Transgenic Plant Journal ©2010 Global Science Books

Papaya Ringspot Virus (PRSV) Coat Protein Gene Virus Resistance in Papaya: Update on Progress Worldwide

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ABSTRACT

Transgenic papayas have been marketed from Hawaii to the US mainland for 11 years and to Canada for 7 years. The resistance provided by a fragment of a *Papaya ringspot virus* (PRSV) coat protein (*cp*) gene enabled Hawaii's papaya industry to recover from destruction. Commercialized in 1998, Hawaii papayas were joined in 2006 by China's containing an untranslatable replicase gene. Worldwide, to our knowledge, these are the only commercialized transgenic papayas although Australia, Brazil, China, India, Jamaica, the Philippines, Taiwan, Thailand, Venezuela, and the US (Florida, Hawaii, US Virgin Islands) developed other transgenic virus resistant papayas. Taiwan's dual *cp* genes protect against PRSV and *Papaya leaf distortion mosaic virus* and India seeks protection against PRSV and *Papaya leaf curl virus*. Florida has petitioned for deregulation of PRSV *cp* gene transformants. In other important papaya growing regions, either PRSV is not a major problem or alternative methods, for example, isolation, use of tolerant cultivars, or introgression of resistance genes from wild relatives, provide useful resistance. Brazil, India, Mexico, and Malaysia rely on broad expanses of buffer zones. In Thailand and the Philippines, tolerance was bred into local papayas and in Australia and the Philippines, resistance genes were introgressed from *Vasconcellea quercifolia*. The latter two examples show the importance of alternatives when hindrances impede adoption of new technologies, for example, conservative precautionary control policies adopted to provide time for government regulators to weigh benefits and detriments. Today, Hawaii supplies local, national, and international markets with high quality transgenic and/or nontransgenic fruit. Nontransgenic papayas are surrounded and protected by transgenic ones making it possible to produce both crops. Someday perhaps a similar scenario will be commonplace worldwide.

Keywords: *Carica papaya*, genetic engineering, introgression, transformation
Abbreviations: **BC**, backcross; **CAPS**, cleavage amplified polymorphic sequence; ***cp***, coat protein; **GMO**, genetically modified organism; **PCR**, polymerase chain reaction; **PRSV**, *Papaya ringspot virus*; **R_0**, first transgenic generation; **RAF**, randomly amplified DNA fragments; **SCAR**, sequence characterized amplified region

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INTRODUCTION

Papayas are a more and more familiar but still exotic breakfast and dessert fruit in the Western world. Consumed year-round, papaya is grown in the tropics and subtropics where the climate resembles that of its center of diversity, Central America (Manshardt 1992). Dried papaya seeds, viable for about four years at room temperature, were readily carried along with ships' cargoes from the Caribbean to distant ports from Malacca to India (Storey 1941) in the 1600s (Purseglove 1968). From Malacca and/or the Philippines, they were dispersed throughout Asia and the South Pacific. Don Francisco de Marin, the Spanish horticulturist who settled in Hawaii, is credited with introducing the large-fruited papayas from the Marquesas to Hawaii between 1800 and 1823 (Storey 1941; Yee *et al.* 1970). They became an export crop from Hawaii in 1948 (Yee *et al.* 1970). Today the top three producers of papayas are Brazil, Mexico, and India (FAOSTAT 2008) where production ranged from 1.9 m to 700 k metric tons, respectively, in 2007. Six other countries, Peru, the Philippines, Venezuela, China, Colombia, and Thailand reported nearly equivalent production values, ranging from 175 to 131 k metric tons, respectively. Papayas are important to smaller locations in the Caribbean, Central and South America, Southeast Asia, Africa, and the US. Selection carried out with the original introductions in each location and with more recent accessions has resulted in a wide variation in fruit size, shape, flavor, and usage.

While most papayas in the Western world are consumed as a nutritious breakfast or dessert fruit high in vitamins A and C (Arriola *et al.* 1980; Wenkam 1990), calcium, and potassium (Wenkam 1990). Papayas are also used in novel

Received: 5 August, 2009. Accepted: 5 May, 2010.

Invited Review

ways. For example, in much of Southeast Asia, green papaya salad is daily staple. Fresh fruit the size of slender watermelons feed whole families at breakfast in Thailand whereas a Hawaiian solo papaya is enough for one person. In China and the Philippines soups are prepared with the green fruit which takes on the texture of cooked squash. Filipinos consume papaya pickles (achara, Mendoza *et al.* 2008). Thai and Filipino businesses dry papayas for trail mix ingredients. Workers at Indian and Sri Lankan papain plantations score the green fruit weekly to collect the milky sap for use in meat tenderizers, digestive medicine, tanning preparations, cosmetics, the manufacture of chewing gum, and beer clarification (Nakasone and Paull 1998). Wine has been made with ripe papaya fruit in Kenya and Hawaii. While the initial highly sulfurous aroma inherent in papaya disturbed some samplers, the subsequent flavors are reminiscent of good Riesling or Viognier wines (Axel Lehrer, pers. comm.). A Hawaiian pineapple and papaya jam made by church bazaar and other organizations and a few small commercial companies is delightful.

Botany and horticulture

Papaya belongs to a monotypic genus *Carica* in the family Caricaceae. Previous members of the genus, about 21 species, were placed into *Vasconcellea* by Badillo (2000, 2001). *Jarilla*, *Horowitziana*, *Jacaratia*, and *Cylicomorpha* are four other small genera that make up the family (Badillo 1971). Caricaceae is mostly a new world family, but one genus, *Cylicomorpha*, is found in Africa (van Droogenbroeck *et al.* 2004).

Papayas are semi-herbaceous trees that can live for 15 or more years. Initially single-stemmed with strong apical dominance, the trees branch after high nitrogen fertilizer input early in the crop cycle or after about one year. Trees in commercial plantings are usually not retained longer than a two year harvest cycle because they become too tall for harvesting.

Orchards are established by multiple planting seeds or seedlings because papayas segregate for sex expression (Storey 1953). Papayas express three sex types, heterozygous male (M_1m) or hermaphrodite (M_2m) and female (mm). In dioecious orchards, usually in cooler climates, 1 male is planted with 5 females for good fruit set (Rod Drew, pers. comm.). In warmer locations hermaphrodites are grown. Segregation is either 1 female to 1 hermaphrodite (perfect flower, self-pollinated) if seed are produced by crossing hermaphrodites and females or 1 female to 2 hermaphrodites if seed are the result of self-pollination. The homozygous male sex type does not develop as a result of probable lethality of the condition. The sex chromosomes of papayas were found to be in an early stage of development toward segregation of sex types (Liu *et al.* 2006).

Orchards are thinned to one hermaphrodite. The labor and expense involved in over-planting and thinning to the preferred sex type are minimized by planting clonally propagated plants, but it is still an expensive alternative involving micropropagation and/or rooted cuttings (Fitch *et al.* 2005a, 2005b; Osvaldo Yamanishi, pers. comm.). Clonal propagation of hermaphrodites or females has been carried out in Taiwan, Australia, China, and Hawaii (Drew and Vogler 1993; Fitch *et al.* 2005a). In Hawaii, clonally propagated papayas bore fruit earlier, lower on the fruit column and with higher yield than thinned seedlings (Fitch *et al.* 2005b). Hainan, China is the only region where the practice of rooting field-grown cuttings appears to be economically viable at a cost of $0.07 per cutting (Osvaldo Yamanishi, pers. comm.).

Pests and diseases

Several bacterial and fungal diseases, arthropod pests, and viral diseases affect papaya (Nishijima 1994). Bacterial diseases include internal yellowing disease of ripe fruit caused by *Enterobacter cloacae* (Nishijima *et al.* 1987) and dieback or bacterial decline caused by *Erwinia caricae* (Ying Kwok Chan, pers. comm.; Ollitrault *et al.* 2007). Fruit, stem, and root rots are caused by *Phytophthora palmivora* and *Pythium* spp. while the skin disease anthracnose is caused by *Colletotrichum gloeosporioides* (Nishijima 1994). Leaf spots result from infestations of black leaf spot (*Asperisporium caricae*) (Nishijima 2002) while powdery mildew (*Oidium caricae*) (Nishijima 1994) and red spider mites (*Tetranychus cinnabarinus*) damage leaves during hot, dry weather (Follett 2000). Control measures include the miticides sulphur, avermectin, and Vendex.

The white peach scale *Pseudaulacaspis pentagona* (Targioni-Tozetti) that weakens trees was discovered in Hawaii in 1997. It is controlled by Sunoil with limited success (Follett 2000). Leafhoppers, *Empoasca stevensi*, are serious pests and the apparent phytotoxicity of the insect fluids results in chlorotic leaf margins. Imidachloprid, pyrethrin, and malathion are used to control infestations.

The most important impediment to production is *Papaya ringspot virus* (PRSV), a potyvirus that eventually kills the plant (Jensen 1949) and for which there is no resistance in *C. papaya*. Symptoms are lightening leaf color and later, characteristic mottling, fruit with ringspots, misshapen fruit, lowered yield, and collapse of the tree. Without vigilant surveillance and rapid removal of infected trees, the aphid vectors, mainly *Myzus persica*, spread inoculum to surrounding trees (Namba and Kawanishi 1966). Control of the aphids does not prevent spread of the virus infection.

PRSV affects papaya production worldwide (Davis and Ying 2004). The methods used to control it enable growers to produce fruit but with the high cost of surveillance and removal or other isolation methods. Another papaya virus, *Papaya leaf distortion mosaic virus* (PLDMV), is important in Okinawa and Taiwan. Also a potyvirus, it causes symptoms similar to PRSV (Maoka *et al.* 1996; Yeh and Bau 2001; Yeh *et al.* 2002). In Okinawa, it is more important than PRSV where 96% of virus infections are PLDMV and only 4% are PRSV. In Taiwan, it is less prevalent than PRSV but a production problem nevertheless. *Papaya leaf curl virus* (PLCV) is important in northern India where the geminivirus and PRSV hinder papaya production (Singh *et al.* 2008).

PRSV CONTROL METHODS

Four methods have been used or are being explored to control PRSV, 1) isolation, 2) cross protection, 3) breeding including intergeneric hybridization, and 4) genetic engineering.

Isolation

Net houses are used in Taiwan to keep the aphid vectors from the annual crops. Since the structures are costly and typhoons damage them regularly, this solution requires high input of capital.

Distance and a wide belt of oil palm separates PRSV infested areas in southern Malaysia (Jodor) from the papaya growing areas in the central and northern parts of the country (Ying Kwok Chan, pers. comm.). Australia implemented a strict quarantine measure to confine PRSV to southeast Queensland (Drew *et al.* 2005b). In Brazil with its expansive landmass, growers move to new locations once the growing region is infested. Similar isolation tactics are followed in Mexico, the Philippines, India, and Hawaii which has virus-free islands. The problem with isolation is often distance from markets and/or treatment facilities as is the case with PRSV-free Kauai Island in Hawaii.

Growers in India use integrated pest management to produce crops despite the presence of PRSV (Chavan *et al.* 2008; Sharma *et al.* 2008; Singh *et al.* 2008). Selection of planting season, border crops, tolerant cultivars, virus-free seedlings, insecticides, roguing infected plants, and regular weeding of orchards were recommended for successful papaya production. Taiwan growers similarly carried out

eradication of diseased plants, cultivation of high-stem barrier crops (e.g., corn), silver mulching, and protection of seedlings with vented plastic bags (Yeh and Kung 2007).

Cross protection

Cross protection was developed that provided economic benefit to growers in Hawaii and later, Taiwan (Yeh and Gonsalves 1984; Wang *et al.* 1987). A Hawaii PRSV isolate, HA, was treated with nitrous oxide as a mutagen to create the mild strain HA 5-1 (Yeh and Gonsalves 1984). It was shown to provide limited protection to some cultivars that growers could market (Mau *et al.* 1989). Eventually trees died after becoming infected with the virulent PRSV strain presumably invading unprotected meristematic tissues. Cross protection was not effective in northeastern Thailand and Taiwan where trees were too weakened for good fruit production or the strain specificity of cross protection using the Hawaii isolate was ineffective (Sakuanringsirikul *et al.* 2005; Yeh and Kung 2007). Inoculation of the cross protecting strain and lower economic yields were two other disadvantages of the protocol.

Breeding

1. Tolerant papaya lines

Tolerance to PRSV was observed in an accession from Colombia and other lines growing in Florida (Conover and Litz 1978). These lines were allowed to cross by open pollination and selection resulted in the tolerant cultivar 'Cariflora' (Conover *et al.* 1986). Resistance was believed to be a quantitative rather than qualitative trait. 'Cariflora' was used for crosses with 'Sunset' in Hawaii where several genetics studies resulted from its progeny (Zee 1985; Sondur *et al.* 1996; Deputy *et al.* 2002). The hybrids had acceptable flavor but the fifth inbred line was lost to root rot (Richard Manshardt, Stephen Ferreira, pers. comm.). A 'Cariflora' derivative from the Hawaii experiment was used by V. Prasartsee in Thailand to develop papayas that farmers were able to market (Gonsalves 1998; Sakuranrungsirikul *et al.* 2005). 'Red Lady' and 'Tainung #5' are other PRSV tolerant cultivars used in Florida (Davis and Ying 2004), Taiwan (Shyi-Dong Yeh, pers. comm.), India (Sharma *et al.* 2008) and Malaysia (Chan 2004). 'Tainung #5,' a cross between a cultivar from Costa Rica and FL-77-5 from Florida (Lin *et al.* 1989), although susceptible to root and fruit rot, was used as a parent in developing PRSV tolerance in the important 'Eksotika' cultivar of Malaysia (Chan 2004; Roff 2007). In the Philippines, the tolerant 'Sinta' was developed from a local PRSV tolerant line, 'Cavite Special,' crossed with 'Cariflora' (Magdalita *et al*. 2004). Tolerant Caribbean and Indian PRSV papaya lines were identified in the US Virgin Islands, e.g., dioecious 'Washington' from India was used to develop tolerant hybrids with fair horticultural traits (Thomas Zimmerman, pers. comm.).

2. Intergeneric hybrids

The products from this long-term effort may prove to be the most widely adopted if the promising introgression protocol provides strong resistance to many PRSV strains. PRSV resistant wild Caricaceae species have been reported (Malaguti *et al.* 1957; Jimenez and Horovitz 1958; Sawant 1958; Riccelli 1963; Conover 1964; Horovitz and Jimenez 1967; Adsuar 1971; Mekako and Nakasone 1975; Khuspe *et al.* 1980; Litz and Conover 1983; Manshardt and Wenslaff 1989a, 199b; Chen *et al.* 1991) and used for resistance gene introgression into papaya. *Vasconcellea cauliflora*, *V. quercifolia*, *V. stipulata*, and *V. pubescens* are resistant to PRSV (Conover 1964; Horovitz and Jimenez 1967). Mekako and Nakasone (1975) produced six interspecific hybrid populations, but none with papaya. Khuspe *et al.* (1980) recovered three seedlings of *C. papaya* X *V. cauliflora*, but lost them before confirming their hybrid nature. Embryo culture was utilized to rescue the hybrid embryos that developed in the absence of endosperm. Litz and Conover (1981b, 1982) cultured ovules from the same cross. The plants were confirmed to be hybrids by isozyme analysis (Moore and Litz 1984).

Intergeneric hybrids (called interspecific in the literature before the *Vasconcellea*s were separated from *Carica*) between papaya and PRSV resistant *V. cauliflora* and *V. quercifolia* had variable levels of resistance. Intergeneric hybrids were embryo rescued because of the failure of endosperm development (Wenslaff and Manshardt 1989a, 1989b; Chen *et al.* 1991). The zygotic embryos were rescued at 3 months after pollination. F_1 hybrids between papaya and *V. pubescens* (= *cundinamarcensis* = *candamarcensis*), *V. quercifolia*, *V. stipulata*, and *V. cauliflora* were recovered (Manshardt and Wenslaff 1989a, 1989b). Field resistance was demonstrated in the papaya x *V. quercifolia* and *V. cauliflora* crosses, but the plants were nearly sterile.

R. Drew's group in Australia and the Philippines carried out introgression studies starting around 1993 and has made good progress (Dillon *et al.* 2005a, 2005b, 2006; Drew *et al.* 2005a, 2005b; 2006a, 2006b; ACIAR 2008; Chris O'Brien and Rod Drew, unpublished results). The group first made hybrids with *V. cauliflora* using highly viable pollen that was produced during the spring, summer, and autumn to obtain approximately 94% embryo germination and normal-looking seedlings (Magdalita *et al.* 1996, 1997a, 1998). *V. cauliflora* and its hybrids with papaya were resistant to Australian PRSV isolates (Magdalita *et al.* 1997b). Only 3 plants survived in southeast Queensland but were infertile; a few plants matured in Los Baños, Philippines but were also infertile (Drew *et al.* 1998, 2005a). Papaya crosses with *V. pubescens* resulted in PRSV resistant F_1 plants but they were infertile (Drew *et al.* 1998, 2005a). While intergeneric hybrids between *C. papaya* and *V. cauliflora* and *V. pubescens* were infertile, *C. papaya* X *V. quercifolia* hybrids had low fertility (Drew *et al.* 1998, 2005a). The improvement in fertility was attributed to the relative genetic closeness of papaya and *V. quercifolia* as determined by isozyme and randomly amplified polymorphic DNA (RAPD) analysis (Jobin-Décor *et al.* 1997).

The resistant F_1 plants were highly sterile and not easy to backcross to papaya for improved fruit quality (Drew *et al.* 2005a). Eleven fertile, PRSV resistant intergeneric hybrids from papaya X *V. quercifolia* were selected from among the 300 progeny that segregated 3:1 for PRSV resistance to three manual inoculations (Drew and O'Brien 2001; Drew *et al.* 2005a). Four F_1 plants from the *C. papaya* X *V. quercifolia* cross were fertile enough to produce backcross 1 (BC_1) embryos. One had variable chromosome counts and 0.2 to 2.2% fertility (Drew *et al.* 2005b). Segregants of the first and second backcross generations were evaluated for resistance to PRSV, fertility, morphology, fruit shape, and quality. All of the BC_1 plants were micropropagated and field tested. Variable PRSV resistance was observed. In Australia, the hybrids were highly resistant to PRSV but in the Philippines there was no resistance. In Australia, one BC_1 plant, a female named clone 54, showed high tolerance to multiple inoculations of PRSV in a glasshouse study. It survived six manual inoculations in the glasshouse and 9 months under virus pressure in the field before it became infected. In the Philippines, however it became infected with PRSV after 2 months in the field. It was difficult to infect the backcross plant by manual inoculation but viruliferous aphids were able to breakdown the resistance.

Despite the breakdown of clone 54 and its BC_2 progeny to Philippine PRSV, additional crosses were made between tolerant Philippine papayas and resistant intergeneric *V. quercifolia* F_1 hybrids, e.g., papaya line 5648 X F_1 line 410 (Drew *et al.* 2006b). Intergeneric F_1 hybrids of papaya X *V. quercifolia* were backcrossed with papaya to create fertile PRSV resistant plants that had potential for use in the Philippines (Sajise *et al.* 2004; Siar *et al*. 2009). Sibling

crosses of BC_3 plants and BC_4 plants were free of PRSV infection (visual symptoms and ELISA) after 11 months of exposure in an infested field (Siar *et al*. 2009).

The research team undertook genetic mapping of a PRSV resistance gene in *V. pubescens* using Randomly Amplified DNA Fingerprint (RAF) markers (Waldron *et al.* 2002; Peace *et al.* 2003; Ramage *et al.* 2003) on a large F_2 population (268 individuals). Several F_1 plants were sibmated from among the embryo rescued *V. pubescens* (PRSV resistant) X *V. parviflora* (PRSV susceptible) progeny. The F_2 plants were manually inoculated with PRSV for segregation of resistance (Dillon *et al.* 2005). A PRSV resistance locus, *prsv-1*, was identified on linkage group 7 in the *V. pubescens* map (Drew *et al.* 1998; Dillon *et al.* 2005, 2006b; Chris O'Brien and Rod Drew, unpublished results). RAF markers OPK41R and OPA115R that flanked the *prsv-1* resistance locus were converted to Sequence Characterized Amplified Region (SCAR) markers and were used to locate PRSV resistant *V. pubescens* X *V. parviflora* F_2 individuals (Dillon *et al.* 2006a). Although the OPA115R SCAR marker segregated perfectly with PRSV resistance in the *Vasconcellea* F_2 plants, it was amplified in papaya and could not be used to identify PRSV resistant hybrids.

The OPK41R SCAR marker was not amplified in papaya and was converted to a co-dominant Cleavage Amplified Polymorphic Sequence (CAPS) marker, *PsiIk4*, after digestion with *Psi* I (Dillon *et al.* 2006b). It mapped to within 2 cM of the resistance locus. The CAPS marker was used to identify resistant plants in 99% of the *V. pubescens* X *V. parviflora* hybrids, backcrosses, and papaya X *Vasconcellea* plants screened (Dillon *et al.* 2006a). However, there was no correlation with the resistant papaya X *V. quercifolia* backcrossed individuals. Thus PRSV resistance in *V. quercifolia* was believed to be different from that in *V. pubescens*.

The CAPS marker may be a useful tool for future marker breeding programs aimed at introgressing PRSV resistance genes from *V. pubescens* into commercially important papaya cultivars (Dillon *et al.* 2006b).

A set of primers, MAMAN ½, based on simple sequence repeats (SSRs), were used to fingerprint the *V. quercifolia* resistance gene(s) in the hybrids (Siar *et al*. 2009). A 150-bp marker was detected in *V. quercifolia* and in the PRSV resistant F_1 hybrids, but not in the BC_2 lines. A unique 100-bp marker, observed in the resistant F_1 hybrids and BC_2 lines, was believed to be a fragment of the 150-bp marker with which it had 53% homology. But the papaya X *V. quercifolia* BC_2 plants did not show the 150-bp marker (Siar *et al*. 2009).

The group is currently crossing the highly tolerant introgressed materials into preferred cultivars in the Philippines (Magdalita *et al.* 2007; Mendoza *et al.* 2008; Siar *et al*. 2009). Very recently, Drew and Siar formed a team with R. Manshardt, S. Ferreira, and M. Fitch in Hawaii to test some of their most resistant introgressed hybrids. Seeds from two backcross 4, the best backcross 3 sibcross, three tolerant Philippine inbred lines and the highly PRSV susceptible Davao Solo seeds were sent to Hawaii for field testing by manual and natural infection. Drew and Siar wish to determine if the new hybrids have PRSV tolerance to strains other than those in Australia and the Philippines (Rod Drew, pers. comm.).

Transgenic approach

The concept of pathogen-derived resistance in transgenic plants was put forth by Sanford and Johnston (1985). R. Beachy's group published the first report on tobacco transformed with the *cp* gene of *Tobacco mosaic virus* (TMV) showing delayed symptom expression (Powell-Abel *et al.* 1986).

The first transgenic papaya calli were produced from leaf disks co-cultivated with *Agrobacterium tumefaciens* (Pang and Sanford 1988). Calli formed but no plants were regenerated. Transgenic papaya plants were developed after *Agrobacterium*-mediated gene transfer using embryogenic calli (Fitch *et al.* 1993). Fitch and Manshardt (1990) generated embryogenic calli and somatic embryos from immature embryos of papaya that were grown on relatively high concentrations of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D). The embryogenic callus cultures of commercial Hawaiian hybrid 'Sunset,' bombarded with transgene constructs based on pGA482G (An 1986) containing the *npt*II (neomycin phosphotransferase II), *uid*A (β-glucuronidase), and the *cp* gene of a mild strain of Hawaiian PRSV, HA5-1 grew into the first transgenic papaya plants (Fitch *et al.* 1990). The transgenic R_0 line 55-1 ('Sunset') was shown in a greenhouse test (Fitch *et al.* 1992) and a proof of concept field test (Lius *et al.* 1997) to be resistant to manual and aphid transmitted virus infection. The homozygous R_3 generation of 55-1 was named 'SunUp.' Later, a field test of 'Rainbow,' an F_1 hybrid between 'SunUp' and the most important local Hawaii cultivar 'Kapoho,' showed that the transgenic plants were resistant to PRSV under commercial field conditions (Ferreira *et al.* 2002). Seven years after resistance was discovered, commercialization was underway following deregulation and licensing efforts (Gonsalves 1998). Several reports describe various aspects of the 'Rainbow' project following commercialization and underscored the fact that other countries attempted to solve their PRSV problems similarly (Manshardt 1992, 1998; Manshardt and Drew 1998; Gonsalves 1998; Fitch 2002; Gonsalves 2002; Fitch 2004; Gonsalves *et al.* 2004; Gonsalves 2006; Manshardt 2007; Manshardt *et al.* 2008). Safety of the transgenic product was reviewed by Fuchs and Gonsalves (2007). Transgenic papaya enabled the Hawaiian papaya industry to recover (Gianessi *et al.* 2002).

The mechanism for the resistance was called post-transcriptional gene silencing (PTGS, Baulcombe 1999). Based on sequence-specific RNA degradation, RNA-dependent RNA polymerase synthesizes short antisense RNA from the transgene mRNA that binds to the complementary strand of the mRNA in the cytoplasm. The RNA duplexes are degraded by dsRNA-specific nucleases (Hamilton and Baulcombe 1999; Dalmay *et al.* 2000). Viral RNA from an infecting virus is similarly degraded and virus infection is eliminated.

There were general trends observed on transgenic plants from the Hawaii materials. Tennant and co-workers (2001) observed that PRSV resistance of hemizygous 'Rainbow' was dependent on developmental stage and *cp* gene sequence homology of HA 5-1 and those of other strains from around the world. Homozygous 'SunUp' had broader resistance to PRSV strains from Hawaii and elsewhere where *cp* sequence homology varied from up to 89.2% (Thailand). 'SunUp' was resistant to all PRSV strains except those from Thailand. Older seedlings of 'SunUp' but not younger ones were resistant to Taiwan strains of PRSV (Chiang *et al.* 2001). This observation corroborated those of Tennant and co-workers (2001) and Gaskill and co-workers (2002) who reported that developmental stage determined resistance, that is, older plants were more resistant than younger ones. Japanese researchers on Ishigaki Island in Okinawa observed that there were no differences between 'Sunset' nontransgenic and 'SunUp' transgenic lines grown side by side there (Komori *et al.* 2002). The data compared were pathogens, growth rate, and soil flora and fauna comparisons.

Data from another transgenic 'Sunset' line, 63-1 with 2 segregating copies of the *cp* gene, showed that the homozygous form of this line was resistant to all PRSV isolates screened (Souza *et al.* 2005; Tennant *et al.* 2005). These data further supported the observation that higher transgene copy number was important for greater resistance (Chiang and Yeh 2001; Gaskill *et al.* 2002).

Chiang and co-workers (2001) described the reactions of recombinant PRSV transcripts on 'SunUp' and 'Rainbow.' They produced recombinants between Taiwan and Hawaii PRSV infectious cDNA clones. The Hawaii HA 5-1 transcript was recombined with several *cp* gene fragments

from the Taiwan PRSV strain YK. 'Rainbow' and 'SunUp' seedlings were infected with the recombinants and the symptoms analyzed. The YK whole *cp* recombinant produced severe symptoms on 'Rainbow' (hemizygous for the *cp* gene), but when a partial YK *cp* was substituted, the symptoms were variable and milder. Recombinants with sequences from the 5′ end of the *cp* gene produced very mild and transient symptoms but those recombinants with YK sequences from the middle of the 3′ parts caused long-lasting and pronounced symptoms on 'Rainbow.' The homozygous 'SunUp' was resistant to all recombinants except for the whole *cp* gene version and the one with the central and 3′ end of the *cp* gene and the 3′ non-coding region. Symptoms were mild. They concluded that the position of the heterologous sequences in the recombinants determined their pathogenicity on the Hawaii transgenic papayas.

1. Hawaii's papaya industry

The Hawaii papaya industry began to grow in the mid 1940s after the large fruit type had been introduced by Marin. The small, single serving type papaya was introduced from Jamaica and Barbados by G. P. Wilder in 1911 (Yee *et al.* 1970). By 1936 only the solo type was grown in Hawaii. The industry developed on the most populous island Oahu but PRSV caused it to relocate to the island of Hawaii in the late 1950s. 'Kapoho' (or 'Kapoho Solo') cultivar became Hawaii's standard commercial cultivar that was grown in lava fields from the 1960s (Hamilton and Ito 1986; Mochida 2007). Hamilton and Ito (1968) developed the internationally popular, red-fleshed 'Sunrise' and 'Sunset' papayas, sibling lines that have become staples in the local and export industries in Brazil and Australia. Later breeding efforts by H. Nakasone, University of Hawaii, resulted in a larger, thick-fleshed cultivar named 'Waimanalo' (X-77) that had 800 g fruit and were borne low on the trunk and selected for lowland soil conditions. 'Waimanalo' was grown and probably outcrossed by K. Kamiya's father whose son developed a new inbred line named 'Kamiya' that consistently commands the highest local prices.

The development of transgenic PRSV resistance by the University of Hawaii and Cornell University team (Fitch *et al.* 1992) just one year prior to the outbreak provided the industry with a means to survive the certain collapse of the local industry. Today the industry is smaller than in days prior to the PRSV outbreak because export markets were lost during the recovery period. But with a farm gate value of about $14 m annually (NASS 2008), the industry continues to try to expand. Current attempts to enter foreign markets have been ongoing (Gonsalves 2002; Gonsalves *et al.* 2004, 2006; Tripathi *et al.* 2006; Suzuki *et al.* 2008; Tripathi *et al.* 2008). Suzuki and co-workers (2008) and Tripathi and co-workers (2008) described research they were required to complete to facilitate deregulation of transgenic papayas in Japan. Recently, the Japanese government moved one step closer toward deregulation, an undertaking that has taken more than 10 years (Stephen Ferreira, pers. comm.).

In 1992 PRSV was discovered in the major papaya growing area in Puna on the Big Island. The region had been virus free for about 30 years although the nearest large population center Hilo, about 30 miles away had backyard infestations of PRSV. Shipments of nontransgenic fruit into new and old markets, Germany and Japan, respectively, can be accomplished partly as a result of the PRSV-buffering effect of transgenic papayas that surround the nontransgenic plantings and provide a medium for aphid vectors with PRSV contaminated mouth parts to rid themselves of the nonpersistent virus before sampling the nontransgenic trees. Problems of organic growers discovering transgenic (not considered organic products) fruit among their plantings are remedied by teaching the growers to cover buds of their hermaphrodite seed bearers prior to anthesis. Although Hawaii hermaphrodite papayas are approximately 98 to 99% self-pollinating (Manshardt *et al.* 2007), carpelloid flowers outcross because the normally tightly closed whorl of 5 petals is more loosely developed. In a pollen drift study, 400 m was determined to be a distance in which pollen drift from transgenic hermaphrodites to nontransgenic hermaphrodites did not occur (Manshardt *et al.* 2007).

2. Other Hawaii PRSV projects

Following commercialization of 'SunUp' and 'Rainbow' papayas, projects aimed at expanding the use of transgenic papayas in Hawaii were undertaken as well as developing other transgenic lines (Fitch 2002). The popular Hawaiian cultivar 'Kamiya' was transformed with an untranslatable *cp* gene of HA5-1 as well as with its replicase gene. An immune hermaphrodite R_0 line was produced using particle bombardment, however since it appeared to contain 8 to 16 copies of the resistance gene, no further work was carried out other than to observe it in the field for natural infection (none), growth form (normal), and to self it for seeds. The replicase transformants were similarly inoculated in the laboratory and greenhouse, found to be immune to PRSV, and planted in the field. Those plants had a high percentage if apparent tetraploids and odd lines that could not be rooted and planted in the field. Since the cost of deregulating, licensing, and commercializing a new transgenic papaya was expected to be high, the researchers also undertook backcrossing of the 'Kamiya' to create PRSV resistant plants for farmers on Oahu where the cultivar is important. Somewhat complex hybrids between 'Kamiya' and two random 'Rainbow' F_2 plants were made and planted in a commercial field in 1998. The farmer liked the first hybrid and continues to use this new commercial hybrid that was named and patented as 'Laie Gold.' Backcrossed 'Kamiya' were made despite the popularity of the complex F_1. An inbred 'Kamiya' backcross 3 is also used by several of the 'Kamiya' growers who continue to command the highest prices at supermarkets, farmers' markets, and small neighborhood shops.

Another backcross project was a 'Rainbow' F_2 crossed with 'Kapoho' to recover the good shipping qualities and other attributes of the original cultivar. 'Kapoho' BC_6 (~99% 'Kapoho') has been developed. A grower anticipating rainy season rot problems was given seed to determine if the PRSV resistant backcross has the somewhat higher *Phytophthora palmivora* tolerance of the original 'Kapoho.' Manshardt (2007) is using other cultivars, 'Saipan Red, 'Waimanalo,' and 'Line 40,' to breed for phytophthora tolerance in transgenic papaya.

Further work on transgene sequences in 'SunUp' and 'Rainbow' was reported (Suzuki *et al.* 2008). The locations of three plasmid sequence fragments were detected in the plants. All the functional transgenes, the PRSV *cp*, *npt*II and *uid*A genes, were found in a single 9,789 base pair (bp) insert. Only two other insertion sites, one consisting of a 290 bp nonfunctional fragment of the *npt*II gene and a 1,533 bp plasmid fragment containing a nonfunctional 222 bp segment of the *tet*A gene were detected in 'Rainbow' and 'SunUp.' Detection of the inserts in plants up to R_8 suggested that the inserts were stably inherited. Five out of the six genomic DNA segments flanking the three inserts were nuclear plastid sequences.

3. Vegetative propagation in Hawaii

Tissue-cultured 'Rainbow' and 'Laie Gold' hermaphrodites have been micropropagated since 1995 and 1998, respectively. A rooted cutting protocol using the micropropagated plants was also developed for technology transfer to farmer and nursery businesses. About 200 stock plants were maintained in a shadehouse. The comparatively clean cuttings (vs. field-grown) were rooted at nearly 100% in spring to late autumn especially with heat mats at 29°C and Oasis® rootcubes (Smithers-Oasis, Kent, OH). The winter season was a challenge with cold and often rainy weather. Field-grown cuttings were rooted in the past but they suffered

from mites, fungal contamination, mealy bugs, and scale and survived and rooted at very low rates of 10 to 20%.

Rooted cuttings of greenhouse grown hermaphrodites were fairly successful. Approximately 15 to 25 cm long shoots 8-15 mm in diameter were cut and the leaves removed except for the topmost 2 or 3 which were trimmed to a circular lamina about 5 cm in diameter. The cut ends were dipped into a rooting mixture, either liquid or dry, e.g., 0.8% indole-3-butyric acid (IBA) in dry powdered talc. The cuttings were potted in a commercial peat: perlite mixture, either 50: 50 or with more peat, in Q-plugs (International Horticultural Technologies, Hollister, CA), Oasis rootcubes, vermiculite, or perlite and the irrigation regime was adjusted to avoid a waterlogged planting medium. Desiccation of the cuttings was restricted by use of plastic domes, plastic bags, or enclosed in humid chambers. The cuttings rooted in 3 to 6 weeks in all of the media examined. They were ready for sale after about 4 to 10 weeks.

Hybrids of PRSV resistant 'Rainbow' F_2 with 'Khaekdom' were produced but growers who were contacted to try the plants were wary of planting transgenic seeds. Several years later however, a grower/businessman heard about the transgenic hybrids and took the researchers to meet the growers who had problems with their trees and surmised it was PRSV. The trees appeared generally healthy, uniform in having elongate fruit that were not the typical large, dark green, rough-skinned Thai 'Khaekdom'. Only two trees had obvious PRSV symptoms. The field looked more underfertilized than suffering from PRSV. With roguing, PRSV did not appear to be much of a threat in this somewhat remote farm area. Seeds of two random elongate hermaphrodite fruits were tested for *uid*A gene expression. Interestingly, one fruit was homozygous for the *uid*A gene and the other was hemizygous for it. The 'SunUp' resistance genes have spread into farming communities where they can actually help free unaware farmers from the threat of PRSV.

4. Florida

PRSV CP virus resistant papayas were developed by Michael Davis' group at the University of Florida (Davis and Ying 2004). *A. tumefaciens* strain LBA4404 was used to transform embryogenic calli of F65 papaya (Known-You Seed Co., Kaohsiung, Taiwan) with the X17-2 *cp* gene of a Florida strain of PRSV. The researchers created and used several construct variations. Unmodified and modified forms of the *cp* gene in translatable and untranslatable forms were used. The wide range of constructs prepared presumably was carried out because the group had developed a highly efficient *Agrobacterium*-mediated transformation method (Ying *et al.* 1999). Sense and anti-sense orientation translatable construct plants were infertile whereas the untranslatable *cp* gene plants, in sense with a frame-shift mutation and sense with three-in-frame stop codons, were highly fertile. A total of 360 transgenic lines was generated (Davis and Ying 2002) and from these, 256 putative transgenic lines were inoculated mechanically with PRSV strain H1K (Davis and Ying 2004). While no R_0 plants were immune, 21 were regarded as highly resistant and crossed with six genotypes to produce 1258 seedlings. Natural infection of the seedlings resulted in 23.3% of the transgenic papayas and 96.7% of the nontransformed plants becoming infected after one year in the field.

Florida is the second American state to petition for deregulation of transgenic virus resistant papayas. The petitioned lines contain the untranslatable construct X17-2 (Federal Register 2008, 2009). The petition was approved by the US Food and Drug Administration (FDA) that determines that the fruit would not be harmful to the consumer (Michael Davis, pers. comm.). The petition was received from the University of Florida by the USDA (APHIS) in 2004 and placed in the Federal Register (2008) in September 2008. It was approved as an unregulated article under its rules by APHIS in September 2009 (Federal Register 2009). The agency is responsible for determining that the new transgenic papayas are not threats to American agriculture. The petition is next slated for EPA evaluation.

5. US Virgin Islands

Zimmerman and co-workers (2007) used *A. tumefaciens* to transform cultivars 'Washington' and 'Yuen Nong' with the *cp* gene of a local strain of PRSV. Resistant R_0 and R_1 lines were identified after mechcanical inoculation with PRSV. They developed a seedling selection method spraying 1000 mg/L kanamycin plus 1 mL/L DMSO to identify PRSV homozygous lines from among R_2 and R_3 seedlings. They surmised that three R_3 papaya seedling lines that did not turn yellow after the kanamycin/DMSO treatment, were probably homozygous for PRSV resistance. Further progress has not been reported.

PRSV cp and other transgene virus resistance worldwide

1. Australia

A quarantine measure has so far spared Australians a PRSV problem in the major growing region in warmer northern Queensland where 80% of the crop is grown (Drew *et al.* 2005a, 2005b). PRSV was discovered in 1991 in Queensland and determined to be a new strain that had recently evolved from PRSV in cucurbits (Bateson *et al.* 1994). Lines and co-workers (2002) developed transgenic papaya containing an Australian PRSV *cp* gene using a particle inflow gun. Two transgenic lines were immune to natural inoculation of PRSV in field testing for 18 months (Drew *et al.* 2005a). The immune plants had up to four copies of the transgene. The government of Australia has not acted on deregulation of the transgenic materials (Rod Drew, pers. comm.).

R. Drew of Griffith University developed highly PRSV tolerant intergeneric hybrids in a collaborative project with S. Siar and V. Villegas of the University of the Philippines, Los Baños (Drew *et al.* 2005a, 2007; Chris O'Brien, unpublished results). The researchers' nearly 20-year effort is described in another section of this review. Their work illustrates the importance of developing more than one solution to an agricultural problem.

2. Bangladesh

CIMMYT in collaboration with Bangladesh initiated a project to help improve papaya for local farmers (CIMMYT 2006). By 2006 funding had run out after plans were put forth for use of 'Kapoho' transformed with a three-segment gene construct made of partial sequences from Hawaii (HA 5-1), Taiwan (YK), and Thailand PRSV *cp* genes. The three sequences were selected as the most diverse for the purpose of providing the broadest possible gene silencing to develop plants that could be used in many if not all papaya growing regions. Plants were near deregulation status but costs and complications from anti-GMO (genetically modified organism) elements made it difficult to continue. Although the plants were quite resistant to a wide range of PRSV strains (Stephen Ferreira, pers. comm.), some of the results reported by CIMMYT (2006) were confusing. In 2005, five transgenic lines were tested from among cultivars 'Kapoho,' 'Sunrise,' and 'Khaekdum.' Some inoculated nontransgenic and transgenic lines did not develop symptoms. The protocol needed to be improved using fresh inocula in the summer of 2005, but no further reports are available.

Hawaii Agriculture Research Center (HARC) became involved in developing PRSV resistant papayas for Bangladesh in 2004 using 4 synthetic gene constructs. The project was initiated with USAID funding in 2004 along with the CIMMYT involvement to assist farmers in Bangladesh. Synthetic PRSV *cp* gene sequences common to the variable and conserved regions of 20 different PRSV strains were

developed. The conserved sequences were about 200 bp long. They were placed in tandom triplicate in pGA482G plasmid vectors and transformed into 'Khaekdom,' 'Sunrise,' 'Khaeknual,' and 'Kapoho' cultivars by particle bombardment and *A. tumafaciens* strain LBA 4404 (Terryl Leong, unpublished results). Out of 65 transgenic lines selected, 33 were regenerated into green plants; some lines were difficult to root and transfer out of tissue culture. Three of the lines from bombardment and two from *Agrobacterium*-mediated transformation contained only the *npt*II gene. They were being rooted and prepared for field planting. The first selection, a 'Sunrise' line in the field for R_1 seed generation and response to PRSV, was hermaphrodite, sterile, and 5 of 6 plants succumbed to natural infection. Other transgenic lines await field planting and R_1 seed generation. The goal of the synthetic gene project was to develop papayas resistant to a broad range of PRSV strains. They were destined for Bangladesh but plans changed and funding was depleted in 2008. Plants are being maintained for field testing at the University of Hawaii by S. Ferreira.

3. Brazil

The Brazilian government's agriculture branch, EMBRAPA, was responsible for 'Sunrise' and 'Sunset' transgenic papayas developed by M. Souza and co-workers (2005). Both translatable and untranslatable constructs of the Brazilian PRSV *cp* genes were transformed into papaya by particle bombardment. Fifty-four transgenic lines were regenerated and inoculated with three different isolates of PRSV, from Brazil, Hawaii, and Thailand. The PRSV *cp* gene transformants, inoculated with PRSV, were resistant in greenhouse testing and field testing consisted of seed production and agronomic evalution. Some lines were resistant to all three isolates. The government has not yet deregulated the plants.

4. China

At Guangzhou's South China Agricultural University (=Huanan Agricultural University) researchers reported that the PRSV replicase gene conferred virus resistance in transgenic papayas (Chen *et al.* 2001). The replicase gene, involved in replication of the viral genome, also imparts virus resistance to transgenic plants (review by Prins *et al.* 2007). This transformant was named 'Huanong #1,' deregulated in 2006, and is now commercialized in Guandong province (Hua Ping Li, unpublished results; Davidson 2006). There were two other groups working on PRSV resistance, at Huazhong Agricultural University in Wuhan, Hubei (Jiang *et al.* 2005) and at Zhongshan University (=Sun Yat-sen University), Guangzhou (Ye *et al.* 2003).

Huanan Agricultural University researchers used the binary vector pBI121 containing an untranslatable replicase gene (Chen *et al.* 2001). The *cp* gene was used earlier but it did not confer resistance to PRSV. The replicase gene had a 35S promoter and a *nos* terminator. Kanamycin resistance was used for selection. The Huanan group observed the single-copy 'Huanong #1' lines for 7 generations with selfing and outcrossing tests. 'Huanong #1' was completely resistant to four PRSV virus strains, Ys, Vb, Lc, and Sm, from different locations in China. Resistance was attributed to post-transcriptional gene silencing. The resistant 'Huanong #1' is a red-fleshed and pyriform papaya. Papayas in southern China are grown in Guangdong, Guangxi, Hainan, Fujian, and Yunnan provinces and in Taiwan.

Safety studies reported by H.P. Li (unpublished results) stated that although replicase is not produced in the papayas (untranslatable construct, no mRNA for the replicase gene was detected), their group tested the protein and found that it was quickly digested in 15 sec (in vitro assay) and was not an allergen according to Genbank, EMBL, PIR, and Swiss Prot database searches. The fruit had the same nutritional characteristics as non-transformed fruits.

The Huazhong group transformed 'Sunset' with *A. tumefaciens* LBA 4404 carrying the binary plasmid pGA482G containing the *cp* and *npt*II genes. They had PCR and Southern hybridization positive plants but no further work has been reported.

Ye and co-workers (2003) reported on the molecular characteristics and field resistance of two transgenic T_1 (=R_1) lines containing a mutant replicase gene but to our knowledge no plants were deregulated or commercialized from the Zhongshan group.

5. India

As the third largest producer of papayas in the world after Brazil and Mexico, India needs to ensure the health of its crop. The most important papaya growing states are Andhra Pradesh, Karnataka, Kerala, Tamil Nadu (southern India), Maharastra, Gujarat (western India), West Bengal, Assam (eastern India), and Bihar (northern India) (Maneesh Mishra, pers. comm.). PRSV is a major problem throughout the papaya growing regions and *Papaya leaf curl virus* (gemini virus) is an additional threat in northern India (Singh *et al.* 2008).

Two Indian institutes are working together to engineer papaya for PRSV resistance, the Indian Council of Agricultural Research (IARI), New Delhi (Jain and Agarwal 2008; Mishra *et al.* 2008) and the Central Institute for Subtropical Horticulture, Lucknow (Chandra and Mishra 2008; Mishra *et al.* 2008). The New Delhi group has developed a dual gene construct, *cp* + replicase, to combat both viruses. The Lucknow group is responsible for transforming and assessing the papayas (Maneesh Mishra, pers. comm.; Chandra and Mishra 2008; Jain and Agarwal 2008; Mishra *et al.* 2008). Besides the dual construct, other constructs for PRSV resistance include a full length *cp* gene (~850 bp) in sense and anti-sense orientation, a truncated *cp* gene (~410 bp), and an inverted repeat *cp* gene (~500 bp) (Jain and Agarwal 2008). The 'Pusa Delicious' transformants developed using *Agrobacterium tumefaciens* are being screened. The Mishra group induced rapid somatic embryogenesis using polyamines and converted the embryos with polyethylene glycol. They also developed a shoot tip transformation system that yielded about 9% PCR positive transformants (Mishra *et al.* 2008). The shoot tip system would enable researchers to directly transform elite cultivars especially hybrids. Since resistance genes in homozygosity are desired, the products could complicate or enhance breeding for new transgenic cultivars. Yang and co-workers (1996) in Taiwan also developed a somatic embryogenesis method to transform petiole tissues from selected hybrids.

A third group at Tamil Nadu Agriculture University in Coimbatore is also working on transforming papayas in collaboration with the Monsanto Company (Maneesh Mishra, pers. comm.).

Researchers in Bangalore at the Indian Institute of Horticultural Research published a study on transient expression of a local *cp* gene to validate the clone with the intent to generate PRSV resistance (Chandrashekara *et al.* 2006). A plant expression construct was electroporated into the meristems of seedlings of 'CO-7' and 'Solo.'

Papaya leaf curl virus (PLCV, gemini virus) is important in northern India (Jain and Agarwal 2008; Maneesh Mishra, pers. comm.). Dual constructs for PRSV and PLCV were developed and are being tested. But *Papaya mosaic virus* and *Papaya apical necrosis virus* are apparently not causes for immediate concern (Gonsalves 1998).

In a different approach to PRSV resistance, Srivastava and co-workers (2008) treated papaya plants with CAP-34, a protein from *Clerodendron aculeatum* to induce systemic antivirus resistance. Control plants showed PRSV symptoms after 20 d in 56% of the plants while CAP-34-treated plants developed no symptoms. Sixty days after infection, 95% of the control plants had symptoms while only 10% of the CAP-34-treated plants had symptoms that were considered mild. Back inoculations of sap onto *Chenopodium quinoa* from inoculated symptomless CAP-34-treated plants did not indicate presence of PRSV. ELISA and RT-PCR

assays on symptomless CAP-34-treated plants also indicated no presence of PRSV while control plants with symptoms showed presence of the virus in the same assays. Suppression of virus replication was suggested by this report.

6. Indonesia

Researchers in Indonesia used co-bombardment with PRSV *cp* and antisense ACC oxidase genes for a two-gene transformant (Damayanti *et al.* 2001 in Mendoza *et al*. 2008). The cultivars 'Bangkok' and 'Burung' were bombarded with a construct p2K7/BICP containing the *cp* gene from a Bogor, West Java PRSV strain. The promoter was 35S. Co-transformation was with pRQ6 containing the *uid*A and hygromycin phosphotransferase (*hph*) genes. No further information is available.

7. Jamaica

Tennant and co-workers (2002; 2005) developed Solo-type transgenic papayas containing translatable and untranslatable *cp* genes using particle bombardment. The papayas were greenhouse and field tested but they are yet to be deregulated. The R_0 translatable *cp* gene lines had 80% field resistance but the R_0 untranslatable *cp* gene transformants had only 44% field resistance.

In Jamaica, R_3 transgenic papayas were assessed for agronomic performance, nutritional composition, and safety of transgenic fruit (Paula Tennant, pers. comm.). Overall, despite variations in agronomic performance and nutritional quality, the transgenic lines appear to be useful for managing the disease. The challenge is to deregulate Jamaican transgenic papayas with acceptable commercial qualities. A biosafety framework is not yet established for Jamaica.

8. Japan (Okinawa)

A group on Ishigaki Island, Okinawa, Japan worked on engineered PRSV and PLDMV *cp* genes. They also evaluated the Hawaii transgenic cultivar 'SunUp' on Ishigaki (Komori *et al.* 2002). They published results (in Japanese) stating that there was no effect of growing transgenic 'SunUp' in Japan as far as alteration of the soil microbial condition. Horticultural differences between nontransgenic and transgenic papayas could not be found.

Papaya leaf distortion mosaic virus (PLDMV, a potyvirus) was reported in Okinawa in 1954 and later in Taiwan (Maoka *et al.* 1996; Chen *et al*. 2002; Komori *et al.* 2002; Yeh and Kung 2007). Like PRSV, since there is no resistance in *C. papaya*, both groups attempted using the transgenic *cp* gene approach (Bau *et al.* 2003, 2004; Tetsuo Maoka, pers. comm.). In Okinawa, PLDMV was discovered in 96% of the virus infected plants; PRSV was only found in 4% of the papayas (Maoka *et al.* 1996). Virus resistant papayas are not yet commercialized in either country. Currently, the Japanese do not conduct transgenic papaya research (Tetsuo Maoka, pers. comm.). PLDMV was discovered as a result of the PRSV transgenic plant field testing in Taiwan and is believed to have been introduced into Taiwan from Okinawa (Chen *et al.* 2002; Yeh and Kung 2007).

9. Malaysia

Pillai and co-workers (2001) reported transformation of papayas for Malaysia. The project was initiated in 1998 together with other Southeast Asian countries in the ISAAA Network on Biotechnology of papaya. The *cp* gene of a local isolate of PRSV was isolated and cloned for both particle gun and *Agrobacterium* transformation of 'Eksotika' papaya. Binary vectors from Monsanto, e.g., pMON54904B, contained both the *cp* and replicase genes. Another vector contained the *cp* gene with a ~250 inverted repeat of the *cp* gene downstream from a stop codon. Field tests of the 87 transgenic lines and R_1 seed generation in net houses were conducted but none of the R_1 seedlings were resistant to PRSV (Ying Kwok Chan, Umi Abu Bakkar, Peng Fatt, pers. comm.).

PRSV was first detected in Johor, the former papaya growing region, in 1991 (Chan 2004). The industry is fortunate that the current papaya growing regions in central and northern Malaysia are buffered with an oil palm plantation belt that keeps PRSV from spreading out of Johor. Researchers have not pursued virus resistance because quality and other pathogen problems are more important for traditional breeding efforts. They have focused on quality for many years, having developed 'Eksotika' and 'Eksotika II' from 'Sunrise' crossed with a local cultivar, 'Subang 6' (Chan 2002). Papayas with nearly blemish-free skin and firm flesh were recent developments (Ying Kwok Chan, pers. comm.). They now have a new devastating bacterial disease caused by *Erwinia caricae* that requires their immediate attention (Ying Kwok Chan, pers. comm.). Additionally, a breeding program for developing papayas with tolerance to PRSV was initiated by the Malaysian government agriculture department, MARDI, in 1991 (Chan 2004). 'Cariflora' was determined to be the most PRSV tolerant cultivar in a screening project (Roff 2007). The tolerant 'Cariflora' and 'Tainung #5 cultivars were crossed with the local cultivar 'Eksotika' (Chan 2004). Single seed descent for F_5 inbred lines was sought. Eleven selections were tested in two heavily PRSV infested areas. Four lines, L248, L41, L90, and L13, were highly tolerant. L248 was the most tolerant and highest yielding while L13 was most similar to 'Eksotika.' Yield and flavor factors were to be improved by further breeding.

10. Mexico

Noa-Carrazana and co-workers (2007) examined different PRSV strains in Mexico. They found wide variation in the populations which suggested that for resistance, careful planning of the *cp* genes to use was important. In 2004, L Herrera-Estrella commented that transgenic papayas were being developed for Mexico (Luis Herrera-Estrella, pers. comm.) but mention was not made of the mode of gene transfer and no further information has been obtained.

11. Philippines

The *cp* gene from a Philippine strain of PRSV was isolated by researchers at the University of the Philippines Los Baños Institute of Plant Breeding (Magdalita *et al.* 2004). Selection of 48 transformants was accomplished after particle bombardment (Magdalita *et al.* 2004). Similar to the experiments in Florida and Malaysia, all of the R_0 transformants were not resistant to PRSV, but resistance was observed in selfed R_1 lines. Since the tolerance is believed to be multigenic, selfing could have resulted in homozygosity and therefore greater tolerance by the protective genes. A second attempt at producing PRSV resistant papayas for the Philippines was initiated by the ISAAA Papaya Biotechnology Network of Southeast Asia (Flasinski *et al*. 2002). *Agrobacterium*-mediated gene transfer using Monsanto constructs resulted in 200 independent transgenic lines containing *cp*, replicase, and inverted repeat *cp* genes.

In 2007, the Los Baños group carried out a confined field trial of three T_3 lines. A total of 135 inoculated seedlings along with 45 uninoculated and 45 control 'Davao Solo' plants was planted and are under evaluation (Lawas and Magdalita 2007).

The collaboration between the Philippines and Australia on introgression of PRSV resistance genes (Drew *et al.* 2006a, 2006b, 2007) could result in useful resistance for papayas before transgenic lines are deregulated, licensed, and commercialized.

12. Taiwan

Taiwan, like Okinawa and India, has at least two important

virus diseases. Control of PRSV, discovered in 1975, was attempted with the various alternative methods discussed earlier in this review. After experimenting with the options, growers opted for the use of net houses, expending large sums to produce their crop. Around 1989, S. Yeh's group started working on transgenic resistance. They developed PRSV resistant papayas using an untranslatable *cp* gene from the Taiwan isolate YK (Bau *et al.* 2003). Four years into the field testing, after they repeatedly tested two immune lines, they found that the resistance was broken by a different potyvirus, PLDMV (Bau *et al.* 2004). After they developed and tested plants with PLDMV resistance, they discovered breakdown in PRSV resistant lines infected with a "super virulent" PRSV (Tripathi *et al.* 2004). They have now created a transgenic papaya with broad resistance to both Taiwanese papaya viruses using an untranslatable construct containing a truncated *cp* gene coding region from a PLDMV isolate and a truncated *cp* gene coding region of a complete 3′ untranslated region of a PRSV isolate (Kung *et al.* 2009). The helper component proteinase HC-Pro gene of PRSV, responsible for many viral processes including suppression of gene silencing, was transformed into papaya and shown to give immunity to a broad range of PRSV strains (Shyi-Dong Yeh, pers. comm.). The government has adopted a very conservative stance toward transgenic papayas and other crops, thus deregulation is not anticipated.

13. Thailand

Papayas are important daily staples in the rural Thai diet (Sakuanrungsirikul *et al.* 2005). Although cross protection did not result in useful PRSV protection, breeding and selection with a tolerant line helped provide trees to farmers in northeastern Thailand. Transgenic papayas for northeastern Thailand were developed starting in 1994 using an untranslatable Thai PRSV *cp* gene (Gonsalves 1998). Tolerant to immune plants in the R_3 generation, evaluated in field plantings and for nutritional and environmental impact data, showed that the transgenic papayas did not differ significantly from nontransgenic papaya (Sakuanrungsirikul *et al.* 2005). One 'Khaekdom' line had 100% field resistance. The papayas have not yet been deregulated.

The Thai National Center of Genetic Engineering and Biotechnology and Plant Genetic Engineering Unit of Kasetsart University, Bangkok, initiated an independent PRSV resistance project using particle bombardment with a Chiang Mai isolate *cp* gene (Phironrit *et al.* 2007, 2008). The Kasetsart team conducted a small scale field trial of an R_2 transgenic line, 'Khaeknual' KN116/5 in 2003 to 2004. The R_0 transgenic line contained 4 copies of the transgene and were 97% resistant in the field after 1 year compared to 100% infection of nontransgenic controls after 2 months. The R_1 progenies contained 2 to 4 copies and showed 20% PRSV resistance to the Chiang Mai strain. Selections of 2-copy R_2 to R_4 plants had 34%, 71%, and 95-100% resistance, respectively. Stability and biosafety assessments of the R_5 generation were ongoing in 2008. The group is preparing their data for publication (Parichat Burns, pers. comm.).

14. Venezuela

In 1993 G. Fermin initiated work at Cornell University to develop PRSV *cp* gene resistant papayas for his homeland (Fermin *et al.* 2004). Two isolates from distant fields in Mérida, Venezuela, EV strain from El Vigía and LA strain from Lagunillas were used for the *cp* gene constructs. *Agrobacterium* was used to transfer the genes into papaya. Four R_0 plants were crossed or self-pollinated to produce R_1 seedlings. The seedlings were resistant to both local PRSV strains as well as to strains from Hawaii and Thailand in greenhouse testing. Field data were not presented. Chin and co-workers (2007) reported on the variation in PRSV strains following a virus epidemic in 2004. Shared similarity was between 88.7 to 98.8%, a wider range than observed in strains collected in 1994. The researchers also compared strains from Jamaica that showed less variation, perhaps because that country is more isolated. They believed that in order to develop transgenic papayas with broad and durable resistance to buffer the relatively rapid rate of change in virus strains over time, synthetic gene constructs composed of sequences common to a wide range of virus strains would be good solutions to breakdown in resistance.

15. Vietnam

Five papaya cultivars, 'Khaekdom,' 'Tim Taiwan,' 'Solo,' 'Mexico,' and 'Local Lansom,' were transformed with *Agrobacterium tumefaciens* using Monsanto constructs pMON65304, pMON65305, pMON65309 (in Mendoza *et al*. 2008). The *cp* gene in sense and antisense orientation and the replicase gene were used. Twenty nine transgenic lines were selected and evaluated under greenhouse conditions. No further information has been reported. Another group reported on *cp* gene sequences from multiple locations in Vietnam (Chu *et al.* 2004). The *cp* gene sequences were 89.7 to 99.8% similar at the nucleotide level and 91.5 to 99.7% at the amino acid level. The strains separated into two groups, the first was composed of isolates from north and central Vietnam and the second group was from Kan Tho and Kon Tom. The third group was from Ho Chi Minh City. The Vietnamese PRSV strains coincided with those in the eastern group of a phylogenetic tree published for 52 sequences from all over the world. They remarked that their work was important for creating transgenic PRSV resistant papayas with sequences common to the most diverse strains for the broadest protection.

Table 1 lists the considerable body of work that was generated after the first transgenic papayas were developed. Other US research organizations and fourteen other countries have shown that similar resistant papayas can be produced to help their growers combat PRSV and other virus pests but only Hawaii and China currently sell commercialized transgenic papayas.

CONCLUDING REMARKS

The Hawaii experience with PRSV *cp* gene virus resistance has helped highlight the value of carrying out basic agricultural research and extending the results toward adoption for the improvement of crops. Without transgenic papayas there would be no Hawaii papayas on the scale available today. After 11 years on the market, the transgenic papayas are readily accepted by local consumers. The fruit shipped to US mainland and Canadian markets face competition with Mexican, Belize, Costa Rican, and Brazilian products. Attempts to deregulate the fruit in Japan are ongoing. Shipment to locations where no apparent deregulation is required, for example, Hong Kong, has not yet been accomplished. Transgenic PRSV *cp* gene virus resistance has provided growers and marketers in Hawaii with livelihoods for the past 11 years. Breeding for improved cultivars has widened the range of fruit types produced such that more growers have been able to make a living. Hawaii consumers are given the opportunity to consume a flavorful, nutritious, and fresh local product to help sustain healthy lifestyles.

Barriers to adoption of virus resistant transgenic papayas are unfortunate and underlie the complex issues that govern societies. Gene transfer from one organism to another occurs in everyday life, from viral inoculations to create disease immunity to bacterial evolution under selective pressure in the presence of antibiotics to plant breeding for new flower colors, all "natural" phenomena that modern society generally accepts. Fortunately for consumers caught in the debates over deregulating transgenic papayas, alternative solutions to the PRSV problem have been developed. Besides traditional isolation methods, breeding more PRSV (and other virus) resistant papayas using tolerance genes from landraces has offered some respite over the years. Recently the exotic introgression breeding of resistance genes

Table 1 Summary of transformation for PRSV resistance.

Papaya virus	Location	Constructs	Method	Results	References
PRSV	Australia	*cp* (UT), 35S, *npt*II	Gun	Resistance	Mahon *et al*. 1996; Lines *et al.* 2002
PRSV	Bangladesh	*cp* (UT), segmented *cp*, synthetic *cp*, 35S, *npt*II	Gun, Agro	Field resistance (segmented)	CIMMYT, 2006
PRSV	Brazil	*cp* (UT), 35S, *npt*II	Gun	Greenhouse resistance, agronomic traits in the field	Souza *et al.* 2005
PRSV	China	Replicase (UT), 35S, *npt*II	Agro	Commercial	Chen *et al.* 2001; Mendoza *et al.* 2008; Hua Ping Li, unpublished results
PRSV, PLDMV	China	Multi-*cp*, 35S, *npt*II	Agro	Southern positive plants	Jiang *et al.* 2004
PRSV, PLCV	India	*cp* + replicase, 35S, *npt*II	Agro	Screening	Mishra *et al.* 2008
PRSV	India	*cp*	Electro-poration	Transient expression	Chandrashekara *et al.* 2006
PRSV	Jamaica	*cp* (UT), 35S, *npt*II	Gun	Field resistance	Tennant *et al.* 2002, 2005
PRSV, PLDMV	Japan	*cp* (UT), 35S, *npt*II	Agro	Discontinued	Tetsuo Maoka, pers. comm.
PRSV	Malaysia	*cp* (UT), 35S, *npt*II	Agro	No resistance	Ying Kwok Chan, pers. comm.
PRSV	Mexico	*cp*, 35S, *npt*II	Gun	Greenhouse tests, 2004; moratorium thereafter	LuisHerrera-Estrella, pers. comm., 2004
PRSV	Philippines	*cp*, 35S, *npt*II	Agro	Field resistance	Mendoza *et al*. 2008
PRSV, PLDMV	Taiwan	*cp* (UT), 35S, *npt*II	Agro	Field resistance	Bau *et al.* 2003, 2004; Yeh and Kung 2007
PRSV	Thailand	*cp* (UT), 35S, *npt*II	Gun	Field resistance	Sakuanrungsirikul *et al*. 2005
PRSV	Thailand USA	*cp* (UT), 35S, *npt*II	Gun	Field test, moratorium	Phironrit *et al*. 2007, 2008
PRSV	Hawaii	*cp*, 35S, *npt*II	Gun	Commercial	Fitch *et al.* 1992; Gonsalves 1998
PRSV	Hawaii	*cp* (UT), replicase, segmented *cp*, synthetic *cp* 35S, *npt*II	Gun, Agro	Field resistance, field testing (synthetic)	Fitch 2002, 2004; CIMMYT 2006; Stephen Ferreira, pers. comm.
PRSV	Florida	*cp* (UT), 35S, *npt*II	Agro	Passed FDA, Petitioned USDA for deregulation	Davis and Ying 2004; Federal Register 2008
PRSV	US Virgin Islands	*cp*, 35S, *npt*II	Agro	Field resistance	Zimmerman *et al*. 2007
PRSV	Venezuela	*cp* (UT), 35S, *npt*II	Agro	Greenhouse resistance	Fermin *et al*. 2004
PRSV	Vietnam	*cp* (UT), 35S, *npt*II	Agro	Selection	Chu *et al.* 2004; in Mendoza *et al.* 2008

cp = coat protein (gene), UT = untranslatable, 35S = 35S promoter, *npt*II = neomycin phosphotransferase II gene

from distant papaya relatives is now reaching an exciting phase after years of good effort. We hope that one or more of the solutions described can help growers and consumers of this healthful fruit.

ACKNOWLEDGEMENTS

I wish to thank the USDA for previous employment during which time I was able to help develop the first transgenic papaya 'SunUp', the University of Hawaii, and Cornell University, the three co-owners of the transgenic plants that we developed. I thank my colleagues Drs. Richard Manshardt, my advisor at the University of Hawaii, College of Tropical Agriculture and Human Resources, Dennis Gonsalves formerly at Cornell University, and Jerry Slightom, formerly at the Upjohn Co. I also thank Dr. Paul Moore at USDA and Dr. Don Heinz at the Hawaiian Sugar Planters' Association (HSPA) for allowing me to use the HSPA laboratory at which I conducted the bulk of my research, and Ms. Stephanie Whalen, Executive Director of the Hawaii Agriculture Research Center (formerly HSPA) where I currently conduct my research. I thank my assistants, Ms. Terryl Leong, Ms. Aileen Yeh, Ms. Peggy Hiraki, Mr. George Yamamoto, Ms. Nancy Saito, Mr. Larry Tochiki, and my volunteers, Ms. Patty Tomasa and Ms. Carol Murakami.

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