CONTROL OF PAPAYA RINGSPOT
VIRUS IN PAPAYA: A Case Study

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ABSTRACT
The papaya crop is severely affected by papaya ringspot virus (PSRV) worldwide. This review focuses on efforts to control the destructiveness of the disease caused by PSRV in Hawaii, starting from the use of cross protection to parasite-derived resistance with transgenic papaya expressing the PSRV coat protein gene. A chronology of the research effort is given and related to the development of technologies and the pressing need to control PSRV in Hawaii. The development of commercial virus-resistant transgenic papaya provides a tangible approach to control PSRV in Hawaii. Moreover, the development of transgenic papaya by other laboratories and employment of a mechanism of effective technology transfer to different countries hold promise for control of PSRV worldwide.

INTRODUCTION
This review covers our efforts to develop control of papaya ringspot virus (PRSV) in papaya, with primary focus on Hawaii. The review traces the evolution of research to control PRSV, the use of transgenic papaya, and its recent commercialization. It is not meant to be an in-depth review of either PRSV or alternate measures in use worldwide to control PRSV in papaya. The information, which is presented chronologically, points out the people involved, interactions of technologies, timing of actions as related to disease occurrence, potentials, and hurdles that must be overcome to use transgenic plants, all in the context of controlling a widespread and severe disease.
THE PAPAYA

Papaya (Carica papaya L.) is an important fruit crop grown widely in tropical and subtropical lowland regions (31). It is largely consumed as a fresh dessert fruit, and the green fruit is often used as salad. Papain is also recovered from the latex of green fruit. The tree is widely planted in home gardens because it is relatively easy to grow from seed; the first mature fruits can be harvested nine months after sowing seeds, and fruit is produced continuously year-round. Although delicious and rich in vitamins A and C (31), the fruit is fragile, a characteristic limiting large-scale exportation to countries in temperate regions. Improvements in postharvest and shipping technologies should enhance commercialization of this crop. In total production, papaya ranks above strawberries and below grapefruit (31). The FAO estimated that about 5.7 million metric tons of fruit were harvested in 1995, almost double the 1980 harvest (18). Brazil, India, and Mexico are the largest producers of papaya. Hawaii is the largest producer of papaya in the United States; about 66% of the total fresh production is exported, primarily to the US mainland and to Japan (33).

Papaya is a herbaceous plant with a single stem bearing a crown of large palmately shaped leaves. Flowers are produced at the axils of the leaf petiole. The plant is polygamous, with male, female, and hermaphrodite plants (31, 36). In the wild, dioecious plants predominate, whereas cultivated plants are both dioecious and hermaphrodites. In breeding cultivars, the latter can be inbred, which results in stable characteristics from generation to generation (31). The Hawaiian Solo types are gynodioecious cultivars (i.e. produce female and hermaphrodite plants) that are nearly isogenic as a result of years of selection and inbreeding. The dominant Solo cultivar grown in Hawaii is the yellow-fleshed Kapoho, followed by the red-fleshed Sunrise, both of which produce small fruit of ca 454–1135 g. Sunrise is also widely grown in Brazil and Jamaica. Numerous other cultivars and selections have also been developed for particular countries. In general, fruit production of hermaphrodite plants is adversely affected by such climatic factors as drought and wide fluctuations of temperature, whereas female plants show more tolerance to these conditions (31).

Papaya trees are fast growing (they can be 12 feet tall in a year), and they produce mature fruit within 9–12 months after seeds are planted. Commercially, when trees are grown at a density of 1500 to 2500 per hectare, annual production can range from 125,000 to 300,000 lbs per hectare. Fruit are harvested for 1 to 2 years, after which the trees are usually too tall for efficient harvesting.

PAPAYA RINGSPOT VIRUS

Papaya ringspot virus (PRSV) is by far the most widespread and damaging virus that infects papaya. The name of the disease, papaya ringspot, is taken from the
ringed spots on fruit of infected trees (25). Trees infected with PRSV develop a range of symptoms: mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, and distortion of young leaves that resembles mite damage (20, 40). Infected plants lose vigor and become stunted. When infected at the seedling stage or within two months after planting, trees do not normally produce mature fruit. Production of fruit by trees infected at progressively later stages is severely reduced and of poor quality, owing to the presence of ringspots and generally lower sugar concentrations.

PRSV is transmitted by numerous species of aphids in a nonpersistent manner to a limited host range of cucurbits and papaya. PSRV also produces local lesions on Chenopodium quinoa and C. amaranticolor. A number of investigations have failed to demonstrate that PRSV is transmitted through seeds of papaya or cucurbits (40), except for one study (5), which showed that 2 of 1355 papaya seedlings from PRSV-infected fruit showed PRSV symptoms. However, seed transmission is not a significant way for spreading PRSV (personal observations).

PRSV is grouped into two types (40): Type P (PRSV-p) infects cucurbits and papaya, whereas type W (PRSV-w) infects cucurbits but not papaya. The latter type was previously referred to as WMV-1 (40). Although both types are serologically closely related, observations suggest that papaya is the major primary and secondary source for the spread of PRSV-p in large plantations and small orchards alike. For example, serological and biological tests on 454 cucurbit plants growing in villages of Northeast Thailand that harbored PRSV-infected papaya showed that while 31% of the cucurbits were infected with PRSV, all were of the W type (V Prasartsee, unpublished results). Similar observations were noted in Hawaii (S Ferreira, personal communication).

Much progress has been made in the molecular characterization of PRSV. Strains of PRSV-p from Hawaii and Taiwan have been completely sequenced (55, 65). The genomic RNA consists of 10,326 nucleotides and has the typical array of genes found in potyviruses (44). The genome is monocistronic and is expressed via a large polypeptide that is subsequently cleaved to functional proteins (64). There are two possible cleavage sites, 20 amino acids apart, for the N terminus of the coat protein (56, 65). These two sites may be functional; the upstream site for producing a functional Nib protein, and the other, to produce an aphid-transmissible coat protein. Nucleotide sequences have been reported for the coat protein genes of 16 PRSV-p and PRSV-w types from different geographic regions (2, 3, 41, 55–57, 65). It is impossible to segregate PRSV-p and PRSV-w types by their coat protein sequences. Within the p-types, however, the coat protein sequences can diverge by as much as 12%. The implication of these sequence differences are discussed in a later section.
The 1991 discovery of PRSV infection of papaya in Southeast Queensland (52), Australia, has provided insights on the possible origins of PRSV-p. Bateson et al (3) proposed that PRSV-p from Australia could have originated through a mutation of the PRSV-w strain, which has been in Southeast Queensland since 1978 (22). Sequence analysis of the coat protein gene of these PRSV-p and PRSV-w isolates showed at least 98% nucleotide sequence identity. Moreover, the coat protein sequences of the PRSV-p and PRSV-w isolates from geographic regions close to Australia (such as Thailand, Vietnam, and Sri Lanka) diverged from PRSV-p from Australia by as much as 12%. These observations also suggest that the Australian PRSV-p type originated from the PRSV-w type present in Australia.

EFFECT OF PRSV ON PAPAYA PRODUCTION

PRSV-p occurs worldwide wherever papaya is grown (40). The effect of PRSV-p in Brazil, Taiwan, and Hawaii are discussed below to illustrate the significance of the virus on the industry and the approaches taken to maintain papaya production.

Brazil is by far the largest producer of papaya (18), accounting for 45% of the 5.9 million metric tons produced worldwide in 1996. The commercial industry started in the 1960s in the southeastern states of Sao Paulo and Rio de Janeiro, where most of the country’s population resides. PRSV was detected in these two states in 1969 (7). The destructive effects of PRSV forced the Brazilian papaya industry to move from Sao Paulo and Rio de Janeiro to the northern, more remote states, Espiritu Santo and Bahia. From 1973 to 1978, Sao Paulo and Rio de Janeiro accounted for 90% of the total papaya acreage, but for only 27% by 1984. By 1993, Sao Paulo and Rio de Janeiro grew only 309 hectares of papaya compared to 4792 hectares in Espiritu Santo and 16,073 hectares in Bahia. This migration of the industry is directly attributable to the effects of PRSV-p. Unfortunately, PRSV has followed the industry, and it is more difficult to establish new farms to temporarily escape the virus; rouging to manage the virus is expensive and not always effective. Moreover, papaya is now grown in regions distant from the major markets of Sao Paulo and Rio de Janeiro. Thus shipping costs to the major markets are much higher, and quality of the fruit is lowered. PRSV-p has been declared the most important problem in papaya cultivation in Brazil.

Unlike Brazil, Taiwan is a small island unable to escape PRSV by shifting its industry away from infected areas. In Taiwan, the industry consists of many farms with fewer than 20 hectares of papaya. PRSV was discovered in 1974 (58) in southern Taiwan and spread throughout the entire island within several years. The destructiveness of PRSV forced farmers to grow papaya as an annual
crop. Seedlings are usually planted in October and November and harvest commences in July or August until December, after which time the trees are removed.

A number of farms in Taiwan have resorted to growing papaya under protective netting to eliminate aphids until the trees have produced a good canopy of fruit. The nets are then removed to allow more sunlight to the trees and thus increase the sugar concentrations of fruit. Trees subsequently become infected, but fruit production is assured for several months. Raising papaya under large net houses is extremely costly but is economically viable because the returns on papaya are very high. As in Brazil, PRSV in Taiwan is the major factor affecting papaya production, aside from other natural disasters such as typhoons.

PRSV IN HAWAII

The effect of PRSV in Hawaii is similar to that in Brazil and Taiwan. The papaya industry started in the 1940s on the island of Oahu, on about 500 acres (11). The virus was discovered in 1945, and the term papaya ringspot virus coined by Jensen in 1949 (25). By the 1950s, production on Oahu was affected and the industry subsequently moved to Hawaii island into the area of Puna, which had no commercial production. Acreage increased to 650 by 1960 and to 2250 in 1990. In contrast, the acreage on Oahu fell to less than 50 by 1990 (11).

Remarkably, Puna remained free of PRSV for over 30 years. One incidence of PRSV was reported in Puna in 1965, but it was eradicated by rapid rouging of trees (MOJ Isherwood, personal communication). Despite the presence of PRSV in Hilo and Keaau, communities only 19 miles away, Puna remained free of PRSV, thanks to an effective physical barrier of lava rocks and to the absence of intervening plantings, which minimized host plants for PRSV. Diligence by the Hawaii Department of Agriculture in surveying and rouging infected trees in the Hilo and Keaau areas kept PRSV from spreading.

ATTEMPTS TO CONTROL PRSV BY CROSS PROTECTION

Although Puna was still free of PRSV-p in the late 1970s, I and personnel at the University of Hawaii were convinced that PRSV-p would inevitably invade Puna. Thus, in 1979, we began to investigate the possibility of using cross protection as a control measure. Cross protection is the phenomenon whereby plants that are systemically infected with a mild strain of a virus are protected against the effects of infection by a more virulent related strain (60, 63). This practice (17) has long been known and has been used to control citrus tristeza (8), tobacco mosaic (42), and zucchini yellow mosaic viruses (54). The key
component in cross-protection programs is the availability of a mild strain that effectively protects against the target virus.

Shyi-Dong Yeh, a graduate student at Cornell University, was sent by the Taiwanese government to investigate PRSV-p because of its devastating effects in Taiwan. His task was to obtain a mild strain of PRSV-p. The attempt to select a mild strain by isolating virus from trees with mild symptoms in heavily infected orchards of Hawaii failed, as did the selection of mild variants through analyses of numerous isolates from local lesions. However, two mild strains (63), designated PRSV HA 5-1 and PRSV HA 6-1, were selected following nitrous acid treatment of leaf extracts of squash infected with PRSV-HA, a severe strain from Hawaii that had been recently characterized (21). Greenhouse experiments showed that both strains were mild on papaya and afforded protection against PRSV HA.

The mild strains were first tried in large-scale field plots in Taiwan (59–61). In preliminary results, plants preinoculated with the mild strains did not show complete protection against the severe strain in the field, but did show a delay in the severe effects of the challenge virus. Although this degree of protection was insufficient for plants under severe disease pressure, economical returns could be obtained by isolating orchards as much as possible and by roguing out severely affected trees until the flowering period. About 100–200 hectares of papaya were protected by PRSV HA 5-1 yearly from 1985 to 1991 (60). Currently, the mild strain is sparsely used, mainly because it does not provide consistent economic returns to the farmers. The failure of PRSV HA 5-1 to completely protect against PRSV in Taiwan is due to differences between the mild strain and the wild-type virus, as shown by greenhouse experiments (51). Similarly, these mild strains (PRSV HA 5-1 and 6-1) did not give good field protection against PRSV strains in Northeast Thailand (V Prasartsee & D Gonsalves, unpublished results).

The mild strains performed much better in Hawaii, presumably because the strains had been derived from a Hawaiian PRSV isolate. A series of field experiments was conducted with the Hawaiian Solo cultivars line 8, Kamiya, and Sunrise on the island of Oahu (11, 34, 39). These studies showed that mild strain PRSV HA 5-1 gave good protection against the local strains, although PRSV HA 5-1 produced noticeable symptoms on leaves and fruit, with the degree of symptom severity markedly dependent on the cultivar. Cultivars line 8 and Kamiya were the least affected and could be grown economically, but Sunrise was too severely affected in fruit appearance. Protection of line 8 has been the most successful (34). However, cross protection has not been widely adopted on Oahu, for several reasons: (a) the adverse affects of the mild strain on Sunrise and to a lesser extent on Kamiya, (b) cross protection requires extra cultural management and care, and (c) the reluctance of farmers to infect their trees with a virus.
DEVELOPMENT OF TRANSGENIC PAPAYA TO CONTROL PRSV IN HAWAII

Parasite-Derived Resistance: A New Approach for Controlling PRSV

The concept of parasite-derived resistance (PDR), conceived in the middle 1980s (43), offered a new approach for controlling PRSV. Parasite-derived resistance is a phenomenon whereby transgenic plants containing genes or sequences of a parasite (in this case, the coat protein gene of a virus) are protected against detrimental effects of the same or related pathogens. The application of PDR for plant viruses was first demonstrated by Beachy’s group (1): Transgenic tobacco expressing the coat protein gene of tobacco mosaic virus was protected against infection by tobacco mosaic virus. Subsequent reports have shown that this approach is effective in controlling many plant viruses (30).

Development of Transgenic Papaya

Our laboratory began utilizing the PDR concept in 1986 by cloning the coat protein gene of PRSV HA 5-1 in collaboration with Jerry Slightom, of The Upjohn Company, who sequenced the gene (41) in an effort to develop virus-resistant transgenic vegetables for the Upjohn’s subsidiary, Asgrow Seed Company. Our objectives were to control the important viruses that infect vegetables, including cucumber mosaic, watermelon mosaic virus 2, zucchini yellow mosaic, and papaya ringspot viruses. Asgrow Seed Company has subsequently developed commercial squash with resistance to zucchini yellow mosaic virus and watermelon mosaic virus 2 (16, 53).

The expertise and reagents obtained from the initial vegetable work were then applied to papaya. As noted above, our specific goal was to control PRSV in Hawaii in anticipation of its invading the Puna district. The cross-protection work had been consistently supported through funds from the USDA Section 406 grant program, which focused on agricultural problems of the Pacific region. Funding from this program had led to cooperative work with scientists from the University of Hawaii: Mamoru Ishii, Ryoji Namba, Mau, and Ferreira. Fortunately, this program also funded the research to use PDR to control PRSV in papaya in Hawaii. Thus, the collaboration of Richard Manshardt and Maureen Fitch from the University of Hawaii, Slightom from The Upjohn Company, and myself from Cornell University was begun. We were also very fortunate to tap the expertise and services of John Sanford at Cornell University, who had recently co-invented the gene gun.

Our target gene of PRSV was the coat protein gene of PRSV HA 5-1, the mild mutant of PRSV HA that had been recently cloned and sequenced for the
vegetable work. The coat protein gene of PRSV HA 5-1 had a 97.7% identity to PRSV-w from Florida (41). Because of various technical difficulties and the requirement that the gene be expressed as a protein, the gene was engineered as a chimeric protein containing 17 amino acids of cucumber mosaic virus at the N terminus of the full-length coat protein gene of PRSV HA 5-1 (28). Whether this would enhance or decrease the chances of obtaining resistant plants was unclear. However, in tobacco this gene construct had expressed high levels of the coat protein as measured by ELISA (28). The transgenic tobacco developed only mild symptoms following infection with tobacco etch virus, a potyvirus unrelated to PRSV (44). The transgenic tobacco could not be tested against PRSV because this virus does not infect tobacco. The cpexp vector was subsequently developed; this vector utilized sequences within the Nco 1 restriction site as the start codon for convenient engineering of coat protein genes into plant expression vectors (45). This vector is widely used today.

The task of transforming papaya was taken up in 1987 by Fitch, a graduate student under the supervision of Manshardt. The target cultivars were the red-fleshed Sunrise, the yellow-fleshed Sunset (a sib selection of Sunrise), and the yellow-fleshed Kapoho, the dominant cultivar grown in Puna. Protocols for transforming and subsequently recovering transgenic papaya plants had not been developed. One report indicated that papaya cells could be transformed, but not regenerated (37). Numerous efforts by Fitch to develop a papaya regeneration system via organogenesis failed. However, a technique to develop transgenic walnuts by transforming embryogenic cultures had recently been reported (35). The research moved rapidly once the decision was made to shift to transforming embryogenic tissue. A technique to produce highly embryogenic tissue starting from immature zygotic embryos was developed (13). In 1988–1989, embryogenic tissue were bombarded with tungsten particles coated with DNA of the PRSV HA 5-1 coat protein gene using the gene gun in Sanford’s laboratory. Transgenic plants were obtained and were growing in the greenhouse 15 months later (14, 15).

What were the important factors that helped to achieve this timely breakthrough in transforming papaya? One was the unending enthusiasm that a graduate student brings into the project. Another was the emphasis on obtaining a sufficient number of transgenic plants to test for resistance, rather than focusing on refining the details of the transformation technique. We wanted to quickly get to the stage of testing transgenic papaya to determine whether PDR would work for controlling PRSV in papaya.

Clones of nine R0 transgenic lines, six Sunset and three Kapoho, were sent to Cornell for inoculation tests with PRSV HA, which is the severe parent of the mild strain PRSV HA 5-1. Inoculations were not done with the homologous
mild PRSV HA 5-1 strain because it produced very mild or no symptoms on papaya, and we assumed that PRSV HA was nearly homologous to PRSV HA 5-1. R0-micropropagated plants of the first line, designated 55-1, that was tested showed excellent resistance to PRSV HA (15). Three other lines showed varying degrees of delay on the onset of symptoms, while the other lines developed symptoms at the same time as control plants. Line 55-1 was female and thus progenies could not be obtained directly from the R0 plants, as would be the case for a hermaphrodite. A two-pronged approach was instituted to move the research ahead aggressively and to determine whether line 55-1 would be resistant to PRSV under field conditions and have suitable horticultural characteristics. First, a decision was made to conduct a field trial using R0 plants instead of waiting a year to obtain R1 plants. Second, R1 plants were obtained by crossing line 55-1 with nontransgenic Sunset under greenhouse conditions both at Cornell University and the University of Hawaii and then the plants were screened in the greenhouse for resistance to PRSV isolates from around world.

Transgenic R1 Plants of Line 55-1 are Highly Resistant to Hawaii Strains But Largely Susceptible to Strains Outside Hawaii

Research headed by graduate student Paula Tennant at Cornell University was done to assess the resistance of R1 plants of line 55-1 against 3 PRSV isolates from Hawaii and 13 isolates from different parts of the world (51). Furthermore, since the coat protein gene in line 55-1 originated from the mild strain PRSV HA 5-1 used in earlier cross-protection studies, experiments were also conducted to determine the cross-protection effectiveness of PRSV HA 5-1 against some of the strains that were being used to challenge plants of line 55-1. Analysis clearly showed that 50% of the progenies were transgenic, with the rest nontransgenic; this confirmed that transgenic plants had one insert of the nptII gene and, presumably, the coat protein gene. These experiments illustrated several important points: (a) Resistance of transgenic plants was not correlated to level of protein expression, (b) R1 plants were highly resistant to Hawaiian isolates, and (c) line 55-1 showed variable levels of resistance (largely susceptible) to non-Hawaiian isolates, (for example, plants inoculated with an isolate from Thailand developed severe symptoms with no delay in symptom appearance, whereas isolates from Jamaica infected line 55-1, but symptoms were delayed and attenuated; isolates from Florida and Mexico infected only a percentage of the plants and symptoms were milder), and (d) the cross-protection results on nontransgenic plants roughly paralleled those observed for transgenic plants. That is, protection was complete against the Hawaiian isolates but not against non-Hawaiian isolates. Also, isolates that rapidly infected line 55-1 also rapidly infected cross-protected papaya. For example, papaya cross
protected with PRSV HA 5-1 showed almost no protection against the Thailand isolate, which also rapidly overcame the resistance of line 55-1. Overall, the results with transgenic papaya reinforced the information previously obtained with cross protection: R1 plants of line 55-1 would not be effective against all isolates of PRSV. However, the results clearly showed that line 55-1 had potential to control PRSV in Hawaii.

Field Trials with R0 Plants Show Line 55-1 Is Effective for Controlling PRSV in Hawaii

In 1991, Animal Plant Health Inspection Service (APHIS) issued a permit for a field trial at the University of Hawaii’s experimental farm at Waimanalo, on Oahu island, under Manshardt’s leadership. The importance of a field evaluation at an early stage cannot be overemphasized because it allowed us to appraise the resistance and horticultural characteristics of line 55-1, to bulk up seeds by crossing line 55-1 with nontransgenic plant cultivars, and to demonstrate the long-term resistance of a transgenic fruit crop to infection.

Plants were set in the field by the end of June 1992. The field trial was designed to determine the resistance of R0 plants to mechanical and aphid inoculations of PRSV. Nontransgenic plants in border rows of the plot were also inoculated with a PRSV isolate from Oahu island to create a high virus inoculum pressure for the field plot. Data were taken on total soluble solid levels of fruit, growth characteristics, and virus symptoms. The transgenic papaya showed excellent resistance throughout the two-year trial (29). Nearly all (95%) of the nontransgenic plants and those of a transgenic line that lacked the coat protein gene showed PRSV symptoms by 77 days after the start of the field trial, whereas none of the line 55-1 plants showed symptoms. Virus was not recovered from line 55-1 plants except for two plants, which showed virus symptoms on side shoots but none on the leaves of the main canopy. Plants grew normally and fruit appearance and total soluble solids of about 13% were within the expected range. Thus, by the mid-1993, the trial had provided convincing evidence that line 55-1 would be useful for controlling PRSV in Hawaii, or at least on Oahu island.

CRISIS IN HAWAII: PRSV INVADES PUNA IN 1992

PRSV Severely Affects Puna Area in Three Years

The inevitable entry of PRSV into the Puna district on Hawaii island was discovered during the first week of May in a papaya field in Pahoa, 1–3 miles from the major papaya growing areas in Puna (23). Apparently, infection had been established in this area for several months, as judged by symptoms on the fruits and the fact that many plants were infected in one location. Surveys
of the immediate area revealed PRSV in abandoned orchards, as well as in young orchards that were not yet producing fruit. PRSV was poised to invade the major papaya growing areas of Puna, which included Kapoho, Opihikau, Kahuawai, and Kalapana.

The Hawaii Department of Agriculture (HDOA) immediately launched an eradication program. The area was surveyed and infected trees were rouged out. A suggestion to destroy all papaya in that area, however, was not approved by the growers. Nevertheless, a HDOA program to mark trees to be rouged by growers was started in 1992 (23). By September 1992, 4915 trees had been rouged in Pahoa, and the number of trees being cut each week had decreased to below 85, providing hope that the virus had been contained (23).

However, the hope of containment was short-lived. The incidence of PRSV increased dramatically in Kapoho, which was closest to Pahoa, as the program of voluntary cutting of trees was not strictly followed and as farmers experiencing high infection rates abandoned their fields, thus creating huge reservoirs of inoculum for aphids to acquire and spread the virus (24). By late 1994, nearly all papaya of Kapoho was infected by the virus. In October 1994, the HDOA declared that PRSV was uncontrollable and stopped the practice of marking trees for rouging. In less than three years, a third of the Puna papaya area was infected. By 1997, Pohoiki and Kahuawai were completely infected. Kalapana was the last place to become heavily infected. In this area, the furthest from the original infection site in Pahoa, the spread of PRSV was slowed by constant rouging of infected plants by farmers, who are encouraged by a small bounty paid by a packing house. Nevertheless, virus infection has increased, and many heavily infected orchards have ceased to eliminate trees. Sixty trees were cut in May 1996, 2905 in July, 4312 in December, and 14,493 in June 1997. By September 1997, rouging was also discontinued in Kalapana (M Isherwood, personal communication). Five years after the onset of the virus in Pahoa, the entire Puna area was severely affected.

**LARGE-SCALE FIELD TRIAL IN DEVASTATED AREA**

*Establishment of Transgenic Field Trial in Kapoho*

By 1994, personnel at the University of Hawaii (e.g. Ferreira and Mau) had recognized that PRSV was out of control in Puna and would significantly reduce the state’s papaya production. To maintain production and eventually reclaim the Puna area for papaya growth, a bold plan was proposed (10): Move new papaya plantings to areas of Hawaii island where PRSV-p had not been detected, completely eradicate papaya and cucurbits from the papaya area in Puna, and place a one-year moratorium on planting papaya and cucurbits. Papaya acreage and thus production would be maintained, and PRSV-p would be eliminated.
from the Puna area. The program was anticipated to be completed in 6–8 years. Funding was provided by USDA to support the initial phases of the plan.

Several factors weighed against the program’s success. Data were needed on whether the Kapoho cultivar would grow well in the areas targeted for expansion, as several observations had suggested that this cultivar does not adapt well outside of Kapoho. More important, complete removal of every papaya tree and cucurbit from the Puna area and maintenance of the area free of infected plants for one year would be a monumental task, requiring widespread cooperation. Nevertheless, this plan of action was a clear declaration of the commitment to control PRSV, to save the papaya industry in Puna, and to maintain papaya production in the state.

Given such a bold initiative, with the Kapoho area virtually abandoned and PRSV out of control elsewhere in Puna, and the papaya industry severely threatened, the stage was set to establish a field trial in Kapoho to determine if the transgenic papaya could be used to rescue the papaya industry. Arguments could be marshaled for and against establishing such a field trial in the middle of a devastated commercial growing region of papaya: Line 55-1 had performed very well in field trials on Oahu island and the line was resistant to PRSV-Panaewa, a greenhouse isolate from Hawaii island; the industry needed drastic actions to survive; the plan for moving the industry might not succeed and PRSV might not be eradicated from Puna; the risk of PRSV-resistant papaya becoming a weed was not relevant because papaya is not a weed in areas that do not have PRSV-p; wild relatives of *C. papaya* are not grown in Hawaii; and the potential benefits of transgenic papaya far outweighed the risks. Mitigating against the field trial were the facts that pollen from the transgenic papaya might contaminate commercial plantings, resulting in the potential sale of commercial fruit with a nonderegulated transgene; preventing pilferage in a trial installed on a farmer’s field would be difficult, and thus there might be serious consequences if stolen fruit ended up in commercial markets.

By late 1994, we submitted an application for a field trial to APHIS. Our previous experience in applying for a field trial permit, combined with the helpful cooperation of APHIS, facilitated the review process. The field trial was allowed with the stipulation that (a) the field must be sufficiently isolated from commercial orchards to minimize the chance of transgenic pollen escaping to nontransgenic material outside of the field test, (b) all abandoned trees in the area must be monitored for the introgression of the transgene into fruits of these trees, and (c) all fruits had to be buried on site.

*SunUp and UH Rainbow Cultivars*

As noted above, early efforts to develop transgenic plants focused on transferring virus resistance into commercial papaya cultivars suitable for Hawaii.
Line 55-1 is a transgenic Sunset, which is a commercial red-fleshed cultivar. Unfortunately, we had not developed virus-resistant Kapoho and thus did not have a yellow-fleshed cultivar as a substitute for this cultivar. Also, there was the question of whether the transgenic plants could grow as well as Kapoho in the Puna area. Manshardt crossed the homozygous transgenic line 55-1 with Kapoho, anticipating the yield of a yellow-fleshed hybrid (because yellow is dominant over red) to substitute for Kapoho until such time that a transgenic Kapoho could be developed. He had made these crosses with plants in the 1992–1994 field trial but had not yet evaluated them for their horticultural characteristics. Thus the proposed field trial became a test not only of virus resistance but also of fruit quality. The homozygous line 55-1 was later named SunUp, the hybrid was made from the cross of the transgenic SunUp, and the nontransgenic Kapoho was named UH Rainbow (32).

Kapoho Field Trial

Approval was obtained in early 1995 and the field trial was set up in Kapoho in October 1995, under the leadership of Ferreira. The trial was set up on the property of a farmer who had ceased growing papaya because of PRSV. One part of the trial consisted of replicated blocks to compare virus-resistance performances of SunUp and UH Rainbow, of cross-protected Kapoho (cross protected with PRSV HA 5-1), and of PRSV-tolerant lines that were being developed by Ferreira and Francis Zee (of USDA Germplasm Repository, Hilo) and other tolerant lines developed in Thailand (V Prasartsee & D Gonsalves, unpublished results). Another part of the trial was established to simulate commercial conditions. A one-square acre solid block of UH Rainbow was planted adjacent to the replicated blocks. Several rows of nontransgenic Sunrise were planted on the perimeter of the replicated and solid blocks. An abandoned papaya field alongside the field plot was used as a primary source of the virus.

The results of the field trial clearly demonstrated the potential value of the transgenic papaya for reclaiming papaya land in Puna (12; S. Ferreira, unpublished results). Except for three plants that showed infection at the beginning of the trial, none of the transgenic plants has become infected as of January 1998, 27 months after starting the trial. In stark contrast, 50% of the nontransgenic control plants within the experiment and in the border rows were infected within four and a half months after transplanting; and all were infected by seven months. The growth differences between the transgenic and nontransgenic trees were remarkable; transgenic plants grew vigorously, with dark green leaves and full fruit columns, whereas nontransgenic plants were stunted, with yellow and mosaic leaves and very sparse fruit columns. In the solid block, UH Rainbow averaged about 100,000 lbs of marketable fruit per acre/year,
whereas nontransgenic plants averaged about 5000 lbs per acre/year. Although these data were from only one trial, observations suggested that UH Rainbow was a higher yielder than Kapoho. Also, the transgenic papaya performed much better than the PRSV-tolerant lines and the cross-protected plants.

Despite their excellent resistance, would farmers accept these cultivars as an acceptable substitute for Kapoho, a mainstay of the Hawaiian papaya industry for several decades? The performance of UH Rainbow was especially critical because it was targeted as the alternative to Kapoho. Taste, production, color, size, and packing and shipping qualities of Rainbow were analyzed. In addition to tests by research personnel, field days were held to allow farmers, packers, politicians, and University personnel to observe the field and fruit at the test site. The consensus was that UH Rainbow is a more than adequate substitute for Kapoho. The fruit is larger than Kapoho, but commercial packers do not see this as a major impediment.

SunUp is a transgenic Sunset, and its properties mirror those of the latter. Although Sunset and Sunrise are more adaptable to different environmental conditions than Kapoho is, these cultivars were not widely grown on Hawaii island. It will be interesting to see if SunUp increases the popularity of red-fleshed cultivars, since it can be grown in areas where PRSV is the limiting factor to economic production.

DEREGULATION AND COMMERCIALIZATION OF TRANSGENIC PAPAYA

To develop a transgenic papaya with virus resistance and excellent fruit qualities represents one hurdle, but to get it deregulated and commercialized is another. The areas of deregulation and commercialization have been in the purview of private companies who stand to benefit financially from developing a product and moving it to the market. Hitherto, University personnel were rarely directly involved, until papaya. First, the ownership of the papaya was placed into the hands of the Papaya Administrative Committee (PAC), which consisted of Hawaii papaya growers who had limited knowledge of these two processes. And second, the researchers involved felt that academic testing of the papaya was insufficient, and therefore the transgenic papaya was moved to the growers in a timely manner. Given the circumstances and interests, the PAC could be expected to follow up on the necessary steps to get the papaya to the commercial stage.

Deregulation

The task of securing deregulation of papaya by APHIS, Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) were taken
up by Richard Manshardt and our lab. APHIS was largely concerned with the potential risk of transgenic papaya on the environment. Two main risks were of heteroencapsidation of the incoming virus with coat protein produced by the transgenic papaya and of recombination of the transgene with incoming viruses. The former might allow non vectored viruses to become vector transmissible, whereas the latter might result in the creation of novel viruses. A third concern—that escape of the transgenic genes to wild relatives might make the relatives more weedy or even make papaya more weedy because of resistance to PRSV—was of no consequence since there are no papaya relatives in the wild in Hawaii, nor is papaya considered a weed there, even in areas were there is no PRSV. In November 1996, transgenic line 55-1 and another recently tested line 63-1 (50) and their derivatives were deregulated by APHIS (47). This action greatly increased the efficiency of the ongoing field trial because fruit no longer had to be buried at the test site, which allowed us to sample and send fruit to various laboratories and to the packing house without undue constraints.

According to the EPA, the coat protein transgenes are a pesticide because they confer resistance to plant viruses. A pesticide is subjected to tolerance levels in the plant. In the permit application, we petitioned for an exemption from tolerance levels of the coat protein produced by the transgenic plant. We contended that the pesticide (the coat protein gene) was already present in many fruits consumed by the public, since much of the papaya eaten in the tropics is from PRSV-infected plants. In fact, we had earlier used cross protection (the deliberate infection of papaya with a mild strain of PRSV) to control PRSV. Fruit from these trees was sold to consumers. Furthermore, there is no evidence to date that the coat protein of PRSV or other plant viruses is allergenic or detrimental to human health in any way. Finally, measured amounts of coat protein in transgenic plants were much lower than those of infected plants. An exemption from tolerance to lines 55-1 and 63-1 was granted in August 1997.

The FDA is concerned with food safety of transgenic products. This agency follows a consultative process whereby the investigators submit an application with data and statements corroborating that the product is not harmful to human health. Several aspects of the transgenic papaya were considered: the concentration range of some important vitamins, including vitamin C; the presence of GUS and nptII genes; and whether transgenic papaya had abnormally high concentrations of benzyl isothiocyanate. This latter compound has been reported in papaya (48). FDA approval was granted in September 1997.

**Commercialization**

In the United States, a transgenic product cannot legally be commercialized unless it is fully deregulated and until licenses are obtained for the use of the intellectual property rights for processes or components that are part of
the product or that have been used to develop the product. The processes in question were the gene gun and parasite-derived resistance, in particular, coat protein–mediated protection. The components were translational enhancement leader sequences and genes (nptII, GUS, and coat protein). This crucial hurdle involved legal and financial considerations beyond our means and expertise. These tasks were taken up by the industry’s PAC and its legal counsel, Michael Goldman.

Several factors favored the PAC efforts to obtain licenses: (a) Although the Hawaiian papaya industry is important to the state, its annual worth is relatively small (annual farm gate value of $17 million); (b) the license holders were not actively working toward developing virus-resistant transgenic papaya; (c) the transgenic papaya was urgently needed to rescue Hawaii’s papaya industry; and (d) licensing the transgenic papaya in a timely manner would demonstrate goodwill in trying to help a distressed industry. Detracting from the possible success of the PAC in obtaining a license was the expense, the relatively small size of the industry, and the extra keen public scrutiny that focuses on transgenic products. Failure by growers or shippers to follow the license agreements could be a source of embarrassment to all involved. Nevertheless, license agreements were obtained from all parties in April 1998, allowing the commercial cultivation of the papaya or its derivatives in Hawaii only. Fruits can be sold outside Hawaii, provided that the importing state or country allows the importation and sale of transgenic papaya. Fruit derived from the licensed transgenic papaya grown outside of Hawaii cannot be sold commercially.

Even before license agreements had been obtained, the PAC had commissioned the Hawaii Agricultural Research Center to produce UH Rainbow seeds so that they would be available when needed. The seeds are being produced on Kauai, where PRSV is not present. Seeds sufficient for 1000 acres are scheduled for distribution in May 1998. Growers should therefore be able to start planting and thus reclaiming abandoned lands in Puna after mid-1998.

WORLDWIDE CONTROL OF PRSV

Now that transgenic papaya appears to be a practical way to control PRSV in Hawaii, what about worldwide control of PRSV with transgenic papaya? The constraints of the license agreement with the germplasm of line 55-1 or their derivatives (e.g. SunUp and UH Rainbow) make it unlikely that line 55-1 will be made available for commercial use outside Hawaii. Moreover, our results with hemizygous plants of line 55-1 showed these plants to be susceptible to PRSV isolates outside Hawaii. These observations suggested that transgenic papaya with coat protein genes specific to targeted PRSV isolates would need to be developed for transgenic papaya to effectively control PRSV worldwide.
However, recent results show that transgenic papaya with resistance to a broader range of PRSV strains can be developed.

**Homozygous Line 55-1 is Resistant to PRSV Isolates Outside Hawaii**

Further infectivity studies on line 55-1 by Tennant gave interesting results showing that resistance was markedly affected by combinations of coat protein transgene dosage (hemizygous vs homozygous), developmental stage of papaya seedlings, and coat protein gene sequence homology of PRSV isolates (49, 50). The results demonstrated that hemizygous plants (e.g. UH Rainbow) are quite susceptible even to some of the Hawaiian isolates when inoculated at a very small seedling stage but are resistant when inoculated at later stages. But as noted earlier, these hemizygous plants are susceptible to a number of isolates originating outside of Hawaii. Strikingly, 55-1 plants that were homozygous for the coat protein gene (i.e. SunUp) were resistant to a larger range of the isolates (except for the Thailand isolate), regardless of plant age. And, large plants of homozygous 55-1 that were inoculated with PRSV-Thailand showed resistance or long delays prior to symptom expression. These observations suggested that resistance of line 55-1 was being governed by RNA-mediated protection (4, 26). Others had clearly shown with other transgenic plants that RNA-mediated protection was affected by gene dosage (38, 46), sequence differences between the transgene and the attacking virus (4, 27), and developmental stage of the plant (38). The underlying mechanism of RNA-mediated protection is likely to be posttranscriptional gene silencing: a phenomenon in which the transgene transcript is degraded following apparently normal levels of transcription in the nucleus (9). In fact, nuclear run-off experiments with isolated nucleic acid from line 55-1 confirmed that the coat protein transgene was posttranscriptionally silenced (49). The narrow protection of the hemizygous line 55-1 and UH Rainbow and the concomitant broader protection of the homozygous SunUp now had an explanation.

**Other Transgenic Papaya Lines Show Broader Resistance**

Our results so far had been obtained from a single line of transgenic papaya. Could transgenic resistance be repeated with other lines and other genes? Preliminary results from other laboratories clearly show that transgenic papaya will be useful for controlling PRSV. Another transgenic line (designated 63-1), generated from the same transformation experiments that resulted in line 55-1, also shows high resistance under field conditions (tested in a replicated plot in the Kapoho field trial). Additionally, this line has broader resistance to PRSV isolates than line 55-1 has (49, 50). We have subsequently produced numerous transgenic plants with the nontranslatable coat protein gene of PRSV HA; a
number of the transgenic plants were resistant to isolates outside Hawaii (19). These results clearly show that resistance is RNA-mediated and that R0 plants with broader resistance than line 55-1 can be obtained. Yeh’s laboratory has also developed transgenic plants with the coat protein gene of PRSV from Taiwan; some of these are resistant to PRSV strains from Taiwan, Thailand, and Hawaii (62). New transgenic lines expressing coat protein genes (translatable or nontranslatable) of PRSV isolates from Brazil, Jamaica, and Thailand (D Gonsalves, unpublished results) and Australia (J Dale, personal communication) have been produced. Recently, transgenic papaya with the Nib gene of PRSV HA 5-1 has been produced (M Fitch, personal communication).

Resistance of 55-1 is Coat Protein Gene Sequence Dependent

RNA-mediated protection clearly suggests that resistance is dependent on the homology of the transgene and the incoming virus. If the sequences of the coat protein transgene are different enough from the coat protein of the incoming virus, the transgenic plant would be susceptible to that virus. Indeed, observations suggest that the resistance of homozygous line 55-1 is overcome by PRSV isolates with coat protein genes with around 90% identity to the transgene. For example, PRSV strains from Thailand and Taiwan have around 90% identity to the transgene and can overcome the resistance of SunUp (3, 56). Can we determine the minimal difference in coat protein sequence identity that will overcome resistance of SunUp?

A major breakthrough in Yeh’s laboratory should enable us to understand the question on sequence homology and resistance that is posed above. His laboratory recently developed infectious transcripts of PRSV HA (6) and other PRSV isolates (SD Yeh, personal communication). Furthermore, he and his coworkers constructed a recombinant hybrid virus (PRSV HA-YKcp) containing the entire genome of PRSV HA except that the coat protein gene (plus about 70 nucleotides of the Nib and the untranslated 3’ region) was exchanged with that of PRSV-YK from Taiwan (SD Yeh, unpublished data). Workers in our laboratory have inoculated UH Rainbow (hemizygous for PRSV HA 5-1 coat protein transgene) and SunUp (homozygous for PRSV HA 5-1 coat protein transgene) with PRSV HA, PRSV YK, and the hybrid virus (PRSV HA-YKcp). Preliminary greenhouse experiments have shown that UH Rainbow and SunUp are resistant to PRSV HA, but susceptible to PRSV YK and PRSV HA-YKcp. These results suggest that differences in the coat protein gene of the attacking virus and the transgene are critical factors that affect resistance in line 55-1. It will be interesting to make virus hybrids with chimeric coat proteins and check their ability to overcome the resistance to line 55-1.
Technology Transfer to Control PRSV Worldwide

Developing countries produce 98% of the world’s papaya crop (31). Can these countries capitalize on the technology to develop virus-resistant transgenic papaya that are horticulturally acceptable locally? Our laboratory at Cornell University is committed to helping other countries obtain this needed technology. Thus far, we have focused on developing a cost-efficient and time-efficient technology transfer program, without the intervention of a large foreign aid donor. The program combines training with collaborative, goal-specific research. The goal is to develop the transgenic plants and conduct the initial screening for virus resistance within 18 months to 2 years. Typically, arrangements are made in collaboration with a government agency from the nation requesting the technology. One example is a program with Thailand’s Department of Agriculture to develop transgenic papaya for the villages of Northeast Thailand. The coat protein gene of PRSV from Thailand was engineered in our laboratory; a Thai scientist, Nonglak Sarindu, was then sent to our laboratory to transform their local papaya cultivars. Regenerated plants were rooted and established in the greenhouse, and then Sarindu conducted the initial testing for resistance. After 20 months in Geneva, New York, Sarindu returned home with promising transgenic plants and embryo cultures. The plants are fruiting in a cage house at a government station in Northeast Thailand. Evaluation of the transgenic plants is continuing in the greenhouse, and eventually they will be tested in the field. Sarindu was trained in all the skills necessary to develop the transgenic papaya and to subsequently advise in the continuing process for dealing with transgenic products. Our primary goal was to develop the transgenic papaya within the targeted time period of a year and a half. Training was important but subsidiary since it could be addressed through hands-on work on the project. The scientist was responsible for completing the application to the regulatory agency for approval to import the transgenic papaya and to test them in the home country. Funding for the project was covered by the foreign government; included were transportation and living expenses for the scientist and a fee to cover the cost of supplies.

Proper technology transfer involves the continuation of the project in the home country. This phase often fails because the developed product is of only academic value and thus there is no pressing need to push to a practical end or there is no commitment to work through the necessary bureaucratic procedures. A well-planned project must objectively anticipate potential impediments to prevent the project from reaching its goal. Perhaps the best guarantee is to have a project that is feasible and yet of demonstrably high importance. Development of PRSV-resistant plants fits this category. This method of technology
transfer can serve as a model system for dealing with other transgenic products at all levels.

The program has worked well so far. Transgenic papaya targeted for Jamaica, Brazil, and Thailand has been developed and shown resistance to local strains of PRSV. A program is also ongoing with Venezuela. However, the success of this approach will be determined four to five years from now, when the transgenic plants are actually in the field and, it is hoped, helping to control PRSV.

SOCIOECONOMIC EFFECT OF TRANSGENIC PAPAYA

Transgenic papaya—SunUp and UH Rainbow—is the first virus-resistant fruit crop to be commercialized in the United States. Over all, it is only the second crop with virus-resistance to be commercialized, next to Asgrow Seed Companies Freedom II squash. For the papaya, results so far hold promise of reviving the Hawaiian papaya industry. Despite extensive documentation of the impact of PRSV on the industry, especially since 1992, few, if any, studies have measured the impact, both sociologically and economically, of a transgenic crop on a local population. Thus, the scenario in Hawaii provides an excellent opportunity to objectively determine what impact a transgenic product has on an industry and its supporting farmers. We hope to measure this impact.

FINAL THOUGHTS

I have attempted to describe a series of events over a number of years that have led up to what appears to be a likely control of PRSV, at least in Hawaii. Proof of success in controlling PRSV in papaya will only be determined in the next several years, as the transgenic papaya is widely planted in Hawaii and as more transgenic papaya are produced and tested worldwide. Work on controlling PRSV should also lead to advancements in fundamental aspects of PDR, cross protection, and management of plant viruses with the use of transgenic plants. Finally, the transgenic papaya will, in my view, allow for the timely assessment of the socioeconomic effect of a transgenic product on agriculture, especially as it relates to farmers in developing countries.

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PAPAYA RINGSPOT VIRUS

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