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# Assessment of Emerging Virus Threats for Application of Transgenic Papaya Resistant to Papaya Ringspot Virus

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## ABSTRACT

The transgenic resistance conferred by the coat protein (CP) gene of a virus has become the most effective method to prevent papaya from infection by the noxious *Papaya ringspot virus* (PRSV). In May 1998, PRSV-CP gene transgenic papaya Rainbow and SunUp were deregulated and granted approval for commercialization, representing the first successful application of a transgenic fruit tree in the world. Although the transgenic varieties are not resistant to most other PRSV strains from different geographic areas, breakdown of the resistance in Hawaii has not been recorded. Apart from Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation. The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity. However, during the 4-year-long field trials, the highly resistant PRSV CP-transgenic papaya lines were found to be susceptible to an unrelated potyvirus *Papaya leaf-distortion mosaic virus* (PLDMV) which has been identified in many places in Taiwan. To

overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene, including parts of the CP genes of PRSV and PLDMV, and conferring resistance against both PRSV and PLDMV, were developed. These transgenic papaya lines with double resistance are considered to have great potential for the control of PRSV and PLDMV in Taiwan. During the field test period, a super strain, PRSV 519, able to break down the resistance of YK-CP transgenic lines, was also noticed. The breakdown of the transgenic resistance by 519 is controlled by a strong gene-silencing suppressor, HC-Pro gene, of the virus in a transgene-homology-independent manner. We suggest that a chimeric construct targeting multiple viral genes, such as the genes of the CP, the replicase and the gene silencing suppressor HC-Pro of a potyvirus, may minimize the chances of the emergence of a super virus strain that can overcome the transgenic resistance, and provides more durable resistance against different viruses.

## INTRODUCTION

There are many common names for papaya (*Carica papaya* L.), including papaw or paw paw (Australia), mamao (Brazil) and tree melon (China). The species is believed to be native to southern Mexico and neighboring Central America and to have been taken to Caribbean countries and South-east Asia during the Spanish exploration in the sixteenth century (Storey 1969). It then spread rapidly to India and Africa. Today, it is distributed widely throughout tropical and subtropical regions of the world. A papaya plant has a single, erect, tree-like herbaceous stem, with a crown of large, palmately and deeply lobed leaves. The main stem is cylindrical and hollow, with prominent leaf scars and spongy-fibrous tissue. Leaves are arranged spirally, with petioles extending horizontally up to 1 m long. Trees contain white latex in all their parts. Flowers are male, female, or hermaphrodite, are found on separate trees and

are borne in the axils of the leaves. The modified cymose inflorescence allows the flowers to be pollinated easily by wind and insects. The type of flowers produced may change on the same tree, depending on age and environmental factors, such as drought and temperature fluctuations. Hermaphroditic trees consistently produce male flowers, but only with few female flowers that produce fruits during warmer or cooler seasons, whereas female trees are more stable and always produce pistillate flowers under these conditions.

Papaya fruits are fleshy berries and superficially resemble melons. Fruits from female trees are spherical, whereas those from hermaphroditic trees are pyriform, oval or cylindrical with a grooved surface. Since the female fruits contain thinner flesh and more seeds in the central cavity, the hermaphrodite fruits are in more demand by consumers. The fruit is a good source of vitamin A and C (Manshardt 1992). Ripe fruits are largely used as a fresh dessert, while green fruits are often used in salads and pickled or cooked as a vegetable. Papain, a proteolytic enzyme present in the latex, collected mainly from green fruits, has various uses in the beverage, food and pharmaceutical industries, e.g. chill-proofing beer, tenderizing meat and in drug preparations for digestive ailments (Chan and Tang 1978). It is also used in bathing hides, softening wool and as soap for washing cloth.

Papaya grows relatively easily and quickly from seeds and can reach up to 10 or 12 ft in height. Fruits are ready to be harvested 9--12 months after planting and a tree can continue producing fruits for about 2--3 years, when the height of the plant is too tall for efficient harvesting. Since plant sex cannot be distinguished before flowering, three to five seedlings are normally planted together and only the most vigorous hermaphrodite ones at flowering are selected and cultivated. In 2005, the FAO

estimated that about 3.9 hundred thousand hectares and about 6.8 million metric tons of fruit were harvested (Table 1). Brazil, Mexico, Nigeria, India and Indonesia yield more than 70% of the total world production. The extensive adaptation of this plant and wide acceptance of the fruit offer considerable promise for papaya as a commercial crop for local and export purposes. Like banana, pineapple and mango, papaya is one of the important cash crops in the tropics and subtropics. However, the production of this economically important fruit crop is limited by the destructive disease caused by *Papaya ringspot virus* (PRSV), and the fragile and perishable nature of the fruit limit large-scale exportation, with the result that papaya lags behind banana and pineapple in world markets.

**Table 1.** World papaya production in 2005. Data from the Food and Agriculture Organization (FAO), Statistical Division, 2005. (http://faostat.fao.org/faostat/)

| Country | Hectares (×1,000) | Metric tons (×1,000) |
|---|---|---|
| Brazil | 36.5 | 1650 |
| Mexico | 26.3 | 956 |
| Nigeria | 91.0 | 755 |
| India | 80.0 | 700 |
| Indonesia | 24.0 | 647 |
| Ethiopia | 11.0 | 230 |
| Congo | 12.5 | 210 |
| Peru | 12.5 | 180 |
| China | 5.8 | 160 |
| Venezuela | 7.5 | 140 |
| Colombia | 7.0 | 137 |
| Philippines | 5.5 | 132 |
| Thailand | 9.0 | 131 |
| Cuba | 11.0 | 120 |
| Kenya | 8.0 | 86 |
| Others | 41.6 | 514 |
| Total | 389.2 | 6753 |

**Worldwide Threat by PRSV Infection**

Production of papaya has been limited in many areas of the world due to the disease caused by *Papaya ringspot virus* (PRSV) (Purcifull et al. 1984). Papaya ringspot disease 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 then became prevalent 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). To date, most of the major papaya plantation areas of the world suffer from devastation by this virus.

PRSV is a member of the genus *Potyvirus* (Purcifull et al. 1984; Murphy et al. 1995), is transmitted non-persistently by aphids and is sap-transmissible in nature. The PRSV genome contains a single-stranded positive sense RNA of about 40 S (De La Rosa and Lastra 1983; Yeh and Gonsalves 1985). Strains of PRSV from Hawaii (Yeh et al. 1992) and Taiwan (Wang and Yeh 1997) have been sequenced; both contain 10,326 nucleotides. The viral RNA encodes a polyprotein that is proteolytically cleaved to generate eight to nine final proteins, including the coat protein for encasidation of the viral genome (Yeh et al. 1992). The virus has a single type of coat protein (CP) of 36 kDa (Purcifull and Hiebert 1979; Gonsalves and Ishii 1980). It induces cylindrical inclusion (CI) (Purcifull and Edwardson 1967) and amorphous inclusion (AI) (Martelli and Russo 1976) in the cytoplasm of host cells. The former consists of a protein of 70 kDa (cylindrical inclusion protein, CIP; Yeh and Gonsalves 1984) and the latter of a protein of 51 kDa (amorphous inclusion protein, AIP; De Mejia et al. 1985a, b). In papaya, PRSV causes severe mosaic and distortion of leaves, ringspots on fruits and water-soaking oily streaks on the upper stems and

petioles. It stunts the plant and drastically reduces the fruit size and quality.

**No Effective Control Measures**

Although tolerant varieties of papaya have been described (Cook and Zettler 1970; Conover 1976; Conover et al. 1986), resistance to PRSV does not exist in *C. papaya*, making conventional breeding difficult (Cook and Zettler 1970; Wang et al. 1978). Tolerance to PRSV has been found in some papaya lines and has been introduced into commercial varieties, but their horticultural properties, such as sweetness, hardness, shape, and shelf-life, are still not commercially desirable (Mekako and Nakasone 1975; Conover and Litz 1978). Other control methods for PRSV, including agricultural practices such as rouging, quarantine, intercropping with corn as a barrier crop and protecting transplanted seedlings with plastic bags, provide only temporary or partial solutions to the problem (Wang et al. 1987; Yeh and Gonsalves 1994).

PRSV HA 5-1, a cross-protecting mild mutant strain of PRSV that was selected following nitrous-acid treatment of a severe strain (HA) from Hawaii (Yeh and Gonsalves 1984), was tested extensively in the field and has been used commercially in Taiwan and Hawaii since 1985 to permit an economic return from papaya production (Wang et al. 1987; Yeh et al. 1988; Yeh and Gonsalves 1994). However, using the approach involving the deliberate infection of a crop with a mild virus strain to prevent economic damage by more virulent strains has several drawbacks, including the requirement for a large-scale inoculation program, reduction in crop yield and losses of cross-protected plants due to super-infection by virulent strains (Stubbs 1964; Gonsalves and Garnsey 1989; Yeh and Gonsalves 1994).

**A Transgenic Approach for Control of PRSV**

The concept of ’pathogen-derived resistance’ (Sanford and Johnston 1985) proposes that transforming plants with a pathogen’s gene would generate resistance to the infection of the corresponding pathogen. By this concept, Powell-Abel et al. (1986) first demonstrated that transgenic tobacco plants expressing the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) conferred resistance to TMV infection. The CP gene-mediated transgenic resistance has been proven effective for protecting tobacco, tomato, potato and other crops from infection by many different viruses (Beachy 1990; Lomonossoff 1995; Goldbach et al. 2003). Thus, the transgenic approach has become the most effective method to protect crops from virus infection.

In order to solve the problems caused by PRSV, Gonsalves’ group at Cornell University and Hawaii started a research project in the late 1980s to develop transgenic papaya. Ling et al. (1991) first demonstrated that the expression of the PRSV HA 5-1 CP gene in tobacco afforded a broad-spectrum of protection against different potyviruses. However, effective gene transfer systems require reliable and efficient procedures for plant regeneration from cells. Fitch and Manshardt (1990) reported that somatic embryogenesis from immature zygotic embryos of papaya could be integrated into a useful gene transfer technology. In the same year, Fitch et al. (1990) incorporated the CP gene of HA 5-1 into papaya via microprojectile bombardment and obtained plants resistant to infection by the severe Hawaii HA strain. Among their transgenic papaya lines, line 55-1 was virtually immune to infection by HA.

**Successful Application of Transgenic Papaya in Hawaii**

The plants of transgenic papaya line 55-1 are highly resistant to Hawaiian PRSV isolates under glasshouse and field conditions (Fitch et al. 1992; Lius et al. 1997). The resistance is triggered by post-transcriptional gene silencing (PTGS), an RNA-mediated specific degradation process of the innate nature of plants against pathogens (Baulcombe 1996, 1999; Hamilton and Baulcombe 1999; Gonsalves 2002). However, the resistance is affected by the sequence identity between the CP transgene and the CP coding region of the challenge virus (Tennant et al. 1994). For example, Rainbow (a CP-hemizygous line derived from SunUp crossed with non-transgenic Kapoho) is susceptible to PRSV isolates outside Hawaii, and SunUp (a CP-homozygous line of 55-1) is resistant to a wider range of isolates from Jamaica and Brazil, but is susceptible to isolates from Thailand and Taiwan (Gonsalves 1998, 2002; Tennant et al. 2001). This characteristic of sequence homology-dependent resistance limits the application of CP-transgenic papaya for controlling PRSV in geographic regions other than Hawaii (Gonsalves 2002).

The field trial of the homozygous line SunUp and hemizygous line Rainbow indicates that both of them offer a good solution to the PRSV problem in Hawaii (Ferreira et al. 2002). By May 1998, Rainbow and SunUp were deregulated by the US Animal and Plant Health Inspection Service and Environmental Protection Agency, and granted approval 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.

**New Threat by an Unrelated Virus for Transgenic Papaya in Taiwan**

A CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation (Cheng et al. 1996). The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity (Bau et al. 2003). Several lines highly resistant to the homologous strain (PRSV YK) provide wide-spectrum resistance to three different geographic strains from Hawaii, Thailand and Mexico (Bau et al. 2003). During four repeats of field trials from 1996 to 1999, transgenic papaya exhibited high degrees of protection against PRSV in Taiwan (Bau et al. 2004). Unfortunately, 18 months after plantation in the fourth field trial, unexpected symptoms of severe distortion on fully expanded leaves, stunting of the apex, water-soaking on petioles, and stem and yellow ringspots on fruit were noticed on PRSV CP-transgenic papaya plants. The causal agent was distinguished from PRSV by host reactions and serological properties (Bau 2000) and later identified as *Papaya leaf-distortion mosaic virus* (PLDMV), a potyvirus originating from Okinawa, Japan, in 1954 (Maoka et al. 1996). All of the PRSV CP-transgenic papaya lines were susceptible to PLDMV infection when evaluated under glasshouse conditions. Therefore, in Taiwan, once PRSV CP-transgenic papaya is widely applied for the control of PRSV, PLDMV will become a serious threat to papaya production.

***A need of* Multiple and Durable Resistance to Different Viruses**

In order to control two or more viruses, transgenic plants with multiple resistances have been generated by combining the entire CP gene of more than one virus, with each gene driven by a promoter and a terminator (Fuchs and Gonsalves 1995). Transgenic lines expressing these chimeric CP constructs were resistant to the

corresponding viruses and protected from mixed infection, such as *Cucumber mosaic virus*, *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* (Fuchs and Gonsalves 1995; Tricoli et al. 1995; Fuchs et al. 1998). Furthermore, the novel approach proposed by Jan et al. (2000) indicated that transgenic plants with resistance to a potyvirus and a tospovirus could be obtained through the PTGS mechanism by fusing a segment of tospoviral N gene to a segment of potyviral CP gene. The same strategy was used to develop double resistance to both PRSV and PLDMV. An untranslatable chimeric construct that contained the truncated PRSV CP and PLDMV CP genes was then transferred into papaya. Through the PTGS mechanism, transgenic papaya plants carrying this chimeric transgene exhibited resistance to both PRSV and PLDMV under glasshouse conditions (S.D. Yeh, unpublished results). These transgenic papaya plants with a double resistance are believed to have considerable potential for the control of PRSV and PLDMV in Taiwan.

**Resistance Breakdown by Super Strains of PRSV with a Strong Gene Silencing Suppressor**

In 4-year field trials, super PRSV strain 5-19 infected transgenic papaya plants were found (Tripathi et al. 2004). The nucleotide identity between the transcript of the CP transgene and PRSV 5-19 RNA is less divergent than that between the CP transgene and other PRSV geographic strains that are not able to overcome the transgenic resistance (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. In order to analyze the role of the gene-silencing suppressor HC-Pro of this super strain, the viral recombinant was constructed by replacing an HC-Pro region of PRSV YK with that of 5-19 and the resistance against the

recombinant was evaluated in transgenic papaya. Results showed that the heterologous HC-Pro region of 5-19 alone was sufficient to break down the transgenic resistance in a transgene sequence-homology independent manner, even though the sequences of the transgene transcript shared 100% identity with the genome of the infecting virus (S.D. Yeh, unpublished results). The breakdown of the transgenic resistance by a strong gene-silencing suppressor of a super strain has strong implications for the application of transgenic crops in virus control. We suggest that a chimeric construct targeting multiple viral genes, including the gene determining viral virulence and gene silencing suppression, such as the HC-Pro gene of a potyvirus, may minimize the chance of the emergence of a supervirus that may overcome transgenic resistance.

**Transgenic Papaya Generated in Other Geographic Areas**

Because of the apparent homology dependence of PRSV CP transgene-associated resistance, the utilization of a CP gene of a local prevalent strain is a prerequisite in order to obtain effective PRSV resistance in transgenic papaya lines for a particular geographic region, as long as genetic variation among virus strains in that region is not a limiting factor (Gonsalves 2002). Using the CP genes of local PRSV isolates to transform local papaya cultivars has been reported in different countries. Lines et al. (2002) used an untranslatable PRSV CP coding region as a transgene to develop two Australian transgenic papaya cultivars which showed immunity to the local PRSV isolate in glasshouse and field tests. Fermin et al. (2004) constructed PRSV-resistant plants by transforming independently with the CP genes of PRSV isolates from two different areas of Venezuela. All the transgenic lines, including $R_0$ and inter-crossed or self-crossed progenies, revealed different levels of resistance to homologous and heterologous isolates from Hawaii and Thailand. In Florida, transgenic papaya lines

carrying the CP gene of the local strain were generated, and the transgenic resistance was introduced to elite papaya cultivars by conventional breeding (Davis and Ying 2004). In addition to the CP gene, the truncated replicase (RP) gene of PRSV was used as a transgene to generate transgenic papaya through *Agrobacterium*-mediated transformation (Chen et al. 2001). PRSV inoculation tests showed that the RP gene conferred resistance to PRSV in transgenic papaya.

## CONCLUSIONS

The transgenic resistance conferred by the CP gene of a virus has become the most effective method to prevent papaya from infection by the noxious PRSV. In the late 1980s, Gonsalves' group at the Cornell University and University of Hawaii started a program to develop transgenic papaya resistant to PRSV, using the PRSV CP gene, through particle bombardment. In May 1998, PRSV-CP gene transgenic papaya Rainbow and SunUp were deregulated and granted approval for commercialization, representing the first successful application of a transgenic fruit tree in the world. Although the transgenic varieties are not resistant to most other PRSV strains from different geographic areas, breakdown of the resistance in Hawaii has not been recorded. Apart from Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation. The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity. However, during the 4-year-long field trials, the highly resistant PRSV CP-transgenic papaya lines were found to be susceptible to an unrelated potyvirus *Papaya leaf-distortion mosaic virus* (PLDMV) which has been identified in many places in Taiwan. To overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene, including parts of the CP genes of PRSV and PLDMV, and conferring resistance

against both PRSV and PLDMV, were developed. These transgenic papaya lines with double resistance are considered to have great potential for the control of PRSV and PLDMV in Taiwan. During the field test period, a super strain, PRSV 519, able to break down the resistance of YK-CP transgenic lines, was also noticed. The breakdown of the transgenic resistance by 519 is controlled by a strong gene-silencing suppressor, HC-Pro gene, of the virus in a transgene-homology-independent manner. We suggest that a chimeric construct targeting multiple viral genes, such as the genes of the CP, the replicase and the gene silencing suppressor HC-Pro of a potyvirus, may minimize the chances of the emergence of a super virus strain that can overcome the transgenic resistance, and provides more durable resistance against different viruses. Transgenic papaya resistant to pathogens other than PRSV have also been developed. It is expected that resistance to insects, tolerance to herbicides, and other important fruit traits will be the next targets of research.

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