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The ability of *Papaya ringspot virus* strains overcoming the transgenic resistance of papaya conferred by the coat protein gene is not correlated with higher degrees of sequence divergence from the transgene

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Abstract

The coat protein (CP) gene mediated transgenic resistance is found to be the best approach for protecting papaya plants against the destructive disease caused by *Papaya ringspot viruses* (PRSV). In order to study the variability of PRSV and the potential threat to the CP-transgenic resistance, five virus isolates were collected from transgenic plants of papaya line 16-0-1, which carry the CP gene of the typical mosaic strain of Taiwan PRSV YK, in an approved test field and fourteen from untransformed papaya plants in different areas of Taiwan. The results of biological, serological, and molecular characterization indicated that all isolates are related to PRSV YK. Among them, the isolate 5–19 from the transgenic line and the isolates CS and TD2 from untransformed papaya were able to overcome the YK CP gene-mediated resistance of papaya lines 18–2–4, 17-0-5, and 16-0-1, which provide high degrees of resistance to different geographic PRSV strains of Hawaii (HA), Mexico (MX), and Thailand (TH). These three isolates were also able to cause symptoms on untransformed papaya plants more severe than those induced by YK. In addition to the host reactions, the variability of the collected 19 isolates was also analyzed and compared with YK and other geographic strains by heteroduplex mobility assay (HMA) and sequence analyses. The results of HMA indicated that the CP genes of isolates 5–19 and TD2 are more divergent than those of other isolates when compared with YK. However, sequence analyses of the transgenic-resistance overcoming isolates 5-19, CS, and TD2 revealed that their CP coding regions and the 3′ untranslated regions (UTRs) share nucleotide identities of 93.9–96.6% and 94.2–97.9% with those of YK, respectively; whereas the other geographic strains of HA, MX, and TH that could not overcome the transgenic resistance share lower nucleotide identities of 89.8–92.6% and 92.3–95.3% with those of YK, respectively. Our results indicate that the ability for overcoming the transgenic resistance is not solely correlated with higher degrees of sequence divergence from the transgene. The possible mechanism for overcoming the transgenic resistance and the potential threat of these PRSV strains to the application of the transgenic papaya lines carrying PRSV YK CP gene are discussed.

Introduction

Papaya ringspot virus (PRSV) is a member of the genus *Potyvirus* of the family *Potyviridae* (van Regenmortel et al., 2000), with flexuous particles of 780 × 12 nm and a genome consisting of single stranded RNA of positive polarity (Purcifull et al., 1984; Yeh et al., 1992). PRSV isolates are transmitted by aphids in a non-persistent manner (Purcifull et al., 1984) and cause the destructive ringspot disease of papaya in many countries (Cook, 1972; Yeh and Gonsalves, 1994). In the late 1970s, PRSV spread throughout Taiwan and has since destroyed most of the commercial papaya orchards (Wang et al., 1978; Yeh and Gonsalves, 1994). Several control measures have been used to protect papaya seedlings from PRSV infection, including selection of planting time to avoid the peak of winged aphids, inter-cropping with barrier crops such as corn, and growing

papaya under netting (Yeh and Gonsalves, 1994). Of these methods only papaya grown under netting provided effective protection against PRSV. However, raising papaya under a large net house is costly and vulnerable to natural risks such as tropical storms.

In order to control the ringspot disease, the mild strain PRSV HA 5-1, a mutant induced by nitrous acid from the severe Hawaii strain HA (Yeh and Gonsalves, 1984), has been used to protect papaya against severe infection by cross protection in Taiwan since 1985 (Yeh et al., 1988). However, the problem of strain-specific protection limits the application of the mild strain in Taiwan and in different areas of the world (Yeh and Gonsalves, 1994). The approach of coat protein (CP)-mediated transgenic resistance (Powell-Abel et al., 1986) avoids many of the potential disadvantages of conventional cross protection as a control measure, such as the cost of inoculation, possible revertants, dissemination of the mild strain to other crops, and adverse effects of the attenuated strains on the host (Yeh and Gonsalves, 1994). The CP gene of HA 5-1 was transferred into papaya and transgenic lines highly resistant to Hawaii strains have been obtained (Fitch et al., 1992; Lius et al., 1997). However, these transgenic lines are susceptible to other PRSV isolates outside Hawaii and are probably not useful in other geographic areas (Tennant et al., 1994). Recently, the transgenic papaya lines, carrying the CP gene of PRSV YK, a severe mosaic isolate of PRSV collected from Yang-Kang County in southern Taiwan (Wang and Yeh, 1992), have been developed (Cheng et al., 1996). They show high levels of broad-spectrum resistance to infection of different geographic PRSV strains under greenhouse conditions (Bau et al., 2003).

Although the local severe PRSV strain from Taiwan was chosen as the working virus to minimize the possible problem generated by strain-specific resistance (Cheng et al., 1996; Bau et al., 2003), it remains important to understand the natural variation of viruses infecting papaya in Taiwan to avoid a possible failure. The heteroduplex mobility assay (HMA) based on genetic differences between viral sequences, as applied to the analysis of genetic relationships of human immunodeficiency virus (HIV) (Delwart et al., 1993), was shown to be a rapid, reliable and cost-effective method for analysis of virus variability. Heteroduplexes are formed between polymerase chain reaction (PCR) amplified fragments of variable regions of different isolates, following their denaturation and reannealing. The difference in migration of heteroduplexes, in nondenaturing gel electrophoresis, results from the effect of primary sequence changes forming mismatches that cause bulges in a dsDNA fragment (Bhattacharya and Lilley, 1989; Delwart et al., 1995; Lin et al., 2000).

In this study, a number of viruses that infected untransformed papaya plants in different areas of Taiwan or YK CP gene-transgenic papaya plants in an approved test field were collected and analyzed by host reactions and ELISA. The sequence divergence of the CP coding regions and 3′ untranslated regions (UTRs) of the identified PRSV isolates was analyzed by HMA using the typical mosaic strain YK as a standard reference. Several PRSV strains variable from YK were identified. Some of them (5-19, CS, and TD2) were found to be able to overcome the transgenic resistance provided by the CP gene of PRSV YK. Results of sequence comparison of the CP coding regions and the 3′ UTRs of these isolates with those of YK and other geographic strains that can not infect the transgenic lines indicate that the ability for overcoming the CP-transgenic resistance of these isolates is not solely correlated to the higher degrees of sequence divergence. The potential threat of these variable PRSV strains that are able to overcome the transgenic resistance in papaya conferred by PRSV YK CP gene is discussed.

Materials and methods

Virus isolation and serological tests

A special permit was obtained from the Council of Agricultural of Taiwan for conducting field trials (1996–2000) for the transgenic papaya lines under isolated conditions at Taiwan Agricultural Research Institute (TARI) at Wufeng since 1996. In 1997–1998, transgenic papayas carrying the CP gene of PRSV YK, a prevalent strain from Taiwan (Wang and Yeh, 1992), were planted in the isolated field of TARI. Diseased leaf samples with mosaic symptom were collected from plants of YK CP-transgenic papaya line 16-0-1 (Bau et al., 2003) and untransformed papaya Tainung No. 2. Also, diseased leaf samples with mosaic symptom from

untransformed plants in papaya orchards located at different areas of Taiwan, including Tsaotun, Chiayi, Chishan, Pintung and Taidong, were collected.

All samples were tested by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using the antiserum against PRSV (Gonsalves and Ishii, 1980). The samples were ground with 0.01 M phosphate (pH 7.0) buffer in a mortar and pestle and the extracts were mechanically introduced to plants of *Carica papaya* var. Tainung No. 2 untransformed seedlings and transgenic line 16-0-1 (Bau et al., 2003), which were maintained in a temperature controlled (23–28 °C) greenhouse. If the virus from a sample caused infection on the transgenic papaya line, it was then mechanically transferred to plants of *Chenopodium quinoa* Wild and a single-lesion transferred virus isolate representing each sample was maintained in Tainung No. 2 papaya seedlings as sources of virus inocula for further assays. A similar procedure was used to obtain virus isolates that caused infection only on untransformed papaya.

The previously characterized PRSV Taiwan strain YK (Wang and Yeh, 1992; Wang and Yeh, 1997; Bau et al., 2003) was used for comparison. In addition, Hawaii strain HA, Mexico strain MX, and Thailand strain TH (kindly provided by Dennis Gonsalves, Cornell University, NY), which were not able to infect the YK-CP transgenic papaya lines (Bau et al., 2003), were also used for comparative studies.

Reactions of CP gene-transgenic papaya lines

In order to study the biological reactions, selected virus isolates were further introduced mechanically to one-month-old micropropagated plantlets of transgenic papaya lines 18-2-4, 17-0-5, and 16-0-1 carrying the CP gene of PRSV YK (Bau et al., 2003), and untransformed papaya plants of Tainung No. 2, and *C. quinoa*. All test plants were kept in a temperature-controlled (25–28 °C) greenhouse and symptoms were recorded 2–4 weeks after inoculation. The experiment was repeated 3–4 times with a total of 10–15 test plants for each assay. Symptoms were recorded and the virus infection was confirmed by DAS-ELISA using the antiserum against PRSV (Gonsalves and Ishii, 1980).

Total RNA extraction

An Ultraspec^{TM} RNA isolation kit (Biotecx, 6023 South Loop East Houston, TX) was used to isolate total RNA from virus-infected leaves of papaya. Fresh leaf tissue (100 mg) was frozen by liquid nitrogen and ground to a fine powder in a 1.5 ml microcentrifuge tube with a glass rod. After the addition of 1 ml Ultraspec^{TM} RNA solution, the mixture was shaken vigorously and stored at 4 °C for 5 min. Following the addition of 0.2 ml of chloroform, the mixture was shaken vigorously for 15 s and placed on ice for 5 min and then centrifuged at 12,000 *g* (4 °C) for 15 min. The aqueous phase was carefully transferred to a new tube and an equal volume of isopropanol was added. The samples were kept at 4 °C for 10 min and then centrifuged at 12,000 *g* (4 °C) for 10 min. The supernatant was removed and the RNA pellet was washed twice with 1 ml of 80% ethanol, briefly dried, and dissolved in 50 µl of Ultraspec^{TM} DEPC water (Biotecx).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to amplify the CP gene of PRSV isolates, following the method of Bateson et al. (1994) with some modifications. A pair of potyvirus degenerate primers, the upstream primer Pot2 and the downstream primer Potl (Colinet et al., 1994), was used to amplify a predicted 1.3 kb fragment which covered the C-terminal region of the NIb gene and the N-terminal region of the CP gene. In addition, four specific primers were also designed from the sequence of the 3′ end of PRSV YK RNA (Wang and Yeh, 1997) at different regions, including YK905, Mo926, Mo928, and Mo1008, which reflected nucleotide positions 9054–9073, 9258–9277, 9278–9297, and 10,077–10,096, respectively (Wang and Yeh, 1997). The corresponding positions of the used primer pairs and the predicted products are indicated in Figure l. For reverse transcription, 1–2 µg of total RNA (as determined by Gene Quant IIRNA/DNA calculator, Pharmacia Biotech, Cambridge, England) extracted from infected leaves was used for reaction. The downstream primer was annealed to total RNA at 70 °C for 5 min and the first-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI) at 42 °C for 1 h. The DNA fragments were amplified

from 2 μl of the cDNA mixture using 2 U of *Taq* polymerase (Protech, Taipei, Taiwan) and 10 pmol each of the downstream and upstream primers. The PCR was performed for 30 cycles with 1 min for denaturation at 94 °C, 2 min for annealing at 55 °C, and 3 min for synthesis at 72 °C. PCR products were analyzed by electrophoresis in a 0.75% agarose gel.

Heteroduplex mobility assay (HMA)

PCR products (0.82 kb) amplified by primers Mo926/Mol008 were further purified using columns of the Clean up-M kit according to manufacturer's instruction (Viogene, Sunnyvale, CA). For HMA, the PCR product (1–2 μ*g* in 15 μl) of the PRSV YK strain was used as a standard and mixed with an equal volume of products amplified from each of the individual isolates. Aliquots of 3.4 μl of l0x annealing buffer (1 M NaCl, 100 mM Tris–HCl (PH 7.8), 20 mM EDTA) were added to the mixture which was denatured at 95 °C for 2 min followed by rapid cooling on ice. The heteroduplex products were separated in 8% polyacrylamide gel with TBE buffer (0.088 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) at 250 V for 2 h and 45 min in an OWL P9DS vertical gel apparatus (OWL Scientific, Inc. Woburn, MA). The gel was treated with ethidium bromide (0.5 $\mu g\,ml^{-1}$) for 20 min and the stained DNA observed on a UV light box.

DNA cloning and sequence analyses

Because the N-terminal region of the CP gene of PRSV contained a poly (A) rich segment, the whole CP coding region and 3′ non-coding region were amplified by the different primer pairs.

The procedure of RT-PCR was conducted to amplify DNA fragments of isolates 5-19 and TD2 (more divergent from YK) and isolates CS and PT91 (less divergent from YK), using the primer pair Mo926/ Mo1008, which amplified a fragment that covered the whole CP coding region except a part of the C-terminal end, and total RNA as template. In addition, the C-terminal end of the CP gene and 3′ UTR region of isolates 5-19, CS, and PT91 was amplified by RT-PCR with the primer pair TL (5′ CTAGATACGCTTTCG 3′)/ Oligo-$dT_{(18)}$NS (5′ AATTGAGCTCGCGC-CCGCTTTTTTTTTTTTTTTT 3′), and those of isolate TD2 amplified with the primer pair TD29758 (5′ CCTTTAATTGAACATGCAA-CTCC 3′)/Oligo-$dT_{(18)}$NS. The PCR products were cloned into pCR-II TOPO vector (Invitrogen, San Diego, CA). Plasmid DNA was purified by the mini-prep method (Sambrook and Russell, 2001), and the correct insert was checked by digestion with *Pst*I and *Eco*RI. DNA sequence was determined using the Sequenase version 2.0 DNA sequencing kit with T7 and Sp6 primers (Amersham Life Science, Cleveland, Ohio). The obtained sequences were analyzed by the programs PILEUP and LINEUP of the GCG package (Wisconsin Package version 9.0, Genetic Computer Group, Madison, WI, USA).

Results

Virus isolation and identification

Five virus isolates (4-20, 5-12, 5-19, 7-8, and 16-18) were collected from diseased YK CP gene-transgenic papaya plants showing mosaic on leaves and five isolates (1-1, 1-4, 1-12, 10-19, and 18-1) from untransformed papaya plants at the experimental farm of TARI at Wufeng. The other nine isolates were collected from untransformed papaya at open orchards located in central, southern, and eastern areas of Taiwan, including TT1 and TT2 from Tasotun (Nantou County), CY1, CY2, CY3, and CY4 from Chiayi, CS from Chishan, PT91 from Pingtung, and TD2 from Taidong. All 19 isolates reacted positively in ELISA with the antiserum specific to PRSV, indicating that they belong to PRSV since no other potyviruses cross react with the PRSV antiserum used in DAS ELISA (Gonsalves and Ishii, 1980; Purcifull et al., 1984).

Host reactions and overcome of transgenic resistance

The results of host reactions of each isolate are presented in Table 1. All isolates induced local lesions (50–100) on the inoculated leaves of *C. quinoa* plants. Among the five isolates obtained from the diseased samples of CP-transgenic papaya plants, only isolate 5-19 infected all plants of transgenic papaya lines 18-2-4, 17-0-5 and 16-0-1 (4 repeats with a total of 15 plants for each line)

Table 1. Host reactions and symptoms induced by isolates of *Papaya ringspot virus* (PRSV) collected from Taiwan and other geographical areas

Isolate	Location	Symptoms[1] on				
		Carica papaya	*Chenopodium quinoa*	PRSV CP-transgenic plants (*C. papaya*)		
				16-0-1	17-0-5	18-2-4
4-20[2]	TARI(Wufeng)	M	LL	–	–	–
5-12[2]	TARI(Wufeng)	M	LL	–	–	–
5-19[2]	TARI(Wufeng)	MW	LL	M	M	M
7-8[2]	TARI(Wufeng)	M	LL	–	–	–
16-18[2]	TARI(Wufeng)	M	LL	–	–	–
1-1	TARI(Wufeng)	M	LL	–	–	–
1-4	TARI(Wufeng)	M	LL	–	–	–
1-12	TARI(Wufeng)	M	LL	–	–	–
10-19	TARI(Wufeng)	M	LL	–	–	–
18-1	TARI(Wufeng)	M	LL	–	–	–
TT1	Tsaotun(Taichung)	M	LL	M	–	–
TT2	Tsaotun(Taichung)	M	LL	–	–	–
CY1	Chiayi	M	LL	–	–	–
CY2	Chiayi	M	LL	–	–	–
CY3	Chiayi	M	LL	–	–	–
CY4	Chiayi	M	LL	–	–	–
CS	Chishan(Kaohsiung)	MW	LL	M	M	M
PT91	Pingtung	M	LL	–	–	–
TD2	Taidong	M,LD	LL	M,LD	M	M, LD
YK	Yung-Kang(Tainan)	M	LL	–	–	–
TH	Thailand	M	LL	–	–	–
MX	Mexico	M	LL	–	–	–
HA	Hawaii	M	LL	–	–	–

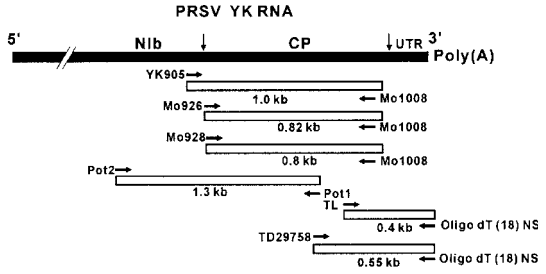
[1] Abbreviations for symptoms: M, mosaic; MW, mosaic and wilting; LL, local lesions; LD, leaf-distortion; "–" represents no symptoms.

[2] Isolates were collected from diseased plants of transgenic papaya line 16-0-1 (Bau et al., 2003) carrying the CP gene of PRSV YK during the field trials at Taiwan Agricultural Research Institute (TARI). Other isolates from TARI were collected from diseased untransformed papaya plants at the same test farm. The remaining isolates were from diseased untransformed papaya plants from different areas of Taiwan. PRSV strain YK from Tainan, Taiwan (Wang and Yeh, 1997), and strains HA, MX and TH from Hawaii, Mexico, and Thailand, respectively, (Bau et al., 2003) were used for comparison.

and induced mosaic symptoms (Figure 1), whereas the viruses extracted from the other four samples did not infect any of the transgenic lines under greenhouse conditions (Table 1). The isolates 4-20, 5-12, 5-19, 7-8 and 16-18, obtained from the five diseased samples induced a typical mosaic symptom on untransformed plants of papaya Tainung No. 2; while the isolate 5-19 caused wilting in addition to the mosaic symptom. The five isolates 1-1, 1-4, 1-12, 10-19 and 18-1 obtained from diseased samples of untransformed papaya plants at the same experimental farm of TARI were able to induce typical mosaic symptoms of PRSV on untransformed Tainung No. 2 papaya plants, but none of them were able to infect the CP-transgenic papaya lines (Table 1).

Among the isolates obtained from diseased samples of untransformed papaya plants from different areas of Taiwan, isolates CS and TD2 from Chishan and Taidong, respectively, were able to overcome the transgenic resistance in all the transgenic papaya lines tested (3 repeats with a total of 12 plants for each isolate) and produce mosaic or mosaic and leaf distortion symptoms (Table 1; Figure 1). The isolates TT1 and TT2 (from Tsaotun), CY1, CY2, CY3, and CY4 (from Chiayi), and PT91 (from Pintung) were unable to overcome the CP gene-transgenic resistance and produced mosaic symptoms similar to that induced by YK on untransformed papaya plants (Table 1).

The isolates 5-19, CS and TD2 capable of overcoming the transgenic resistance induced



Isolate	RT-PCR product using primer pair			
	Pot2/Pot1 (1.3 kb)	YK905/Mo1008 (1.0 kb)	Mo926/Mo1008 (0.82 kb)	Mo928/Mo1008 (0.8 kb)
1-1	+	+	+	+
1-4	+	+	+	+
1-12	+	+	+	+
4-20	+	+	+	+
5-12	+	+	+	+
5-19	+	+	+	+
7-8	+	+	+	+
10-19	+	+	+	+
16-18	+	+	+	+
18-1	+	+	+	+
TT 1	+	+	+	+
TT 2	+	+	+	+
CY 1	+	+	+	+
CY 2	+	+	+	+
CY 3	+	+	+	+
CY 4	+	+	+	+
CS	+	+	+	+
PT 91	+	+	+	+
TD 2	+	+	+	+
YK	+	+	+	+
TH	+	+	+	+
MX	+	-	+	+
HA	+	-	+	-

Figure 1. The symptoms induced by PRSV isolates YK, 519, CS, and TD2 on YK CP gene-transgenic plants (papaya line 16-0-1) and untransformed plants (Bau et al., 2003). (A) The isolate YK causes systemic mosaic and stunting of an untransformed papaya plant but no symptoms on YK CP gene-transgenic papaya plants. (B) The isolate 5-19 induced a systemic mosaic and narrowing of leaves of an untransformed papaya; and mosaic, and leaf narrowing and distortion on YK CP-transgenic plant. (C) The isolate CS induced mosaic on an untransformed papaya plant and mild mosaic on a CP-transgenic plant. (D) The isolate TD2 caused severe leaf narrowing and distortion on an untransformed plant and YK CP-transgenic plant.

severe symptoms consisting of mosaic with wilting and leaf distortion. These symptoms were different from those of YK and other non-transgenic-resistance infecting isolates (mosaic only) of PRSV induced on untransformed papaya plants (Figure 1). Among the isolates that overcome the transgenic resistance, the isolate 5-19 induced symptoms 2–3 days earlier on CP-transgenic lines than the other isolates did.

PRSV YK was used in this study as a control and it did not infect any of the transgenic lines tested (Figure 1). Similarly, the PRSV strains from Hawaii (HA), Mexico (MX), and Thailand (TH) did not infect the YK CP gene-transgenic lines under greenhouse conditions (Table 1). These results indicated that the YK CP gene-transgenic lines tested were resistant to not only the YK isolate but also PRSV isolates from other geographical areas of the world as previously reported (Bau et al., 2003).

RT-PCR amplification

Results of the RT-PCR reaction are summarized in Figure 2. Using the potyvirus-specific primer pair Pot2/ Pot1, a specific PCR product of 1.3 kb was amplified from all isolates including strains HA, MX, and TH. This DNA fragment corresponded with the predicted span between the NIb and CP coding regions of potyviruses. Expected DNA fragments of 1.0 kb were amplified by YK905/Mo1008 from PRSV YK and TH strains but the primer pair failed to amplify MX and HA strains. Using Mo928/Mo1008, a specific PCR product of 0.8 kb was amplified from all isolates except HA. The primer pairs Mo926 /Mo1008 amplified a specific DNA fragment of 0.82 kb from all isolates and strains, and were therefore used for comparing diversity with the PRSV YK strain.

Heteroduplex mobility assay (HMA)

When the 0.82 kb DNA fragments covering the N-terminal variable region and most of the other remaining CP coding region were used for HMA, the slower migrating bands were considered as heteroduplexes formed between divergent DNA molecules during the processes of denaturing and reannealing. These heteroduplexes were not formed when the PCR products were reannealed with homologous DNA. In this experiment, PRSV YK was used as a standard (Figure 3, lane 1) for comparison. When annealed with PRSV YK strain, heteroduplexes formed by the isolates 5-19 and TD2 (Figure 3 lanes 7 and 18, respectively) migrated much slower than those of other isolates. The slightly slower migration of heretoduplex bands was observed in combinations of 17 other isolates (Figure 3). Therefore, the CP genes of 5-19 and TD2 isolates were considered more diverse from YK CP gene than those of the other isolates collected.

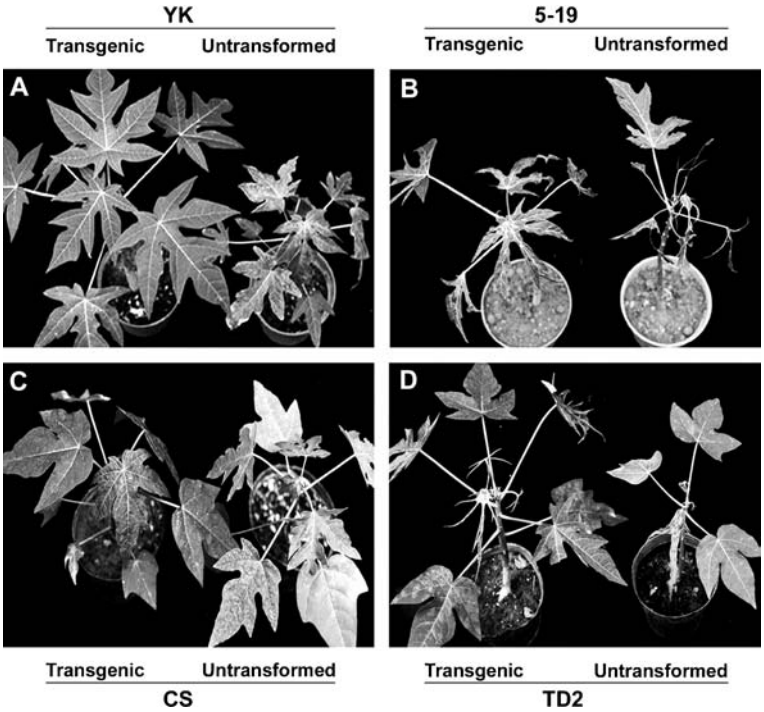


Figure 2. Primer pairs used for reverse-transcription polymerase chain reaction (RT-PCR) and their corresponding positions in the genomic region of PRSV YK strain. The locations and expected sizes of the RT-PCR products are indicated. Among these primers, YK905, Mo926, Mo928, Mo1008, TL, TD29758 and Oligo $dT_{(18)}$ NS were specific primers designed from the sequence of YK strain (Wang and Yeh, 1997), and Pot1 and Pot2 were degenerate primers designed for potyviruses (Colinet et al., 1994). "+" represents the specific DNA fragments amplified by RT-PCR with the two primers used. "–" represents no DNA fragments amplified.

Cloning and sequence comparison

Because of the high divergence in HMA and the infection on YK CP-transgenic papaya lines, isolates 5-19, CS, and TD2 were chosen for cloning and sequencing. A less divergent isolate PT 91 that did not infect the transgenic lines was also chosen for comparison. The nucleotide and amino acid identities of the CP coding region of isolates 5-19, CS, TD2, and PT91 compared with those of YK, HA, MX, and TH are shown in Tablele 2. Analyses revealed that the CP coding regions of isolates 5-19, CS, TD2, and PT91 share identities of 95.9% (95.0%), 96.6% (96.8%), 93.9% (94.4%), and 96.9% (95.4%), respectively, at the nucleotide (amino acid) level with that of YK, whereas the CP coding regions of strains HA, MX, and TH share lower identities of 91.2% (95.4%), 89.8% (93.0%), 92.6% (95.8%), with that of YK. Among the four isolates from Taiwan, PT91 that could not overcome the transgenic resistance shares the highest nucleotide (96.9%) identity and CS that overcomed the transgenic resistance shares the highest (96.8%) amino acid identity with PRSV YK. Whereas, TD2 has the lowest sequence (93.9% nucleotide and 94.4% amino acid) identity with YK out of the three Taiwan isolates infecting the CP-transgenic papaya lines.

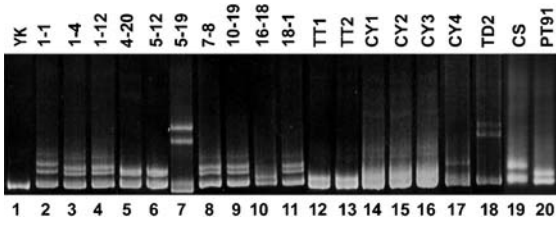


Figure 3. Heteroduplex mobility assay (HMA) of 19 isolates of PRSV using YK strain as a standard (lane 1). Heteroduplexes formed between products of RT-PCR from the two vireses were analyzed by electrophoresis in an 8% polyacrylamide gel. Lanes 2 to 20 are test samples.

Analyses of the 3′ UTR revealed that all the four isolates, 5-19, CS, TD2, and PT91, share

higher nucleotide identities (94.2–97.9%) with that of PRSV YK than the strains from other geographic locations (92.3–95.3%) (Table 2). The isolate 5-19 had the highest nucleotide identity of the 3′ UTR with that of strain YK (97.9%), whereas TD2 had the lowest nucleotide (94.2%) identity (Table 2).

Alignment of the nucleotide sequences of the CP genes and 3′ UTRs of isolates 5-19, CS, TD2, PT91, and YK showed no distinct patterns of nucleotide variability within the CP coding region and/or 3′ UTR. The variable nucleotides were found randomly distributed all over the CP coding regions and 3′ UTRs. However, six additional nucleotides were noted at the N-terminal region of CP gene of the isolate TD2 when compared with other PRSV isolates (Figure 4).

Discussion

The transgene conferring the resistance to PRSV contains the translatable frame of the CP sequence and the untranslatable 3′ UTR of PRSV YK (Bau et al., 2003). The molecular analyses indicated that the resistance to different geographic strains of PRSV is conferred by RNA-mediated gene silencing rather than CP-mediated functional interference (Bau et al., 2003). In this investigation, results of biological, serological, RT-PCR characterization indicate that all 19 isolates belong to PRSV. Some isolates that were able to overcome the transgenic resistance conferred by the CP gene of YK in papaya were identified and their CP coding regions and 3′ UTRs were found to be closely related to PRSV YK. The phenomenon of strain-specific resistance has been reported for transgenic papaya lines carrying the CP gene derived from Hawaii isolate. The transgenic papaya provides immunity to HA strain, but is susceptible to strains from other areas of the world (Fitch et al., 1992; Tennant et al., 1994; Gonsalves, 1998). In spite of the broad-spectrum resistance to strains HA, MX and TH of PRSV from other geographical areas provided by our CP gene-transgenic papaya lines (Bau et al., 2003), PRSV isolates 5-19, CS and TD2 overcame the resistance of these transgenic lines. Upon natural selection,

Table 2. Comparison of nucleotide and amino acid identities of CP coding regions and 3′ UTRs of PRSV isolates from Taiwan and other geographic areas

Isolate	Transgenic-resistance infecting isolates			Non trasgenic-resistance infecting isolates				
	519	CS	TD2	PT91	YK	HA	MX	TH
519	–	96.1	94.1	95.5	95.9	90.7	89.4	92.8
	–	94.7	94.7	98.0	97.9	91.8	94.2	97.1
CS	96.8	–	94.0	96.5	96.6	91.0	90.1	93.0
	–	–	90.9	94.7	95.2	88.5	90.9	92.8
TD2	93.6	95.4	–	93.1	93.9	91.0	89.7	92.0
	–	–	–	94.7	94.2	89.4	91.8	93.7
PT91	93.6	95.8	93.3	–	96.9	91.1	89.6	92.0
	–	–	–	–	97.6	91.8	96.1	96.1
YK	95.0	96.8	94.4	95.4	–	91.2	89.8	92.6
	–	–	–	–	–	92.3	93.7	95.3
HA	94.0	95.8	95.8	94.2	95.4	–	94.6	90.1
	–	–	–	–	–	–	93.7	92.8
MX	91.9	92.6	93.0	91.2	93.0	94.7	–	88.9
	–	–	–	–	–	–	–	93.3
TH	92.6	95.1	92.6	93.3	95.8	93.0	90.2	–
	–	–	–	–	–	–	–	–

Percent identities of CP genes (upper rows) and 3′ UTRs (lower rows) are presented for the nucleotide sequences (above the diagonal) and the amino acid sequences (below the diagonal). YK represents the typical mosaic strain of PRSV collected from Taiwan (Wang and Yeh, 1992); HA, MX, TH represent a severe strains from Hawaii, Mexico, and Thailand, respectively; (Bau et al., 2003), and 519, CS, TD2, and PT91 isolates were collected from different areas of Taiwan. Sequence comparison was conducted with SeqWeb version 2.1 of GCG package (Wisconsin package version 9.0, Genetics Computer Group, Madison, WI, USA). Sources for sequences: PRSV YK (Wang and Yeh, 1997); HA (Yeh et al., 1992), MX and TH (Bau et al., 2003); 519, CS, TD2, and PT91 (this investigation).

these isolates will become prevalent strains once our transgenic papaya lines are widely planted. The potential impact on PRSV ecology and emergence of new recombinant viruses infecting YK CP transgenic papaya lines can not be overlooked. These isolates of PRSV can survive and replicate as well in transgenic resistant papaya plants as in susceptible untransformed papaya plants on which they induce very severe symptoms of mosaic and wilting. The killing nature of these isolates displayed on the untransformed plants implies a major risk of the application of YK CP-transgenic papaya in Taiwan.

Although isolates 4-20, 5-12, 5-19, 7-8 and 16-18 were collected from diseased YK CP gene-transgenic papaya plants during the field trial at TARI, only isolate 5-19 overcame the transgenic resistance of papaya lines 18-2-4, 17-0-5 and 16-0-l under greenhouse conditions. All the leaves collected from the diseased plants showed typical symptoms of mosaic and distortion without conspicuous difference. Although ELISA was employed to detect the presence of PRSV, there was no significant difference in the relative levels of virus accumulation in the collected samples. The isolation of several cultures from transgenic papaya line tested might be due to the situation that the transgenic resistance provided by the transgene was weakened by some biotic (attack of other pathogen, insects, nematodes etc.) or abiotic (nutrients, temperature, humidity etc.) factors under the field conditions at the time of the virus infection. Moreover, plant developmental stages, transgene doses and transgene homology play an important role in overcoming the transgenic resistance in papaya in Hawaii conditions (Tennat et al., 2001). In other cases, although the crude extracts from the diseased samples were first transferred on the transgenic line 16-0-1, the possibility of losing a more virulent isolate present in a mixed population can not be ruled out. Also, the possibility that a less virulent virus may evolve during the single-lesion transfer from *C. quinoa* to papaya can not be excluded. Among the other five isolates (1-1, 1-4, 1-12, 10-19 and 18-1) collected from untransformed diseased papaya plants in the same field of TARI, none of them was able to infect the CP-transgenic papaya lines (Table 1). Our observations suggest that there is a selection effect provided by the transgenic papaya for more virulent strains under natural field conditions. Nevertheless, analyses on more samples collected from transgenic and untransformed papaya plants under the same field conditions are needed to confirm this suggestion.

The isolates of PRSV from the geographical areas other than Taiwan, such as from Hawaii (HA), Mexico (MX) and Thailand (TH), were not able to overcome the transgenic resistance clearly demonstrated that the criterion of the broad-spectrum resistance of YK CP gene-transgenic lines was similar to that described previously (Bau et al., 2003). Host reactions of the isolates HA, MX and TH were similar to those of other non-transgenic-resistance infecting isolates of Taiwan (Table 1). However, among other nine isolates collected from different areas of Taiwan in the open orchards of papaya, two isolates, CS and TD2, were able to overcome the transgenic resistance of papaya lines 18-2-4, 17-05, and 16-0-1, indicating the complexity of PRSV strains in Taiwan. This complexity was further demonstrated by host reactions and induction of more severe symptoms on untransformed papaya plants by isolates 5-19, CS and TD2 (Figure 1). Variability among the isolates was also revealed by RT-PCR. HA and MX were not amplified by the YK-sequence-derived primer pair YK905/Mo1008 and HA was not amplified by the primer pair Mo928/Mol008 (Figure 2) indicating the existence of sequence variability in the CP of HA and MX relative to YK.

HMA was used for rapid detection of the sequence divergence of the CP genes to compare the relationships of PRSV isolates with the YK strain. The transgenic papaya lines used for determination of resistance against PRSV isolates collected carry the entire CP coding region with the complete 3′ UTR of severe Taiwan strain YK, transcribed from a CaMV 35 S promoter (Wang and Yeh, 1997). The broad-spectrum resistance of YK CP gene-transgenic lines seems to be CP sequence-homology dependent as reflected in HMA analysis by which the heteroduplexes of the isolates 5-19 and TD2 formed with YK strain migrated much slower than the other isolates. However, the sequences of the CP coding regions and 3′ UTRs of isolates 5-19, CS and TD2 were less divergent to YK strain than those of other geographical strains of HA, MX and TH. In spite of narrower sequence diversities in the CP coding regions and the 3′ UTRs than the non-infecting strains HA, MX and TH, the three isolates are capable of overcoming

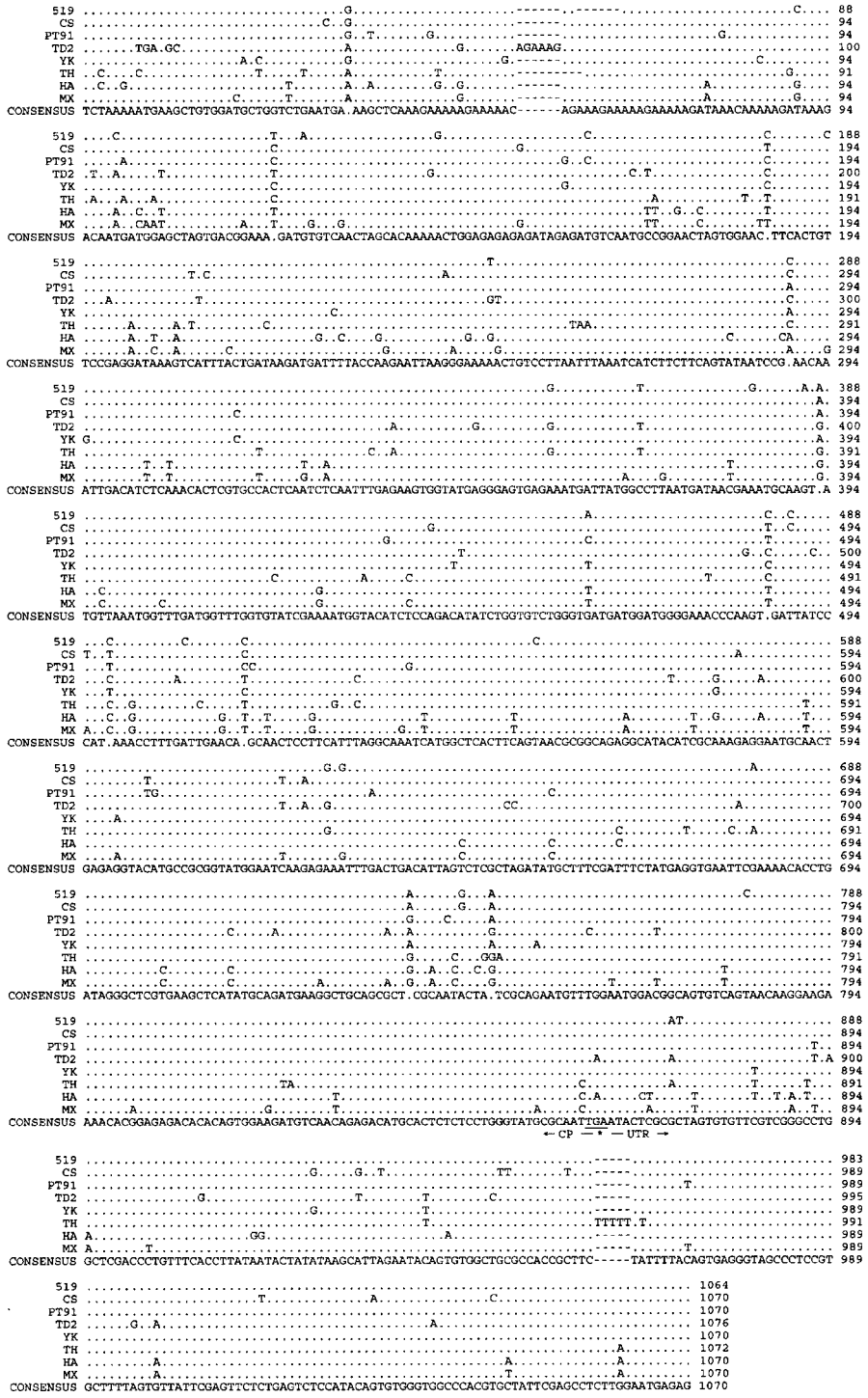


Figure 4. Alignments of nucleotide sequences of the CP coding regions and the 3′ UTRs (in the DNA form) of isolates 5–19, CS, PT91, TD2 and strains YK, TH, HA, and MX of PRSV. The source of the sequence of Hawaii strain HA was from Yeh et al. (1992, accession X67673) and the sequence of Taiwan strain YK from Wang and Yeh (1997, accession X97251). The sequences of Thailand strain TH and Mexico strain MX were from Bau et al.(2003). Nucleotides that differ from the consensus sequence are shown; "." indicates the conserved residues; "-" indicates gaps in the sequence; and "*" indicates the termination codon of the CP gene. Sequence alignment was conducted by the PILEUP program of the GCC package (version 9.0, Genetics Computer Group, Madison, WI).

the YK CP gene-mediated transgenic resistance in papaya (Table 1). Thus, our results indicate that the ability of the PRSV strains for overcoming YK CP gene-mediated transgenic resistance was not solely dependent on the higher degrees of sequence divergence from the transgene. The random distribution of differences in the nucleotide sequences of the CP coding regions and 3′ UTRs compared also suggests that the sequence homology with the transgene may not be critical for overcoming transgenic resistance. Therefore, in these three isolates, there may be other region(s) of the genome involved in overcoming the transgenic resistance in papaya provided by the YK CP transgene.

In a previous study, Maki-Valkama et al. (2000) showed that transgenic resistance to PVY^{O} associated with post-transcriptional gene silencing (PTGS) of P1 transgene is overcome by PVY^{N} strains that carry highly homologous P1 sequences. Also, in the analysis of comparative reactions of recombinant papaya ringspot viruses with chimeric CP genes and wild-type viruses on CP-transgenic papaya, it was suggested that virus sequences or genes that do not correspond to the transgene may affect the phenotypic reaction of the transgenic plant (Chiang et al., 2001). Our investigation supports the previous suggestion that sequence homology to the transgene is not the only viral factor involved in overcoming transgenic resistance mediated by RNA silencing.

Several reports indicate that the HC-Pro gene acts as a general pathogenicity enhancer mediating synergism (Pruss et al., 1997; Shi et al., 1997) and also has the capability to suppress PTGS (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Isolates 5-19, CS and TD2 share high degrees of sequence homology with the YK CP transgene; however, these isolates induced more severe symptoms on untransformed papaya plants than other isolates do (Table 1; Figure 1). We think that the resistance of transgenic YK CP lines overcome by isolates 5-19, CS and TD2 may be due to the involvement of the HC-Pro gene as a silencing suppressor and virulence enhancer. Whether the HC-Pro gene of these isolates are actually responsible for overcoming the transgenic resistance conferred by the YK CP gene remains to be further investigated. Studies about the SiRNAs would be informative about this inference in understanding the PRSV-transgenic resistance or resistance-breaking.

The identification of isolates capable of overcoming the transgenic resistance in papaya plants provided by the CP gene of PRSV and the analysis on the variability of the isolates with the transgene in this investigation provide useful insights for the study of the mechanism for overcoming transgenic resistance and for generating a broader spectrum resistance to avoid possible failures.

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