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RESISTANCE TO PAPAYA RINGSPOT VIRUS IN TRANSGENIC PAPAYA BREEDING LINES

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Abstract. **The resistance of transgenic papaya breeding lines to *Papaya ringspot virus* (PRSV) was examined. Resistance was conferred by non-translatable transgenes derived from the coat protein (CP) gene of a PRSV isolate (H1K) from Florida. To render the CP gene non-translatable, either a stop-codon (D6 lines) or frame-shift (X17-2 lines) mutation had been introduced into the CP gene. Non-transgenic and transgenic papaya lines (R_3 generation) were mechanically inoculated with three isolates (H1A, H1C, and H1K) of PRSV representing the genetic diversity of the virus in Florida. The mean severity of symptoms evaluated weekly for 8 weeks post-inoculation was consistently lower in the transgenic lines regardless of the PRSV isolate, and transgenic resistance to the different virus isolates did not differ noticeably. Ten or more plants each of 12 transgenic papaya lines and 23 non-transgenic accessions, including named varieties and selections, were planted in a**

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field in May 2003 and evaluated for the incidence and severity of PRSV following natural infections. Within 8 months, all of the non-transgenic papaya plants became infected by PRSV and exhibited moderate to high levels of disease severity. In contrast, only a few plants of four of the 12 transgenic lines developed mild symptoms of PRSV. Thus, although not immune to PRSV infection, especially when mechanically inoculated, transgenic lines exhibited a high level of resistance to natural infection in the field.

Papaya ringspot virus (PRSV) causes one of the most economically important diseases of papaya in the world and is a major limiting factor in papaya production in Florida (Conover 1964; Gonsalves, 1998). Genetic transformation of papaya with translatable or non-translatable constructs of the coat protein (CP) gene of PRSV provides an effective means to generate PRSV-resistant plants (Bau et al., 2003; Fitch et al., 1992; Lines et al., 2002). Transgenes for PRSV resistance have been incorporated into the 'Rainbow' and 'Sun-up' varieties presently being commercially grown in Hawaii (Tennant et al., 2001), and we have embarked on a program to develop transgenic PRSV-resistant papaya varieties for Florida (Davis et al., 2003). We have been developing entirely new transgenic papaya lines because licensing restrictions prohibit growing the Hawaiian PRSV-resistant transgenic varieties outside of Hawaii, and the possibility that the Hawaiian transgene might provide inadequate protection against isolates of the virus present in Florida (Davis and Ying, 2004). The CP of a Florida isolate of PRSV was used to create the transgenic lines. All transgenic lines were female, and selected PRSV-resistant lines were crossed with six papaya genotypes. The first generation (R_1) was installed in the field in 2001, and three successive generations, derived from self-pollinated hermaphrodite selections, have since been installed in the field. The frequency of natural infection by PRSV in the transgenic lines has been considerably less than that for non-transgenic plants in the same fields (Davis et al., 2003; Davis and Ying, 2004).

The mechanism of the resistance is thought to be RNA-mediated, homology-dependant, post-transcriptional, gene silencing (Lines et al., 2002; Tennant et al., 2001). Thus, the level of plant resistance is dependent upon a substantial level of RNA homology between the transcribed RNA of the CP transgene and the native gene of the attacking PRSV isolate. When the degree of homology is adequate, transcribed PRSV RNA for CP is destroyed stopping viral replication and, thus, conferring plant resistance. Isolates of PRSV have been shown to vary in the degree of homology between their CP genes (Bateson et al., 1994; Davis and Ying, 1999). Often, this variation is greater for isolates in different widely separate geographic locations than it is for isolates in the same location. Homology among CP genes of PRSV isolates from Florida was greater than that between the same genes and those of isolates elsewhere in the world, and furthermore the greatest homology with isolates outside of Florida was with isolates from the Caribbean region.

Although our original transgenic selections all exhibited a high degree of resistance to PRSV following mechanical inoculation with the same isolate used to obtain the CP transgene, some of the progeny of these lines were susceptible to natural infection by PRSV in the field (Davis et al., 2003; Davis and Ying, 2004). This susceptibility appeared to vary with both the transgenic line and the parentage of the plants. Thus, selective breeding for a high level of resistance should help to overcome this problem. However, a possibility exists that the transgenic lines might be more susceptible to infection by isolates of PRSV other than the one originally tested. Investigations reported here were conducted to evaluate the resistance of our transgenic lines to different isolates of PRSV from Florida and to directly compare natural infection with PRSV in 12 of our R_3 transgenic lines to that in 23 non-transgenic varieties or selections varying from highly susceptible to tolerant in their reaction to PRSV.

Materials and Methods

Plant materials. The non-transgenic papaya varieties and selections used in this study are listed in Table 1, and the transgenic lines are listed in Table 2. Seedlings were produced as previously described (Davis and Ying, 2004). For PRSV inoculation experiments, four to six week-old seedlings were transplanted to Pro Mix BX soil mix (Premier Horticulture, Ltd., Dorval, Quebec) amended with 14-14-14 Osmocote (Scotts-Sierra Horticultural Products Co., Maryville, Ohio) at 6.7 kg m^{-3} of soil mix in 1-liter pots and fertilized biweekly with a 1 g L^{-1} solution of Miracle-Gro fertilizer (Scotts Miracle-Gro Products, Inc., Port Washington, N.Y.). Plants were maintained in a shade house at ambient temperature.

For the field study, seedlings were transplanted in May 2003 to raised beds that had been covered with plastic mulch and fumigated with methyl bromide. Plants were fertilized weekly through the drip irrigation system. A randomized block design with two blocks was used. Each transgenic or non-transgenic selection was installed in at least one plot in each block, and each plot contained five plants.

PRSV inoculations and evaluations. PRSV isolates from Florida were collected and genetically characterized as previously described (Davis and Ying, 2002). Three isolates, H1A. H1C, and H1K, representing the diversity of PRSV found in Florida, were used. Newly expanded leaves on six-week-old seedlings were inoculated mechanically with virus isolates, using inoculum prepared by grinding infected leaves with a mortar and pestle in 0.01 M potassium phosphate buffer, pH 7.2, containing Carborundum as previously described (Davis and Ying, 2004). Plants of two transgenic and two non-transgenic selections were inoculated. Controls consisted of non-inoculated plants and buffer-inoculated plants of each selection. Plants were grown in a shade house at ambient temperatures. Plants were evaluated for PRSV severity on newly expanded leaves weekly for eight weeks post-inoculation using the following ratings: 0 = no symptoms; 1 = questionable or very mild mosaic leaf symptoms; 2 = severe mosaic leaf symptoms; 3 = leaf distortion symptoms; 4 = shoot tip dieback; 5 = dead plant. There were five plants per treatment and the entire experiment was repeated once.

The plants in the field experiment were evaluated for PRSV incidence and severity every four to six weeks for five months beginning two months after the plants were installed in the field. The severity ratings were: 0 = no clear symptoms (vein-clearing, mosaic, or leaf distortion); 1 = definite mild symptoms confined to a small part of the crown; 2 = moderate systemic symptoms and/or ring spots on the fruits; 3 = severe symptoms throughout the crown; 4 = stunted crown with very severe symptoms; 5 = defoliated and/or dead.

Results and Discussion

The papaya varieties and transgenic selections inoculated with different PRSV isolates from Florida were chosen to provide materials with similar genetic backgrounds for compari-

Table 1. Papaya cultivars and breeding selections.

Cultivar or breeding selection[z]	Source of seed	Origin
Solo (802)	Brooks Tropicals, Miami, FL	Brazil
Cariflora (905)	B. R. Brunner, UPR[y]	Florida
Experimental No. 15 (900)	Known-You Seed Co., Kaohsung, Taiwan	Taiwan
Glades Native, wild (883)	M. J. Davis, TREC	Florida
Know-You No. 1 (864)	Known-You Seed Co., Kaohsung, Taiwan	Taiwan
Know-You No. 1 (867)	Aloha Seed & Herb, Paia, HI	Taiwan
Maradol (870)	Aloha Seed & Herb, Paia, HI	Mexico
Oropeza (985)	J. H. Crane, TREC	unknown
PR6-65 Dwarf (906)	B. R. Brunner, UPR	Puerto Rico
PR6-65 red selection (903)	B. R. Brunner, UPR	Puerto Rico
Red Lady (869)	Aloha Seed & Herb, Paia, HI	Taiwan
Red Lady (863)	Known-You Seed Co., Kaohsung, Taiwan	Taiwan
Solo 40 (907)	B. R. Brunner, UPR	Puerto Rico
Solo China (909)	R. Olszak, Homestead, FL	China
Solo Sunrise (861)	Known-You Seed Co., Kaohsung, Taiwan	Hawaii
Solo Sunrise (865)	Aloha Seed & Herb, Paia, HI	Hawaii
Solo Sunrise (908)	B. R. Brunner, UPR	Hawaii
Solo Sunset (868)	Aloha Seed & Herb, Paia, HI	Hawaii
Tainung No. 5 (902)	B. R. Brunner, UPR	Taiwan
Tainung No. 1 (862)	Known-You Seed Co., Kaohsung, Taiwan	Taiwan
Tainung No. 1 (904)	Aloha Seed & Herb, Paia, HI	Taiwan
TREC 11B19-02	this study	Florida
TREC 15A21-02	this study	Florida
TREC 15A5-02	this study	Florida
TREC 15B13-02	this study	Florida
TREC 15B8-02	this study	Florida
TREC 17A11-02	this study	Florida
TREC 19B30-02	this study	Florida
TREC 1A4-02	this study	Florida
TREC 2B28-02	this study	Florida
TREC 4B16-02	this study	Florida
TREC 6B9-02	this study	Florida
TREC 7B23-02	this study	Florida
Waimanolo (866)	Aloha Seed & Herb, Paia, HI	Hawaii
Washington No. 5 (901)	B. R. Brunner, UPR	India

[z]Accession numbers at the Tropical Research and Education Center (TREC), Homestead, Florida are in parentheses.
[y]University of Puerto Rico, Lajas Substation, Lajas, Puerto Rico.

son. The "F65" papaya that was transformed to produce our original PRSV-resistant transgenic papaya lines was a breeding selection obtained by a grower in Florida from the Known-You Seed Co. (Kaohsung, Taiwan). The grower had been informed that 'F65' was a recent ancestor of the 'Red Lady' variety that is currently planted widely. We have not been able to confirm this reported relationship nor have we been able to obtain new seed for 'F65.' Consequently, we selected 'Red Lady' for comparison with transgenic lines in the inoculation studies. "Red Lady' is tolerant to PRSV. "Solo Sunrise' was selected as our other choice, because it is highly susceptible to PRSV, thus providing the contrast between tolerant and intolerant varieties, and because transgenic lines derived from original crosses with 'Solo Sunrise' were available for comparison. During the course of this study, one transgenic line, TREC 7B23-02 (Table 2), was determined by PCR analyses to contain some plants that lacked the modified CP gene but retained the *npt*II gene conferring kanamycin resistance (data not shown), and although only a few of these plants were available for comparison, they provided us an almost genetically identical set of plants for comparison with those of the original transgenic line.

Inoculations with all three PRSV isolates clearly demonstrated that transgenic plants containing the modified CP gene of the H1K isolate were not only resistant to the homologous PRSV isolate but also to the other two PRSV isolates from Florida (Fig. 1). All plants inoculated with PRSV became infected regardless of the isolate used. This confirmed our previous results indicating that the transgenic plants were not immune to infection following mechanical inoculation of young plants (Davis and Ying, 2004). After inoculation, the non-transgenic varieties developed symptoms faster and to a consistently greater extent. The 'Red Lady' variety was more tolerant to PRSV than the 'Solo Sunrise' variety, as expected, but the difference was much less than that between the non-transgenic varieties and PRSV-resistant transgenic lines. Both of the non-transgenic varieties eventually developed severe disease; whereas, symptoms in the PRSV-resistant lines usually never surpassed being mild. The variants within a transgenic line that lacked the CP gene but contained the *npt*II gene reacted very similarly to the non-transgenic varieties, strongly indicating that the presence of the modified coat protein gene in the transgenic plants was largely responsible for the observed limitation to disease progress. Furthermore, there

Table 2. Transgenic breeding lines used in this study. All original transgenic lines (R_0) were female plants derived by regeneration of somatic embryos of the 'F65' cultivar. These original lines were crossed with different germplasm selections, and selected progenies were self-pollinated to obtain the R_3 generation used in this study.

Breeding line	Original transgenic line[z]	Origin hermaphroditic progenitor[y]
TREC 2B28-02	D6	Red Lady
TREC 4B16-02	D6	Tainung No. 5
TREC 11B19-02	D6	PR 6-65
TREC 15A5-02	D6	Solo Sunrise
TREC 15A21-02	D6	Solo Sunrise
TREC 19B30-02	D6	Solo 40
TREC 1A4-02	X17-2	Experimental No. 15
TREC 15B13-02	X17-2	PR 6-65
TREC 17A11-02	X17-2	Solo Sunrise
TREC 6B9-02	X17-2	Tainung No. 5
TREC 7B23-02	X17-2	PR 6-65
TREC 15B8-02	X17-2	PR 6-65

[z]The D6 line had a stop-codon mutation of the PRSV coat protein transgene, and the X17-2 line had a frame-shift mutation of the PRSV coat protein transgene.
[y]Pollen for listed progenitor (see Table 1) obtained from B. R. Brunner for cross.

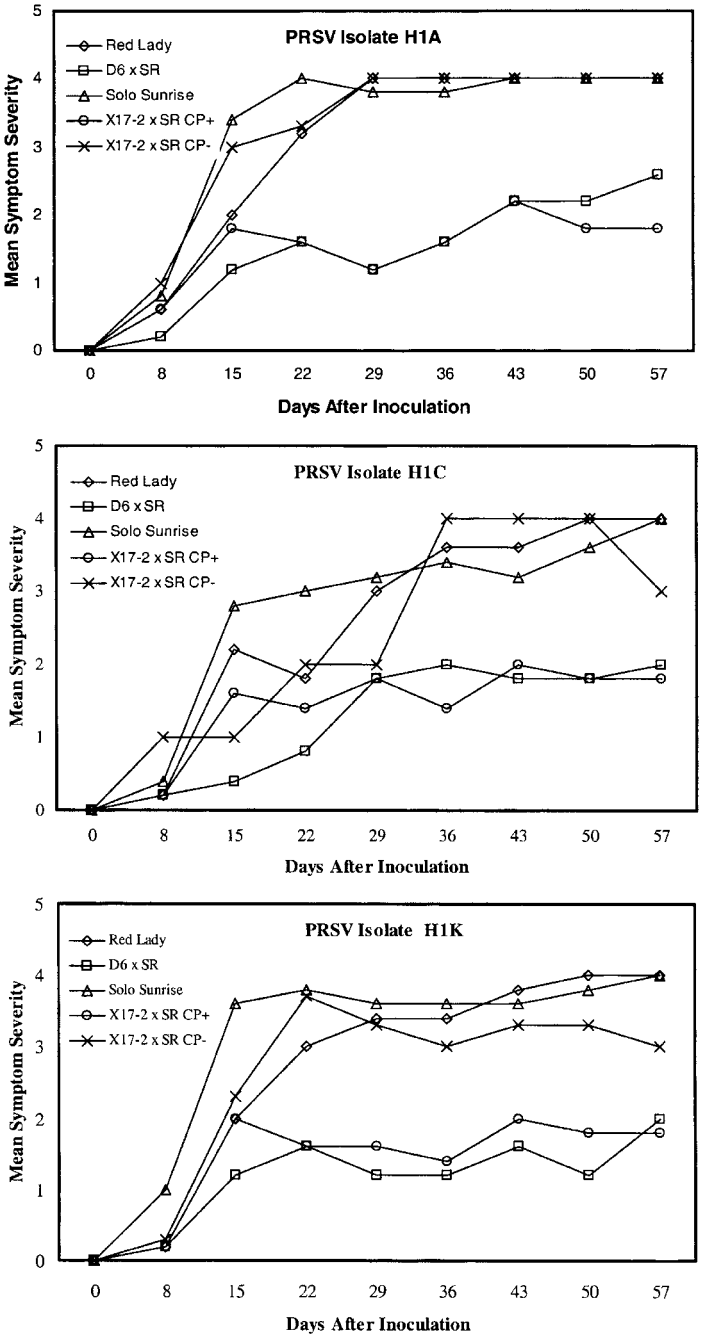

Fig. 1. Disease progress in transgenic and non-transgenic papaya seedlings following inoculation with different PRSV isolates representing the diversity of the virus found in Florida. The TREC 15A21-02 transgenic line (D6 × SR) was derived from a cross between the D6 transgenic line and the 'Solo Sunrise' non-transgenic variety. The TREC 7B23-02 transgenic line (X17-2 × SR) was derived from a cross between the X17-2 transgenic line and the 'Solo Sunrise' non-transgenic variety, and plants both with the coat protein transgene (CP+) and without the coat protein transgene (CP-), but still containing the *npt* II gene, were evaluated. Plants of the 'Solo Sunrise' and 'Red Lady' non-transgenic varieties were evaluated for comparison.

was no consistent difference in the level of protection conferred by the non-translatable CP transgenes with either the stop-codon mutation (D6 line) or frame-shift mutation (X17-2) when the results for the inoculations with all three PRSV isolates are considered. Essentially the same results were obtained when the experiment was repeated (data not shown).

Although the transgenic lines are susceptible to PRSV infection following mechanical inoculation, the lines continued to exhibit a high degree of resistance to natural infection by PRSV in field plantings (Table 3), as reported for previous generations (Davis and Ying, 2004). The reason for this resistance to infection in the field is unknown. Presumably, aphid vectors are responsible for natural inoculations in the field. The inoculum dose provided by aphids might be considerably less than that of the mechanical inoculations and unable to overcome the transgenic resistance in most instances. The transgenic resistance might be overwhelmed by the inoculum dose used in mechanical inoculations. Another possibility is that the transgenic plants became more resistant as they grew older. Increased resistance in older transgenic PRSV resistant papaya plants has been reported (Tennant et al., 2001).

Eight of the 12 transgenic breeding lines planted in the field for comparison with non-transgenic varieties and breeding selections did not develop PRSV during the eight month evaluation period (Table 3). Some plants of the four other transgenic lines developed PRSV, but PRSV in these transgenic lines appeared at a slower rate than in the non-transgenic plants. Tolerance to PRSV was evident in some of the non-transgenic varieties; 'Red Lady,' 'Cariflora,' 'Tainung No. 5,' and 'Washington No. 5' were among the most tolerant varieties, agreeing to a large extent to a previous evaluation of non-transgenic materials in Florida (Crane et al., 1995). The 'Solo' varieties were the most susceptible to PRSV.

The results of this study indicate that the CP transgenes present in the PRSV-resistant papaya breeding lines confer resistance to different strains of the virus in Florida, and, although this resistance does not prevent infection following mechanical inoculation, the resistance is adequate to prevent natural infection in the field. Continued inbreeding and selection of the transgenic lines has resulted in the majority of them being homozygous for the transgenes (data not shown), which might impart an increased level of resistance, as found in the Hawaiian transgenic papaya varieties (Tennant et al.,

Table 3. PRSV incidence and mean disease severity in transgenic breeding lines and non-transgenic varieties and breedings elections subjected to natural inoculation in the field.

Source[z]	No. plants	Months after planting: 2		4		6		8	
		% infected	Mean severity	% infected	Mean severity	% infected	Mean severity	% infected	Mean severity
TREC 2B28-02	14	0	0.0	0	0.0	0	0.0	0	0.0
TREC 11B19-02	18	0	0.0	0	0.0	0	0.0	0	0.0
TREC 15A21-02	15	0	0.0	0	0.0	0	0.0	0	0.0
TREC 15B13-02	10	0	0.0	0	0.0	0	0.0	0	0.0
TREC 15B8-02	15	0	0.0	0	0.0	0	0.0	0	0.0
TREC 7B23-02	9	0	0.0	0	0.0	0	0.0	0	0.0
TREC 17A11-02	15	0	0.0	0	0.0	0	0.0	0	0.0
TREC 6B9-02	20	0	0.0	0	0.0	0	0.0	0	0.0
TREC 4B16-02	16	0	0.0	0	0.0	6	1.0	19	2.3
TREC 15A5-02	15	0	0.0	0	0.0	40	1.8	53	3.4
TREC 19B30-02	15	0	0.0	0	0.0	7	1.0	7	4.0
TREC 1A4-02	8	0	0.0	0	0.0	13	3.0	13	4.0
Red Lady (869)	5	20	1.0	100	1.4	100	2.0	100	2.0
Cariflora (905)	10	10	1.0	60	1.0	90	2.6	100	2.5
Red Lady (863)	10	20	1.0	90	1.6	100	2.4	100	2.7
Tainung No. 5 (902)	10	20	1.0	100	2.0	100	2.7	100	3.0
Oropeza (985)	10	30	1.0	100	2.1	100	2.7	100	3.0
Experimental No. 15 (900)	10	10	1.0	100	1.7	100	2.4	100	3.1
Washington No. 5 (901)	10	20	1.0	100	1.9	100	2.5	100	3.1
Known You No.1 (864)	10	30	1.0	100	1.8	100	2.5	100	3.1
Maradol (870)	5	20	1.0	100	3.0	100	3.2	100	3.2
Tainung No. 1 (862)	10	0	0.0	100	2.0	100	2.5	100	3.5
Tainung No. 1 (904)	10	40	1.0	100	2.1	100	3.3	100	3.5
PR 6-65 Dwarf (906)	10	10	1.0	100	2.2	100	3.0	100	3.5
Glades Native, wild (883)	10	70	1.9	100	3.1	100	3.1	100	3.6
Known You No. 1 (867)	5	40	1.0	100	2.2	100	2.6	100	3.6
PR 6-65 Red (903)	10	0	0.0	90	2.0	100	2.8	100	3.7
Solo (802)	10	40	1.0	100	2.9	100	3.4	100	3.8
Solo China (909)	4	0	0.0	100	3.0	100	3.3	100	3.8
Solo Sunrise (861)	9	11	1.0	100	3.1	100	3.6	100	3.9
Solo Sunrise (908)	10	20	2.0	100	3.2	100	3.2	100	3.8
Solo Sunrise (865)	5	60	1.0	100	3.2	100	4.0	100	4.0
Solo Sunset (868)	5	40	1.0	100	2.8	100	3.4	100	4.0
Waimanolo (866)	5	20	1.0	100	3.0	100	4.0	100	4.0
Solo 40 (907)	10	20	2.0	100	3.5	100	3.9	100	4.2

[z]The seed source and origins of the accessions are given in Table1, and the pedigree of the transgenic lines is given in Table 2.

2001). The fourth generation of our transgenic, PRSV-resistant papaya breeding lines is presently being evaluated in the field and has the potential to produce several new papaya varieties in the near future.

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