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CP-Transgenic and non-Transgenic Approaches for the Control of Papaya Ringspot: Current Situation and Challenges

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ABSTRACT

In the last decade, successful cases of managing plant virus diseases using the transgenic approach have been reported, with the best known example being the *Papaya ringspot virus* (PRSV)-resistant transgenic papayas in Hawaii. Use of the coat protein (*cp*) gene has proven effective with not only papaya, but with various plant-virus systems such as squash. Although other viral sequences are equally effective in conferring resistance, few transgenic plants engineered with these sequences have made their way into the market. In addition, opposition to genetic manipulation of crop plants has prevented wide application of the technology, despite the fact that many countries (including Jamaica, Brazil and Venezuela) have produced and characterized several generations of resistant transgenic papayas. Using the papaya-PRSV system as a case study, we examine the transgenic cropping systems available, constraints to the adoption of transgenic papayas in various countries, as well as the impact the technology has made on world production of this fruit crop. Alternative non- *cp* and non- transgenic approaches of managing PRSV are also presented.

Keywords: *Carica papaya*, biotechnology, GM, transgenic fruits
Abbreviations: ***cp***, coat protein gene; **DAS-ELISA**, double antibody sandwich enzyme linked immunosorbent assay; **HC-Pro**, helper component-protease; ***npt*II**, neomycin phosphotransferase type II gene; **PRSV**, *Papaya ringspot virus*; **PTGS**, post transcriptional gene silencing; **siRNA**, small interfering RNA; ***uid*A**, β-glucuronidase gene

CONTENTS

INTRODUCTION

Papaya (*Carica papaya* L.), the best known and most widely distributed species of the family Caricaeae, is cultivated mainly for its nutritious fruits in tropical and subtropical regions (Manshardt 1992). Among its pathogens, *Papaya ringspot virus* (PRSV) represents the most serious threat to production (Tripathi *et al*. 2008), with losses of up to 100% reported in some regions (Tennant *et al*. 2007). Although PRSV was first described in Hawaii in 1945, it has been recognized as a major threat to papaya production in many tropical and subtropical areas including South and Central America, Africa, Asia and the Caribbean (partially reviewed by Tripathi *et al*. 2008) before the 1940s. Moreover, PRSV still continues to be reported "for the first time" in many countries where, most likely, it has been overlooked for decades, as in the case of Mexico (Noa-Carrazana *et al*. 2006). Arguably the multiple names assigned to the etiological agent and the disease over the years has clouded the clear identity of isolates. **Table 1** summarizes the "first report" of PRSV in different regions worldwide between 1910 and 2006. It is worth noting that in at least two cases the presence of PRSV in certain regions antecedes the "first report". In Australia, the virus was assumed to be reported for the first time in 1991 (Thomas and Dodman 1993), but recently Lima *et al.* (2001) note that a disease attacking papayas in the early 1929s could most likely be attributed to PRSV. Not surprisingly, in the decade of the 1930s many different reports in the Caribbean assumed that the virus was already present in Venezuela, Barbados and Jamaica (see **Table 1**). Interestingly, India, despite being reputed as a center of origin of the virus, has apparently served poorly as a source of the virus to neighboring countries since the disease only became evident in surrounding regions in the late 1970s (Thailand) or 2000s (Iran). Based on molecular analysis of PRSV isolates, Gibbs *et al.* (2008) argue that the most plausible explanation for the PRSV diaspora is the transmission of the virus by seeds (but not necessarily via papaya), an event that has apparently occurred at least three times, and probably 300 years ago to the Americas. Mutation, in addition to movement of PRSV, appears to be significant in the molecular evolution of the virus. Recombination, however, is emerging as an important factor affecting the genome architec-

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Invited Review

Table 1 Examples of "first reports" of *Papaya ringspot virus* in selected countries.

Year	Country	Reference
Circa 1930	Florida	The description of a viral disease of squash resembles that of *Watermelon mosaic virus* 1 (a previous synonym of PRSV), Anderson 1954
Circa 1930	Jamaica and Minor Antilles	In Jamaica in 1929 according to Jensen (1949), and the beginning of the 1930s as reported elsewhere (Marte and Thomas 1984)
1931	Tanganyka	Wallace 1936 (possibly PRSV)
Circa 1937	Venezuela	Muller 1941
Circa 1940	Puerto Rico	Adsuar 1947; according to others (Marte and Thomas 1984), most probably during the 1930s
1949	Hawaii	Jensen 1949
1952	Colombia	Torres and Giacometti 1966
1958	India	Capoor and Varma 1958, cited by Jain *et al.* 2004
1969	Brazil	Costa *et al.* 1969
1975	Mexico	Téliz-Ortiz *et al.* 1991
Circa 1975	Thailand	Charoensilp *et al.* 2003
1991	Australia	Thomas and Dodman 1993
1929		According to symptomatology as reported by Shukla and Ward (1989) and re-interpreted by Lima 2001
2000	Iran	Pourrahim *et al*. 2004
2002	St. Kitts	Chin *et al.* 2007
2004	Bangladesh	Jain *et al*. 2004
2004	Cook Islands	Davis 2004
2006	Ivory Coast	Diallo *et al*. 2006

ture of PRSV (Mangrauthia *et al*. 2008).

All commercial and non-commercial papaya cultivars and types, respectively, are susceptible to PRSV. Diseased plants develop the classic symptoms of stunting, drastically reduced yield and fruits with the diagnostic water-soaked ringspot blemishes (Purcifull *et al.* 1984; Gonsalves 1994). The pathogen is transmitted predominantly by several species of aphids in a non-persistent manner and is not considered seed-borne (Purcifull *et al*. 1984). Bayot *et al.* (1990) however, raised the possibility that PRSV might be transmitted through seeds (0.15%), which would help explain the easy dispersion of the virus throughout the tropics. Even if not transmitted via papaya seeds, seeds from cucurbits or other hosts may play a role in the epidemiology of the disease. In addition, there is at least one report on the potential transmission of PRSV by birds (Trujillo *et al*. 1989).

Attempts at managing PRSV by conventional means have proven difficult, especially under high disease pressure, but there are a few successful cases that will be discussed later in this review. Resistance against the virus does not exist in *C. papaya*. Resistance genes from species belonging to other genera in the Caricaceae family have been identified, but the resistance appears to be variable and dependent on the geographic origin of the virus and environmental conditions (Gonsalves *et al*. 2005). Recent advances in the generation of intergeneric hybrids using the well known *Vasconcellea* species (see below), however, promise to offer a reliable way to broaden the papaya's genetic base.

Transgenesis was initiated in the mid 1980s when Fitch *et al*. (1993) successfully transformed and regenerated virus resistant transgenic papaya plants. By 1998, two PRSV resistant papaya cultivars, 'SunUp' and 'Rainbow', were released to growers in Hawaii (Fitch *et al*. 1992; Manshardt 1998). To date, these transgenic papayas have offered durable resistance to PRSV and have controlled the disease in Hawaii (Ferreira *et al.* 2002). Other countries, like Brazil, Jamaica, Venezuela, Thailand, Australia (Lines *et al*. 2002), Taiwan (Bau *et al.* 2003), and more recently the Philippines and Vietnam (Tecson Mendoza *et al*. 2008), have since used the technology and the virus *cp* gene from their region to develop their own transgenic varieties. These transgenic papaya varieties are at various stages of development and evaluation.

In this review, we analyze the resistance mediated by the virus *cp* gene in transgenic papaya, in comparison with that of transgenic plants engineered with other virus derived sequences, and provide an update on the progress and constraints in the adoption of transgenic papayas in selected countries. The use of alternative non- *cp* (chimeric and synthetic transgenes) and non- transgenic approaches of managing PRSV is also discussed. Finally, but no less importantly, we argue that *although not strictly required to control the disease*, transgenic papayas are an example of biotechnology making good on its promise. Furthermore, work with this crop plant, along with a number of other transgenic crops, is widening the offering of biological systems which will facilitate analysis of biological phenomena and advance basic and applied science.

PAPAYA'S WORLD PRODUCTION IS NON-TRANSGENIC

Based on FAO's statistics of area harvested, yield and gross production (2010), 59 countries worldwide produced some 9, 095, 875 MT of papaya in 2008 (**Fig. 1**). As shown in **Fig. 2**, world production of papaya has been increasing steadily, in an exponential fashion, over the last 48 years. However, scrutiny of the production levels in individual countries during this period reveals considerable variation.

In the period analyzed, papaya production increased by 687.58% or almost 7 times, presumably due to improved yields (that amounted to ca. 203.21% increase or 2 times), and to an augmentation in the area devoted to papaya cultivation (3.38 times increase). Of note, the strategy of *growing more papaya* differed between countries. **Table 2** lists the major producers of papaya during the last nine years, from 2000 to 2008. The first 20 producers for 2008 account for 96% share of global production and none of these countries, apart from China perhaps, which is responsible for 1.32% of world production, cultivates transgenic papayas. **Fig. 3** summarizes the production, yield and cultivated area of the major, mid- and minor producers of papaya. It is worth noting, for example, that in India (the first largest papaya producer) a surprisingly high increase in production can be entirely attributed to a huge increase of area harvested in response to a tremendous decrease in yield in the 1980s (that it stills needs to recover the good figures in yield of the early 60s). In contrast, two other major producers, Brazil and Mexico, seemingly increased production by means of improving yields in combination with a moderate increase in the area harvested. Similarly, the mid-producers Venezuela and China employed much the same strategies to increase papaya production. On the contrary, the yield has remained more or less the same during the last 15 years (after a short period of increased yields) in the Philippines and this complements the trend of increased areas devoted to the cultivation of papaya. Finally, among selected small producers, the most notable case is the United States. The strategy adopted in the US involved the development and the introduction of transgenic papaya cultivars

Fig. 1 Major papaya producing countries in the world as 2008, based on the production data available from FAO (May 5, 2010). The 5 shading patterns indicate the levels of production: black, countries producing more than 500,000 MT per year (e.g., Brazil); dark grey, countries producing 100,000-499,999 MT per year (e.g., China); grey, countries producing 50,000-99,999 MT per year (e.g., Cuba); light grey, countries producing 10,000-49,999 MT per year (e.g., US); and faint grey, countries producing less than 10,000 MT per year (e.g., Argentina). Countries in white do not produce papayas, or the production data is not available.

Table 2 The 20 major papaya producer countries during the last 10 years, in tonnes, according data gathered from FAO.*

Countries	2000	2001	2002	2003	2004	2005	2006	2007	2008
India	1796960	2590400	2147200	1692100	2535100	2139300	2482100	2685900	2685900
Brazil	1439712	1489324	1597700	1714590	1612348	1573819	1897639	1811540	1900000
Nigeria	748000	748000	755000	755000	755000	755500	759000	765000	765000
Indonesia	429207	500571	605194	626745	732611	548657	643451	621524	653276
Mexico	672376	873457	876150	955694	787663	709477	798589	919425	638237
Ethiopia	197300	223000	226000	230540	260000	260000	260000	260000	260000
Congo	213000	206222	210305	212180	214070	215980	217900	219840	223770
Colombia	112627	110764	86912	91608	103870	140346	164606	223945	207698
Guatemala	24040	39000	54000	69000	84000	99000	113277	184530	184530
Philippines	75896	77417	127680	130764	133876	146628	157120	164234	182907
Peru	159622	158910	172669	189793	193923	171055	175428	157771	157771
Venezuela	114234	130204	152738	148030	131753	118063	151353	132013	132013
Thailand	119000	120000	120000	125000	125000	131000	131000	131000	131000
China	154222	159207	162572	164559	157620	118475	151283	117914	120359
Bangladesh	41000	44000	48000	47505	50615	240000	105245	95785	103609
Cuba	95503	135128	107240	120100	119000	91797	90309	89700	89400
Kenya	63410	77822	81811	86491	86000	87000	86000	86000	86000
Malaysia	60000	65000	70000	78000	75000	72000	72000	72000	72000
El Salvador	3000	3000	40000	53413	60470	63456	67264	65295	71172
Costa Rica	28786	27239	26458	31125	33815	35565	31090	41042	58408
World	6954812	8207372	8089108	7930846	8594281	8066114	8913064	9210748	9095875

*http://faostat.fao.org (May 5, 2010)

in Hawaii in the late 1990s. Although not all papayas grown in the US are transgenic, it is quite surprising that the yields have been fluctuating since the introduction of the transgenic cultivars. Apart from an initial recovery in 2000, papaya production has not reached its maximum. Decreases in both total area harvested and gross production are evident. A similar trend is observed with the yield data. It is pertinent to highlight that papaya consumption in the US has increased steadily after an explosive demand in the early 1990s. Moreover, the strategy selected to deal with the demand included increased importation of the fruit rather than increased internal production. Hawaiian papayas play a minor role in the economics of the crop and its consumption in the US (Suiyanata 2002); that is, in terms of total production. *The vast majority of papayas consumed in the US are apparently non-transgenic*.

It would then appear that the statement made by many that only through the cultivation of transgenic cultivars can papaya be grown worldwide in the presence of the decimating PRSV, lacks support: transgenic papayas are grown commercially in very few countries (US, and maybe China), the area devoted to cultivating transgenic papayas is negligible, and most countries worldwide have learnt to cope with the economically devastating disease. Notwithstanding, as shown in the following sections, transgenic papayas have proven effective in controlling the virus and have furthered our knowledge of plant-virus interactions and the many ways scientific knowledge can be employed to solve practical issues related to agricultural production.

cp TRANSGENIC PAPAYAS

In 1985, Sanford and Johnston proposed the concept of pathogen-derived resistance (PDR) as a method of developing resistance against pathogens, such as viruses. It was theoretised that the expression of a portion of the pathogen's

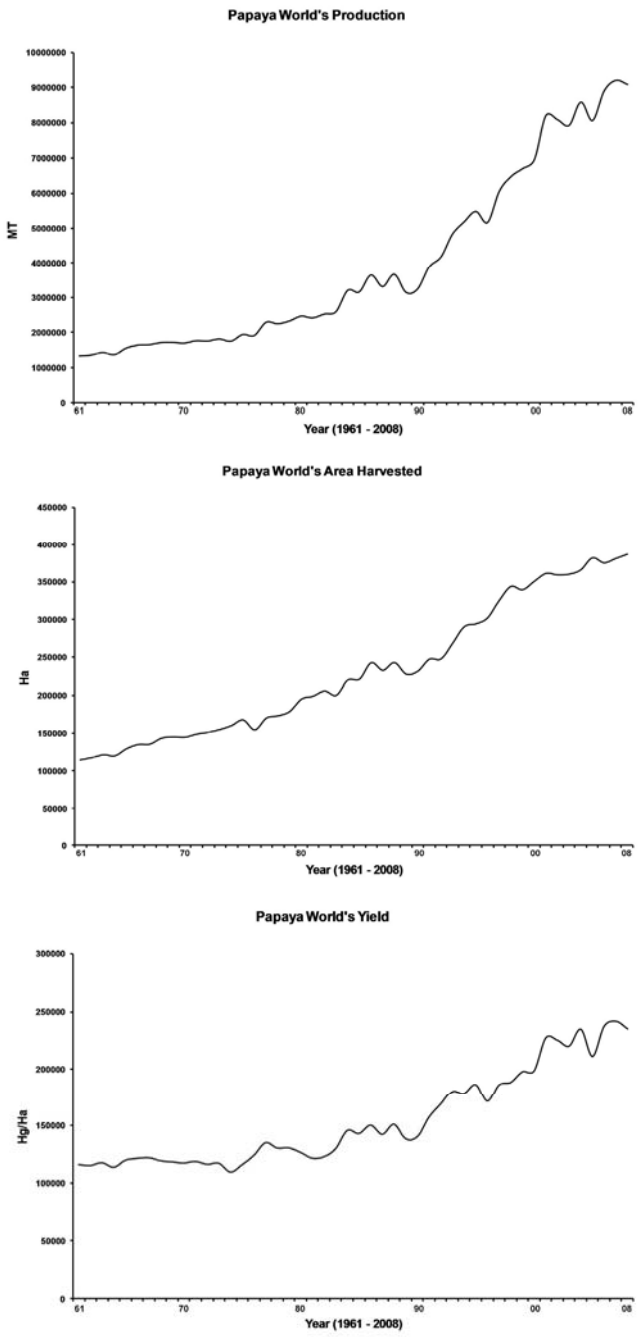

Fig. 2 Forty eight year span of the world's papaya production, area harvested and yield according to data gathered from FAO (May 5, 2010). The graphs were produced using available data from all world countries.

genetic material, albeit in a dysfunctional form, could inhibit the pathogen. Subsequent studies initially examined the effects of functional and later dysfunctional forms of the virus coat protein gene (*cp*) gene in transgenic plants. Powell-Abel *et al*. (1986) demonstrated that transgenic tobacco plants expressing the *cp* gene of *Tobacco mosaic virus* (TMV) exhibited phenotypes ranging from asymptomatic to attenuated or delayed symptom expression following inoculation with TMV. Later experiments by Lindbo and Dougherty (1992) with transgenic tobacco expressing mutant (untranslatable) *cp* of *Tobacco etch virus* (TEV) also demonstrated resistance against the challenge progenitor virus, therefore suggesting the involvement of the transgene RNA in conferring resistance. It was posited that sequence-specific RNA degradation induced by viral transgenes target RNA species sharing sequence identity with the transgene, resulting in virus resistance (Lindbo *et al*. 1993).

This model, coupled with the discovery that dsRNA induces a form of post transcriptional gene silencing (PTGS, Waterhouse *et al*. 1998), expanded and confirmed the original conception of PDR. Control of virus infections by the induction of a conserved, RNA-based plant antiviral defense response is achieved through small-interfering RNAs (siRNAs). Various reviews summarize the history of gene silencing research that contributed to our present understanding of plant virus resistance (Lindbo and Dougherty 2005; Prins *et al*. 2008; Eamens *et al*. 2008; Csorba *et al*. 2009).

Given the successful development of virus resistant transgenic plants, coupled with the complete characterization of PRSV and the difficulties associated with the introgression and nature of PRSV resistance in relatives of papaya (Gonsalves *et al.* 2005), transgenesis of papaya was initiated by a research group at Cornell University and the University of Hawaii. The group, in applying the concept of PDR, used the virus *cp* gene. Other research groups soon followed suit, with some 14 counties documenting the development and characterization of transgenic papaya varieties with indigenous PRSV isolates. **Table 3** summarizes the characteristics of the transgenic varieties reported in the literature.

Similar *cp* constructs were used in the development of the transgenic lines in the respective countries. That is, translatable versions of the *cp* starting beyond the NIa-protease cleavage site of the virus polyprotein. Translation signals of β-glucuronidase (*uidA*) or *Cucumber mosaic virus* (CMV), linked to the *Cauliflower mosaic virus* 35S promoter, were engineered to the *cp* in the region of the glutamine-serine (Q/S) cleavage site of the indigenous severe strain. The Hawaiian transgenic papaya, however, carry a slightly different construct consisting of 48 the nucleotides of the white leaf strain of CMV linked to the structural sequence of the mild cross protection strain, PRSV HA 5-1, a laboratory mutant derived from the severe PRSV HA strain.

The majority of the research groups have used microprojectile bombardment as the DNA delivery method into *in vitro* papaya materials and, consequently, the copy numbers and a range in insertion patterns, truncations and re-arrangements of the transgenes are reported with transgenic lines from different regions (e.g. Lines *et al*. 2002; Kertbundit *et al*. 2007; Ruanjan *et al*. 2007). Complete single *cp* constructs have been obtained after bombardment, and in the case of the transgenics from Hawaii, nonfunctional, truncated inserts of the marker genes, neomycin phosphotransferase (*npt*II) and antibiotic (*tet*), resistance, were also found (Fermin 2002; Suzuki *et al*. 2008). Bau *et al*. (2003) reported on 1 to 2 copies following *Agrobacterium* transformation. Quite surprisingly, greater than 2 *cp* insertions were found in transgenics obtained by *Agrobacterium* transformation in Florida (Davis and Ying 2004). The insertion site in the papaya genome was characterized with the Hawaiian transgenic papaya. In keeping with reports of other transgenic plants (e.g. *Arabidopsis* and rice, Sawasaki *et al*. 1998; Matsuo *et al*. 2005), the sequences flanking the transgenes were identified as plastid DNA derived sequences in the nuclear genome (nuclear plastid sequences, nupts). Given the prokaryotic-like gene transcription and translation, it was deduced that these sequences do not represent functional or expressed genes (Fermin 2002; Suzuki *et al*. 2008), and thus, the transformation event did not result in a disruption of endogenous gene expression. This was further substantiated in field studies where the agronomic and nutritional performance of transgenic trees and fruits were assessed (Ferreira *et al*. 2002). Superior agronomic performance under virus disease pressure with yields three times higher than the industry average and fruits with percent soluble solids above the minimum (11%) required by commercial fruit were noted.

Untranslatable constructs of PRSV *cp* containing *cp* sequences engineered with a stop codon or frame shift mutation have also been used in the generation of transgenic papaya (Lines *et al*. 2002; Tennant *et al*. 2002; Davis and Ying 2004; Tennant *et al*. 2005). Untranslatable constructs derived from a mild strain of PRSV from Hawaii, PRSV

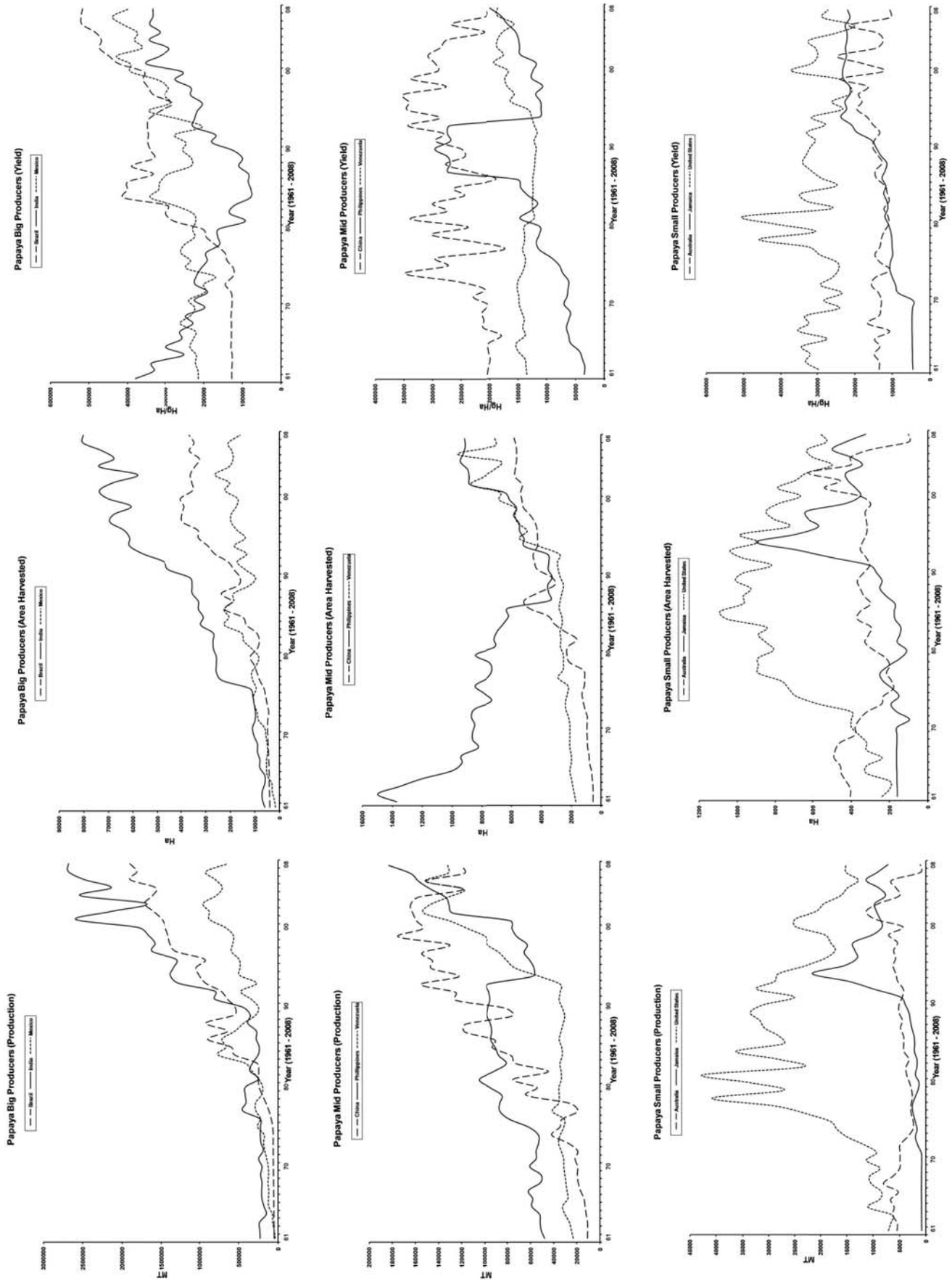

Fig. 3 Forty eight year span of papaya production, area harvested and yield in major, mid and minor producers according to data gathered from FAO (May 5, 2010).

HA 5-1, were developed by engineering a frame shift mutation in the *cp* gene. Somatic 'Sunrise' papaya embryos were transformed with non-linearized pGA482GG harboring the PRSV HA 5-1 untranslatable *cp* gene (Cai *et al*. 1999). Embryogenic kanamycin-resistant clusters were treated as independent lines that allowed the subsequent establishment of 83 plants in the greenhouse. All 83 R_0 transgenic lines were sequentially challenged twice with the nearly homolo-

gous, severe PRSV HA (99.8% similarity with the transgene). Each R_0 line was represented by the fully developed plant plus its clonal cuttings. Different reactions were detected and subsequent generations were challenged with other isolates from the Bahamas, Brazil, Jamaica, Mexico and Thailand, and an additional heterologous Hawaiian isolate (C. Gonsalves, unpublished results, Fermin 2002). Untranslatable *cp* transgene-mediated resistance to PRSV was reported due to PTGS: no transgene-derived protein was detected *in vitro*, nor *in planta*, in a selected line which showed a high level and wide range of resistance against diverse geographical isolates of PRSV, and a high rate of *cp* transgene nuclear transcription was observed but its cytoplasmic mRNA was barely detectable. Finally, as observed in other transgenic papayas (Tennant *et al.* 1994; Chiang *et al.* 2001; Tennant *et al.* 2001) the level and range of resistance was largely homology-dependent. This work demonstrated that an untranslatable *cp* gene is able to confer resistance to the homologous strain of the virus, and also to some close geographical isolates of PRSV by PTGS. More

Table 3 Summary of the characteristics of transgenic papaya developed by various research groups.

Country	Cultivar	Construct	Transformation	Transgene copy number	Transgene expression	Resistance testing		Reference
						Greenhouse	Field	
TRANSLATABLE *cp*								
Australia	Local variety	*uidA* leader + CaMV 35S pro+ PRSVBridgeman Downs (Queensland) *cp* gene from Q/S start with stop codon in the middle of sequence	Biolistics	1- 4 reported on truncations & rearrangements of the *cp* (no correlation copy number & level of R but to level of degraded RNA in northerns)	CP not detected in ELISA and low levels of *cp* detection in northerns	Truncated 1 copy 0% R 4 copies 100% 3 copies 100% R 3 copies 15% R	Truncated 1 copy 0% R 4 copies 100%R 3 copies 80% R (15% in greenhouse)	Lines *et al*. 2002
Brazil	Sunrise solo & Sunset solo	CaMV 35S + CMV leader+ PRSV Bahia *cp* from Q/S start CaMV 35S + CMV leader+ PRSV Bahia *cp* from E/S start	Biolistics	nt	Low to high levels CP protein detected in ELISA	R_1 46% R to PRSV Cruz das Amas R_1 0% R to PRSV Bahia, HA & TH	na	Souza Junior *et al*. 2005
Florida	cv. F65 (ancestor of F65)	*uidA* leader + CaMV 35S pro+ PRSV H1K cp gene from Q/S start	*Agrobacterium*	1-> 2	*cp* not detected in northern analysis	5- 13%	nt	Davis and Ying 2004
Hawaii	Sunset solo	CMV leader + 16 aa CMV *cp* + PRSV HA 5-1 *cp* gene from Q/S start	Biolistics	1- 2 (correlation between R and sequence similarity with *cp* transgene, copy number, plant age)	Low to high levels CP protein & transcript detected in ELISA & northern analysis	55-1: R_0 NS; R1 NS but showed symptoms against PRSV Oahua 63-1: R_1 40-52% resistance against Hawaiian isolates, but 26-39% resistance against BR, JA & TH isolates	55-1: R_1 (Rainbow) & R_3 NS for 12 mo 63-1: R_0 resistant in field for 12 mo	Fitch *et al*. 1992; Tennant *et al*. 1994; Ferreira *et al*. 2002; Tennant *et al*. 2005; Souza *et al*. 2005
Jamaica	Sunrise Solo	CaMV 35S + CMV leader+ PRSV Caymanas *cp* from Q/S start	Biolistics	1-3	*cp* RNA detected in northern analysis	R_0 29-40% R	R_0 50-89%	Cai *et al*. 1999; Tennant *et al*. 2002, 2005
Taiwan	Tainung No. 2	*uidA* leader + CaMV 35S pro+ PRSV YK cp gene from Q/S start	*Agrobacterium*	1-2 (R plants had 2 copies; highly R had one copy)	*cp* transcript detected in Northern (a relationship between R and the detection of CP & *cp* transcript)	4 categories of reactions: delay & then symptoms (40%), mild mottling (70-80%), immunity, susceptible	70-80% R (no correlation between R and sequence similarity with *cp* transgene)	Bau *et al*. 2003; Tripathi *et al*. 2004
Thailand	Khak Dum	CaMV 35S + *uidA* leader+ PRSV Ratchaburi province *cp*	Biolistics	Multiple insertions with rearrangements & deletions	CP detected in western analysis in 2 of 8 lines	All lines susceptible except for one line (G2)	nt	Kertbundit *et al.* 2007; Ruanjan *et al.* 2007
Venezuela	Tailanda roja (Thailand red)	CaMV 35S + CMV leader+ PRSV EV & VE from Q/S start	*Agrobacterium*	1	CP not detected in ELISA and low levels of *cp* detection in northern analysis.	All R_0 plants with LA or EV *cp* R to PRSV LA and EV R_1 EV *cp* 7% R, 50-73% R & 60-60% R to PRSV EV, La and HA R_1 EV+LA cp 0% R, 31% R & 38% R to PRSV EV, LA and HA R_2 EV cp 22-32% R	nt	Fermin *et al*. 2004

Table 3 (Cont.)

Country	Cultivar	Construct	Transformation	Transgene copy number	Transgene expression	Resistance testing		Reference
						Greenhouse	Field	
UNTRANSLATABLE *cp*								
Brazil	Sunrise solo & Sunset solo	CaMV 35S + CMV leader+ PRSV Bahia *cp* from Q/S start	Biolistics	nt	nt	R_1 100% R to PRSV Bahia & PRSV HA, 72% R PRSV TH	na	Souza Junior *et al*. 2005
Florida	cv. F65 (ancestor of F65)	*uidA* leader + CaMV 35S pro+ PRSV H1K *cp* gene from Q/S start in antisense	*Agrobacterium*	1-2	*cp* not detected in northern analysis	12- 15%	Nt	Davis and Ying 2004
		uidA leader + CaMV 35S pro+ PRSV H1K *cp* gene from Q/S start with frame shift mutation			*cp* not detected in northern analysis	4- 42%	71- 90%	
		uidA leader + CaMV 35S pro+ PRSV H1K *cp* gene from Q/S start with 3 in frame stop			*cp* not detected in northern analysis	8-34%	12-90%	
Hawaii	Sunset solo	CaMV 35S + CMV leader+ PRSV Caymanas untranslatable *cp*	Biolistics	1-3	*cp* RNA detected in R_0 by Northern analysis	3 categories of reactions: 28 lines HA resistant (100%); 22 lines mixed R (i.e. 49% showed R & S); 33 lines S	nt	Cai *et al*. 1999; Fermin 2002
Jamaica	Sunrise Solo	CaMV 35S + CMV leader+ PRSV Caymanas untranslatable *cp*	Biolistics	nt	*cp* RNA detected in R_0 by Northern analysis	R_0 15-29% R	R_0: 10% R or delay in symptom expression; 18-66% delay & mild symptom expression R_1: 0% R, 25-100% delay, mild symptom expression	Tennant *et al*. 2002, 2005

R resistant; S susceptible; nt not tested; na not available

research is needed, however, to fully understand the biochemistry of viral resistance conferred by a transgene, that from its conception, is tagged for degradation just as nonsense mediated decay can also target untranslatable messengers for their destruction (Hilleren *et al.* 1999; Isshiki *et al.* 2001; Lykke-Andersen 2001).

One benefit of plants engineered with untranslatable transgenes is that a protein of viral origin is not present in the final product and thus the risks associated with potential allergenicity of transgenic products are avoided (Ruibal-Mendieta *et al*. 1997). Surprisingly, the NPTII protein was also not detected in some of the transgenic lines described, even though the plants showed high level of resistance to PRSV. A transcriptionally silenced *nptII* gene could account for the negative results by DAS-ELISA. It can also be that transgene mRNA degradation by PTGS is playing a role in limiting the level of the NPTII protein below the detection of DAS-ELISA. Recently, it has been demonstrated that plants harboring the same construct at the very same position display different patterns of transgene expression, and that the silencing of the introduced transgenes may be a stochastic event concomitant to plant transformation (Day *et al.* 2000). Alternatively, many epigenetic phenomena can be explained by transgene methylation (Matzke and Matzke 1998a, 1998b), but experiments were not performed to assess if the *nptII* gene was transcriptionally, instead of post-transcriptionally silenced (van Houdt *et al.* 2000). In the case under discussion some of the engineered transgenic papayas produce neither CP nor NPTII as translational products of their respective transgenes. The lack of a CP produced *in planta* precludes the possibility of heterocapsidation (transcapsidation) that has been claimed as a source of environmental concern when dealing with transgenic plants transformed with viral sequences (Robinson 1996; Hull 1998; Hammond *et al.* 1999).

With the development of transgenic papaya harboring similar *cp* constructs from different regions and seemingly different insertions, a number of conclusions on the phenomenon of *cp* transgenic virus resistance in papaya have been reported. It appears that no one set of characteristics contribute to the level of resistance, but rather an interplay of *in planta*, virus and external factors. Firstly, transformation with either translatable or untranslatable forms of the *cp* gene confers resistance against PRSV. However, higher levels of resistance (29-40% *vs* 15-29%) were obtained with transgenic plants harboring the translatable *cp* form in the Jamaican transgenic papaya (Tennant *et a*l. 2005), whereas the converse was observed with transgenic papaya lines in Florida carrying translatable or untranslatable *cp* (5-12% *vs* 4.2-41.7%; Davis and Ying 2004).

Tennant *et al*. (2001) summarized virus and *in planta* factors that complicate transgenic virus resistance in papaya. It was reported that transgene dosage, plant developmental stage and sequence identity (> 89.5%) between transgene and virus isolate populations can affect the level of transgenic resistance. Young and older Hawaiian hemizygous PRSV HA 5-1 *cp* plants were resistant to the homologous PRSV HA (99.8% homology to *cp* transgene), while only

older plants were resistant to the other Hawaii isolates (96.7% homology). However, all inoculated hemizygous *cp* plants were susceptible to PRSV isolates collected from Jamaica, Brazil, and Thailand. In contrast, homozygous *cp* plants were resistant to all PRSV isolates, except the isolate from Thailand, regardless of the plant developmental stage. Resistance to the Thailand isolate, which shares 89.5% homology to the transgene, was observed only with plants inoculated at an older stage. Tripathi *et al*. (2004) later showed that isolates with greater than 95% sequence similarity with the *cp* transgene could break down resistance of transgenic lines. In addition, breaches in resistance by related but more aggressive strains of the virus, or by viruses belonging to emerging pathotypes of non-homologous viruses have been reported. Bau *et al*. (2008) report on a Taiwanese strain of *Papaya leaf mosaic distortion virus*, which is quite different from the previously reported Japanese strain, that was able to break resistance in PRSV transgenic plants although the *cp* gene from both viruses are similar enough to trigger PTGS.

Other factors have been purported to influence the levels of resistance observed in transgenic papaya plants. Bau *et al*. (2003) suggested the involvement of the virus helper component-protease (HC-Pro gene), a general pathogenicity enhancer with the ability of suppressing PTGS (Kasschau and Carrington 1998) in cases where there was no correlation of resistance to higher degrees of sequence similarity with the transgene. It was further speculated that environmental conditions, notably low temperatures, high soil moisture, and infections with other pathogens (root rot fungi) affected the physiological status of the transgenic plants and under these suboptimum conditions, the mechanism of PTGS was affected and virus infection ensued (Bau *et al*. 2004). Tennant *et al*. (2005) and Ruanjan *et al*. (2007) showed that *cp* transgene insert and PRSV resistance are not mutually inherited in the progeny. The onset of virus symptoms corresponded with a progressive decrease in the levels of siRNA in the R_1 progeny of transgenic lines transformed with the *cp* gene of a severe Thai isolate (Ruanjan *et al*. 2007). Evidence indicates that resetting of silencing occurs in plants following the generation of seeds, is dependent on a threshold level of transgene expression and the stabilization of nascent transcripts (Rovere *et al*. 2002).

One of the limitations of deploying homologous resistance in disease control is the inconvenience of resistance which is not universally useful in regions afflicted by different geographical isolates of the virus. Thus, in order to generate multiple resistance against viruses of the same group, or isolates of the same virus, transformations with either independent transgene constructs consisting of viral genes or a single transgene harboring more than one *cp* gene were investigated. In the case of tospoviruses, for example, multiple resistance was achieved initially by transforming tobacco plants with the nucleocapsid gene of three different tospoviruses (under the control of independent promoters) to which the transgenic plants gained resistance (Prins *et al.* 1995). Later investigations extended this model and showed that any single fragment derived from the N gene of *Tomato spotted wilt virus* (TSWV) was able to confer resistance against TSWV (Pang *et al.* 1997). A minimum length was required to trigger the gene silencing mechanism, but even below that threshold, resistance was attainable if the short transgene (less than 100 bp) was fused to a carrier DNA (Jan *et al.* 2000). This simple experiment opened the doors to more practical ways of engineering multiple viral resistance. In fact, by the late 1990s two different fragments from two different tospoviruses were fused, the chimeric transgene put under the control of a single promoter, and resistance was achieved against both viruses (Jan *et al*. 2000). The possibility existed, then, that by cloning different fragments of different geographical isolates of the virus, resistance could be widened – at local, national or even continental levels. This strategy was subsequently used to engineer resistance against three different isolates of PRSV in a single transformation experiment (Chiang *et al.* 2001).

By the late 1990s, the manipulation of the mechanism of gene silencing using fragments of virus genes of minimum sequence similarity as a means of strengthening and widening the level of resistance against different viruses was clearly demonstrated. However, the converse approach was also developed, that is, plants were transformed with a single synthetic gene derived from a manipulated N gene sequence of two distantly related tospoviruses (Fermin 2002). Initial experiments involved manipulating the sequence similarity between the transgene and its target virus. Gene constructs derived from the third fourth of the nucleocapsid *N* gene (3/4*N)* nucleotide sequence of *Tomato spotted wilt virus* (TSWV), differing in sequence similarity and location of nucleotide changes, were used to transform *Nicotiana benthamiana* explants. Leaf explants were transformed with constructs designed and synthesized with 5, 10, 15 and 20% changes evenly scattered along the transgenes, or with constructs (5 and 15% changes) with nucleotide changes clustered at the 5′ or 3′ end, or the middle of the transgene. R_0 plants were tested for resistance. The construct with 5% scattered changes gave 2-5 fold more resistance than those with 10, 15, or 20% changes. Only the 5% scattered construct had 20 nt long stretches that were identical to the challenge 3/4*N* gene of TSWV. In contrast, constructs with clustered changes conferred similar levels of resistance as the transgene that had no changes. Subsequently, a custom-designed construct with 10% of changes, not evenly scattered but strategically located in the construct to have 2-3 stretches at least 23 nt long identical to different tospoviruses 3/4*N* genes was designed. This synthetic, short transgene was 90% similar to the TSWV and *Groundnut ringspot virus* (GRSV) 3/4*N* genes, that otherwise share only a 79% similarity for this sequence. This construct was able to confer resistance to TSWV and GRSV demonstrating the feasibility of engineering multiple virus resistance with short, synthetic constructs (Fermin 2002). Further applications will tell whether the strategy can be successfully used to engineer multiple resistant transgenic papaya plants transformed with a short, synthetic transgene.

FACTORS CONTRIBUTING TO THE ADOPTION OF TRANSGENIC PAPAYA

Despite the potential of transgenic varieties for the control of plant virus diseases, less than 0.1% (~8,500 ha) of some 117 million ha under cultivation with transgenic crops is planted with virus resistant transgenic crops (James 2008). Transgenic papaya varieties as well as tomato and sweet pepper resistant to CMV and papaya resistant to PRSV have been released and adopted in the US and China, respectively (Stone 2008). Reports out of Hawaii describe a high adoption rate by farmers (Gonsalves *et al.* 2007). Farmers who have acquired seeds of the transgenic papaya varieties tout excellent performance of trees under virus pressure. Some produce transgenic papaya for sale or use the transgenic papaya as a buffer zone to assist in the economical production of non-transgenic papaya (Gonsalves *et al*. 2004; Tripathi *et al*. 2007). Despite the benefits, the rate of adoption of virus resistant transgenic crops is extremely low. Moreover, 14 countries have developed their own transgenic papaya varieties utilizing the *cp* gene from their region (Tecson Mendoza *et al*. 2008). Challenges facing the adoption of the technology as it relates to papaya appear to be associated with the application of the biotechnological protocols for the development of the transgenic product as well as the subsequent stages involving the development of a commercially viable product, notably regulatory issues and trade regulations.

A number of steps are critical to the development of a virus resistant transgenic crop; identifying the gene of interest, transformation of the crop plant, characterization of the new phenotype and multiplying seed. Unlike the development of the other transgenic varieties that involves the search for a suitable herbicide tolerant gene or a combina-

tion of genes capable of conferring adequate levels of insect resistance, the development of virus resistant transgenic papaya involves the isolation of the virus gene and engineering a transgene functional *in planta*. Most challenging is the following step, notably the delivery of the transgene to *in vitro* plant materials and obtaining a viable number of transformation events. Whereas reasonable transformation efficiency rates of 5-30% are reported with herbaceous crops (tomato and maize, Ishida *et al*. 1996; Frame *et al*. 2002; Cortina and Culiáñez-Macià 2004), lower rates of about 1% are generally obtained with papaya (Cabrera-Ponce *et al*. 1995; Cai *et al*. 1999). Thus, most of the research groups involved in transgenic papaya technology have formed collaborations with groups in the US (either the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the Gonsalves laboratory or Australia (Australian Centre for International Agricultural Research ACIAR), in order to easily and successfully pursue the development of a transgenic papaya product. The collaborations, typically involving the initial steps of the tissue culture of the progenitor plant species and transformation with the engineered virus gene in the host laboratory, have been successful in transferring the technology; that is, transgenic plants were regenerated and characterized. Manpower, facilities and funding in these situations are not the issue (Tecson Mendoza *et al*. 2008), but rather the final steps leading to field testing and the commercial release of the transgenic product; namely the biosafety regulatory issues and social acceptance of the technology.

Fermin *et al*. (2004) compared the adoption of transgenic papaya in three countries, Hawaii, Jamaica and Venezuela. The product was similarly developed, that is, papaya embryos were transformed with a translatable version of the *cp* gene from the respective countries. However, the rates of adoption in the respective countries vary. Fermin *et al*. (2004) attribute the differences in adoption to demand-side factors, the presence of an established biosafety regulatory framework as well as the acceptance of the technology by the consumer.

The Hawaiian story is well known. The papaya industry was in crisis in wake of the movement of PRSV in 1992 from the island of Oahu to the Puna region of the Hawaii island, the largest producing region of the country that accounted for 95% of total production. Within two years after the detection of PRSV in this region, the disease was rampant and efforts at eradication were abandoned by the Hawaiian Department of Agriculture. Concurrently, transgenic varieties, carrying the *cp* of a mild strain of PRSV were generated from 'Sunset' papaya embryos and the first field trial with one transgenic line was established in 1992. The transgenic papayas proved highly resistant to virus field infections and subsequent efforts were focused on stabilizing the line and the development of a commercially acceptable product. R_3 generations were obtained, thus creating the 'SunUp' variety which is homozygous for the *cp* gene and crosses were made with the R_1 'Sunset' and 'Kapoho', the preferred yellow flesh variety in Hawaii (Gonsalves 1998; Manshardt 1998). Given the excellent resistance against field isolates, deregulation of the transgenic papaya was initiated by the research team to government agencies, USDA's Animal and Plant Health Inspection Service, under the Plant Protection Act (APHIS), Environmental Protection Agency (EPA) and the Food and Drug Administration Agency (FDA). The legal considerations, that is licenses for the use of the intellectual property rights for the processes (gene, PDR) and components (*nptII*, *uidA*, *cp*) used in the development of the transgenic product, and financial considerations were addressed by the industry's papaya administrative committee and its legal counsel (Gonsalves 1998). Within three years, transgenic seeds were distributed to farmers in Hawaii. The rapid development and adoption of the transgenic papaya in less than ten years in Hawaii is attributed to the dedication of the research team as well as the dedication of and acceptance by the farmers. Moreover, and most significant, the industry was in crisis; all available commercial varieties were highly susceptible to the virus which was now widely distributed, mild strain cross protection strategies did not translate to long lasting economic benefits, neither did tolerant varieties and no significant progress had been achieved in the introgression of resistance genes from other members of the Caricaceae family (Gonsalves 2005). The transgenic varieties appeared to be the only available solution for continued production of the crop in Hawaii.

Jamaica ranks first in the production of papaya in the Caribbean (FAO Statistics 2010) and is one of the few countries that supplies its domestic and international markets. Papaya is regarded as one of the cash crops in the agricultural industry that is competitive globally and can provide food security. Yields higher than those reported in other Caribbean countries are obtained in Jamaica (e.g. > 220,184/ ha, FAO Statistics 2010) and the fruit is esteemed for its high quality. First described in Jamaica in 1929 (Jensen 1949), PRSV remained a disease of minor importance until outbreaks in the traditional papaya producing regions in the late 1980s. By the mid 1990s, the disease spread to all papaya-growing regions in Jamaica. Based on the reported success in Hawaii, the industry moved forward by initiating collaborations with the Gonsalves laboratory and began the transformation of 'Sunrise solo' *in vitro* materials. Engineering of the transgenes and transformation of the plant materials were conducted at Cornell University. Subsequent field evaluations were conducted in Jamaica along with substantial risk assessment involving nutrient composition analysis and toxicity studies using rat models (Tennant *et al*. 2005; Powell *et al*. 2008; Roberts *et al*. 2008).

Notwithstanding, at the start of the project in the mid 1990s government deregulating agencies did not exist in Jamaica. That is, there were no legislative or regulatory mechanisms for *overseeing* the *research* and safety trials of genetically modified plants before their formal approval for *commercialization or for the commercialization of the research outcomes*. Thus, the local collaborators approached the National Commission on Science and Technology (NCST) on obtaining a permit for the importation of the transgenic plants from Cornell University and by 1997 a National Biosafety Committee (NBC) was established. The NBC was given a statutory mandate under the Plants (Importation) Control Regulations (1997) and oversight responsibilities for the importation and research on transgenic plants. Within one year, permission was obtained and field-testing of genetically modified papaya varieties initiated. The results were not as dramatic as in Hawaii; however, resistance against PRSV was identified in some transgenic varieties as well as horticultural characteristics that could be manipulated in later generations for the development of a commercial product (Tennant *et al*. 2005). Further, the findings of the safety assessments suggested that the Jamaican transgenic papayas may not have adverse effects as regards the nutritional and toxicological parameters considered (Powell *et al*. 2008; Roberts *et al*. 2008). Thus, the next stage of the project was to field test subsequent generations, examine their performance on farmers' orchards and to build seed supply. However, the legislative and regulatory mechanisms needed to facilitate this stage, and the later stages of commercialization, were still not in place. In preparing to ratify the Cartagena Protocol, which was signed in 2001, there was the development of the National Biosafety Framework, National Biosafety Policy and National Biosafety Act. Nonetheless, public consultations were not conducted, nor documents presented to the Political Directorate. Thus, the deregulation of transgenic papaya cannot proceed until these mechanisms are in place. Moreover, unlike the situation in Hawaii in the mid 1990s, production of papaya is still possible in Jamaica without transgenic papaya. Production has decreased by 48% because of PRSV (STATIN 2006), but the industry has maintained viability and a few farmers continue to satisfy domestic markets and exports to the US, Canada, and other Caribbean countries, ensuring markets and prices without much competition. Management

practices involving vigilant scouting coupled with the prompt removal of infected trees in their immediate vicinity have contributed to this sustained production in regions with low disease pressure. Given the hurdles, deregulation costs and possible trade restrictions with Europe, it may be a while before transgenic papayas become common place in Jamaica.

Quite another situation exists in Venezuela where papaya is mainly a cash crop grown for local consumption. Over 7,000 ha are under cultivation and 130,000 tonnes produced on average (FAO Statistics 2010). Following the engineering of the *cp* gene constructs in collaboration with the Gonsalves lab, *Agrobacterium* transformations were initiated in Venezuela and transgenic 'Thailand Red' were developed in house (Fermin *et al.* 2004). A special permit was obtained from the Ministry of Health in 2001 for a small field trial with transgenic plants. Special permission was required because there are no biosafety regulations and guidelines for research, development and transboundary movement of genetically modified crops and their products in the country. In the absence of a National Biosafety Committee (still under creation), the Ministry of Health was the principal agency involved in biosafety related matters (it is presently the Ministry of Environment). Once in the field, resistance data against the virus were collected for 8 months, but 4 months following, opponents of the technology gained access to the secure plot and destroyed all the plants. Data on the durability of resistance and horticultural characteristics were not collected after eight years of work and expenditure in excess of $40,000. The production of papaya continues in Venezuela. Similar to the situation in Hawaii in the early outbreaks of PRSV, the farmers continually move out of heavily infected areas to virus-free areas and establish new papaya orchards. Additionally, as in many other countries (for instance Ecuador; Convenio MAG/IICA 2001, and Mexico, Rivas-Valencia *et al.* 2008), papaya is treated as an annual or biannual crop, after reaching its reproductive stage, and all general cultural practices associated with these crops are employed. Given the land mass, this may prove a viable method of growing papaya for a number of years to come.

Of note, some transgenic crops have made their way to the fields in Latin America. For example, cotton in Argentina and Mexico, corn in Argentina and Uruguay, and soybean in Argentina, Brazil, Mexico, Paraguay and Uruguay. The great imponderable question is why have some crops been accepted while others, such as papaya, remain as regulated articles, have not received clear acceptance from the government, and granted permission for limited greenhouse or field testing of the article (Bárcena *et al*. 2004; FORAGRO/IICA 2006). In the case of Venezuela it might be that the acceptance of the transgenic papayas is considered by some GMO opponents as the Trojan horse to include and accept others developed outside the country or in the country itself (for example, rice, plantain, coffee, cassava, mango and papaya). In the case of Brazil, it appears that the non-delivery of transgenic papaya is because of the slow process of turning technology into viable and approved seed products. The transgenic papayas were developed by a national Agricultural Research Corporation in collaboration with scientists in the US. The project started in the early 1990s. A transgenic product was developed and taken to Brazil. After molecular analysis and preliminary field evaluation of agronomic characteristics in Brasilia, transgenic populations are being incorporated into the papaya-breeding program at Embrapa Cassava and Tropical Fruits in Bahia. Biosafety studies needed for release in the market are yet to be conducted (Avila *et al*. 2001), most probably during 2010. Transgenic soybean varieties were however delivered as completed transgenic products to Brazil.

Gonsalves *et al*. (2007) cited other factors influencing the adoption of transgenic papaya that are relevant to developing-country producers, i.e. product and process standards, and market impacts. Import approvals are required by the importing country. For the Hawaiian transgenic papaya, these permits were easily procured from Canada, but regulatory clearance in Japan has not been obtained. The Japanese government has required additional risk assessment data on the transgenic product, thus increasing the cost of obtaining regulatory approval. Further, production of transgenic commodities will invariably require the implementation of testing and monitoring systems in accordance with international standards in quality assurance. Moreover, product prices may decline as a result of aggregate yield, certainly in the initial stages of adoption.

Thus, in Hawaii, the rapid adoption of the transgenic papaya was driven by demand-side as well as supply-side factors. In other countries, the demand-side factors do exist and have facilitated the development of the transgenic product by various research teams, but the demand is apparently not sufficient to bring other key players, such as farmers, consumers and policy makers, on board. Moreover, a seemingly more complex interplay of social, political and trade factors have constrained the application of the technology into these agricultural systems.

NON-TRANSGENIC APPROACHES

Even though transgenic papayas have proven feasible for controlling PRSV, their limited use on a global scale implies that there must be other ways of fighting against the disease. It would appear that most of the resources have been directed towards the development of transgenic cultivars in some regions precluding active study into understanding other ways that can be used for controlling PRSV. But what many may have seemingly overlooked has been addressed in various Latin American countries that account for more than 50% of global papaya production. These regions have directed much effort to increasing and improving papaya production. Many scientific publications are available, albeit to a limited audience as they are written in Spanish or Portuguese.

For more than 20 years one of the authors has visited papaya orchards south of Lake Maracaibo and only recently located small, healthy papaya orchards free of PRSV (**Fig. 4**) despite the presence of infected fields less than 20 km away. To rule out the presence of illegal transgenic plants, samples were collected and tested for the *cp* transgene. In all polymerase chain reactions conducted, using appropriate controls and primers directed to different parts of the *cp* gene, amplification of PRSV-derived sequences were negative (Castro and Fermin, unpublished results). Apparently the culture of intercropping plantain in alternate rows with papaya is a potentially useful management strategy against non-persistent PRSV disease and warrants further investigation. In other regions of Venezuela, papaya is grown as an annual or bi-annual crop since capital return is guaranteed.

In Brazil, non-transgenic control of the disease relies mainly on avoidance, cucurbit eradication, rouging, and cross protection (Lima *et al*. 2001). Similar strategies are adopted in Colombia, particularly the rouging of cucurbits. Researchers found that both commercially grown cucurbits and weeds, like *Momordica charantia*, in close proximity to commercial papaya orchards were positive for PRSV. Apparently the cucurbit plants tested served as feeding and virus acquisition hosts for *A. gossypii* (Arango *et al*. 2000). Also in Colombia, this time in the Caribbean region, early rouging, along with the use of grass barriers and the elimination of yellow leaves have been applied with some success (Paéz 2003). El Salvador, which according to FAO (2010) touts the highest yield for papaya production, achieves control primarily through careful selection of land, use of barriers plants, crop rotation, careful selection and germination of seeds (not necessarily certified seeds), rouging, avoidance of aphids by planting seedlings when the vector population is at its lowest, weed eradication, balanced fertilization and the use of insect traps (Rodríguez 2004). Epidemiological studies in Mexico clearly show that transplanting schedules may serve to delay the onset of

Fig. 4 Healthy 1 ½ year old papaya plants intercropped with plantain (**A, B**) in close proximity to orchards severely affected by PRSV (less than 20 km apart). Typical symptoms of the disease (leaf deformation, chlorosis, ring spots on very few remaining fruits) are visible on plants in the latter orchards (**C**).

epidemics, and hence, reduce the incidence of the disease (Mora-Aguilera *et al*. 1996), as do rouging and crop low density (Hernández-Castro *et al*. 2003).

In Puerto Rico, management practices are directed towards the aphid vector. Work with plastic mulch has proven effective in decreasing aphid populations in papaya orchards, and hence disease incidence. Trees in plots with protective mulching showed delayed appearance of symptoms, but more importantly increased yields (measured as marketable fruits) of more than a 100% (Robles *et al*. 2006). The study of the biology of the aphids that vector PRSV can contribute to the development of strategies for controlling the PRSV disease in papaya. It is well known that aphids do not colonize papaya plants; however, it is during the initial the feeding probe that the virus is acquired on the insects' stylet. In this regard it is necessary to evaluate the different aphids that vector PRSV and subsequently devise a strategy to target these populations. In Venezuela, Vegas *et al*. (1985) reported that efficacy in transmitting PRSV varies largely between *Myzus persicae* and *Aphis gossypii* (80% transmission), *Toxoptera aurantii* (40%), and a group comprised by *A. craccivora*, *A. nerii*, *Pentalonia nigronervosa* and *Rhopalosiphum maidis* (20% transmission). More recently Kalleshwaraswamy and Krishna Kumar (2007) re-evaluated the efficiency of three aphid species in transmitting PRSV and demonstrated that this varied among the species analyzed (*A. gossypii*, *A. craccivora* and *M. persicae*). Based on their work in India, efforts will have to be directed to the main vector, *A. gossypii*, in the subcontinent.

Barrier plants have also been tested as deterrents of plant virus vectors in many crop management systems (Hooks and Fereres 2006), including papaya. In Mexico, for example, Rivas-Valencia *et al.* (2008) demonstrated that barrier plants interfered with the efficiency of PRSV transmission by delaying the beginning and progress of disease epidemics. The authors proposed the use of a number of different species (e.g. corn [*Zea mays*] and roselle [*Hibiscus sabdariffa*]) rather than the use of a single species. It was recommended that a combination of different varieties be used at different times during the papaya crop cycle, in addition to applications of non-damaging-to-the-plant "insecticides" (e.g., mineral oils like Safe-T-Side®). The cultivation of organic papayas, a growing trend in countries like Mexico (20,551 tonnes per year), is setting an example for other countries in Central America and the Caribbean (Pohlan *et al*. 2007).

Since symptom development in PRSV-infected papayas is dependent on temperature, it is very difficult to envision a strategy aimed at manipulating this abiotic factor to cope with the virus. Mangrauthia *et al*. (2009) reported that the expression of symptoms is maximal between 26 and 31°C, which is also the ideal temperature range for growing papaya. The authors also analyzed the fate of virus induced siRNAs and their interaction with HC-Pro and discovered a temperature-regulated host–virus relationship. A study on manipulating the activity of HC-Pro through crop nutrition and other abiotic factors may generate novel ways of knocking out counter-silencing strategies in favor of plant defense against virus attack. Along the same vein, since HC-Pro is an important factor in facilitating infections of alternate hosts by PRSV-W (Yap *et al*. 2009), investigations into controlling the activity of its ortholog in PRSV-P might be helpful for limiting PRSV infections of papaya (and cucurbits).

In some regions, cross protection is used to produce papaya commercially. This strategy is based on the assumption that infection with an attenuated strain of the virus protects the challenged plant from the detrimental effects of a second infection by a more virulent variant of the same virus. Cross protection in papaya has been tried in the past in Hawaii, Taiwan (Yeh and Gonsalves 1984, 1994; Sheen *et al*. 1998), Brazil (Lima *et al*. 2001), and Venezuela (Vegas *et al*. 2000; González *et al*. 2002) with mixed results. In almost all cases, cross protection must be accompanied with other control measures to be effective. The main reasons for limited adoption of the strategy have been summarized elsewhere (Yeh *et al*. 2007): the need of a large-scale inoculation program, break down of resistance by more virulent isolates of the virus, financial constrains of small producers, and, we must add, national legislation overseeing controlled infections in the field. Cross protection must be reevaluated, however, since a comprehensive analysis of the epidemiology of the disease under this kind of management has not been conducted as thoroughly as in the successful case of cross protection against *Citrus tristeza virus* and *Barley yellow dwarf virus* (Zhang and Holt 2001).

Finally, in a recent report Srivastava *et al.* (2009) showed that PRSV infections can be suppressed through the use of a systemic antiviral protein isolated from the prickly myrtle plant, *Clerodendrum aculeatum* (Srivastava *et al.* 2009). Although preliminary, these results are encouraging since the lack of symptom development in mechanically challenged papaya plants seems to be due to an inhibition of virus replication *in planta*.

Other regions, namely the US and Australia, are focused on developing genetic resistance against papaya ringspot. Genetic resistance continues to be the first choice for disease control. When resistance genes are not present in the commercial cultivars, breeders opt for introgression from related species by means of controlled crosses. With papaya this approach has always been difficult because of reproductive barriers. Recent advances offer the promise of overcoming reproductive isolation of the species. Although some PRSV tolerant papaya lines have been developed by introgression to selected cultivars, for example "Cariflora" (Conover *et. al*. 1986), the pursuit of fully resistant cultivars has proven to be more difficult. The most consistent efforts at breeding resistant papayas have been in Australia and some artificial interspecific hybrids between *C. papaya* and

Vasconcellea cauliflora, *V. quercifolia* and *V. cundinamarcensis* (Magdalita *et al*. 1997a, 1997b; Drew *et al*. 2005) have been recovered after embryo rescue and micropropagation (Manshardt and Weslaff 1989). However, the F_1 plants regenerated were infertile except for the progeny of crosses with *V. quercifolia* (Drew *et al*. 1998). These plants showed a level of fertility that facilitated backcrossing with *C. papaya*, giving rise to male plants resistant to PRSV. In a different study published in 2009, O'Brien and Drew reported on the results of several interspecies crosses between *C. papaya* and diverse *Vasconcellea* (*C. papaya* x *V. pubescens*; *C. papaya* x *V. parviflora* (F_1); *V. pubescens* x *V. parviflora* (F_1); *V. pubescens* x *V parviflora* (F_2); *V. pubescens* x *V parviflora* (F_3 RR); *C. papaya* x [*V. pubescens* x *V parviflora* (F_2 RR]); (*V. pubescens* x *V. parviflora* (F_2 RR) x *V. parviflora* (BC_1); {(*V. pubescens* x *V. parviflora* F_2 RR) x *V. parviflora*} BC_1 x *V. parviflora* (BC_2). Many of the hybrids were morphologically normal and a few showed resistance to PRSV upon mechanical inoculation. In others, the presence of molecular markers (cleaved amplified polymorphic sequences [CAPS]) linked to resistance was demonstrated (Dillon *et al.* 2006). Although *V. parviflora* is not resistant to PRSV, the species is being used as a bridge to introgress resistance genes from *Vasconcellea* species to *C. papaya* (O'Brien and Drew 2009). The generation of hybrids through intergeneric crosses is ongoing in Venezuela by Vegas *et al*. (2003) with *C. papaya* and *V. cauliflora* using the methods of Magdalita *et al.* (1997a, 1997b) involving embryo or ovule rescue. Intergeneric hybrids have proven useful in obtaining not only resistance against PRSV, but also in the introgression of other useful genes into *C. papaya*.

Another way of dealing with the papaya ringspot disease involves the analysis and use of tolerant varieties, which do exist for papaya. In pioneering efforts to analyze PRSV tolerance in papaya, Conover and Litz (1978) evaluated 95 papaya accessions from around the world. It was demonstrated that tolerance was inherited quantitatively, not qualitatively, and dependent on the phenotype of the parents used in the cross. However, crosses required to generate true breeding tolerants are unpredictably cumbersome and has precluded or impeded consistent efforts to develop new varieties. Nonetheless, success was attained in Mexico with the development of the PRSV- tolerant hybrid 'Azteca' using genotypes derived from accessions collected in Tabasco in 2003. These plants appear to be performing well under ongoing field assessments (Mirafuentes and Azpeitia 2008).

CONCLUDING REMARKS

Plant disease management is a human activity that is as complex as it is unavoidable in agriculture (Gilligan 2008), particularly with virus diseases (Jones 2009). Almost every available management strategy has been employed in attempts to control PRSV; some measures are more effective than others and various countries have deployed different strategies against the disease, including transgenic cultivars. The latter strategy has been touted as a biotechnological success although few regions have embraced the technology and the world area devoted to the cultivation of transgenic papayas is virtually negligible. As examined earlier, a set of fortuitous circumstances, as well as the application of the technology in a country already driven by the easy acceptance of innovation, influenced the quick route of transgenic papayas from the lab to the consumer's table. For the most part, various analyses have focused on why other countries have not adopted a good working strategy, rather on how have these countries dealt with the disease as successfully, even more so, than Hawaii. Although papaya production on a global scale is steadily increasing, continued development and application of versatile and effective strategies against the virus are required in order to sustain this trend. Invariably the strategies will have to be weighed against the individual country's realities and capabilities, and as suggested elsewhere for the control of *Tospovirus* diseases (Pappu *et al*. 2009), they should be available on a national level, or even regional level, rather than a few targeted isolated growers.

Notwithstanding the poor adoption of transgenic papayas resistant to PRSV, the technology behind the creation of the transgenic papaya is sound and effective, and has opened the door to other applications that include traits not related to virus resistance, e.g. ripening, as reported by López-Gómez *et al*. (2009) and many more to come (Akhond and Machray 2009). The research has furthered our knowledge of the popular tropical fruit culminating in the sequencing of its genome (Ming *et al*. 2008). These later accomplishments, and future developments in papaya engineering, as well as a guaranteed increase of the knowledge of its basic biology, are due in part to the timely application of a nascent technology to a plant otherwise poorly utilized by science. While also noting the advances in knowledge of gene regulation by RNA interference and the ensuing impact on our understanding of basic processes dealing with virus biology, study of basic aspects of virus-vector-plant interactions has been ignored. In the case of PRSV, for instance, there are many unanswered questions regarding the true origin of PRSV, the molecular forces that shape virus populations in different parts of the world, the potential of virus transmissibility through seeds, virus interaction with known vectors and the epidemiology of the disease.

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