Generation of a Papaya Hybrid Variety with Broad-Spectrum Transgenic Resistance to *Papaya ringspot virus* and *Papaya leaf-distortion mosaic virus*

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ABSTRACT

The aphid-borne potyvirus, *Papaya ringspot virus* (PRSV), is the major limiting factor for papaya production worldwide. Transgenic resistance conferred by the PRSV-coat protein (CP) gene and based on the mechanism of post-transcriptional gene silencing (PTGS), has become the most effective method to protecting papaya from infection by the noxious virus. The PRSV-CP transgenic lines of cultivars 'Rainbow' and 'SunUp' have been commercialized in Hawaii since 1998, and to date, they have retained their resistance against the virus. However, another aphid-borne potyvirus, *Papaya leaf-distortion mosaic virus* (PLDMV), which occurs in Okinawa and Taiwan, has emerged as a serious threat for PRSV-CP transgenic papaya. To deal with the new emerging problem, double resistance in transgenic papaya carrying a chimeric construct containing partial CP genes of PRSV and PLDMV was generated. In addition, a super strain of PRSV was recently identified, that contains a stronger gene silencing suppressor capable of effectively shutting off PTGS and single or double CP-transgenic resistance in a homology-independent way. To solve this problem, transgenic resistance generated by an untranslatable construct targeting the PRSV HC-Pro gene was developed and the transgenic papaya lines provided broad-spectrum resistance against the PRSV super strain and PRSV isolates from different geographical locations. The event-specific molecular markers, derived from the flanking sequences of the transgene integration, in combination with the sex-linked markers, significantly fastened the molecular breeding process for pyramiding of single, double and super transgenic resistance into a commercial hybrid papaya cultivar. The super commercial hybrid cultivar of papaya with broad-spectrum resistance to different strains of PRSV and PLDMV has a great potential for application in different geographic regions of the world.

CONTENTS

WORLDWIDE THREAT ON PAPAYA PRODUCTION BY PRSV INFECTION

Papaya (*Carica papaya* L.) is believed to be indigenous to Southern Mexico and neighboring Central America. It was introduced in Caribbean countries and South-east Asia during the Spanish exploration in the 16th century (Storey 1969). Because of its palatable fruits, the papaya crop spread rapidly to the Indian subcontinent and Africa, and today it is distributed widely throughout tropical and sub-

Invited Review

tropical regions of the world.

Ripe papaya, a rich source of vitamin A and C (Manshardt 1992; Chandrika *et al.* 2003), is consumed as fresh fruit or used to prepare several food products, including jam and jelly, while the unripe fruit is used as vegetable or pickled. The latex-rich unripe fruit and other parts of papaya, including leaves, which are rich in several active components such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates (Seigler *et al.* 2002) have food processing, pharmaceutical and cosmetic applications (Chan and Tang 1978; Osato *et al.* 1993; Otsuki *et al.* 2010).

Brazil, Mexico, Nigeria, India and Indonesia account for more than 70% of the global papaya production. With a global productivity of 6.85 million metric tons, papaya is one among the ten most important fruit crops of the world, the others being apple, avocado, banana, grapes, mango, pears, pineapple, strawberry and tomato. However, the relatively fragile and perishable nature of papaya fruit causes it to lag behind several of these major fruits in production, value and export. Of the global annual quantum of papaya fruit production, only a small fraction of nearly 2% is being exported (Primary data source: FAOstat Agriculture 2007-http://www.faostat.fao.org/; Bapat *et al.* 2010).

Papaya productivity is limited in many areas of the world due to the disease caused by *Papaya ringspot virus* (PRSV) (Purcifull *et al.* 1984), which is the major obstacle to large-scale commercial production of papaya (Yeh and Gonsalves 1984). PRSV was first reported in Hawaii in the 1940s (Jensen 1949a), and subsequently its prevalence in Florida (Conover 1964), Caribbean countries (Adsuar 1946; Jensen 1949b), South America (Herold and Weibel 1962), Africa (Lana 1980), India (Capoor and Varma 1948; Singh 1969), the Far East (Wang *et al.* 1978) and Australia (Thomas and Dodman 1993) was noticed. To date, most of the papaya plantation areas of the world suffer devastation by this virus.

CHARACTERISTICS OF PRSV AND LACK OF NATURAL RESISTANCE

PRSV is a species of the genus *Potyvirus* (Purcifull *et al.* 1984; Murphy *et al.* 1995). PRSV is transmitted non-persistently by aphids and it is sap-transmissible in nature. The virion is a flexuous particle of 780×12 nm with a positive sense single-stranded RNA genome (De La Rosa and Lastra 1983; Yeh and Gonsalves 1985). The genomic RNAs of several strains of PRSV, including a few from Hawaii (Yeh *et al.* 1992) and Taiwan (Wang and Yeh 1997), have been sequenced. The 10.3 kb PRSV genomic RNA encodes a single polyprotein, which is proteolytically processed by three virus coded proteases into 10 final proteins, including the 36 kDa coat protein (CP) for encapsidation of the viral genome (Purcifull and Hiebert 1979; Gonsalves and Ishii 1980; Yeh *et al.* 1992) and the proteins of cylindrical (Purcifull and Edwardson 1967) and amorphous (Martelli and Russo 1976) inclusions in the cytoplasm of the infected host cells. The cylindrical inclusion protein (CIP) and the amorphous inclusion protein (AIP) are 70 kDa (Yeh and Gonsalves 1984) and 51 kDa (De Mejia *et al.* 1985a, 1985b), respectively. The uncapped genomic RNA, possessing viral genome-linked protein (VPg) covalently attached to the 5′ end and a polyadenylate tail at the 3′ end, has a genetic organization of 5′ leader, P1 protease (P1), helper component protease (HC-Pro), P3 protein (P3), CIP, 6 kDa protein (6K), nuclear inclusion protein a (NIa, which is processed further into VPg and NIa protease), nuclear inclusion protein b (NIb), CP and 3′ non-coding region (Dougherty and Carrington, 1988; Yeh *et al.* 1992).

In papaya, PRSV causes severe mosaic and distortion of leaves, ringspots on fruits and water-soaked oily streaks on the upper stems and petioles. It stunts the plant and drastically reduces the size and quality of fruits. Apart from the predominant mosaic strains of PRSV, certain strains of PRSV cause severe wilting and death of the infected plants

(Chang 1979).

Natural resistance to PRSV does not appear to exist in *C. papaya*, making conventional breeding difficult (Cook and Zettler 1970; Wang *et al.* 1978). However, PRSV tolerant lines of this crop have been described (Cook and Zettler 1970; Conover 1976; Conover *et al.* 1986). Although the PRSV tolerance trait has been introduced into several papaya cultivars, the horticultural properties of the resultant lines were not commercially desirable in some instances (Mekako and Nakasone 1975; Conover and Litz 1978). Other control measures against PRSV, including agricultural practices such as using pesticides against PRSV-transmitting aphids, rouging, quarantine, intercropping of papaya plants with corn as a barrier crop, protecting transplanted seedlings with plastic bags and covering entire orchards with plastic nets provide only transient and inadequate protection from PRSV infection (Wang *et al.* 1987; Yeh and Gonsalves 1994). In the late 1980s, in an attempt to find an effective solution to the severe yield loss caused by PRSV, Gonsalves and coworkers at Cornell University and the University of Hawaii initiated cross protection trials with papaya using attenuated strains of PRSV and the development of CP transgenic papaya lines resistant to PRSV.

CONTROL OF PRSV BY CROSS PROTECTION WITH ATTENUATED PRSV STRAINS

McKinney (1929) showed that plants infected with a virus would develop resistance against subsequent infection by a related virus. Attenuated strains of virus species are useful in cross protecting crop plants against severe strains of the virus, which otherwise cause high yield losses. The lack of effective control strategies against PRSV and the resultant severe economic loss necessitated the inclusion of other strategies such as cross protection in PRSV management. Cross protection is a practical strategy, in which healthy crop plants are deliberately infected with an attenuated strain of a virus to protect the crop against economic damage by severe strains of the same virus (Gonsalves and Garnsey 1989). PRSV HA 5-1, an attenuated mutant strain of PRSV, which was selected following nitrous-acid mutagenesis of a severe strain HA originated from Hawaii (Yeh and Gonsalves 1984), was tested extensively in the field and used commercially in Taiwan and Hawaii during 1985-1994 to permit economic return from papaya production (Wang *et al.* 1987; Yeh *et al.* 1988; Yeh and Gonsalves 1994). However the strategy has several drawbacks, including non-availability of efficient and genetically stable attenuated strains, superinfection with virulent strains, requirement for a large-scale inoculation facility and insufficient improvement in the yield (Stubbs 1964; Gonsalves and Garnsey 1989; Yeh and Gonsalves 1994). Therefore, genetically stable attenuated virus strains with broad-spectrum protection ability may be screened from natural sources or engineered in the laboratory. These protective strains may be used along with other control measures, according to the dominant viruses and agricultural conditions of the geographical regions, to reduce the loss from severe infection.

CONTROL OF PRSV BY A CP-TRANSGENIC APPROACH

The concept of pathogen-derived resistance (Sanford and Johnston 1985) proposes and describes the use of genetic elements from a pathogen's genome to confer resistance on a host originally susceptible to the pathogen. The concept of pathogen derived resistance was validated by the *Tobacco mosaic virus* (TMV) resistance of the transgenic tobacco lines carrying the coat protein (CP) gene of TMV (Powell-Abel *et al.* 1986). CP gene-mediated transgenic resistance has been proven effective in protecting tobacco, tomato, potato and other crops from infection by many different RNA viruses (Beachy 1990; Lomonossoff 1995; Goldbach *et al.* 2003). Further, many transgenic horticultural crops

have demonstrated excellent resistance to several viruses in the field. PRSV CP transgenic papaya lines resistant to PRSV (Gonsalves 1998) was deregulated and released in the United States. The PRSV NIb (replicase gene) transgenic papaya lines of the cultivar 'Huanong No. 1' has been approved in the People Republic of China (Gottula and Fuchs 2009; Guo *et al.* 2009).

In Hawaii, under greenhouse and field conditions, the plants of the PRSV CP transgenic papaya line 55-1 were highly resistant to infection by PRSV isolates originating from Hawaii (Fitch *et al.* 1992; Lius *et al.* 1997). The resistance of the plants of this line was shown to be triggered by a post-transcriptional gene silencing (PTGS) mechanism, a process of specific degradation of messenger RNA responsible for transgenic resistance to plant pathogens (Baulcombe 1996; Baulcombe 1999; Hamilton and Baulcombe 1999; Gonsalves 2002). However, the degrees of resistance against a virus by PTGS mechanism may be affected by the degrees of sequence homology between the transgene and the corresponding gene of the infecting viral strain, such as that between the CP transgene and the CP coding region of the challenge virus (Tennant *et al.* 1994). In the case of papaya, a CP transgenic hemizygous line of the cultivar 'Rainbow', a hybrid derived by crossing a CP transgenic homozygous line of 'SunUp' with non-transgenic plant of the cultivar 'Kapoho', was susceptible to PRSV isolates exotic to Hawaii. However, the CP transgenic homozygous line of the cultivar 'SunUp' was resistant to a wider range of isolates from Jamaica and Brazil, though it was susceptible to isolates from Thailand and Taiwan (Gonsalves 1998; Tennant *et al.* 2001; Gonsalves 2002). The characteristic of sequence homology-dependence of transgenic resistance apparently limits the application of CP-transgenic papaya for controlling PRSV in geographic regions other than Hawaii (Gonsalves 2002).

The field trials of the CP transgenic homozygous line of 'SunUp' and CP transgenic hemizygous line of 'Rainbow' indicated their efficacy in managing PRSV in Hawaii (Ferreira *et al.* 2002). By May 1998, these CP transgenic lines of 'Rainbow' and 'SunUp' cultivars of papaya were deregulated by the United States Animal and Plant Health Inspection Service and Environmental Protection Agency, and approval was granted from the Food and Drug Administration for commercial application (Gonsalves 2002). This is the first successful case of a transgenic fruit tree being commercialized in the world.

EMERGING THREAT OF PLDMV INFECTING PRSV CP-TRANSGENIC PAPAYA

In Taiwan, PRSV CP gene of a native strain PRSV YK was used to generate PRSV-resistant transgenic lines from the hybrid papaya cultivar 'Tainung No. 2' by *Agrobacterium*-mediated transformation (Cheng *et al.* 1996). The transgenic lines showed various levels of resistance, ranging from a period of delay in symptom development to complete immunity (Bau *et al.* 2003). Several lines, which were highly resistant to the homologous strain (PRSV YK), also provided wide-spectrum resistance to strains from three different geographic regions, Hawaii, Thailand and Mexico (Bau *et al.* 2003). In the field trials during 1996-1999, these PRSV CP transgenic papaya lines exhibited high degrees of consistent protection against PRSV in Taiwan (Bau *et al.* 2004). However, 18 months after establishment in the fourth field trial, unexpected symptoms of severely distorted fully expanded leaves, stunting in growth, water-soaking on petioles and stem, and yellow ringspots on fruit, were noticed on the plants of these lines. The causal agent was distinguished from PRSV by its different host reactions and serological properties and identified as a P type strain *Papaya leaf-distortion mosaic virus* (PLDMV) (Bau *et al.* 2008), a potyvirus originating from Okinawa, Japan in 1954 (Maoka *et al.* 1996). PLDMV P-TW-WF, the first reported P type strain from Taiwan, was found to be distinct from the P type strains from Japan because of its inability to infect several cucurbit hosts (Bau *et al.* 2008), which were previously reported as hosts of the Japanese strains (Maoka *et al.* 1996; Maoka and Hataya 2005). Since all of the PRSV CP-transgenic papaya lines were susceptible to PLDMV P-TW-WF infection under glasshouse conditions, PLDMV was considered as an emerging threat for the application of the transgenic papaya in Taiwan and other regions (Bau *et al.* 2008).

THE APPROACH TO GENERATE DOUBLE RESISTANCE AGAINST PRSV AND PLDMV

The PLDMV infection of PRSV CP transgenic papaya lines resistant to PRSV infection necessitated the urgent development of strategies for controlling the newly emerging potyvirus PLDMV along with PRSV in Taiwan and elsewhere (Bau *et al.* 2008). Transgenic crops with resistance to multiple viruses can be generated by engineering the plants with CP genes from more than one virus. Such multi-viral CP transgenic crops include transgenic squash resistant to *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) (Fuchs and Gonsalves 1995; Tricoli *et al.* 1995; Fuchs *et al.* 1998) and transgenic cantaloupe (*Cucumis melo* L. var. cantalupensis Naud.) resistant to CMV, ZYMV and WMV (Fuchs *et al.* 1997). However, the multi-viral CP transgenes in these cases were under the control of independent sets of *cis* elements. Although this approach promises multiple virus resistance, it does not guarantee balanced expression of individual transgenes and their faithful co-segregation to progenies. The expression- and segregation-related handicaps can be prevented by designing single-promoter-controlled chimeric transgene possessing sequence elements from multiple virus genes. A single-promoter-controlled chimeric transgene comprising full length CP sequence of *Turnip mosaic virus* and partial nucleocapsid (N) gene sequence of *Tomato spotted wilt virus* has been transferred to plants of *N. benthamiana* to generate resistance to these two viruses (Jan *et al.* 2000). The efficacy of this approach in conferring effective broad-spectrum virus resistance has also been demonstrated by a composite transgene comprising segments of the N gene elements from four economically important tomato-infecting tospoviruses (Bucher *et al.* 2006). Similarly, the efficacy of an untranslatable composite transgene comprising PRSV W and ZYMV CP gene segments in providing resistance against these viruses has been demonstrated by Wu *et al.* (2010).

In generating transgenic papaya lines with resistance to both PRSV and PLDMV, an untranslatable chimeric construct comprised of untranslatable segments of PRSV YK CP and PLDMV P-TW-WF CP genes was constructed and transferred into papaya cultivar 'Thailand' by *Agrobacterium*-mediated transformation (Kung *et al.* 2009). The transgenic papaya lines carrying the chimeric construct were regenerated and micropropagated. Several of the generated PRSV-PLDMV CP transgenic papaya lines exhibited resistance against both PRSV and PLDMV under greenhouse conditions. Molecular analysis of these plants revealed that the transgenic resistance of these plants to PRSV and PLDMV is triggered by the PTGS mechanism. Three lines (10-4, 14-1 and 14-3) showed high degrees of resistance not only to PLDMV P-TW-WF and PRSV YK, which were the transgene donors, but also to the heterologous strains of PRSV MX, TH, and HA, originating respectively from Mexico, Thailand and Hawaii (Kung *et al.* 2009). These transgenic papaya plants with PRSV and PLDMV resistance are considered to have a great potential for the control of PRSV and PLDMV in Taiwan and elsewhere (Kung *et al.* 2009).

TRANSGENE INTEGRATION EVENT-SPECIFIC MARKERS FOR GMO IDENTIFICATION AND MOLECULAR BREEDING

Despite continuing remonstrations by scientific and socio-political groups and stringent regulations by governments (Singh *et al.* 2006), more and more crops are being genetically modified (GM), and the global area of GM crop cultivation has been increasing in a fast pace (James 2008). Governmental regulations and measures to uphold consumer rights and intellectual property rights necessitate strategies to detect and characterize GM organisms (GMOs).

Detecting GMOs and characterizing them for GM-caused host genomic structure/expression changes and GM-derived molecules are challenging tasks requiring knowledge and expertise. The methods of GMO analysis range from simple phenotypic identification and bioassay to highly advanced DNA-, protein- and metabolite-based strategies, the speed, sensitivity and precision of which are dependent upon methods and detection devices. Excellent reports reviewing the existing strategies of GMO identification and characterization with proposals and suggestions for improvement, standardization, and harmonization, and future speculations (Michelini *et al.* 2008; Holst-Jensen 2009; Gašparič *et al.* 2010; Gryson 2010) have been published. Analyzing previously available information of the reported detection methods for GMOs, Dong *et al.* (2008) recently launched a useful interactive GMO database.

The widely used DNA-based method of polymerase chain reaction (PCR) was introduced in GMO identification in the mid 1990s. The improvements in the PCR technique and applications, such as multiplex PCR assays and real-time PCR, and the advancements in separation and detection devices, have greatly improved the speed, efficiency and precision of GMO identification and quantification. Microarrays are being used for nucleic acid hybridization-based direct analysis of GMO samples or their analyses after PCR. Apart from PCR and hybridization analyses, improvements in DNA sequencing technology have made possible the high throughput sequencing analyses of GMO samples. Moreover, GM-derived proteins and metabolites can also be detected by immunological techniques and mass spectroscopy. Tengs *et al.* (2009) characterized transgene-caused modifications in *Arabidopsis thaliana* genome using high throughput sequencing of transcripts of transgenic and non-transgenic individuals and subsequent computational subtraction to identify transgene construct-derived messengers. They also validated the usefulness of the crop cDNA sequences and expressed sequence tags (ESTs) available in cDNA/EST databases for such studies.

From the available papaya cultivar SunUp EST collection, the largest EST collection with >75000 sequences, generated as a part of a papaya genome/transcriptome program (Ming *et al.* 2008), 23 CP-transgene construct-derived ESTs were identified (Tengs *et al.* 2009). Guo *et al.* (2009) demonstrated the applicability of chymopapain gene, a single copy papaya-specific gene lacking allelic variations, as the endogenous reference gene in the conventional and real time quantitative PCR analyses of GM papaya. PCR-based protocols for transgenic event-specific characterization of GMO have also been established. PCR of restriction enzyme-digested adaptor-ligated total genomic DNA fragments of a transgenic organism with a primer directed towards a sequence element of the transgene construct and a primer directed towards the ligated adaptor can amplify DNA product(s), the sequencing analyses of which reveals genomic context(s) of transgene integration. Application of such strategy in our recent analysis of PRSV CP transgenic papaya lines identified several transgene integration event-specific markers (Fan *et al.* 2009), which can be used to fulfill regulatory requirements, to protect intellectual property rights of a particular transgenic line and to monitor molecular breeding to improve transgenic crops.

IMPORTANCE OF SEX-LINKED DNA MARKERS AND HIGH DENSITY GENETIC MAPS

Carica papaya is a diploid ($2n$ = 18) trioecious species comprising individuals of male, hermaphrodite and female sexes. Horticulturally, the male plants are solely pollinators with low economic value. The female plants, which produce spherical fruits of thinner flesh with more seeds are commercially less desirable than the hermaphrodite plants, which produce pyriform fruits of thicker flesh with fewer seeds. Given these preferences, it is imperative to develop efficient methods for papaya sex identification at a very early seedling stage to avoid growing individuals of less desired sexes in large proportions in papaya orchards.

Based on the segregation ratios from crosses among three sex types, Storey (1938) and Hofmeyr (1938) proposed that sex of papaya was determined by a single gene represented by three alleles, M, M^h, and m. Males (Mm) and hermaphrodites (M^hm) are heterozygous and females (mm) are homozygous recessive. MM, M^hM^h and MM^h are embryonic lethal (Hofmeyr 1938; Storey 1938), resulting in a 2:1 segregation of hermaphrodites to females from hermaphrodite crossing. The papaya sex locus has been genetically mapped to a specific linkage group (Sondur *et al.* 1996).

Reliable molecular markers showing strict co-segregation with sex-phenotype(s) can be used for sex identification of papaya at the seedling stage to facilitate papaya cultivation and breeding. In papaya, several molecular markers linked to sex have been reported (Sondur *et al.* 1996; Parasnis *et al.* 1999; Deputy *et al.* 2002; Urasaki *et al.* 2002; Chen *et al.* 2007). A sex-linked randomly amplified polymorphic DNA (RAPD)-based 450 bp papaya sex determination marker (PSDM) is present in male and hermaphrodite plants, but absent in female plants (Urasaki *et al.* 2002). Based on such RAPD-based PSDM sequences, specific SCAR (sequence-characterized amplified region) primers can be designed to produce more specific SCAR markers for accurate sex identification (Deputy *et al.* 2002). Simple sequence repeat (SSR) markers similar to CPM1815 (identified in the cultivar 'SunUp'), which distinguish hermaphroditic individuals from females (Chen *et al.* 2007) can also be used for papaya sex identification.

Cloning and characterization of the sex determination genes and understanding the sex determination process have profound applications in papaya breeding and cultivation. Papaya possesses a primitive Y chromosome, with a male-specific region that accounts for only about ~10-13% of the chromosome, but has undergone severe recombination suppression and DNA sequence degeneration (Liu *et al.* 2004; Ming *et al.* 2007). Physical mapping and sample sequencing of the non-recombination region led to the conclusion that sex determination is controlled by a pair of primitive sex chromosomes with a small male-specific region of the Y chromosome. Ming *et al.* (2007) postulated that two sex determination genes control the sex determination. A feminizing (stamen suppressing) gene causes stamen abortion before or at flower inception and a masculinizing (carpel suppressing) gene causes carpel abortion at a later flower developmental stage.

High-density genetic maps assist cloning specific genes of interest, such as those for sex determination and other important traits. Detailed physical mappings reveal structural details about the sex determination region and sequencing is expected to uncover candidate sex determining genes. Ma *et al.* (2004) constructed a high-density genetic map of papaya using 1498 amplified fragment length polymorphic (AFLP) markers mapping into 12 linkage groups, covering a total genetic length of 3294 cM, with an average distance of 2.2 cM. The genetic map revealed severe recombination around the sex determination locus with a total of 225 markers co-segregating with sex types. A sequence-tagged high-density microsatellite genetic map of papaya (Chen *et al.* 2007) and a physical map of the papaya genome with integrated genetic map and genome sequence

(Ming *et al.* 2008; Yu *et al.* 2009) have been constructed for comparative structural evolutionary genomics of papaya.

MICROPROPAGATION AND TRANSFORMATION OF HERMAPHRODITIC PLANTS OF ELITE PAPAYA CULTIVARS

In most world markets, fruits from hermaphroditic plants are commercially desirable, for they contain less seeds and thicker flesh (Yeh *et al.* 2007). A breeding program for commercially viable papaya cultivars is preferably linked with the complex traits of disease resistance, fruit quality and hermaphroditism. In such efforts, genetic transformation should be conducted on selected papaya cultivars with commercially desired traits to avoid the time-consuming breeding process to incorporate the transgenic resistance to a commercial variety.

Somatic embryos derived from immature zygotic embryos are the most commonly used materials for papaya transformation (Fitch and Manshardt 1990; Cabrera-Ponce *et al.* 1995, 1996; Cheng *et al.* 1996; Cai *et al.* 1999). Such somatic embryos are considered to be the most effective explants for both biolistic gene delivery and *Agrobacterium*-mediated transformation. However, dissection to extricate immature zygotic embryos requires skill, and their availability and efficacy are affected by seasonal factors. Moreover, the sex types (i.e., male, female or hermaphrodite) and other horticultural traits of transgenic papaya lines can be determined only after flowering and fruit production. Despite the time and effort spent, only a subpopulation of plants will be represented by hermaphroditic plants with desired fruit quality.

Alternatively, papaya somatic embryos can also be developed from adventitious roots of *in vitro* shoot-origin, the development of which is technically simple and independent of external seasonal variations. From the *in vitro* shoot stage, the total duration required for development of genetically transformable papaya somatic embryo with excellent regeneration potency is nearly four months, as shown for hermaphroditic plants of the hybrid papaya cultivar 'Tainung No. 2' (Lin and Yang 2001). Using a chimeric transgene construct comprising PRSV and PLDMV CP gene sequences, Kung *et al.* (2010) transformed somatic embryos developed from adventitious roots of *in vitro* shoots of hermaphroditic plants of the cultivar 'Tainung No. 2', and developed PRSV-PLDMV resistant hermaphroditic papaya lines. This strategy was also used to produce transgenic lines of the cultivars 'Thailand' and 'Sunrise', the parental cultivars of the hybrid 'Tainung No. 2' (Kung *et al.* 2010). The hermaphroditic sex-linked RAPD marker amplifiable by the primers SDP1 and SDP2 (Urasaki *et al.* 2002) was used to ascertain the hermaphroditic criterion of the transgenic plants. The floral and fruit phenotypes of the double-virus resistant hermaphroditic papaya plants of these three cultivars were similar to those of non-transgenic control plants (Kung *et al.* 2010).

Following this new transformation procedure, single-virus (PLDMV) resistance and double-virus (PRSV and PLDMV) resistance in transgenic papaya lines of different papaya cultivars with hermaphrodite sex and desired horticultural properties have been developed in our laboratory (Kung *et al.* 2010). The commercially valuable 'Tainung No. 2' papaya hybrid variety, the most popular variety in Taiwan, with double-virus resistance to PRSV and PLDMV can be directly used for practical application via micropropagation without any further breeding. Therefore, this new approach is a fast and efficient transformation method for different papaya varieties and it can significantly shorten the time-consuming breeding program.

SEQUENCE HOMOLOGY-INDEPENDENT BREAKDOWN OF PRSV-PLDMV TRANSGENIC RESISTANCE BY A PRSV SUPER STRAIN AND THE SOLUTION

In Taiwan, apart from the above described unexpected PLDMV infection of PRSV CP transgenic papaya lines, we also encountered an extremely virulent PRSV strain, designated PRSV 5-19, infecting the PRSV-resistant PRSV CP transgenic lines (Tripathi *et al.* 2004) and the recently developed PRSV-PLDMV resistant transgenic lines (You 2005; Kung *et al.* 2009). The super strain PRSV 5-19 was able to breakdown the resistance of the transgenic lines carrying PRSV coat protein (CP) gene, which share over 95% nucleotide identity with the CP of 5-19. This level of sequence identity is less than that of other PRSV strains which are not able to overcome the transgenic resistance conferred by PRSV CP transgene (Tripathi *et al.* 2004), indicating that the breakdown of the transgenic resistance is not correlated to the sequence divergence between the infecting virus and the transgene.

The multifunctional protein HC-Pro (Urcuqui-Inchima *et al.* 2001) is the gene silencing suppressor of a potyvirus (Anandalakshmi *et al.* 1998). It can counteract the host defensive reaction of PTGS and help the establishment of the invading virus. Our recent and ongoing studies with the PRSV super strain PRSV 5-19 reveal that the PRSV 5-19 super strain contains a stronger gene silencing suppressor that suppresses PTGS-mediated transgenic resistance conferred by the CP transgene in a sequence homology-independent manner (You 2005 and unpublished data). The breakdown of the transgenic resistance by a stronger gene silencing suppressor of a super strain has strong impacts on the application of transgenic crops for virus control. Hence, the PRSV strains like 5-19 are regarded as potential threats to the CP gene-mediated resistance of transgenic papaya lines.

In order to disarm the counteracting ability of the invading super strain against the host defensive PTGS reaction, new transgenic lines of the papaya cultivar 'Sunrise' carrying the untranslatable full, N-, and C-terminal region of HC-Pro coding sequences have been developed by *Agrobacterium*-mediated transformation of somatic embryos derived from selected hermaphroditic individuals. Several transgenic lines show high levels of resistance to both the super strain PRSV 5-19 and other severe strains from Taiwan, Hawaii, Thailand and Mexico (unpublished data). In the highly resistant lines, low levels of mRNA accumulation and higher levels of accumulation of siRNA of the transgene were observed, suggesting that a post-transcriptional gene silencing targeting the HC-Pro gene to abort the function of gene silencing suppression was the underlying mechanism for resistance. Our results indicated that papaya lines carrying an untranslatable silencing suppressor gene HC-Pro of a PRSV virulent strain can solve the problem resulting from PRSV super strains that overcome the transgenic resistance in a homology-independent manner (unpublished data).

PYRAMIDING OF SINGLE, DOUBLE, AND SUPER TRANSGENIC RESISTANCE IN A COMMERCIAL HYBRID

Apart from our earlier PRSV CP transgenic papaya lines resistant to PRSV, we have recently developed different PRSV-PLDMV CP transgenic papaya lines of 'Thailand' and 'Sunrise' cultivars, which are the parental lines of the most famous commercial hybrid in Taiwan, with double-virus resistance to both PRSV and PLDMV (Kung *et al.* 2009). Using the strategy to generate transgenic resistances in horticulturally desirable hermaphroditic plants, we have also generated HC-Pro transgenic lines of the cultivar 'Sunrise' with resistance to PRSV super strain 5-19, to overcome the problem resulted from homology-independent breakdown of single or double CP-transgenic resistance (unpub-

lished data). For broad-spectrum resistance to strains of PRSV and PLDMV, a super commercial papaya hybrid 'New Tainung No. 2' is currently being generated by pyramiding the transgenes the parental lines Thailand and Sunrise carrying various transgenes. Our breeding procedure involves (i) selection of transgenic lines with high degrees of resistance to PRSV and PLDMV, (ii) fixation of the transgenes in parental lines as homozygote by selfing of double-virus resistant papaya line of Thailand and super-PRSV resistant papaya line of Sunrise. (iii) As demonstrated recently by us (Fan *et al.* 2009), appropriate transgenic event-specific primers are used to monitor the transgene-genotypic status of the parental lines and progenies during breeding and to confirm pyramiding of the transgenes in the final hybrid of "New Tainung No. 2". In this way, a new hybrid with commercially desirable traits and broad-spectrum resistance to strains of PLDMV and PRSV can be generated within two years. During the process of pyramiding, appropriate sex-linked markers, as suggested by Urasaki *et al.* (2002), is used to confirm the hermaphroditic nature and hemizygotic/homozygotic status of each papaya line and its progeny. The development of this super transgenic papaya hybrid variety should have a great potential for global application for controlling different strains of PRSV and PLDMV in different geographic regions.

CONCLUDING REMARKS

The transgenic resistance conferred by the viral CP gene has become the most effective method to prevent papaya from infection by the noxious PRSV. In 1998, PRSV-CP gene transgenic papaya cultivars 'Rainbow' and 'SunUp' were deregulated and granted approval for commercialization, representing the first successful application of transgenic fruit tree in the world. Although the transgenic lines are not resistant to most of the PRSV strains from other different geographic areas, the breakdown of the transgenic resistance to PRSV strains indigenous to Hawaii has not been noticed. This is probably due to the fact that Hawaii islands are isolated by thousand miles of ocean and the virus strains are quite homogenous. Since the mid 1990s, several transgenic papaya lines with broad-spectrum resistance to PRSV have been developed in Taiwan (Cheng *et al.* 1996; Bau *et al.* 2003; Kung *et al.* 2009, 2010). Several of these transgenic papaya lines were evaluated under isolated-field trials (Bau *et al.* 2004) and assessed for their biosafety for many years to meet the strict regulations of the country. However, owing to the conservative attitude of Taiwan towards GM crops, similar to most European countries, several evaluated GM crops are awaiting for deregulation allowing cultivation and commercialization within Taiwan.

Other than the excellent performance of PRSV CP-transgenic papaya in Hawaii, the highly resistant PRSV CP-transgenic papaya lines developed in Taiwan were found susceptible to an unrelated potyvirus, PLDMV, which has been identified in various regions of Okinawa and Taiwan. To overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene comprising parts of the CP genes of PRSV and PLDMV, and conferring resistance against both PRSV and PLDMV have been developed in our laboratory. These transgenic papaya lines with double resistance were considered having great potential for the control of PRSV and PLDMV in Taiwan and elsewhere.

However, an unexpected breakdown of the transgenic resistance of PRSV CP transgenic lines and PRSV-PLDMV CP transgenic lines by a super strain, PRSV 5-19, was encountered. The breakdown of the transgenic resistance by PRSV 5-19 is due to the stronger gene-silencing suppression ability of the HC-Pro of the virus strain, which can shut off PTGS and abort the transgenic resistance completely, and not the result of the divergence between viral CP gene and the transgene. To disarm the stronger gene silencing suppression ability of the invading super strain, new transgenic papaya lines carrying the untranslatable sequences targeting HC-Pro gene have been developed by

Agrobacterium-mediated transformation of papaya somatic embryos from the selected hermaphroditic individuals of elite hybrid cultivars and their parental lines. Several transgenic lines show high degrees of resistance to the super strain PRSV 5-19 and other severe strains originating from different geographic origins. We believe that through the pyramiding of single, double and super transgenic resistance in a commercial hybrid should provide a more sustainable wide-spectrum resistance for application of transgenic papaya in different geographic regions.

The recent advances in transformation of somatic embryos derived from the adventitious roots of elite papaya cultivars provide a prompt way to generate transgenic papaya lines with desired horticultural properties and hermaphroditic sex, for direct application or shorten the time-consuming process of breeding. The molecular markers derived from the flanking sequences of the transgene integration significantly fasten the molecular breeding process for pyramiding of single, double and super transgenic resistance into a commercial hybrid papaya cultivar.

The advances in the molecular biology of papaya are still at the early stage. Although several genes of latex enzymes and fruit-ripening have been studied, molecular analyses related to horticulturally important traits such as ripening control, flavor, flesh color, hardiness and shape of fruits remain to be investigated thoroughly for future breeding. The sex-linked DNA markers and genetic determinants of sex provide a good basis for molecular breeding and for the selection of the desirable hermaphroditic individuals for papaya plantation. With the elucidation of the complete genome information of papaya, more advances in functional genomics of papaya will surely benefit this unique tropical fruit as one of the shining stars in the world market.

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