide sequences of the CP gene, and were more distantly related to 23 other isolates from throughout the world (11). Isolates from Puerto Rico and Mexico appeared more closely related to the Florida isolates than were isolates from more distant locations. These results indicated that transgenic PRSV-resistance based on the CP gene of a Florida isolate might be successfully used to manage PRSV in Florida and other locations in the Caribbean region. To aid in the development of transgenic PRSV-resistant papaya, we developed a method for efficient *Agrobacterium*-mediated genetic transformation and rapid regeneration of papaya plants (40). Because transgene constructs with nontranslatable CP genes of other potyviruses have been shown to confer virus resistance in plants (22,28,35), we developed and tested different constructs with the CP gene of Florida PRSV isolate H1K in both translatable and nontranslatable forms in the present study. CP gene constructs were prepared in the sense or antisense orientations, and in the sense orientation with either a frame-shift mutation or a three-in-frame stop codon mutation. Following *Agrobacterium*-mediated transformation with the different constructs, papaya plants were obtained with each construct that exhibited high levels of resistance to PRSV. Selected transgenic lines were crossed with elite papaya genotypes to initiate a breeding program for development of PRSV-resistant papaya cultivars. Progeny from selected crosses were installed in a field planting and evaluated for natural infection by PRSV.

MATERIALS AND METHODS

Transgene construction. A PRSV CP gene from the Florida isolate H1K was amplified by reverse-transcription polymerase chain reaction (RT-PCR), cloned into pGEM-T vector, and sequenced (GenBank AF 196839) as previously reported (11). The primers used in transgene construction were F0 (5′-AGA**ACTAGT** CCCGGGTGGTCAGTCCCTT*ATG*TCCA AAAATGAAGCTGTGGATG-3′), F00 (5′-AGAAGATCTCCCGGGTGGTCAGTCC CTTATGTCCAAAAATGAAGCTGTGG ATG-3′), F01 (5′-AGA**ACTAGTC**CCGG GTGGTCAGTCCCTT*ATG*TTCCAAAAA TGAAGCTGTGGATG-3′), F02 (5′-AGA **ACTAGT**CCCGGGTGGTCAGTCCCTT *ATG*TAGTGATAAAATGAAGCTGTGGA TGCTG-3′), R01 (5′-TACAGATCTACC CTCACTATAAAATAGAAGC-3′), and R02 (5′-TAC**ACTAGT**ACCCTCACTAT AAAATAGAAGC-3′) (the *Bgl*II site is underlined, the *Spe*I site is in bold type, and the first initiation codon *ATG* is in italic type). The cloned CP gene was used as a template. Based on reports by Cheng et al. (5,6), the *uid*A leader was more ef-

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This work was supported in part by grant no. 2001-34135-11150 from the USDA, CSRS, Tropical-Subtropical Agriculture Research Program.

This article is published as Florida Agricultural Experiment Stations Journal Series No. R-09447.

Accepted for publication 21 November 2003.

Publication no. D-2004-0120-03R

fective for transformation than a homologous virus leader and enhanced the expression of the PRSV CP. Therefore, the *uid*A leader with an initiation codon (5′-CTAGTCCCGGGTGGTCAGTCCCTT*ATG*-3′) was placed at the 5′ end of all PRSV CP constructs by inclusion of the sequence in the appropriate primer. The sense open reading frame (ORF) of the CP gene was amplified with primers F0 and R01. The antisense fragment was amplified with primers R02 and F00. The frame-shift fragment was amplified with primers F01 and R01. The three-in-frame stop codon fragment was amplified with primers F02 and R01. PCR was performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). The conditions for PCR reaction were described previously (12). The PCR parameters were 40 cycles of 94°C for 1 min, 52°C for 1.5 min, and 72°C for 1 min, followed by a final extension 72°C for 10 min. All four fragments were confirmed by sequencing as described by Ying et al. (39).

The binary vector pBI121 (BD Biosciences Clontech, Palo Alto, CA) was double-digested with *Sma*I and *Sac*I, blunted with T4 DNA polymerase, and then relegated with T4 DNA ligase to produce pBI121zy (W/O-CP; Fig. 1A). Linearized pBI121zy was produced by digestion with *Xba*I and *Bam*HI. The four PRSV-CP fragments were separated by agarose gel (0.7%) electrophoresis, isolated from the gel using Wizard PCR Preps DNA Purification kit (Promega Corp., Madison, WI), double-digested with *Bgl*II and *Spe*I, and then cloned into the linearized pBI121zy (Fig. 1).

Generation of transgenic plants. The procedure for generation of transgenic papaya plants was described previously by Ying et al. (40). Immature zygotic embryos were obtained from papaya seed (*Carica papaya* L. cv. F65; Known-You Seed Co., Kaohsiung, Taiwan). Approximately 1 g of embryogenic culture derived originally from five immature zygotic embryos was used for *Agrobacterium*-mediated transformation, with each construct using *Agrobacterium tumefaciens* LBA4404 cells purchased from Life Technologies (Grand Island, NY).

PCR and Southern blot analyses. Total DNA was extracted from approximately 50 mg of fresh young leaves using plant DNAzol (Life Technologies). The primers used for amplification of an 840-bp fragment of the *npt*II gene were 5′-ATAATCG GATCCGGATCTGGATCGTTTCGCATG -3′ and 5′-ACCCCAGATCTCCGCTCA GAAGAACTCGTCAAG-3′ and those for amplification of a 555-bp fragment of the PRSV CP gene were 5′-ACCATGGGA

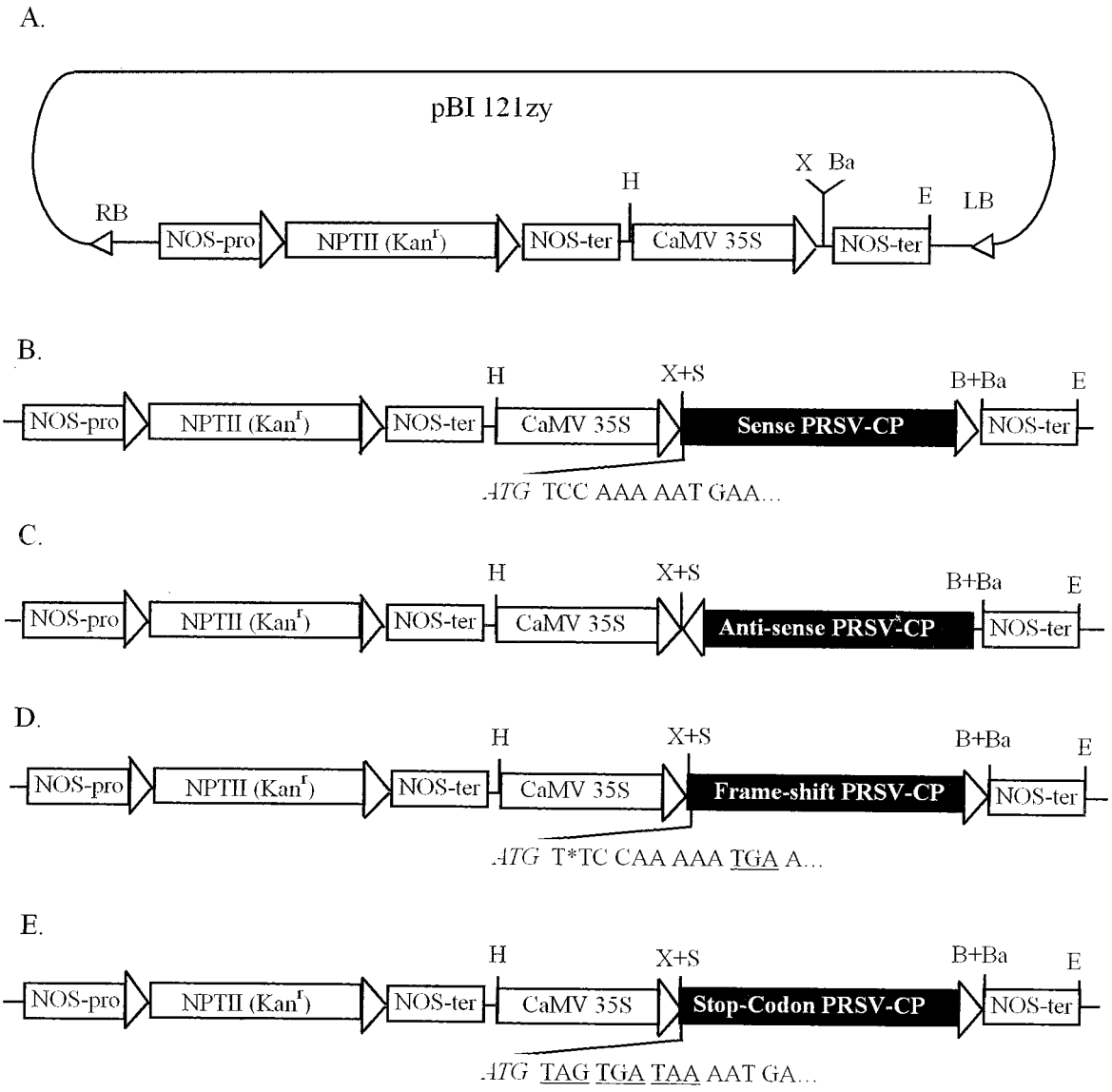

Fig. 1. Diagrams of constructs used for *Agrobacterium*-mediated transformation of papaya. **A,** pBI121zy without coat protein (CP) gene (W/O-CP). **B,** Sense *Papaya ringspot virus* (PRSV) CP (S-CP) construct. **C,** Antisense (AS-CP) construct. **D,** Frame-shift (FS-CP) construct with a "T" inserted immediately after the initiation codon *ATG*. **E,** Three in-frame stop codons (SC-CP) construct with the stop codons underlined. The first five codons of each CP transgene are indicated. The first initiation codons (*ATG)* are italicized. Restriction enzyme sites: Ba, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; at X+S, both ends of *Xba*I and *Spe*I were ligated; and at B+Ba, the ends of *Bgl*II and *Bam*HI ligated. RB, Right border; LB, left border.

ACTTTCACTGTTCCGAG-3′ and 5′-AGCTTTCATCTGCATGTGAGCTTCGC-3′. PCR was performed as described above. PCR products were separated by electrophoresis on 1.5 and 1.8% agarose gels, respectively.

For Southern analysis, papaya nuclear DNA was isolated using Floraclean (Q.Biogene, Carlsbad, CA) and digested with *Eco*RI or *Hin*dIII that each cut all transgene constructs once. The digested DNA was electrophoresed in a 0.6% agarose gel in 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and transferred onto nylon membranes (Maximum Strength Nytran, 0.2-µm pore size; Schleicher & Schuell, Keene, NH), as described by Ying et al. (38). A 555-bp fragment of the PRSV CP gene was generated by PCR using cloned PRSV CP DNA as a template. The fragment was digoxigenin-labeled (Boehringer Mannheim, Indianapolis, IN), and used as a hybridization probe. The conditions for Southern hybridization were described previously (38).

Evaluation of resistance to PRSV. PRSV isolate H1K was maintained in papaya plants in a greenhouse for inoculum production. Young, fully expanded leaves (approximately 2 g) from infected plants were excised, put into a cold mortar with 20 ml of 10 mM sodium phosphate buffer (pH 7.0, 4°C), and ground with 2 g of Carborundum (600 mesh) on ice. For challenge inoculations, the entire upper surfaces of two young leaves of each plant were rubbed gently with a pestle wetted with inoculum. Inoculations were repeated once after 2 weeks. The plants were grown in a greenhouse and were approximately 10 weeks old when first inoculated. Disease severity ratings were recorded for two adjacent newly expanded leaves as follows: nonsymptomatic = 0; only a few, tiny chlorotic spots = 1; weak mosaic symptoms = 2; moderate mosaic symptoms = 3; severe mosaic symptoms without leaf distortion = 4; and very severe mosaic symptoms with leaf distortion = 5. Data were taken starting 14 days after the first inoculation and then on days 18, 23, 28, 32, 36, 41, 45, 50, and 58.

Analysis of R_1 generation for field resistance to PRSV. All transgenic lines (R_0) were pistillate. Twenty-one transgenic lines were pollinated by hand to produce seed for the R_1 generation. The plants were grown in 40-gallon pots in a greenhouse. Pollen was obtained from male flowers of gynodioecious cultivars that were being grown commercially in Florida or being grown experimentally at the Lajas Substation of the University of Puerto Rico. The local cultivars were Red Lady and Experimental No. 15. The cultivars grown in Puerto Rico were selections of B. R. Brunner (Department of Horticulture, University of Puerto Rico, Mayaguez) and were 'Puerto Rico 6-65,' 'Tainung No. 5,' 'Solo 40,' and 'Sunrise.' Red Lady and Tainung No. 5 are tolerant to PRSV, and Puerto Rico 6-65 and Sunrise are highly sensitive to PRSV (10). The relative sensitivities to PRSV of the other cultivars used in this study have not been reported. A camel-hair brush was used to transfer pollen to the stigmatic surfaces of female flowers at anthesis, and the pollinated flowers then were labeled with tags for identification. Seed were collected from the fruit at maturity, dried at room temperature after removing the sarcotesta by hand, and stored at 4 to 8°C.

For germination, seeds were soaked for 3 h in a 1-g/liter solution of Miracle-Gro fertilizer (Scotts Miracle-Gro Products, Inc., Port Washington, NY) and then planted in 98-well seedling trays containing Pro Mix BX soil mix (Premier Horticulture, Ltd., Dorval, Quebec, Canada) amended with 14-14-14 Osmocote (Scotts-Sierra Horticultural Products Co., Maryville, OH) at 6.7 kg/m^3 of soil mix. Seeds were germinated and seedlings grown in a greenhouse at 28 to 34°C. When seedlings were 3 to 4 weeks old, they were sprayed to run-off with kanamycin sulfate (Agri-Bio, Miami, FL) at 2 mg/ml in water with 0.02% SilWet L77 (Setre Chemical Co., Memphis, TN). Seedlings were sprayed using a high-pressure spray gun at 80 psi at a distance of 10 to 20 cm from the plants. Each tray of seedlings was put immediately into a black garbage bag and kept out of direct sunlight for 12 to 14 h overnight to allow the kanamycin solution to be absorbed by the plants. The trays were removed from the bags and placed back on the greenhouse bench. Chlorosis of foliage of seedlings without the *npt*II gene was seen after approximately 7 days. Chlorotic seedlings were discarded, and nonchlorotic seedlings were transplanted to 24-well seedling trays.

Seedlings were transplanted to the field on 15 March 2001. Seedlings were planted in rows in groups of three plants with a spacing of 30 cm between plants. The groups were planted in a 2-m-wide zigzag pattern with 2 m between centers of adjacent groups within each row. After flowering (4- to 5-month-old plants), one plant in each group, the most vigorous hermaphrodite when available, was retained. Rows were 3.65 m apart. Each row had eight plots containing eight plants each. One to five plots per cross were planted randomly within the field. Nontransgenic controls also were planted in the field and consisted of the cvs. Cariflora, Puerto Rico 6-65, Puerto Rico 6-65 Dwarf, Maradol, Tainung No. 5, and Solo 40. Data on PRSV incidence was recorded for 1 year after planting.

RESULTS

Generation of PRSV transgenic plants. After transformation with each transgene construct (Fig. 1), 10 clumps of embryogenic culture originating from 25 immature zygotic embryos produced an average of 7.7 kanamycin-resistant shoots from each clump (approximately 100 mg of embryogenic culture). Of 385 putatively transgenic shoots, 360 (93.5%) developed roots upon transfer to rooting medium and subsequently were established in soil. The numbers of putatively transgenic plants produced were 68 with the sense CP (S-CP) construct, 42 with the antisense CP (AS-CP) construct, 83 with the frame-shift CP (FS-CP) construct, 107 with the stop codon CP (SC-CP) construct, and 60 with the construct without a CP (W/O-CP). All the regenerated plants appeared normal, except for two that appeared dwarfed.

PRSV resistance evaluation. Of the 300 putatively transgenic lines derived by transformation with the different CP constructs, 256 of the most vigorous lines were inoculated with PRSV H1K. Reac-

Table 1. Distribution of *Papaya ringspot virus* (PRSV) resistance among putative transgenic lines inoculated with PRSV isolate H1K from Florida and evaluated for disease severity every 4 to 5 days for 2 months

		Frequency of plants in PRSV resistance groups[a]							
		Susceptible		Weakly resistant		Moderately resistant		Highly resistant	
Construct[b]	No. of plants	No.	Percent	No.	Percent	No.	Percent	No.	Percent
W/O-CP	10	10	100	0	0	0	0	0	0
S-CP	64	43	67.2	10	15.6	8	12.5	3	4.7
AS-CP	34	17	50.0	8	23.5	5	14.7	4	11.8
FS-CP	72	24	33.3	15	20.8	30	41.7	3	4.2
SC-CP	86	32	37.2	18	20.9	29	33.7	7	8.1

[a] PRSV-resistance groupings were based on mean severity ratings: susceptible, ≥4.0; weakly resistant, >2.0 but <4.0; moderately resistant, >1.0 but ≤2.0; and highly resistant, ≤1.0.

[b] Transgene constructs: W/O-CP = construct without a CP, S-CP = CP genes were in the sense orientation, AS-CP = antisense orientation, FS-CP = sense orientation with a frame-shift mutation, and SC-CP = sense orientation mutated with three-in-frame stop codons.

tions to PRSV varied among these plants from highly susceptible to highly resistant (Table 1). In contrast, typical PRSV symptoms developed in all PRSV-inoculated control plants, including both vector controls and nontransformed controls. PRSV symptoms began to appear within 1 week after inoculation of susceptible plants but were delayed for various times in the more resistant lines. During the first month after inoculation, all 10 of the nontransgenic control plants and all 10 control plants transformed with the W/O-CP construct developed severe PRSV symptoms, including both mosaic and leaf distortion symptoms. Likewise, 116 of the 256 (45.3%) plants tested that were putatively transformed with CP constructs also developed severe PRSV symptoms, and the reaction to PRSV in these plants was indistinguishable from those in the controls both in respect to rate of development and severity. Susceptibility was not due to the absence of the CP transgene in all of these plants, because the transgenes were detected by PCR in 67 of 113 (59.3%) plants tested (*data not shown*). The remainder of the PRSV-inoculated transgenic plants exhibited some level of resistance to PRSV. Fifty-one (19.9%) of the plants were weakly resistant. These weakly resistant lines displayed a reduced rate of symptom development, but eventually developed severe mosaic and leaf distortion symptoms. Symptoms in the 89 remaining lines, including 72 (28.1%) moderately and 17 (6.6%) highly resistant lines, typically did not develop past a mild mosaic in affected leaves and often were remittent during the 2-month evaluation period after inoculation.

PCR and Southern analyses. Total DNA from three putative transgenic plants for each of the four PRSV-CP transgenes was tested by PCR for the presence of both the *npt*II and PRSV CP transgenes, and all 12 plants were positive for both transgenes. As would be expected, results for a plant transformed with the vector without CP genes (W/O-CP) were positive only for the *npt*II gene, but not for a CP gene.

After inoculation with PRSV isolate H1K, 85 lines within the S-CP, AS-CP, FS-CP, and SC-CP groups, which for the most part appeared moderately to highly resistant to PRSV, were subjected to Southern analyses to determine the number of copies of the transgenes present. Transgenes were detected in 84 of the lines. One to nine transgene copies were found in the transgenic plants, and the insertions appeared random (*data not shown*). Results for a nontransgenic control plant were negative. In all, 46.4% (39/84) of the plants had single copies, 32.1% (27/84) had two copies, and only 21.4% (18/84) had more than two copies (Table 2).

Inheritance of transgenes. Twenty-one transgenic lines were selected to initiate a papaya cultivar development program. The selections were based on plant vigor, resistance to PRSV, and, in most cases, having a single copy of the transgenes. All selections ranged from moderately to highly resistant to PRSV. All plants regenerated from somatic embryos, including those transformed with the CP constructs, were pistillate. Newly opened flowers on the selected lines were pollinated with pollen obtained from nontransgenic gynodioecious papaya cultivars. All lines with the SC-CP and FS-CP transgenes were fertile; these lines produced an average of 352.5 seed per fruit (59,218 seed total) and germination was greater than 80% overall tested. However, the lines with the S-CP and AS-CP transgenes were practically infertile; they only produced an average of 5.0 seed per fruit (121 seed total) and germination was only 3.3% overall.

After spraying the seedlings with kanamycin, 49.6% of the seedlings failed to develop chlorotic leaves or have other adverse effects, indicating that they had inherited the *npt*II gene that conferred resistance to kanamycin (Table 3). The nontransgenic seedlings without the *npt*II gene became chlorotic within 2 weeks of treatment, and the extent of chlorosis varied from a few bleached areas on newly expanded leaves to bleaching of all new foliage. Chlorotic tissues did not recover. The R_0 transgenic plants had been crossed with nontransgenic plants; therefore, the segregation of transgenes was expected to vary with the number of transgenes in each of the R_0 plants (Table 3). The segregation of transgenes in the progenies of R_0 plants with one transgene copy agreed well with the expected 1:1 ratio (50% of progeny with transgene; $\chi^2 = 17.84$, $P = 0.91$, overall progeny with single transgene copy number). However, the numbers of kanamycin-resistant seedlings were generally much less than expected in the progenies of R_0 plants with more than one transgene copy.

Table 2. Number of transgene copies in transgenic papaya lines exhibiting moderate to high levels of *Papaya ringspot virus* (PRSV) resistance[a]

		No. of plants with indicated transgene copy number[b]		
Construct[c]	No. tested by Southern analysis	1	2	>2
S-CP	12	6	2	4
AS-CP	12	5	4	3
FS-CP	29	15	11	3
SC-CP	32[d]	13	10	8
Total	85	39	27	18

[a] Reaction to PRSV was evaluated after inoculation of putatively transgenic lines with PRSV isolate H1K.
[b] Consensus copy number was determined by Southern analyses.
[c] Transgene constructs: S-CP = coat protein genes were in the sense orientation, AS-CP = antisense orientation, FS-CP = sense orientation with a frame-shift mutation, and SC-CP = sense orientation mutated with three-in-frame stop codons.
[d] Transgenes were not detected in one plant of the SC-CP group.

Table 3. Segregation of transgenes in the R_1 progenies of lines[a]

Transgenic line[b]	No. of seedlings	Kan[r] (%)	Transgene copy no.	χ^{2c}	*P*
SC-CP-3	95	52.6	1	0.2632	0.6080
SC-CP-4	86	52.3	1	0.1860	0.6662
SC-CP-6	1,406	50.4	1	0.0711	0.7897
SC-CP-46	191	50.8	1	0.0471	0.8282
SC-CP-62	482	70.7	2	4.6501	0.0311
SC-CP-63	180	28.3	2	238.41	0.0000
SC-CP-68	196	46.9	1	0.7347	0.3914
SC-CP-72-1	234	49.1	3	314.73	0.0000
SC-CP-75	1,566	49.0	2	565.47	0.0000
SC-CP-88	174	64.4	2	10.490	0.0012
SC-CP-95	1241	45.4	3	2,007.3	0.0000
SC-CP-101	189	55.6	1	2.3333	0.1266
FS-CP-17-1	191	49.2	1	0.0471	0.8282
FS-CP-17-2	1,258	50.1	1	0.0032	0.9550
FS-CP-26	852	43.5	2	449.74	0.0000
FS-CP-33	185	48.1	1	0.2649	0.6068
FS-CP-87	166	48.8	1	0.0964	0.7562
Total	8,701	49.6	…	…	…

[a] Seedlings were sprayed with kanamycin sulfate (2 mg/ml) to detect the absence of the *npt*II gene in susceptible progeny by the appearance of bleached leaves.
[b] SC-CP = coat protein gene in sense orientation mutated with three-in-frame stop codons and FS-CP = sense orientation with a frame-shift mutation.
[c] The R_0 lines had been crossed with nontransgenic plants; therefore, transgenes were expected to segregate at a 1:1, 3:1, and 8:1 ratio for lines with 1, 2, and 3 copies of the transgenes, respectively. The χ^2 value and probability for goodness of fit to the predicted segregation level are given.

Progenies of 54 crosses representing 17 of the transgenic lines were transplanted to the field in March 2001 and evaluated for 1 year. Within 1 year of planting, 293 of 1,258 transgenic plants (23.3%) became naturally infected by PRSV based on the development of visual symptoms (Table 4). In comparison, 29 of 30 nontransgenic control plants (96.7%) in the same planting became infected. The incidence of nonsymptomatic plants varied from 61.1 to 95.4% among transgenic progeny of the six male parents, and from 12.1 to 89.6% among progeny of the 17 transgenic lines. Thus, although not apparently immune to PRSV, the transgenic papaya lines appeared to be resistant to natural infection by the virus.

DISCUSSION

Because of the severe constraints imposed by PRSV on papaya cultivation in Florida, papaya cultivar development in the area has been limited to the development of PRSV-tolerant papaya and has resulted in one notable success, namely the development of cv. Cariflora (9). Cariflora is a rare source of genetic tolerance to PRSV and used widely to provide genes for PRSV tolerance in papaya cultivar development programs throughout the world. However, papaya production still is limited substantially by PRSV even when using tolerant cultivars (18). Freed from the constraints imposed by PRSV, papaya cultivar development could be focused more effectively on improvement of other characteristics that would enhance production in different geographic areas, such as resistance to other diseases and insect pests, and cold tolerance in Florida. The successful development of the papaya cvs. SunUp and Rainbow with transgenic resistance to PRSV that are now being grown commercially in Hawaii has demonstrated that pathogen-derived resistance can be used effectively for cultivar development and PRSV management.

Advances in the protocols for developing transgenic papaya (3–5,40) have overcome problems incurred earlier due to low rates of transformation, high frequencies of abnormal plants, and long periods required for regeneration of somatic embryos (15,16). In the present study, transgenes were delivered efficiently into papaya somatic embryos by *Agrobacterium*-mediated transformation following procedures that we developed previously (40). Thus, we produced 360 papaya plants transformed with genetic constructs containing either the *npt* II gene or both the *npt*II gene and different forms of the CP gene of a local strain of the PRSV. Our success in obtaining a relatively high efficiency of transformation, and consequently a three or more times greater number of transgenic papaya plants than in previous studies, was probably due, in part, to the use of cv. F65 in addition to refinements in the transformation protocol. Embryogenic cultures derived from F65 zygotic embryos readily produced somatic embryos that were amenable to transformation, apparently unlike the materials used in some other studies (15,23). F65 is an experimental cultivar and a recent ancestor in the pedigree of Red Lady, which is a PRSV-tolerant cultivar that is widely grown in Florida and elsewhere. We decided to use F65 because of its propensity toward somatic embryogenesis and transformation, and then develop new cultivars to meet various horticultural and marketing criteria.

Initial transformations of papaya with the CP gene of PRSV, either by microprojectile bombardment (15) or by *Agrobacterium*-mediated transformation (5), utilized CP genes from local isolates in the sense orientation, and the genes were expressed in the transformed plants. Subsequently, untranslatable CP genes also were engineered into papaya that either had been intentionally mutated by creation of an in-frame shift after the third amino acid (2) of the CP gene or had been produced inadvertently by a base change in the CP gene creating a stop codon mutation (23). In all of these cases, transformation of papaya with either translatable CP genes or untranslatable CP genes conferred resistance to PRSV. Similar results were obtained in the present study using translatable and untranslatable forms of the CP gene of a Florida PRSV isolate. In addition to using untranslatable CP gene constructs with frame shift and stop codon mutations, we also used a construct with the CP gene in the antisense orientation. Resistance conferred by both translatable and untranslatable PRSV CP genes appears to be RNA-mediated and due to post-transcriptional gene silencing (2,23,30). We assume that the same resistance mechanism was functional in our plants, because Northern analyses indicated that RNA transcripts were either undetectable or degraded in resistant plants transformed with each of the four CP gene constructs (*results not shown*).

Faced with the need to screen a large number of transgenic papaya lines for resistance to PRSV, we elected to inoculate the R_0 lines without first multiplying them. Because PRSV is not seed transmissible, we reasoned that PRSV would be eliminated in subsequent generations. Previous studies used either micropropagation (16,23) or crossing with nontransgenic

Table 4. Incidence of natural infections by *Papaya ringspot virus* PRSV in field plots of the R_1 generation of transgenic papaya lines containing the coat protein (CP) gene of PRSV strain H1K made nontranslatable with either a stop-codon (SC-CP) or frame-shift (FS-CP) mutation[a]

	No. with PRSV/no. of plants for progeny of the given male parent							
Line[b]	No. 15	Puerto Rico 6-65	Red Lady	Solo 40	Sunrise	Tainung No. 5	Grand total	Resistant (%)
SC-CP-3	…	…	2/16	…	…	…	216	87.5
SC-CP-4	…	…	2/16	…	…	…	2/16	87.5
SC-CP-46	…	…	…	…	11/17	9/16	20/33	49.4
SC-CP-6	…	0/41	0/40	0/41	11/40	34/40	45/202	87.8
SC-CP-62	4/16	3/16	2/17	…	7/16	6/16	2,281	72.8
SC-CP-63	…	…	…	…	6/8	0/8	6/16	62.5
SC-CP-68	…	…	…	…	11/16	10/16	21/32	45.2
SC-CP-72-1	…	…	0/8	…	13/17	10/17	23/42	55.2
SC-CP-75	3/40	2/41	1/33	1/32	8/41	14/41	29/228	87.3
SC-CP-88	…	…	…	…	8/16	6/16	14/32	56.2
SC-CP-95	0/34	0/24	1/32	…	8/40	8/32	17/162	89.5
SC-CP-101	…	…	…	…	12/16	17/17	29/33	12.1
FS-CP-17-1	…	…	…	…	3/16	3/16	6/32	81.2
FS-CP-17-2	4/16	6/40		5/32	15/49	5/41	35/178	80.3
FS-CP-26	0/16	…	1/33	…	4/16	6/41	11/106	89.6
FS-CP-33	…	…	…	…	5/16	1/16	6/32	81.2
FS-CP-87	…	…	…	…	1/8	4/9	5/17	70.6
Total	11/122	11/162	9/195	6/105	123/332	133/342	293/1,258	76.7
Resistant (%)	91.0	93.2	95.4	94.3	63.0	61.1	76.7	…
Control	…	…	…	…	…	…	29/30	3.3

[a] Plants were in the field for 1 year.
[b] SC-CP = sense orientation mutated with three-in-frame stop codons and FS-CP = sense orientation with a frame-shift mutation.

plants for seed production (2,16) to multiply transgenic lines before challenge inoculation of a representative portion of the resulting transgenic plants. Screening transgenic lines by inoculation as we did saves time and effort but is subject to potential problems. Tenant et al. (30) found that the transgenic cv. Rainbow, which is hemizygous for the CP gene of HA 5-1 PRSV isolate from Hawaii, was immune to the homologous PRSV isolate but more susceptible to heterologous PRSV isolates when younger than 17 weeks old. Although our lines were inoculated with the homologous H1K PRSV isolate, they were less than 10 weeks old when inoculated. Fortunately, large portions of our transgenic lines were substantially resistant to PRSV despite their young age. Another concern was whether the PRSV infection might negatively affect the fertility of the lines, making subsequent propagation difficult. Two lines each with the sense and antisense CP genes were found to be infertile following repeated pollination attempts with pollen from several different papaya cultivars. The transgenes alone or in combination with PRSV infection might have affected fertility. Testing to determine if other lines with the sense and antisense CP transgenes also were infertile was not possible because they had been discarded by the time the infertility had been detected. Infertility has not been associated with CP transgenes in other studies, and all our lines with the frame-shift or stop codon mutations of the PRSV CP gene remained fertile even after inoculation with PRSV.

The level of protection imparted by the different CP transgenes in the present study varied, presumably due to such factors as insertion site of the transgenes (position effect) in the papaya genome and the number of insertions. Similar variation in protection has been observed previously (2,16,23). None of our transgenic lines were immune to infection by PRSV, although immunity to infection by PRSV isolates has been reported previously (3,23). These immune lines frequently had multiple copies of the transgenes, but lines with single copies of the CP transgene have been found to be immune at least to the homologous PRSV isolate (16). There was no obvious correlation between transgene copy number and the level of protection against PRSV conferred in our R_0 lines; however, even if such a relationship existed, the differences in protection due to position effects at different transgene locations presumably might have interfered with detection of the relationship. Furthermore, we made an effort to select transgenic lines without multiple transgene copies to avoid the unpredictable segregation of multiple transgenes in subsequent generations, and this preference would have biased any subsequent evaluation of a potential relationship between copy number and the level of transgenic protection. Interestingly, the presence of multiple copies presented another problem possibly due to silencing of the *npt*II gene. In general, the *npt*II gene segregated as expected in the progeny of R_0 lines with single copies of the transgenes as indicated by the frequencies of kanamycin resistance, but lower than expected frequencies of kanamycin resistance in progenies were observed for multiple-copy R_0 lines. If such gene silencing exists when multiple copies of the *npt*II gene are present, then using kanamycin resistance as a marker to aid in the detection of transgenic progenies would favor selection of plants with single-copy transgenes. Consequently, any protective advantage of having multiple copies of the CP transgene might be lost.

In south Florida, the incidence of PRSV usually is very high in any field planting of papaya, as was observed in the nontransgenic papaya plants in our field test. Consequently, protection against natural infection by PRSV in the R_1 progenies of crosses between selected transgenic lines and nontransgenic genotypes was readily observed in the field test without the need for challenge inoculations. The results again indicated that the CP transgenes with both the frame shift and the stop codon mutations conferred substantial protection against PRSV in some lines. However, some R_1 lines appeared to be very susceptible to natural infection by PRSV in the field, even though their transgenic parents had appeared to have moderate to high resistance in the initial screening of the transgenic lines. In a previous study, we demonstrated that genetic variability existed among PRSV isolates in south Florida, although the isolates apparently were more closely related to one another than to isolates from other geographic regions (11). It is possible that this genetic variability might relate to pathogenic variability, but this has not been evaluated. Such pathogenic variability, however, might account for the differences observed between the results of challenge inoculations with the isolate from which the transgenes were originally derived and the result of natural infections with unknown isolates. Furthermore, the level of protection conferred by the CP transgenes appeared to be influenced by the genetic background provided by the nontransgenic male parent. Progenies derived from crosses of transgenic lines with Sunrise and Tainung No. 5 genotypes appeared to be more susceptible to PRSV infection than those having other nontransgenic genotypes as male parents. This can be understood for the Sunrise progenies because this cultivar is very susceptible to PRSV. Apparently, however, the progenies of Tainung No. 5 were susceptible to infection despite its reported tolerance to PRSV.

Although we would like to have found immunity to PRSV in some of our transgenic lines, the level of resistance found in some of the lines is promising. Further improvement in protection against PRSV might be obtained by selection for homozygosity of transgenes in subsequent generations. The SunUp papaya cultivar presently being grown in Hawaii is homozygous for the CP gene derived from line 55-1 and exhibits greater resistance to different PRSV isolates and at a younger age than does the hemizygous Rainbow cultivar derived from the same 55-1 line (30). We hope that, with careful selection of homozygous transgenic lines, papaya cultivars with substantial resistance to PRSV similarly can be developed for commercial production in Florida and elsewhere in the Caribbean region.

ACKNOWLEDGMENTS

We thank X. Yu for technical assistance and B. R. Brunner for providing papaya pollen from his genotype selections.

LITERATURE CITED

1. Bateson, M. F., Henderson, J., Chaleeprom, W., Gibbs, A. J., and Dale, J. L. 1994. Papaya ringspot potyvirus: isolate variability and origin of PRSV type P (Australia). J. Gen. Virol. 75:3547-3553.
2. Bau, H.-J., Cheng, Y.-H., Yu, T.-A., Yang, J.-S., and Yeh, S.-D. 2003. Broad spectrum resistance to different geographic strains of papaya ringspot virus in coat protein gene transgenic papaya. Phytopathology 93:112-120.
3. Cabrera-Ponce, J. L., Vegas-Garcia, A., and Herrera-Estrella, L. 1995. Herbicide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. Plant Cell Rep. 15:1-7.
4. Cai, W., Gonsalves, C., Tennant, P., Fermin, G., Souza, M., Jr., Sarindu, N., Jan, F.-Y., and Zhu, H.-Y. 1999. A protocol for efficient transformation and regeneration of *Caricia papaya* L. In Vitro Cell Dev. Biol. 35:61-69.
5. Cheng Y.-H, Yang, J.-S., and Yeh, S.-D. 1996. Efficient transformation of papaya by coat protein gene of papaya ringspot virus mediated by *Agrobacterium* following liquid-phase wounding of embryogenic tissues with carborundum. Plant Cell Rep. 16:127-132.
6. Cheng, Y.-H., Yang, J.-S., and Yeh, S.-D. 2000. Construction and evaluation of transgenic tobacco plants expressing the coat protein gene of papaya ringspot virus with different translational leaders. Bot. Bull. Acad. Sin. 41:1-10.
7. Chiang C.-H., Wang, J.-J., Jan, F.-J., Yeh, S.-D., and Gonsalves, D. 2001. Comparative reactions of recombinant papaya ringspot viruses with chimeric coat protein (CP) genes and wild-type viruses on CP-transgenic papaya. J. Gen. Virol. 82:2827–2836.
8. Conover, R. A. 1964. Distortion ringspot, a severe disease of papaya in Florida. Proc. Fla. State Hortic. Soc. 77:440-444.
9. Conover R. A., Litz, R. E., and Malo, S. E. 1986. 'Cariflora'—a papaya ringspot virus-tolerant papaya for South Florida and the Caribbean. HortScience 21:1072.
10. Crane, J. H., Dorey, A. J., Schaffer, B. A., and McMillan, R. T, Jr. 1995. Comparison of papaya ringspot virus effects on 23 cultivars and 18 selections of papaya (*Carica papaya*) in South Florida. Proc. Fla. State Hortic. Soc. 108:354-357.
11. Davis, M. J., and Ying, Z. 1999. Genetic diversity of the papaya ringspot virus in Florida. Proc. Fla. State Hortic. Soc. 112:194-196.
12. Davis, M. J., Ying, Z., Brunner, B. R., Pantoja, A., and Ferwerda, F. H. 1998. Rickettsial relative associated with papaya bunchy top dis-

ease. Curr. Microbiol. 36:80-84.
13. De La Rosa, M., and Lastra, R. 1983. Purification and partial characterization of papaya ringspot virus. Phytopathol. Z. 106:329-337.
14. Drew, R. A., O'Brien, C. M., Magdalita, P. M., and Drew, R. A. 1998. Development of *Carica* interspecific hybrids. Acta Hortic. 461:285-291.
15. Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9:189-194.
16. Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1992. Virus resistant papaya plants derived from tissue bombarded with the coat protein gene of papaya ringspot virus. Bio/Technology 10:1466-11472.
17. Gianessi, L. P., Silvers, C. S., Sankula, S., and Carpenter. J. E. 2002. Viral resistant papaya. Page 8 in: Plant Biotechnology: Current and Potential Impact for Improving Pest Management in the U. S. Agriculture, An Analysis of 40 Case Studies. National Center for Food and Agricultural Policy. Online publication.
18. Gonsalves, D. 1998. Control of papaya ringspot virus in papaya: A case study. Annu. Rev. Phytopathol. 36:415-437.
19. Herold, F., and Weibel. J., 1962. Electron microscopic demonstration of papaya ringspot virus. Virology 18:307-311.
20. Jensen, D. D. 1949. Papaya ringspot virus and its insect vector relationships. Phytopathology 39:212-220.
21. Lana, A. F. 1980. Transmission and properties of virus isolated from Carica papaya in Nigeria. J. Hortic. Sci. 55:191-197.
22. Linbo, J. A., and Dougherty, W. G. 1992. Pathogen-derived resistance to a potyvirus: Immune and resistance phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.
23. Lines, R. E., Persley, D., Dale, J. L., Drew, R., and Bateson, M. F. 2002. Genetically engineered immunity to papaya ringspot virus in Australian papaya cultivars. Mol. Breed. 10:119-129.
24. Lius, S., Manshardt, R. M., Fitch, M. M. M., Slightom, J. L., Sanford, J. C., and Gonsalves, D. 1997. Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus. Mol. Breed. 3:161-168.
25. McMillan, R. T., Jr., and Gonsalves, D. 1988. Effectiveness of cross-protection by a mild mutant of papaya ringspot virus for control ringspot disease of papaya in Florida. Proc. Fla. State Hortic. Soc. 100:294-296.
26. Purcifull, D. E., Edwardson, J. R., Hiebert, E., and Gonsalves, D. 1984. Papaya ringspot virus. CMI/AAB Descr. Plant Virus 292.
27. Singh, A. B. 1969. A new virus disease of Carica papaya in India. Plant Dis. Rep. 53:267-269.
28. Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. 1994. Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs. Plant Cell 6:1441-1453.
29. Story, G. E., and Halliwell, R. S. 1969. Identification of distortion ringspot virus disease of papaya in the Dominican Republic. Plant Dis. Rep. 53:757-760.
30. Tennant, P. F., Fermin, G., Fitch, M., Manshardt, R., Slightom, J. L., and Gonslaves, D. 2001. Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. Eur. J. Plant Pathol. 107:645-653.
31. Tennant, P. F., Gonsalves, C., Ling, K. S., Fitch, M., Manshardt, R., Slightom, J. L., and Gonslaves, D. 1994. Different protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. Phytopathology 84:1359-1366.
32. Thomas, J. E., and Dodman, R. L. 1993. The first record of papaya ringspot virus-type P in Australia. Aust. Plant Pathol. 22:2-7.
33. Wang, C. H., Bau, H.-J., and Yeh, S.-D. 1994. Comparison of the nuclear inclusion b protein and coat protein genes of five papaya ringspot virus strains distinct in geographic origin and pathogenicity. Phytopathology 84:1205-1210.
34. Wang, H. L., Wang, C. C., Chiu, R. J., and Gonsalves, D. 1978. Preliminary study on papaya ringspot virus in Taiwan. Plant Prot. Bull. (Taiwan) 20:133-140.
35. Waterhouse, P. M., Graham, M. W., and Wang, M. B. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA 95:13959-13964.
36. Yeh, S.-D., and Gonsalves, D. 1994. Practices and perspective of control of papaya ringspot virus by cross protection. Adv. Dis. Vector Res. 10:237-257.
37. Yeh, S.-D., Gonsalves, D., Wang, H.-L., Namba, R., and Chiu, R.-J. 1988. Control of papaya ringspot virus by cross protection. Plant Dis. 72:375-380.
38. Ying, Z., Janney, N., and Houtz, R. L. 1996. Organization and characterization of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit $^{\varepsilon}$N-methyltransferase gene in tobacco. Plant Mol. Biol. 32:663-671.
39. Ying, Z., Mulligan, R. M., Janney, N., and Houtz, R. L.. 1999. Rubisco small and large subunit N-methyltransferase: Bi- and monofunctional methyltransferase that methylate the small and large subunits of Rubisco. J. Biol. Chem. 274:36750-36756.
40. Ying, Z., Yu, X., and Davis, M. J. 1999. A new method for obtaining transgenic papaya plants by *Agrobacterium*-mediated transformation of somatic embryos. Proc. Fla. State Hortic. Soc. 112:201-205.