MOLECULAR PLANT PATHOLOGY (2008) **9**(3), 269–280 DOI: 10.1111/J.1364-3703.2008.00467.X

Pathogen profile

Papaya ringspot virus-P: characteristics, pathogenicity, sequence variability and control

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SUMMARY

Taxonomy: *Papaya ringspot virus* (PRSV) is an aphid-transmitted plant virus belonging to the genus *Potyvirus*, family *Potyviridae*, with a positive sense RNA genome. PRSV isolates belong to either one of two major strains, P or W. The P strains infect both papaya and cucurbits whereas the W strains infect only cucurbits.

Geographical distribution: PRSV-P is found in all major papaya-growing areas.

Physical properties: Virions are filamentous, non-enveloped and flexuous measuring 760–800 × 12 nm. Virus particles contain 94.5% protein and 5.5% nucleic acid. The protein component consists of the virus coat protein (CP), which has a molecular weight of about 36 kDa as estimated by Western blot analysis. Density of the sedimenting component in purified PRSV preparations is 1.32 g/cm^3 in CsCl.

Genome: The PRSV genome consists of a unipartite linear single-stranded positive sense RNA of 10 326 nucleotides with a 5′ terminus, genome-linked protein, VPg.

Transmission: The virus is naturally transmitted via aphids in a non-persistent manner. Both the CP and helper component (HC-Pro) are required for vector transmission. This virus can also be transmitted mechanically, and is typically not seed-transmitted.

Hosts: PRSV has a limited number of hosts belonging to the families *Caricaceae*, *Chenopodiaceae* and *Cucurbitaceae*. Propagation hosts are: *Carica papaya*, *Cucurbita pepo* and *Cucumis metuliferus* cv. accession 2459. Local lesion assay hosts are: *Chenopodium quinoa* and *Chenopodium amaranticolor.*

Control: Two transgenic papaya varieties, Rainbow and SunUp, with engineered resistance to PRSV have been commercially grown in Hawaii since 1998. Besides transgenic resistance, tolerant varieties, cross-protection and other cultural practices such as isolation and rogueing of infected plants are used to manage the disease.

Virus code: 00.057.0.01.045.

Virus accession number: 57010045.

Useful link: http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/57010045.htm

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INTRODUCTION

Papaya ringspot virus (PRSV), a member of the aphid-transmitted genus *Potyvirus*, is the cause of a destructive disease and a major limiting factor for papaya and cucurbit cultivation worldwide (Purcifull *et al.*, 1984). PRSV is grouped into papaya-infecting type-P (PRSV-P) and non-papaya -infecting type-W (PRSV-W). PRSV-P isolates infect plants in the families *Caricaceae*, *Cucurbitaceae* and *Chenopodiaceae* whereas isolates of PRSV-W infect plants in the families *Cucurbitaceae* and *Chenopodiaceae.* PRSV-P and PRSV-W are serologically indistinguishable and are transmitted non-persistently by several species of aphids. PRSV-P infection is typically characterized by the production of ringspot symptoms on fruit of infected papaya trees (Jensen, 1949). In addition to ringspots, PRSV produces a range of other symptoms such as leaf mosaic and chlorosis, water-soaked oily streaks on the petiole and upper part of the trunk, distortion of young leaves that sometimes results in shoestring-like symptoms that resemble mite damage, stunting of infected plants, and flower abortion (Fig. 1). Consequently, fruit production can be severely decreased, and fruit sugar levels reduced by 50% or more. Symptoms on cucurbits are similar to those on papaya. PRSV is able to infect members of the family *Chenopodiaceae* such as *Chenopodium amaranticolor* and *Chenopodium quinoa* but the disease symptoms are restricted to local lesions on the leaves (Purcifull *et al.*, 1984). Infection assays on these 'local lesion hosts' are used as supplementary information for evaluation of PRSV pathogenicity. The virus can be propagated and

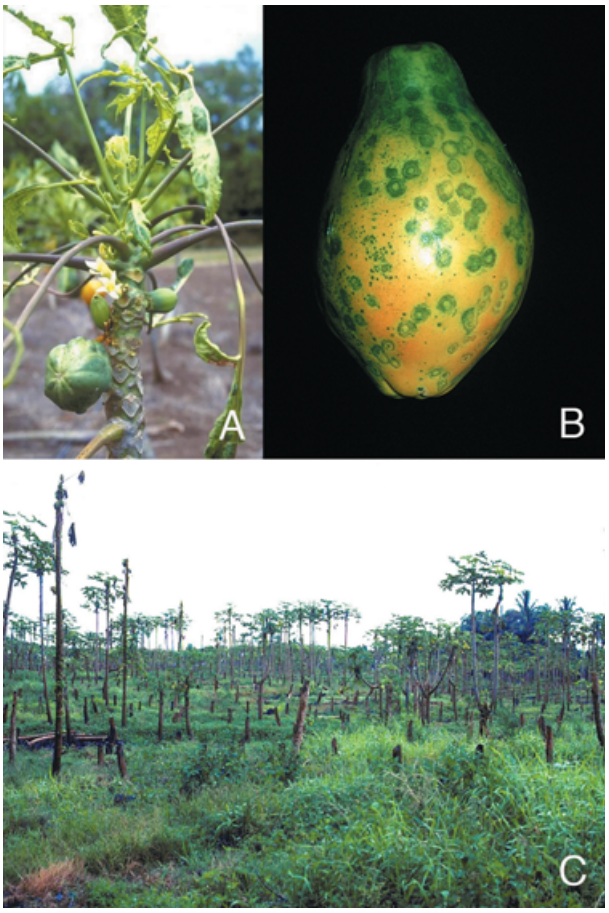

Fig. 1 Symptoms of PRSV on papaya. (A) PRSV-infected papaya tree, (B) ringspot symptoms on fruit, and (C) PRSV-infected papaya orchard in Puna area of Hawaii in 1994.

maintained for experimental studies on *Carica papaya*, *Cucurbita pepo* and *Cucumis metuliferus*, although in the case of *Cucumis metuliferus* it will proliferate on some accessions but not others (cv. Accession 2459 but not, for example, PI 292190) (Yeh *et al.*, 1984). Recently, *Momordica charantia* (a member of the family *Cucurbitaceae*) is reported to be a climbing weed host reservoir for PRSV-P in Jamaica with infected plants exhibiting vein clearing symptoms (Chin *et al.*, 2007).

The devastating nature of PRSV on commercial crops is exemplified by the near destruction of the Hawaiian papaya industry in the 1990s (Ferreira *et al.*, 2002; Gonsalves, 1998, 2006; Gonsalves *et al.*, 2006, 2007a). This pathogen profile reviews the current knowledge on the biology of PRSV, pathogenesis, genome, isolate/sequence diversity and development of disease control strategies. An overview on the control of PRSV by development of genetically engineered virus resistance in papaya has been included as it is the most successful approach to combat PRSV and it has been commercially implemented in Hawaii. However, PRSV remains a threat to the economic production of papaya and cucurbits worldwide (Fermin and Gonsalves, 2003; Fermin *et al.*, 2004; Fuchs and Gonsalves, 2007; Gonsalves, 1998, 2006; Gonsalves and Fermin, 2004; Gonsalves and Ferreira, 2003; Gonsalves *et al.*, 2004a,b, 2006, 2007a,b; Suzuki *et al.*, 2007; Tripathi *et al.*, 2006).

GENOME ORGANIZATION

Papaya ringspot virus belongs to the genus *Potyvirus*, family *Potyviridae*. The virus consists of non-enveloped flexuous filamentous particles which consist of a positive-sense, single-stranded, unipartite RNA genome encapsidated by the genome-encoded coat protein (CP). The genomic RNA of PRSV is 10 326 nucleotides long followed by a tract of polyA sequence (A_n) (Fig. 2). An open reading frame starting at nucleotide 86 and ending at nucleotide 10 120 encodes a polyprotein of 3344 amino acids from which all the proteins of the virus are derived. As with other potyviruses, several proteins are produced through a combination of cotranslational, post-translational, autoproteolytic and transproteolytic processing by three virus-encoded endoproteases, P1, HC-Pro and NIa (Adams *et al.*, 2005; Yeh and Gonsalves, 1985; Yeh *et al.*,

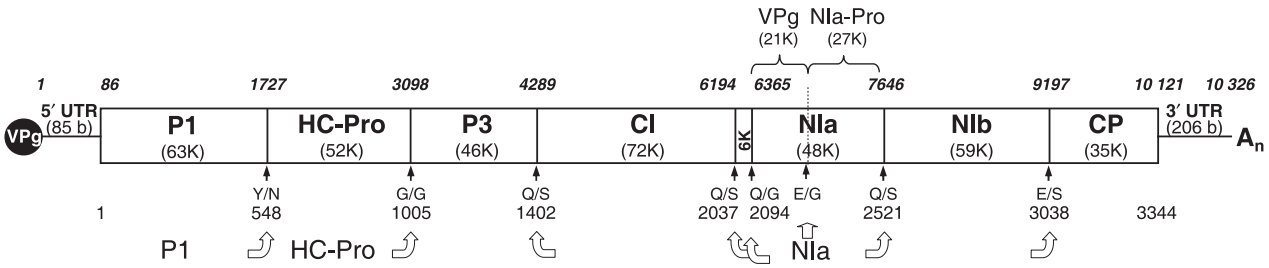

Fig. 2 PRSV genome map of the Hawaiian isolate HA (GenBank accession number S46722). Numbers at the top indicate nucleic acid position. Numbers at the bottom indicate the amino acid position in the polyprotein. Molecular weights of the individual viral proteins are shown below the viral protein name in parentheses. The 5′ and 3′ untranslated regions (UTR) are marked along with their lengths (in bases, b) in parentheses. Amino acids flanking the cleavage sites of proteases P1, HC-Pro and NIa (shown with block arrows) are indicated below black arrows marking the relative cleavage site position. The solid black circle labelled VPg represents the genome-linked protein.

1992). The viral genes [name (product size)], listed in the order of their occurrence (5′ to 3′), in the PRSV genome are: P1 (63k), helper component (HC-Pro, 52k), P3 (46k), cylindrical inclusion protein (CI, 72k), 6K (6k), nuclear inclusion protein a (NIa, 48k), nuclear inclusion protein b (NIb, 59k) and coat protein (CP, 35k) (Fig. 2) (Quemada *et al.*, 1990; Wang and Yeh, 1997; Yeh and Gonsalves, 1985; Yeh *et al.*, 1992) . The P1 protein of PRSV is 18–34k larger than that found in other potyviruses. Several of the virus-encoded proteins have been identified by the non-virion complexes they form in infected plant cells, including the HC-Pro (also known as the amorphous inclusion or AI protein), the CI associated with cylindrical or pinwheel inclusions, and NIa and NIb proteins associated with nuclear inclusion bodies.

Interestingly, two cleavage sites at the N-terminus of the CP have been predicted based on PRSV sequence analysis. The first, VFHQ/SKNE (Quemada *et al.*, 1990), produces a CP of 33k, smaller than the 36k estimated from authentic CP (Gonsalves and Ishii, 1980), and an NIb protein containing 537 amino acids, which is about 20 amino acids larger than those of other potyviruses. The second, VYHE/SRGTD (Yeh *et al.*, 1992), produces a CP and Nb of similar size to other potyviruses. There is no firm evidence to suggest that only one cleavage site is used for processing. Heterogeneous CP products would be expected if both sites were used in polyprotein processing. This may explain why purified CP preparations that have been stored frequently contain the major 36k form as well as CPs that are 2–5k smaller.

The 85-base, 5′ untranslated region (UTR) of the PRSV genome is rich in A and U residues, similar to other potyviruses, but is 63–121 bases shorter. The 5′ UTR shows sequence similarity to the 5′ UTR of a number of other potyviruses, suggesting that this region may serve a common functional role (Yeh *et al.*, 1992).

The virus-encoded VPg protein that is derived from the autoproteolytic processing of the NIa protease in potyviruses is a covalently bound component in a number of plant and animal viral genomes. The role of the VPg is related to priming of RNA synthesis during replication of the viral genome and possibly packaging of the virion. Evidence from studies of similar positive-strand viruses suggest that viral genome replication occurs via intermediate complexes, which include the virus-encoded NIb (RNA-dependent RNA polymerase or RdRp) and minus-strand templates (Riechmann *et al.*, 1992).

HOST SPECIFICITY DETERMINANTS, PATHOGENESIS AND VECTOR TRANSMISSION

As with other potyviruses, PRSV is typically stylet-borne and is transmitted by many species of aphids (mainly *Myzus persicae* and *Aphis gossypii*) in a non-persistent manner. The acquisition and transmission of infectious PRSV virion particles occurs during the brief period when the aphid superficially probes into the plant. Two virus-encoded proteins, CP and HC-Pro, are required for this process (Maia *et al.*, 1996; Peng *et al.*, 1998; Pirone, 1991; Pirone and Blanc, 1996).

The development of a system to produce infectious viral transcripts from recombinant PRSV *in vitro* (Chiang and Yeh, 1997) has provided a unique opportunity to begin to identify viral-encoded gene segments involved in various viral functions, including pathogenicity, host range and vector transmission (Chen *et al.*, 2001b; Lee *et al.*, 2001), as well as to probe the mechanisms involved in induced virus resistance caused by cross-protection and transgene-induced post-transcriptional gene silencing (Chiang *et al.*, 2001; You *et al.*, 2005). The results of bioassay studies with *in vitro* transcripts of recombinant PRSV-W with its *CP* gene replaced with that of a *CP* gene from PRSV-P demonstrated that the *CP* gene is not a determinant for infection of papaya (Chen *et al.*, 2001b). Instead, similar experiments with PRSV-W containing PRSV-P segments from the region including the *NIa* gene and a portion of the *NIb* gene were required for papaya infection. Mutations in the PRSV-P genome at two amino acid positions in the NIa-Pro region, which are conserved within isolates of the same host range type but differ between PRSV-P and PRSV-W, 2309 (K→D) and 2487 (I→V), demonstrated that these two residues are critical for conferring PRSV pathogenicity of papaya (Chen *et al.*, 2003).

In order to identify the genetic determinants for pathogenicity across host species, recombinants were generated between a severe PRSV-P strain (PRSV HA) and a nitrous acid mutant derived from it (PRSV HA5-1). Mutations in the *P1* and *HC-Pro* genes of the severe strain engineered to match those found in the mild strain resulted in the attenuation of both PRSV HA symptoms in papaya as well as reduced local lesion formation on *Chenopodium quinoa* (Chiang *et al.*, 2007; Lee *et al.*, 2001). The results also indicated that of the two genes, *HC-Pro* was the major determinant for local lesion formation in *Chenopodium quinoa*.

Understanding the interactions occurring between the host and viral pathogen and between multiple viruses and their host during mixed infections is of fundamental biological interest as well as a key to developing strategies to control or manage disease. In the case of PRSV, the use of cross-protection, a tool which has been used to protect a plant from the severe symptoms of a virulent virus by prior inoculation with an attenuated form of the same virus, has provided a clear demonstration that these biological interactions exist. Although cross-protection is not clearly understood at the molecular level, studies suggest sequence-specific RNA-mediated natural defence, which is similar to post-transcriptional gene silencing (PTGS) (Ratcliff *et al.*, 1999). Experiments to evaluate the efficacy of cross-protection against virulent PRSV-P strains differing in geographical origins suggest that cross-protection is more effective against challenge strains of the virus that are related more closely

related to the virus used for cross-protection (Gonsalves and Garnsey, 1989; Yeh and Gonsalves, 1994), consistent with the notion that sequence specificity plays a role in the efficacy of protection. Further evidence for the involvement of viral homology and the importance of extent and position of this homology in the phenomenon of strain-specific cross-protection was provided in a study using the original and recombinant versions of the attenuated PRSV-P HA5-1 on *Cucumis metuliferus* followed by infection with virulent strains PRSV-W or PRSV-P. While the attenuated PRSV mutant HA5-1 provided 90–100% protection against the severe parental strain PRSV HA under greenhouse and field conditions, it provided only 20–30% protection against the PRSV-W from Taiwan (Yeh and Gonsalves, 1984, 1994). By contrast, the recombinant HA5-1 carrying both the heterologous CP and the 3′ UTR from PRSV-W significantly enhanced the protection against PRSV-W in cucurbits (You *et al.*, 2005). In particular, the heterologous 3′ UTR was found to be critical for enhancement of the protection against PRSV-W in *Cucumis metuliferus*. Interestingly, the same recombinant HA5-1 virus carrying the heterologous PRSV-W CP or 3′ UTR reduced the effectiveness of protection against PRSV HA in papaya, indicating that both the CP and the 3′ UTR are also important in cross-protection in papaya (You *et al.*, 2005).

SEQUENCE DIVERSITY AND EVOLUTION

Knowledge of sequence diversity among isolates of a virus and their distribution has the potential to deepen our understanding of viral origins, development, dispersion and disease etiology. This information would be useful in developing effective virus disease management programmes. With PRSV, most studies have focused on examining sequence variation in the *CP* gene. Initially data from the USA and Australia (Bateson *et al.*, 1994; Quemada *et al.*, 1990) suggested that there was little sequence variation among the *CP* gene from PRSV isolates within these countries. However, recent sequence data of PRSV *CP* genes of isolates from India (Jain *et al.*, 2004) and Mexico (Silva-Rosales *et al.*, 2000) suggest greater sequence variation among the local populations of PRSV isolates in other countries. A phylogenetic tree illustrating the relationship of PRSV isolates from different countries utilizing published *CP* gene sequences (Bateson *et al.*, 2002; Jain *et al.*, 2004) is shown in Fig. 3. Reported *CP* sequence diversity at the amino acid (10%) and nucleic acid (14%) levels were highest among the Asian populations of PRSV isolates (Jain *et al.*, 2004). Interestingly, this variation is considerably less than that found among isolates of other potyviruses, such as *Yam mosaic virus* (YMV), where the nucleotide sequence diversity was reportedly as high as 28%. The PRSV isolates collected within India were as different from each other in *CP* gene sequence (0–11%) as they were to the *CP* gene sequences of isolates collected in Bangladesh (9–11%), other Asian countries (4–14%), Australia (5–11%) and the Americas (5–11%). A study by Bateson *et al.* (2002) also described the high levels of diversity in PRSV isolates from the Indian subcontinent and proposed that they probably represented the oldest population of PRSV, and based on their basal position according to phylogenetic analysis, the origin of PRSV might have been South Asia.

Some of the variation observed between PRSV-P isolates was due to differences in the *CP* gene length, which varied between 840 nucleotides encoding 280 amino acids for the KA2 isolate from India (Jain *et al.*, 2004) and 870 nucleotides encoding 290 amino acids for the VNW-38 isolate from central Vietnam (Bateson *et al.*, 2002). All described differences in *CP* length were confined to the first 50 amino acids at the amino terminal end of the CP, a region of noted variability due to changes in the number of so-called EK repeats (Bateson *et al.*, 2002; Jain *et al.*, 2004). The sequence differences in this region of the virus genome lead mainly to conservative amino acid substitutions, which probably preserves CP function. At present, variation in length of the *CP* gene has been described from among PRSV populations within countries such as India (Jain *et al.*, 2004) and Vietnam (Bateson *et al.*, 2002) but not from Thailand (Bateson *et al.*, 2002).

An interesting question regarding PRSV is the origin of types P and W. A number of viral diseases on cucurbits were initially associated with *Watermelon mosaic virus* 1 (WMV-1) but not with any viral diseases of papaya. Later, serological and molecular characterization showed that WMV-1 was virtually identical to PRSV. Did type P originate from W, or conversely, did they evolve independently? A sequence study of PRSV-P and PRSV-W from Australia suggests that the recent outbreak of PRSV-P came from the population of PRSV-W already present in Australia rather than as a new introduction (Bateson *et al.*, 1994, 2002). This proposal is also supported by the diversity of cucurbit-infecting potyviruses and virus isolates that are phylogenetically closer to PRSV.

CONTROL STRATEGIES: CONVENTIONAL TO GENETICALLY ENGINEERED RESISTANCE

PRSV disease management practices include quarantine, eradication, avoidance by planting papaya in areas isolated from the virus, continual rogueing of infected plants, use of tolerant lines to lower the economic losses caused by PRSV, cross-protection and transgenic resistance. The Hawaiian papaya story is described here because it involves all of the above efforts and ultimately transgenic resistance in papaya was most successful in controlling the virus.

Development of PRSV-resistant cultivars through conventional breeding met with limited success because of difficulties in overcoming intergeneric reproductive barriers of wild, related species of papaya (Gonsalves *et al.*, 2006; Manshardt, 1992; Manshardt and Wenslaff, 1989). In addition, partial loss of tolerance in back-crosses with the commercial papaya parent also limits the

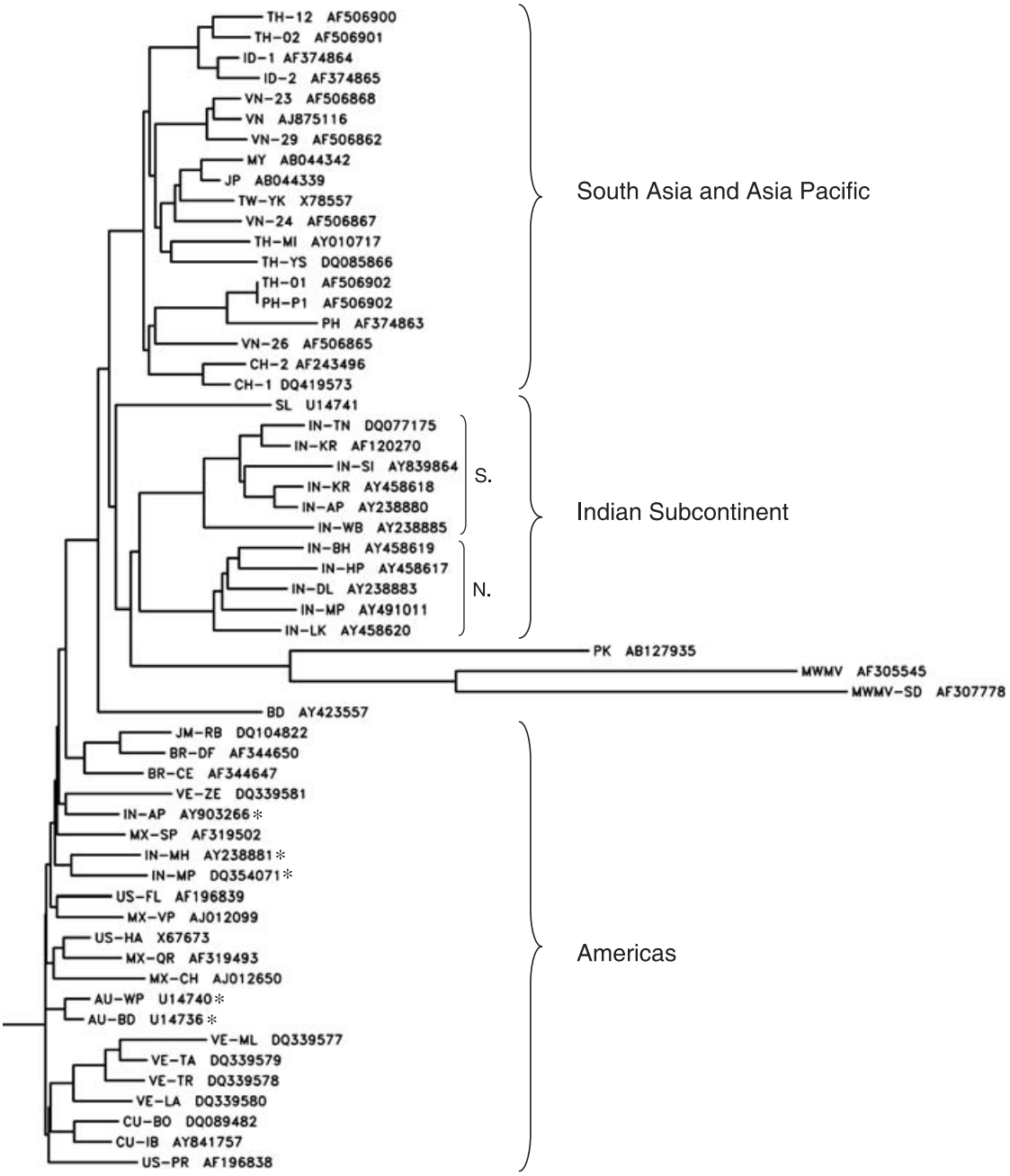

Fig. 3 Neighbour-joining tree showing the relationships between *CP* gene sequences of PRSV isolates from various countries. The tree was constructed with Clustal W and Drawgram programs using CP sequences of PRSV isolates from AU (Australia), BD, (Bangladesh), BR (Brazil), CH (China), CU (Cuba), ID (Indonesia), IN (India), MX (Mexico), MY (Malaysia), JM (Jamaica), JP (Japan), PH (Philippines), SL (Sri Lanka), TW (Taiwan), TH (Thailand), US (USA), VE (Venezuela) and VN (Vietnam). A CP sequence from a PRSV-W type isolate from Pakistan (PK) and *Moroccan watermelon mosaic virus* (MWMV) *CP* genes were used as outgroup sequences. N. and S. denote North and South India, respectively. Asterisks denote PRSV CP sequences of isolates that are not from the Americas, but cluster with sequences of PRSV from countries of that region.

usefulness of this approach. As a control strategy for PRSV in papaya, cross-protection was initiated in 1979 and used briefly in Hawaii, Florida, Mexico, Taiwan and Thailand (Wang *et al.*, 1987; Yeh and Gonsalves, 1994; Yeh *et al.*, 1988). Cross-protection involves the use of a mild virus strain to protect plants against economic damage caused by the severe strain of the same virus or related virus (Gonsalves and Garnsey, 1989; Yeh and Gonsalves, 1984). Mild strain HA5-1 was selected from severe strain

HA after nitric acid mutagenesis and used to cross-protect papayas in Hawaii (Yeh and Gonsalves, 1984). Although used for about 5 years, relatively severe virus symptoms occurred on certain cultivars, such as Sunrise, and it was thus not widely adopted (Ferreira *et al.*, 1992; Mau *et al.*, 1989; Pitz *et al.*, 1994). In Taiwan, cross-protection was evaluated extensively, but it proved to be only marginally effective. The value of the mild strain HA5-1 was more fully appreciated when it was used later as the source of the *CP* gene for genetically engineering resistance. Use of the *CP* gene from the mild PRSV strain would probably mitigate against the impact of potential viral recombination possibly associated with *CP* gene transformation.

Although other laboratories have engineered resistance against PRSV using the *CP* (Bau *et al.*, 2003; Cheng *et al.*, 1996; Davis and Ying, 2004; Lines *et al.*, 2002) or the replicase (Chen *et al.*, 2001a) genes of PRSV, we will emphasize here the Hawaii transgenic papaya case because the resultant Rainbow and SunUp cultivars are the only transgenic papayas to be deregulated and commercialized, and to be sold and consumed in the mainland US and Canada (Gonsalves, 1998, 2006; Gonsalves *et al.*, 2004b, 2007a; Suzuki *et al.*, 2007; Tripathi *et al.*, 2006).

Hawaii transgenic papaya case

Although first reported in 1945 (Jensen, 1949), PRSV was probably observed as early as 1937 (Parris, 1938) on the island of Oahu in Hawaii. PRSV appeared to cause a relatively mild disease of papaya until the middle 1950s when a strain of the virus causing yellow mosaic disease was reported (Ishii and Holtzmann, 1963). This new strain was initially described as a new papaya virus (*Papaya mosaic virus*) but shown later to be a different strain of PRSV, suggesting that the new yellow mosaic strain had either evolved from the original milder strain of the virus or that a second more severe strain was introduced in the mid-1950s. Interestingly, within 10 years of its occurrence, the new yellow mosaic strain of PRSV quickly became widespread and severely affected production. On Oahu papaya acreage declined from a peak of just over 243 ha in 1956 to less than 16 ha in 1968.

With the decline in papaya production on Oahu due to both PRSV and encroaching urbanization, the Puna district on the island of Hawaii became the major production centre. By the early 1990s, production averaged about 25 000 tonnes of fruit annually, and represented about 95% of Hawaii's papaya production (Ferreira *et al.*, 1992).

By 1971 PRSV had become established on the island of Hawaii in home-grown papaya in the Hilo and Kona districts; it quickly became established in Keaau also, about 6.5–8 km from Hilo. A strict quarantine was imposed prohibiting the movement of papaya seedlings between the Hilo/Keaau region and commercial production areas in Puna. In effect, a PRSV-free buffer of about 30 km kept commercial production relatively isolated from PRSV. In this buffer zone, only occasional backyard or homeowner plants were noted, and remained PRSV free.

In 1992, PRSV was observed in Puna (Ferreira *et al.*, 2002). Within 3 years, commercial production in Puna was no longer possible (Fig. 1C), except for a few relatively isolated areas.

By the autumn of 1995, growers attempted to 'outrun' the virus by relocating production along the Hamakua coast, just north of Hilo, in former areas of sugarcane production where PRSV was absent. In this way, papaya production on the island of Hawaii was partially spared the total impact of PRSV, as production declined from 23 600 tonnes to 11 600 tonnes from 1992 to 1999.

As the PRSV epidemic played out in Puna, efforts to develop a solution were well underway, having been initiated about 6 years earlier. The concept of pathogen-derived resistance (PDR), conceived in the mid-1980s (Sanford and Johnston, 1985), offered a new approach for controlling virus diseases. In 1986, Gonsalves' laboratory at Cornell University in collaboration with the Upjohn Company and the University of Hawaii started work to engineer papaya with the *CP* gene of the mild PRSV strain HA5-1. The *CP* gene construct was originally designed with the concept that protein expression was required for resistance. The PRSV CP is produced by post-translational protease cleavage of a PRSV polyprotein and does not contain a native translation signal specific to CP or even a 5′ UTR. Therefore, a chimeric PRSV *CP* transgene construct was made utilizing the translation signals found in the leader sequence (5′ untranslated RNA translational enhancer and initial 16 amino acid coding sequences) of the *Cucumber mosaic virus* (CMV) *CP* gene fused in-frame to the structural sequence of the PRSV *CP* including the Q/S protein cleavage site and 70 nucleotides of the non-coding region. This was accomplished by cloning the PRSV HA5-1 *CP* structural sequence from plasmid pPRV117 between the CaMV 35S double enhancer promoter—translational leader sequence and CaMV 35S terminator of a CMV expression cassette (Quemada *et al.*, 1990, 1991). This PRSV *CP* expression cassette was finally cloned into pGA482GG, a modified version of the *Agrobacterium* transformation vector pGA482 (An *et al.*, 1988) that contained the *nptII* (neomycin phosphotransferase II) gene behind a nopaline synthase promoter and a *uidA* (GUS or β glucuronidase) gene behind a CaMV 35S promoter, used for kanamycin selection and colorimetric screening of transformants, respectively (An, 1986; Ling *et al.*, 1991).

The red-fleshed cultivars Sunrise and Sunset, and the yellow-fleshed Kapoho were transformed with PRSV HA5-1 *CP* biolistically (Fitch *et al.*, 1990). Nine selected transgenic lines from original transformants, six Sunset and three Kapoho, were screened for resistance with PRSV HA from which the mild strain HA5-1 had been derived. R_0-micropropagated plants of the first transformant line designated 55-1 showed excellent resistance to PRSV HA (Fig. 4A) (Fitch *et al.*, 1992).

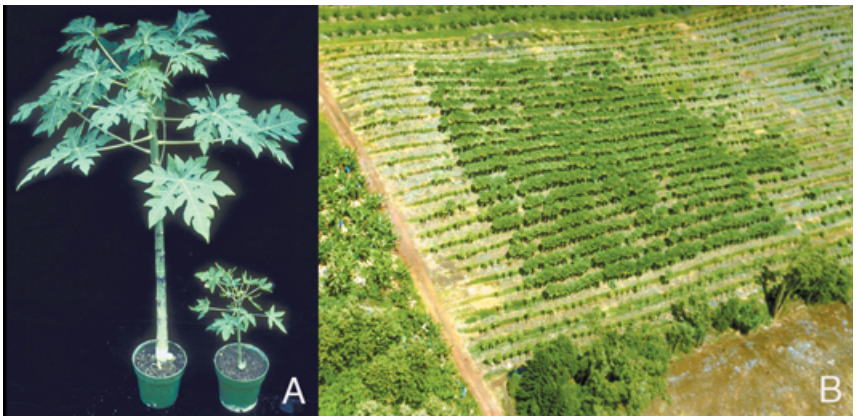

Fig. 4 Evaluation of transgenic papaya for PRSV resistance. (A) R_0-transgenic papaya line 55-1 (left) and nontransgenic control (right) six months after inoculation with PRSV HA in the greenhouse. (B) Aerial photograph of the 1-acre plot of Rainbow papaya in Puna (Hawaii) 28 months after transplanting. The Rainbow block was surrounded by non-transgenic susceptible Sunrise plants, which are severely infected by PRSV.

In 1991, the first field assessment of line 55-1 was made (Lius *et al.*, 1997). Nearly all (95%) of the non-transgenic plants and those of a transgenic line that lacked the *CP* gene were infected by 77 days after the start of the field trial, whereas none of the line 55-1 plants was ever infected. PRSV was not recovered from line 55-1 plants except for two plants, which showed virus symptoms on side shoots but none on the leaves of the main canopy. Plants of line 55-1 grew normally and produced fruit with total soluble solids of about 13%, within the expected range for fruit from uninfected plants. Thus, by mid-1993, the trial had provided convincing evidence that line 55-1 would be useful for controlling PRSV in Hawaii (Gonsalves, 1998).

Given the imposing presence of PRSV on the island of Hawaii and the performance of line 55-1 and its R_1 derivatives in the greenhouse and field trials, an active attempt to utilize this new transgenic germplasm for the development of commercial cultivars was made. Because the major cultivar grown in Puna was the yellow-fleshed Kapoho, an F1 hybrid of Kapoho and a homozygous selection of line 55-1 (SunUp) was made to produce a yellow-fleshed, hemizygous cultivar, later named Rainbow (Manshardt, 1998). The resulting Rainbow cultivar bore pear-shaped fruit with yellow-orange flesh as anticipated and together with SunUp was ready to be tested in field trials in Puna in late 1995.

The results of the 1995 field trial were excellent and clearly demonstrated the potential value of transgenic papaya (Fig. 4B) for reclaiming papaya production in Puna (Ferreira *et al.*, 2002; Gonsalves *et al.*, 2004b). Of the non-transgenic controls plants, 50% showed virus symptoms within 4.5 months after transplanting, and all were infected by 11 months. The growth differences between the transgenic and non-transgenic trees were remarkable (Fig. 5); transgenic plants grew vigorously, with dark green leaves and full fruit columns, whereas non-transgenic plants were stunted, with yellow and mosaic leaves and very sparse fruit columns. Fruit production data of Rainbow and SunUp in field tests showed that the yields were at least three times higher than the industry average while maintaining the percentage soluble solids above the minimum requirement for commercial fruits (Ferreira *et al.*, 2002).

Fig. 5 View of PRSV disease progress in the field test in Puna (Hawaii) at 18 and 23 months (top and bottom, respectively). In each view the susceptible Sunrise variety is shown on the left and resistant transgenic Rainbow on the right.

The US governmental agencies involved with deregulation of a transgenic product are the Animal and Plant Health Inspection Services (APHIS), Food and Drug Administration (FDA) and Environmental Protection Agency (EPA). By 1997, approvals from

all three agencies were granted and in April 1998, licence agreements from all parties were obtained, allowing the commercial cultivation of papaya 55-1 or its derivatives in the State of Hawaii. The deregulation of Hawaiian transgenic papaya has been previously reviewed (Gonsalves, 1998; Gonsalves *et al.*, 2007a; Suzuki *et al.*, 2007). At present, transgenic papaya is widely sold in Hawaii and exported to the mainland US and Canada. The transgenic papaya has clearly demonstrated a great impact on controlling the destructive disease caused by PRSV, enabling an increase in non-transgenic papaya production as well as transgenic papaya production and diversification of papaya cultivars in Hawaii. This is perhaps a good model of engineered resistance in papaya for disease control, and biotechnology and technology transfer to other regions.

Although just over 70% of the acreage grown in Puna is transgenic (Rainbow), a significant amount of the non-transgenic (susceptible) Kapoho cultivar continues to be grown for sale in Hawaii's most important market in Japan. The use of the transgenic cultivars ensures that a significant amount of non-transgenic papayas can continue to be produced in areas previously devastated by PRSV.

Potential threat to transgenic resistance

Since its release in 1998, the transgenic papaya has had great socio-economic impact on the Hawaiian papaya industry (Gonsalves, 1998, 2006; Gonsalves *et al.*, 2007a). This success can largely be attributed to both the stability/durability of the transgenic resistance, and the desirable horticultural and fruit quality attributes of the cultivars, Rainbow and SunUp.

However, because the PRSV population in Hawaii is relatively homogeneous, the papaya industry in Hawaii is in a precarious position should resistance breakdown occur due to the emergence or arrival of divergent PRSV strains.

Greenhouse inoculation studies by Tennant *et al.* (1994) showed that the resistance of R_1 transgenic papaya of line 55-1 was narrow. R_1 plants were resistant to isolates from Hawaii but susceptible to PRSV strains occurring in other countries. Rainbow, which like the 55-1 R_1 plants is hemizygous for the *CP* transgene, shared a similar narrow resistance, whereas SunUp (homozygous) showed good resistance to isolates from Hawaii as well as to isolates from Jamaica and Brazil (Table 1). These results suggest that doubling transgene dosage will broaden transgenic resistance (Tennant *et al.*, 2001).

Additional work with line 63-1 (Tennant *et al.*, 2005) further confirms the relationship between transgene dosage and broader resistance (Souza *et al.*, 2005). Line 63-1 is similar to line 55-1, but had double insertion of the *CP* gene, and was resistant to a range of isolates of PRSV not only from Hawaii (HA) but also from Jamaica (JA), Thailand (TH) and Brazil (BR) (Souza *et al.*, 2005; Tennant *et al.*, 2005).

Table 1 Coat protein (*CP*) nucleotide sequence homologies of PRSV isolates to PRSV HA5-1 and summary of reactions of different isolates to Rainbow and SunUp papaya.

PRSV isolates	% Homology to transgene *CP*					Reaction to isolates	
	N	Core	C	3′ncr	Overall	Rainbow	SunUp
Hawaii-HA	99.3	99.8	100	100	99.8	R	R
Hawaii-OA	97.3	98.0	100	95.7	97.9	sR	R
Hawaii-KA	95.3	97.1	98.3	93.6	96.7	sR	R
Hawaii-KE	95.3	97.1	98.3	93.6	96.7	sR	R
Jamaica-JA	89.3	95.0	91.5	69.6	92.5	S	R
Brazil-BR	84.4	93.9	98.3	73.3	91.6	S	R
Thailand-TH	83.7	90.7	91.5	89.4	89.5	S	sR

N = 199 nucleotides of the amino terminus, core = 641 nucleotides of the core region, C = 59 nucleotides of the carboxy terminus, and 3′ncr = 35 nucleotides of the non-coding regions following the stop codon. R = resistant; sR = susceptible at young stages and resistant at older stages; S = susceptible. Table is modified from Tennant *et al.* (2001).

Comparison of the *CP* gene sequences from the various PRSV isolates suggested that resistance is positively correlated with the degree of homology between the *CP* of the infecting virus and the transgene. PRSV isolates from Hawaii showed 97–100% sequence homology to the transgene *CP,* whereas the isolates from elsewhere showed 89–93% *CP* sequence homology to the transgene. Among all isolates of PRSV from outside of Hawaii, the *CP* gene of the Thailand PRSV had the least homology to the transgene *CP* (Table 1) and in turn had the most severe symptom expression.

CP expression data from tested transgenic papaya plants were consistently lower in homozygous SunUp than hemizygous Rainbow. Nuclear run-on experiments were done to determine whether the lower expression levels were due to decreased transcription or PTGS. These results showed clearly and consistently higher transcription in homozygous (SunUp) than in hemizygous (Rainbow) plants. In contrast, Northern assays showed that the steady-state levels of the transgene transcript in SunUp were lower or equal to levels in Rainbow. In another study by Souza *et al.* (2005), resistant plants of line 63-1 had lower CP expression levels than susceptible plants. These results strongly suggest that the mechanism of transgenic resistance is sequence homology-dependent and RNA-mediated via PTGS (Tennant *et al.*, 2001). This requirement for transgene homology limits the applicability of transgenic resistance in different geographical regions that may harbour PRSV strains which are molecularly diverse.

Although there appears to be a strong relationship between transgene and challenge virus *CP* gene homology for resistance to be expressed, other factors also influence the host's response. Experiments on the infectivity of recombinant PRSV HA with

different segments of the *CP* gene and/or its 3′ UTR replaced with the cognate segment from PRSV isolates that can overcome transgenic resistance suggest that position effects and homology within the *CP* gene are also important (Chiang *et al.*, 2001).

Studies with recombinant PRSV transcripts carrying other heterologous PRSV segments showed that the *HC-Pro* gene of the challenge strain plays a role in determining virus pathogenicity and virulence on transgenic papaya. In addition, HC-Pro acts as a suppressor of the plant's gene silencing defence mechanism, which in turn influences effectiveness of resistance (Bau *et al.*, 2004b; Tripathi *et al.*, 2003, 2004; Yeh *et al.*, 2005).

Other studies have shown that the absence of transgene homology with the challenge virus *CP* gene does not always correlate with ability of a PRSV strain to breakdown resistance in transgenic papaya (Chen *et al.*, 2002; Ruanjan *et al.*, 2007; Tripathi *et al.*, 2004; Yeh *et al.*, 2003). Agro-transformation of papaya with the *CP* gene of a native Taiwanese isolate, PRSV YK (Cheng *et al.*, 1996), showed good resistance in several field trials from 1996 to 1999 (Bau *et al.*, 2003, 2004a). Several lines were highly resistant to the homologous strain (PRSV YK) and also to three different geographical PRSV isolates from Hawaii, Thailand and Mexico (Bau *et al.*, 2003). Interestingly, during the fourth field test in 1999, PRSV infection of this line was observed and the PRSV strain isolated from infected transgenic line was designated PRSV 5-19 (Chen *et al.*, 2002; Tripathi *et al.*, 2004). Further characterization of the virus revealed that the nucleotide identity between the transcript of the *CP* transgene and PRSV 5-19 RNA was less divergent than that between the *CP* transgene and other geographical strains of PRSV that were not able to overcome the transgenic resistance (Tripathi *et al.*, 2004).

This observation suggests that the breakdown of the transgenic resistance was not correlated with the sequence divergence between the infecting virus and the transgene. To study the potential role of silencing suppressor HC-Pro in the resistance breakdown, an infectious viral recombinant was constructed by replacing the HC-Pro region of PRSV YK with that of PRSV 5-19 and the resistance against the recombinant was evaluated in transgenic papaya lines. These results demonstrated that the heterologous HC-Pro region of 5-19 alone was sufficient to breakdown the transgene-linked resistance, even though the sequences of the transgene transcript shared 100% identity with the genome of the infecting virus (Yeh *et al.*, 2005). This resistance breakdown by the PRSV 5-19 of PRSV was most probably due to the involvement of the *HC-Pro* gene as a silencing suppressor and/or virulence enhancer (Tripathi *et al.*, 2004; Yeh *et al.*, 2005).

The above observations show that resistance in transgenic papaya can be overcome by PRSV with distant homology to the transgene, or by PRSV strains with HC-Pro that can sufficiently suppress the silencing mechanism of transgenic papaya. It would therefore be important to develop transgenic papaya that could mitigate against the impact of these PRSV strains.

CONCLUSION AND FUTURE PROSPECTS

Papaya ringspot virus belongs to one of the largest and most economically important plant virus groups. Utilizing the concept of CP-mediated resistance, transgenic papaya cultivars Rainbow and SunUp were developed and released in a timely manner to save Hawaii's papaya industry from damage caused by PRSV. Evidence suggests that transgene conferred PRSV resistance is mediated by RNA via homology-dependent post-transcriptional gene silencing or PTGS. While the transgenic resistance in Rainbow and SunUp has proven durable for nearly 10 years in Hawaii, resistance breakdown could occur with the emergence of new viral strains locally or by the introduction of divergent strains from regions where the virus exists, particularly from localities where the suppression of the gene silencing mechanism by HC-Pro has been demonstrated. This potential for resistance breakdown suggests that it is important to monitor the PRSV population for its diversity and the arrival or emergence of new and more virulent strains in nature. The recent increase in cumulative data on PRSV gene sequences, including analyses on isolate variability and phylogenetic relationships between different geographical isolates will be helpful for devising effective PRSV management strategies such as design for the most effective transgene for specific regions.

In fact, evidence that transgenes could only protect against closely related virus strains stimulated the development of a next generation of transgenics that used a new approach of combining different transgene sequences to produce plants that are resistant to multiple viruses or virus isolates. Broad-spectrum resistance to diverse strains of PRSV will benefit small-scale farming worldwide. Advances in our basic knowledge of pathogenicity and infectivity determinants of PRSV, the mechanism of gene silencing and gene silencing suppression in relation to transgenic resistance and its breakdown should also contribute to transgene design and other viral resistance strategies.

However, technology is not the major hurdle in commercializing virus-resistant transgenic plants for other regions. Although Rainbow and SunUp papaya have had a great socio-economic impact in Hawaii and the fruit has been consumed in Hawaii, the US mainland and Canada without any reported adverse effects on human health or the environment, other issues remain as major concerns limiting the commercialization of transgenic plants in many countries. In fact, several countries have developed papaya resistant to PRSV, but deregulation and governmental acceptance are the biggest and last remaining challenges limiting its application in the field and real adoption by all farmers.

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