Comparison of the Nuclear Inclusion b Protein and Coat Protein Genes of Five Papaya Ringspot Virus Strains Distinct in Geographic Origin and Pathogenicity

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ABSTRACT

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A complementary DNA library was constructed to the genomic RNA of an isolate of papaya ringspot virus (PRSV), PRSV YK (YK), that represents the most prevalent mosaic strain of Taiwan. A 2.96-kb clone, pYKB9, corresponding to the 3′-region of YK RNA was selected by immunoscreening with the antiserum to the coat protein (CP). The clone had 2,960 nucleotide residues and represented part of the nuclear inclusion a (NIa) gene, the complete nuclear inclusion b (NIb) gene, the entire CP gene, and the 3′-noncoding region of YK RNA. The nucleotide sequence was compared to those of the 3′ regions of the published Hawaii severe strain, PRSV HA (HA), the Hawaii mild strain PRSV HA 5-1 (HA 5-1), the nonpapaya-infecting Florida strain, PRSV W(fl) (W(fl)), and the nonpapaya-infecting Australia strain PRSV W(aust) (W(aust)). The NIb region had 83.1% nucleotide identity and 95.7% amino acid identity compared to that of HA, with a total of 262 nucleotide differences resulting in only 22 amino acid changes. A similar trend also was found when the NIb region of YK was compared to the partially elucidated NIb regions of HA 5-1 and PRSV W(fl), with 84.7 and 85.2% nucleotide identities (147 and 151 nucleotide differences), respectively: both resulting in 97.6% amino acid identity (only eight amino acid changes). Most of the nucleotide differences between the Asian and American strains occurred in wobble bases and, thus, conserved the amino acid residues, suggesting a common selection pressure to keep the same protein conformation. The CP region showed 89.6–90.3% nucleotide identities and 93.2–94.8% amino acid identities with the compared strains and the 3′ noncoding region showed 91.4 and 96.4% identities. Analysis of the N terminus of YK CP supported the cleavage site as previously predicted, VYHE/SRGT; however, a possible alternative site at VFHQ/SKNE, 20 amino acids downstream, also was present. The different amino acid residues among the CP of PRSV strains were located mostly at the N-terminal regions of the CP. Alignment of the CP of five sequenced PRSV strains indicated that the variation followed the differences in geographic origins rather than host specificity.

Additional keywords: amino acid sequence, potyvirus.

Papaya ringspot virus (PRSV) is a member of the plant potyvirus group, with flexuous particles of 780 × 12 nm and a genome consisting of a single-stranded (ss)RNA of positive polarity (12,37). PRSV has a single type of coat protein (CP) of 36 kDa (22,38) and induces both cylindrical pinwheel (36,56) and amorphous inclusions (10,32) in the cytoplasm of host cells. Most PRSV isolates belong to one of the two major groups, type P or W (37). Type P, papaya-infecting isolates, cause serious problems in papaya throughout tropical and subtropical regions,

whereas type W isolates, previously described as watermelon mosaic virus 1, are economically important on cucurbits throughout the world (37). Most isolates of these two pathotypes are serologically indistinguishable when tested by their CP antisera (53). However, the host range of PRSV type W is limited in Chenopodiaceae and Cucurbitaceae, whereas type P isolates also infect Caricaceae (papaya) (37,53).

PRSV HA (HA) was originally isolated from Hawaii and is a typical severe strain of type P (22). This virus has been well characterized in host range (37,53), serology (53), gene expression (51), and genetic organization (55). PRSV HA 5-1 (HA 5-1), a mild strain induced by nitrous acid mutation from HA (50), causes infection in papaya without conspicuous symptoms and has been widely used for control of PRSV in papaya by cross-protection (48,54).

During the late 1970s, PRSV spread throughout Taiwan and destroyed most of the commercial papaya orchards (47). Two major strains of PRSV on papaya were found in Taiwan: One caused mosaic symptoms on papaya, and the other caused wilting symptoms (9). The mosaic strain was considered the most prevalent strain in Taiwan (48). Although the severe Taiwan mosaic strain; the severe Hawaii strain, HA; the mild Hawaii strain, HA 5-1; and the nonpapaya-infecting Florida PRSV W(fl) (W(fl)) strain are distinct in pathogenicity, their differences in CP cannot be distinguished by serology (53). The nucleotide sequences of the 3′-terminal regions of type W W(fl), HA 5-1 (40), and the type W Australia strain, PRSV W(aust) (W(aust)) (4) have been determined, and they showed 97.2-99.3% amino acid identity in their CP regions (4,40). Recently, the complete nucleotide sequence and genetic organization of the RNA genome of HA has been reported (55). The CP gene of HA is almost identical to that of HA 5-1 and W(fl), with amino acid identities of 99.3 and 97.9%, respectively (46). In this study, the nucleotide sequence of the CP region of a Taiwan mosaic strain, PRSV YK (YK), was elucidated. To analyze the differences in the CP genes among the PRSV strains distinct in pathogenicity and geographic origins, the nucleotide sequences of the nuclear inclusion b (NIb) and the CP genes of YK were compared with those of the severe strain, HA, the mild strain, HA 5-1, and the nonpapaya-infecting Florida, W(fl), and Australian, W(aust), strains. The differences in CP relating to the cross-protection effectiveness of HA 5-1 are discussed.

MATERIALS AND METHODS

Virus purification and RNA extraction. YK, collected from southern Taiwan, is a stable isolate with biological properties similar to the most prevalent mosaic strain of Taiwan (9,53). YK was propagated in *Cucumis metuliferus* (Naud.) Mey. (35). Virus particles were purified by Cs_2SO_4 centrifugation according to the method of Gonsalves and Ishii (22). Virus RNA was extracted by sodium dodecyl sulfate-proteinase K treatment and sucrose density-gradient centrifugation as described by Yeh and Gonsalves (51).

Complementary DNA synthesis and cloning. Complementary (c)DNA synthesis was based on the method of Gubler and Hoffman (23). The primer, the adaptor, and the enzymes used were from Stratagene (La Jolla, CA). Oligo-d$(T)_{18}$ with an *Xho*I site was used as a primer to initiate first-strand cDNA synthesis from the YK RNA template with Moloney murine leukemia virus reverse transcriptase. The second-strand cDNA was synthesized from the first strand by the addition of RNase H and DNA polymerase I. The double-stranded DNAs were blunted by T4 DNA polymerase and then ligated with *Eco*RI adaptors. After digestion with *Xho*I, they were unidirectionally ligated to Uni-ZAP XR lambda phage vector (Stratagene) at the *Eco*RI/*Xho*I site. Recombinant phage DNAs were encapsidated by a Gigapack Gold packaging extract (Stratagene), plated on *Escherichia coli* strain PLK-F′ and amplified in *E. coli* strain XL 1-Blue according to the instructions provided by Stratagene.

Immunoscreening and in vivo excision. Insert-bearing clones were identified by immunoscreening (30). The materials and conjugate used were from Bio-Rad (Richmond, CA). Plaques grown on a XL 1-Blue bacterial lawn were transferred to nitrocellulose (NC) paper, reacted with the antiserum to the CP of HA (53), and horseradish-peroxidase conjugated by secondary antibody. The NC papers were finally stained with 3,3′-diaminobenzidine tetrahydrochloride substrate, and positive plaques were selected.

The selected lambda recombinants were converted to phagemids by in vivo excision according to the procedure of Short et al (43). Cells of *E. coli* XL 1-Blue were coinfected with a selected lambda recombinant and a helper phage, R408, to excise the phagemid containing the cDNA insert from the lambda DNA. Bacterial colonies containing Bluescript plasmid were recovered by plating the phagemid-infected XL 1-Blue cells on ampicillin plates. Minipreparations of plasmids were done according to the method of Davis et al (11), and the purified plasmids were digested with *Not*I and *Xho*I to determine the size of cDNA inserts. All restriction enzymes used in this study were obtained from Boehringer GmBH (Mannheim, Germany).

Nucleotide sequencing and comparison. The clone pYKB9, initially immunoselected with CP antiserum, was converted to plasmid and used for sequencing. Different orientations of the clone were constructed in pBluescript II KS(+) or KS(−) (Stratagene). The plasmids were unidirectionally deleted by *Exo*III/mungbean-nuclease treatment (24,25,39). Nucleotide sequencing was performed by the dideoxynucleotide chain termination method (42) with ssDNA templates generated by the helper phage VCSM13 (Stratagene). Sequence data were assembled and analyzed by MicroGenie software (Beckman Instruments, Fullerton, CA). The nucleotide sequence of the 3′-terminal region of YK was compared with the published nucleotide sequences of the 3′-terminal regions of HA (46), HA 5-1, W(fl) (40), and W(aust) (4). Multiple sequence alignments of amino acids of CP of PRSV were accomplished by CLUSTAL (version 1.2) of PC/Gene software (IntelliGenetics, Mountain View, CA) designed by Higgins and Sharp (26,27), and the distance data matrix obtained from CLUSTAL was used to construct a dendrogram.

RESULTS

Nucleotide sequence of clone pYKB9. The complete sequence of clone pYKB9, which reflected the 3′-terminal 2,960 nucleotides upstream of the poly(A) tract, is shown in Figure 1. Computer analysis revealed one open reading frame (ORF) consisting of 2,751 nucleotides with the presumed initiation codon upstream. The ORF was followed by a 3′-untranslated region of 209 nucleotides and a polyadenylated tract (28 A residues in pYKB9). This sequence included part of the nuclear inclusion a (NIa) gene, the complete NIb gene, the complete CP gene, and the entire 3′ noncoding region.

Comparison of the NIb region. Potyviral genomes were translated into polyproteins that were then cleaved at specific sites to generate final products (6–8,14,16,17). Analysis of other potyviruses showed that the NIb gene was adjacent and upstream of the CP gene (16). The cleavage site between the NIa and the NIb proteins was located at VFEQ/SGSRWL, which is similar to VFEQ/SGGRWL of the HA isolate and those established for other potyviruses (14,16,55). Clone pYKB9 also reflected 93 amino acids of the C-terminal region of the NIa protein (Fig. 1).

The original position of the N terminus of the CP was first predicted at site VFHQ/SKNE (40), resulting in a 33-kDa protein that is smaller than the molecular mass of 36 kDa estimated from the authentic CP (22,38). However, a new position of the N terminus of HA CP was predicted at site VYHE/SRGT based on the sizes of the NIb gene, the CP, and the cleavage rule for the NIa protease (55). YK had a cleavage site similar to HA at the position of VYHE/SRST and also generated a predicted NIb protein consisting of 517 amino acids (Fig. 1).

The size of potyviral NIb genes ranges from 1,536 to 1,563 nucleotide residues for a protein of 512–521 amino acid residues (1,55). The NIb region of the pYKB9 clone upstream of the CP

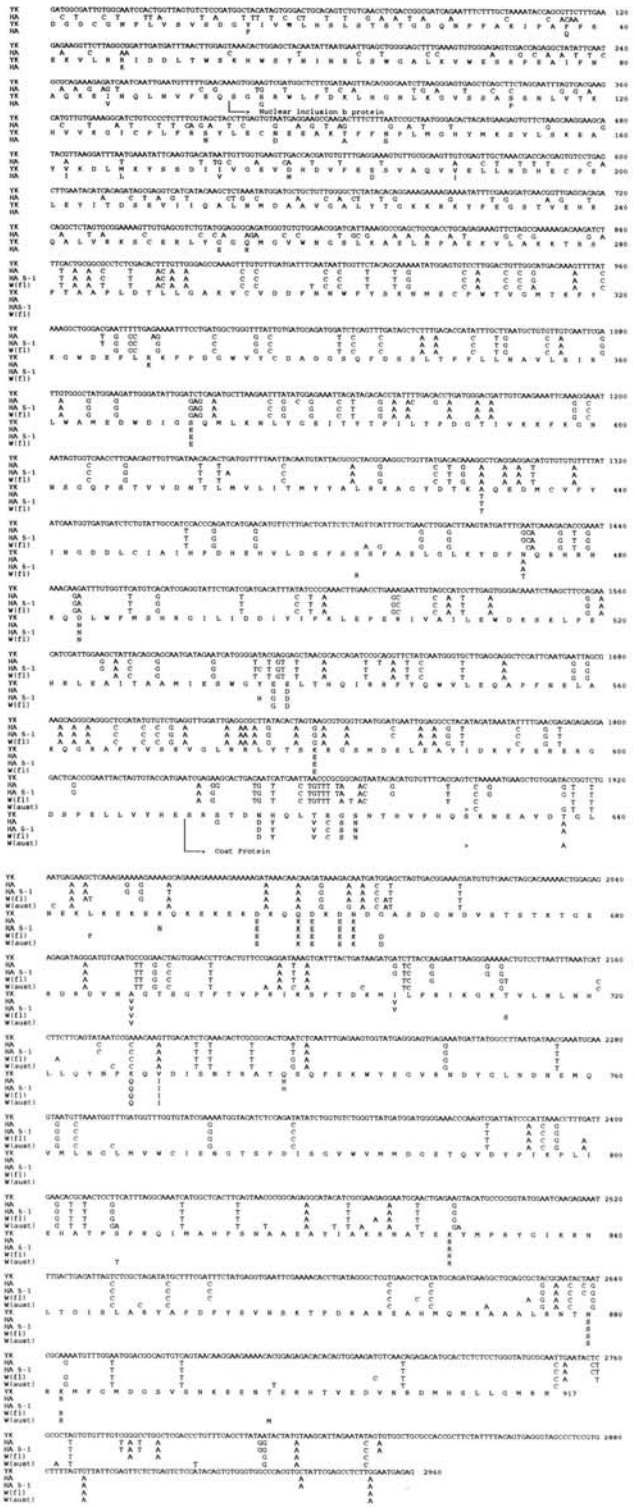

Fig. 1. The complementary DNA sequence and the predicted amino acid sequence of the nuclear inclusion a (NIa) and b (NIb) genes, the coat protein (CP), and the 3′-untranslated regions of a Taiwan mosaic strain of papaya ringspot virus (PRSV YK). Comparison of YK with the same regions of strains HA (severe from Hawaii), HA 5-1 (mild from Hawaii), and W(fl) (type W nonpapaya-infecting from Florida), and W(aust) (type W nonpapaya-infecting from Australia) also is shown. The YK sequences were derived from clone pYKB9 (EMBL Accession X78557). The source of the sequences of HA was Yeh et al ([55], EMBL accession X67673), of HA 5-1 and W(fl) was Quemada et al ([40], EMBL accession D00594, D00595), and of W(aust) was Bateson and Dale ([4], EMBL accession S89893). The arrows indicate the cleavage sites for liberating the N terminus of the NIb protein or the CP, and > indicates the first residue of the CP of W(aust).

gene contained 1,551 nucleotides with a coding capacity of 517 amino acids, which was within the size of other potyviruses.

The complete NIb region of YK had 83.1% nucleotide identity and 95.7% amino acid identity with that of HA. There are 262 nucleotide differences in the NIb gene that resulted in 22 amino acid changes (Fig. 1; Table 1).

Because the NIb regions of HA 5-1 and W(fl) were only partially sequenced (40) and that of W(aust) was not available (4), only the 990 nucleotide residues of the NIb region of YK, reflecting two-thirds of the C-terminal region of the protein, were compared with those available for HA 5-1 and W(fl); the results showed 84.7 and 85.2% nucleotide identity, respectively. However, the amino acid sequences of this portion of the NIb proteins of the three strains were highly conserved, and the YK strain has an identity of 97.6% with each of the other two strains (Table 1). Although there are 151 and 147 nucleotide differences in the NIb protein regions, only eight amino acids were different between YK and the other two strains (Table 1; Fig. 1). The consensus motifs YCDADGS, GNNSGQPSTVVDNT(S)LMV, and NGDDL-X_{34}-K, responsible for the putative RNA polymerase function of HA (55), also were conserved in YK.

Comparison of the CP genes. Comparison of the YK CP with those of HA, HA 5-1, and W(fl), showed 89.7, 89.9, and 89.6% nucleotide identity, and 93.2, 93.5, and 93.5% amino acid identity, respectively. There were 93–96 nucleotide residues different in the CP regions of YK and the other strains, resulting in changes at 20 or 21 amino acid positions (Table 1). Among the different amino acids, there were 16 amino acids conserved in the American strains of HA, HA 5-1, and W(fl) (46), which were mostly located at the N-terminus of the CP (Fig. 1). The N-terminal sequence of the Taiwan strain was highly variable from the American strains, with seven amino acid differences from the first 13 residues (Fig. 1, amino acid position 331–343) and five amino acid differences from the 46th to the 53rd amino acid residue of the CP (Fig. 1, amino acid position 376–383). However, the corresponding regions were conserved among the three American strains (Fig. 1).

A dendrogram of the relationships among the CP of YK and the other PRSV strains, analyzed by the multiple alignment program CLUSTAL of the PC/Gene software, is shown in Figure 2. Because only 861 nucleotides of the CP region of W(aust) were reported, only the 287 amino acids among five strains of PRSV CP were compared. Comparison of 287 amino acids of the YK CP region with that of W(aust) showed they shared 90.3% nucleo-

TABLE 1. Sequence comparison of the 3′-terminal region (3′-NTR) of RNA of papaya ringspot virus (PRSV) isolate YK (a mosaic strain from Taiwan, 2,960 nt) with those of isolates HA (a severe strain from Hawaii, 2,960 nt), HA 5-1 (a mild strain induced from HA, 2,120 nt), W(fl) (a nonpapaya-infecting strain from Florida, 2,120 nt), and W(aust) (a nonpapaya-infecting strain from Australia, 1,070 nt)

	Percent sequence identity[a]				
	Partial NIb		CP		3′-NTR
Isolate	Nt	A.A.	Nt	A.A.	Nt
HA	83.1 (262)[b]	95.7 (22)[b]	89.7 (95)	93.2 (21)	92.3 (16)
HA 5-1	84.7 (151)[c]	97.6 (8)[c]	89.9 (93)	93.5 (20)	91.4 (16)[d]
W(fl)	85.2 (147)[c]	97.6 (8)[c]	89.6 (96)	93.5 (20)	94.7 (11)
W(aust)	NA[e]	NA	90.3 (83)[f]	94.8 (15)[f]	96.2 (8)

[a] NIb = nuclear inclusion b protein; CP = coat protein. Percent identity was analyzed by Beckman MicroGenie software. Numbers in parentheses indicate the total numbers of different nucleotide (Nt) or amino acid (A.A.) residues in the compared region. Source for the sequence of HA was Wang and Yeh (46), of HA 5-1 and W(fl) was Quemada et al (40), and of W(aust) was Bateson and Dale (4).

[b] The complete NIb gene was compared.

[c] Only the 330 amino acids of the NIb gene were compared; the first 187 amino acids at the N-terminus were not available.

[d] The 3′-extreme of YK contained two more nucleotides, AG, than did HA 5-1 before the poly(A) tail.

[e] Not available.

[f] Only the 287 amino acids of the CP were compared; the first 20 amino acids at the N-terminus were not available.

tide identity and 94.8% amino acid identity (Table 1). The relationships among the examined PRSVs suggested the existence of two branches: One branch contains YK, and the other branch contains the other four strains of PRSV.

Comparison of the 3′-untranslated regions. The length of the 3′-untranslated region of YK was identical to that of HA (46), with 209 nucleotides followed by a poly(A) tail. When compared with the 3′-untranslated regions of HA, HA 5-1, W(fl), and W(aust), it showed 92.3, 91.4, 94.7, and 96.2% nucleotide identity, respectively, and there were 16, 16, 11, and eight different nucleotides, respectively (Table 1; Fig. 1).

DISCUSSION

The original prediction by Quemada et al (40) of VFHQ/SKNE for the liberation of the CP would generate a highly variable C-terminus of the NIb protein for the YK strain (Fig. 1), and, thus, would be contradictory to the conservation trend of the NIb protein at the C-terminal regions of potyviruses (31,41,55). The cleavage site predicted by Yeh et al (55) generates a NIb protein with 517 amino acids, which agrees with the sizes of other potyviruses (512–521 amino acids) (1,55) and a variable sequence located at the N-terminal region of the CP gene. Analysis of the YK RNA sequence supports the cleavage site to liberate the N terminus of HA CP at VYHE/SRGT. However, the original prediction of VFHQ/SKNE, 20 amino acids downstream of the VYHE/SRGT cleavage site (40), also is present in the coding sequence of the YK CP (Fig. 1) and fits the general rule for cleavage mediated by PRSV NIa protease (55). The VFHQ/SKNE cleavage also fits the expected positioning of the DAG triplet close to the N-terminus as suggested by the aphid transmissibility hypothesis (3). Thus, it is possible that VFHQ/SKNE is essential for the generation of an aphid-transmissible CP, and VYHE/SRGT is mainly for the generation of a functional NIb protein. If both sites are used, the N terminus of the CP will be heterogeneous. This may explain why the purified CP of PRSV, either prepared fresh from purified virus or after storage at 4 C, is associated frequently with some degraded forms of CP 2–5 kDa smaller than the major 36-kDa protein (22,37,55). This phenomenon also was reported in W(aust) (4) and other potyviruses (28,29,34). To further determine the cleavage site of the N terminus of PRSV CP, serological analysis with antiserum against a synthetic peptide that contains the first 20 amino acids of the N terminus of YK and mutagenesis studies at the two possible cleavage sites are required.

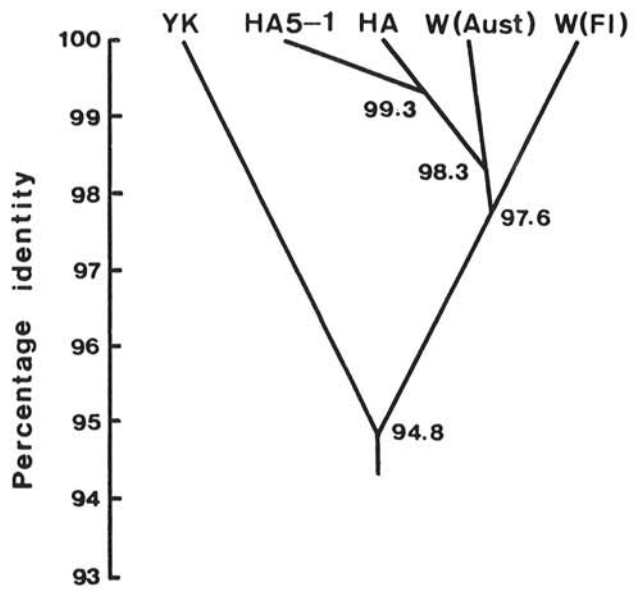

Fig. 2. A dendrogram showing the relationships of the 287 amino acids of coat proteins of five strains of papaya ringspot virus (PRSV). The tree was constructed from multiple protein alignment by the CLUSTAL program of PC/GENE software (26,27). The source for the sequences of PRSV strains HA (severe from Hawaii) was Wang and Yeh (46), of HA 5-1 (mild from Hawaii) and W(fl) (type W nonpapaya-infecting from Florida) was Quemada et al (40), and of W(aust) (type W nonpapaya-infecting from Australia) was Bateson and Dale (4).

The primary function of potyviral CP is, obviously, to encapsidate viral RNA, a process in which the highly conserved central and C-terminal regions, which are the major common characteristics of potyviral CP, are likely to be involved (44). Overall identity of YK CP with those of other strains ranged from 93.2 to 93.5%. This is above the threshold of 90% identity for classifying distinct strains of the same virus (45). Thus, YK should be considered a strain of PRSV. The N-terminal region of YK CP is highly variable when compared to other American strains; this also agrees with the general trend that the N-terminal regions of the CP among different strains of the same potyvirus are variable (40,44, 45,49,55). The relationships among the aligned CP of five PRSV strains showed that the W(aust) strain is closer to the Hawaii type P strain, HA, than to the Florida type W strain, W(fl). It is interesting to note that the degree of variation of the CP does not follow major differences in host specificity, such as papaya- or nonpapaya-infecting but is more closely related to geographic distribution.

Recently, a hypothesis has been proposed that there is a specific involvement of a conserved (I/V) DAG block, present at the N-terminus of CP, in virus transmission by aphids (2,3,20,21,31). The DAG triplet of HA is changed to DTG in YK and W(fl) (Fig. 1), but YK is highly aphid transmissible. Thus, the change at the DAG triplet among these four strains does not correlate to their aphid transmissibilities.

HA 5-1 has been widely used for control of PRSV by cross-protection in several locations worldwide (54). The mild strain, HA 5-1, provided a high degree of protection in papaya against the severe strain, HA, in Hawaii, but the effectiveness of cross-protection varied with the isolates. Different degrees of cross-protection effectiveness were observed when HA 5-1 was used against PRSV strains from Florida, Mexico (33), Taiwan, or Hawaii (48). Under greenhouse conditions, a very high degree of protection (90–100%) against the parental severe HA was observed (50), but relatively low protection rates (50–60%) were obtained when the mild mutant, HA 5-1, was used to protect papaya against the mosaic or wilt strains from Taiwan (48). Under field conditions, protection in Hawaii lasted longer than 2 yr, but a high frequency of protection breakdown in Taiwan still limited papaya to an annual crop (54). Apparently, strain-specific protection limits the application of HA 5-1 in different areas of the world. CP of the protective virus strain is thought to be involved in interference with uncoating of the challenge virus or with sequestering the RNA genome of the challenge virus both in conventional cross-protection (13) and CP-moderated resistance in transgenic plants (5). Recently, Fitch et al (18) successfully incorporated the PRSV CP gene of HA 5-1 into papaya via microprojectile bombardment and obtained plants that expressed the CP gene. The transgenic plants were tested for resistance against 13 isolates of PRSV, and their results showed a strain specificity similar to that observed with classic cross-protection (52).

Recently, we generated several YK CP-transgenic papaya lines that are resistant to YK and other Taiwan mosaic type isolates, but that are only moderately resistant or not at all resistant to HA (S. D. Yeh, *unpublished data*). Sequence homology of the CP between the severe strain, HA, and the mild strain, HA 5-1, has a closer relationship (99.3%) than that between the Taiwan mosaic strain YK and the mild strain HA 5-1 (93.5%). It appears that the lower degrees in conventional cross-protection or CP-mediated protection are related to the lower homologies of the CP genes. Thus, to minimize the strain-specific protection problem, a useful PRSV mild protective strain for cross-protection should be derived from a locally prevalent strain. In addition, a CP gene from a locally prevalent strain should be used for CP-mediated resistance in transgenic papaya.

Comparison of the five strains analyzed showed that there are significant differences in the nucleotide level, but that amino acid

sequences are quite conserved. Differences in nucleotide similarities might be due to distinct evolutionary pathways in different geographic regions; most of the nucleotide differences occur in the wobble bases of codons. The NIb protein is the putative potyviral polymerase (15) and the most conserved protein of potyviral products (55). It is possible that there is a common selection pressure to keep the correct conformation for the essential function of the NIb protein, such as viral replication. The higher degree of identity of the NIb proteins between PRSV strains indicates that the NIb protein is highly conserved among PRSV strains of different geographic origins.

Frenkel et al (19) proposed that the 3′-untranslated regions of potyviral genomes are useful for taxonomic purposes. They determined that percentages of sequence similarities between accepted virus strains ranged from 83 to 99%, whereas similarities between distinct viruses ranged between 39 and 53%. Comparison of the 3′-untranslated region of YK with those of HA (46), HA 5-1, W(fl) (40), and W(aust) (4) indicates that this region of PRSV strains is relatively conserved. The 3′-untranslated regions of different potyviruses are highly variable (55). Thus, the sequence of the 3′-untranslated region of PRSV RNA may be valuable as a marker for distinguishing PRSV from other potyviruses.

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