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Control of Papaya Ringspot Virus by Cross Protection

Papaya (*Carica papaya* L.) is one of the most widely grown and economically valuable fruits of the tropics and subtropics. This large herbaceous, dicotyledonous plant with a single stem and a crown of large, palmately lobed leaves grows fast and yields fruit 8–10 months after being transplanted in the field. The extensive adaptation of the plant and the wide acceptance of the fruit offer considerable promise for local and export markets. Like banana, pineapple, and mango, papaya is one of the important cash crops in the tropics and subtropics.

A destructive disease caused by papaya ringspot virus (PRV) is a major obstacle to wide-scale planting of this fruit tree. PRV has been reported as a major limiting factor for growing papaya in areas of Hawaii, Florida, the Caribbean countries, South America, Africa, Australia, and the Far East (12). In papaya, the virus causes mottling and distortion of leaves (Fig. 1A), ring spots on fruit (Fig. 1B), and water-soaked streaks on stems and petioles. The disease stunts the plant and drastically reduces the size of the fruit.

PRV is a member of the potyvirus group, with flexuous, filamentous particles about 780 × 12 nm. The virus induces cylindrical (pinwheel) inclusions and amorphous inclusions in the cytoplasm of host cells. It is transmitted mechanically and by many species of aphids in a nonpersistent manner. PRV has a narrow host range that includes species of three dicotyledonous families: Caricaceae, Chenopodiaceae, and Cucurbitaceae. The virus is serologically indistinguishable from watermelon mosaic virus-1 (WMV-1), which recently was reclassified as papaya ringspot virus-W and is of economic importance wherever cucurbits are grown (12).

PRV was first recorded in southern Taiwan in 1975, and within 4 years, the virus had destroyed most of the papaya production in commercial orchards along the west coast of the island (Fig. 2) (14). The total yield of papaya dropped from 41,595 t in 1974 to 18,950 t in 1977. During the same period, the wholesale price increased sixfold, from $3.67 to $20.70 per kilogram Taiwan dollars (from $0.04 to $0.24 per pound U.S. dollars). Because of the attractiveness of the high price of the fruit, the total planting area of papaya increased from 1,658 ha in 1975 to 4,498 ha in 1985. Soon after the disease became widespread on the west coast of the island, the government encouraged farmers to grow papaya on the east coast, isolated from the west by a central range of high mountains. Despite strict quarantine measures to prevent movement of papaya seedlings from the west, the virus soon became established on the east coast, where it is now widespread. As a result, Taiwan has lost the chance to export the fruit to Hong Kong and Japan, and even the domestic supply is insufficient. Moreover, papaya tress are now grown as annuals or biannuals instead of perennials because of severe virus infection.

Cross Protection for Control

Cross protection, discovered by McKinney in 1929 (7) with tobacco mosaic virus (TMV), is a phenomenon in which plants systemically infected with one strain of a virus are protected from the severe effects of a second related strain of the same virus. Cross protection for control of TMV was not adopted widely until Rast (13) produced a mild mutant (MII-16) from a common tomato strain of TMV by nitrous acid mutagenesis. This mutant has been used commercially and has been applied to a high proportion of glasshouse-grown tomato crops in the Netherlands and the United Kingdom since 1970 (3). Successful control of tomato mosaic disease with an attenuated mutant (L11 A), isolated from the tomato strain of TMV in plants treated with high temperature, was also reported in Japan (11). Cross protection has been used on a large scale to control citrus tristeza virus (CTV), a closterovirus that is important worldwide. Naturally

Fig. 1. Symptoms of papaya ringspot disease on (A) papaya plant, showing mosaic and deformation of leaves and general stunting, and (B) papaya fruit.

Fig. 2. Papaya orchard in Taiwan infected with papaya ringspot virus.

occurring mild strains of CTV have been selected and offer protection in the field (9). In Brazil, the number of protected sweet orange trees exceeded 8 million in 1980, and no breakdown in protection was observed (2).

Several attempts to develop effective control measures for PRV in Taiwan have proved either ineffective or only of marginal benefit. Such practices include avoiding the disease by planting papaya in the seasons of few alate aphids, intercropping with a high-stem barrier crop such as corn, eradicating diseased plants in orchards, spraying with mineral oil and systemic insecticides, protecting young seedlings with plastic bags, and mulching the orchard soil with reflective silver-colored plastic sheets after transplanting. Although tolerant selections of papaya have been described, resistance to PRV does not occur within *C. papaya* (1). Some species of *Carica* are resistant to PRV, but unfortunately they are incompatible with *C. papaya* and conventional interspecific hybridization has been unsuccessful (8). A diligent roguing program has been practiced successfully in Hawaii to suppress the spread of PRV in certain areas of the state (10). Roguing is not a permanent solution for an area that is not geographically isolated, however, and eradicating sources of the virus from Taiwan, where the disease has become endemic, is impossible.

The severe crop losses, the unavailability of PRV-resistant papaya varieties, the difficulty of eradication, and the restrictive host range of PRV make cross protection an attractive method of controlling this disease.

Search for Mild PRV Strains

The key for practical application of cross protection is the availability of a useful protective virus strain. One mild strain, PRV Su-mm, selected from 230 PRV isolates in Taiwan offered a certain degree of protection to papaya against infection by severe strains (6). Unfortunately, this naturally occurring strain was not mild enough and not satisfactory for practical use in virus disease control. Attempts to select mild strains from 116 isolates collected from Hawaii were also unsuccessful (16). Although some PRV isolates obtained by single-lesion selection from natural populations caused various degrees of symptom severity on papaya seedlings, none proved to be a useful strain. Thus, the isolation of mild PRV strains from the natural population seems to be a difficult task. This contrasts with citrus tristeza virus, in which mild strains easily collected from the field are used for the practical application of cross protection (9).

Because efforts to select naturally occurring mild strains of PRV failed, the endeavor was shifted to artificial mutagenesis. Nitrous acid, a powerful chemical mutagen for plant RNA viruses, was used to induce mutants from PRV HA, a severe strain isolated from Hawaii (5). Crude sap from PRV-infected squash was treated with nitrous acid (ph 6.0) at 20 C for 30 minutes and used to inoculate *Chenopodium quinoa* Willd., a local lesion host for PRV. Single lesions that developed on *C. quinoa* 20–30 days after inoculation were transferred to papaya seedlings. Two attenuated mutants, designated as PRV HA 5-1 and PRV HA 6-1, were obtained from 663 single-lesion isolations in July 1982 (16). Papaya seedlings inoculated with these two isolates remained symptomless or showed diffuse chlorotic spots with no reduction in plant size. When tested with double-antibody-sandwich (DAS) ELISA using the antiserum against PRV (5), the plants showed strong positive reactions similar to those of other plants inoculated with the severe PRV HA strain, indicating that symptomless infection was not due to low concentration or slow replication of the virus. Moreover, the mutants caused symptomless or mild infection to the most susceptible cucurbitaceous plants, *Cucumis metuliferus* (Naud.) Mey. (horned cucumber) and *Cucurbita pepo* L. (squash), and behaved similarly in melons, watermelons, cucumbers, and pumpkins (15). Because the systemic hosts of PRV in nature are limited only to Caricaceae and Cucurbitaceae (12), these results indicated that the possiblility of damaging cucurbitaceous crops in the vicinity of papaya orchards protected with the mild mutants would be minimal.

Tests in the Greenhouse

To test the effectiveness of the mild mutants, PRV HA 5-1 was used to protect papaya plants against challenge inoculation with a severe PRV HA strain under greenhouse conditions (16). The tests were conducted at Cornell University from October 1982 to April 1983. When papaya seedlings at the five- to six-leaf stage were preinfected with PRV HA 5-1 by rubbing individual leaves and then challenged with a severe PRV HA strain after different time intervals, a high proportion (79–93%) of the plants remained symptomless even 2–3 months after challenge inoculation when the interval between protective and challenge inoculation was more than 26 days (16). In contrast, papaya seedlings mock-inoculated with buffer first and reinoculated with PRV HA showed severe symptoms 15 days later (Fig. 3).

Cross protection between PRV HA 5-1 and PRV HA was investigated by imposing challenge inoculations on leaves in different positions of protected plants. A high proportion (90%) of plants that were challenge-inoculated on fully expanded leaves remained with no or only mild symptoms even 3 months after challenge inoculation (16). However, a majority (50–80%) of the plants that were challenge-inoculated on the upper nonexpanded young leaves or all leaves showed severe symptoms 60–90 days after challenge inoculation (16), indicating that the upper young leaves around the apex are susceptible to invasion by the severe strain.

When plants preinfected with PRV HA 5-1 were continually challenged at different fully expanded leaf positions 30, 32, 34, and 36 days after the protective inoculation, nearly all plants remained symptomless 3 months after the first challenge inoculation (16). Moreover, PRV HA 5-1 also protected the very susceptible *C. metuliferus* against PRV HA (16) (Fig. 4). Partial protection (i.e., delay in expression of severe symptoms) against PRV type W, previously watermelon mosaic virus-1 (12), was also observed in *C. metuliferus* preinfected with PRV HA 5-1 (16).

In general, either complete or a high degree of protection was observed when PRV HA 5-1 was used to protect papaya against the severe effects of infection by the parent strain HA under various mechanical challenge treatments. The results indicated the attenuated mutants had a good potential for controlling PRV.

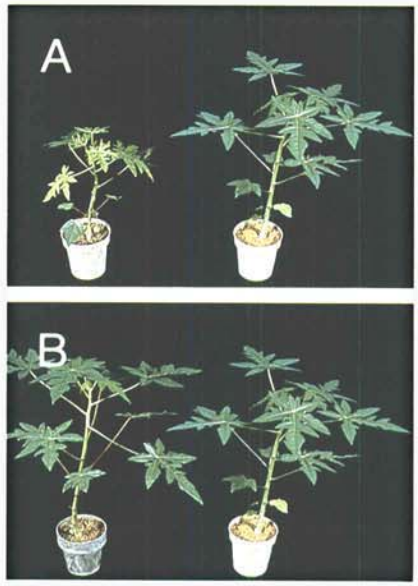

Fig. 3. Cross-protection effectiveness of PRV HA 5-1 (symptomless) against PRV HA (severe) in papaya: (A) Papaya infected with PRV HA showing severe symptoms (left) and healthy plant (right). (B) Papaya first infected with PRV HA 5-1, then challenged with PRV HA, showing no reduction in growth 45 days after challenge inoculation (left) and healthy plant (right).

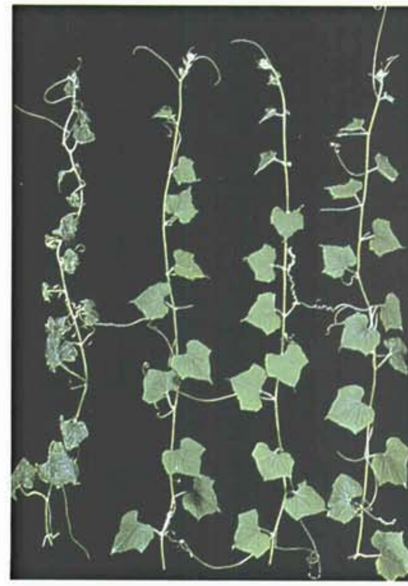

Fig. 4. Cross-protection effectiveness of PRV HA 5-1 (symptomless) against PRV HA (severe) in *Cucumis metuliferus*: (Left to right) Plant infected with PRV HA, showing severe symptoms; plant first infected with PRV HA 5-1, then challenged with PRV HA, showing no reduction in growth 30 days after challenge inoculation; plant infected with PRV HA 5-1 alone; and healthy plant.

Fig. 5. After mass inoculation with a mild strain of PRV by pressure spray (A), papaya seedlings are kept in a screenhouse (B) and checked by ELISA in 3–4 weeks to confirm infection.

Method of Mass Inoculation

Papaya is normally propagated by seed, so an efficient method of mass inoculation of seedlings was developed for the practical use of cross protection. Inocula were prepared from infected *C. metuliferus* plants 3–4 weeks after inoculation with the mild mutant. Leaves were blended in cold potassium phosphate buffer (0.01 M, pH 7.0, 1 g/20 ml), and the extracts were strained through cheesecloth, then mixed with Carborundum (600 mesh, 40 g/1.5 L) in a metal tank connected to a spraying gun (nozzle diameter 1.2 mm) (Fig. 5A). Infection rates of 100% were achieved and mechanical damage was avoided by using pressures of 4–6 kg/cm^2 at a distance of 10 cm or 8 kg/cm^2 at 20 cm. The method was highly efficient—one person could inoculate 10,000 seedlings in 2 hours. Seedlings were inoculated at the four- to five-leaf stage and grown singly in 10 × 8 cm plastic bags containing soil and manure. Individual seedlings in bags were placed in wooden boxes (60 × 40 × 16 cm, 100 bags per box) and kept in the screenhouse (Fig. 5B). In Taiwan, papaya seedlings are routinely raised in plastic bags and arranged in wooden boxes for easy handling during transplanting, so the additional cost of spraying was minimal.

Because no conspicuous symptoms developed after inoculation and because assay of each seedling was not practical, randomly selected seedlings (1%) were tested by ELISA to ensure that the seedlings were infected by mild mutants. Also, seedlings were inoculated twice to increase the chance of infection.

Field Trials

In the spring of 1983, a small test was performed in Hawaii to evaluate the effect of PRV HA 5-1 on the growth of papaya under field conditions. Growth and fruit-set of plants inoculated with HA 5-1 were normal. The horticultural properties of papaya were not affected by the mild mutant, although the fruit of some plants had a few ring spots; these became less apparent at maturity and did not affect the quality of the fruit. In another trial, HA 5-1 protected plants and healthy plants were subject to natural infection by aphids. This test showed that HA 5-1 gave good protection against the Hawaii strains of PRV under field conditions (Fig. 6); large field trials could not be conducted in Hawaii, however.

HA 5-1 and HA 6-1 were introduced to Taiwan from Cornell University in 1983. Tests were conducted under greenhouse and field conditions at the Feng-Shan Tropical Horticultural Experiment Station to study the potential of cross protection for control of papaya ringspot disease in Taiwan (15). Under greenhouse conditions, both mutants caused only mild or symptomless infection on test plants in the Chenopodiaceae and Cucurbitaceae families and on the major papaya cultivar of Taiwan, Tainung No. 2. Also under greenhouse conditions, HA 5-1 and HA 6-1 provided a high degree of protection in papaya against the severe effects of the wilting and the mosaic strains of PRV prevalent in Taiwan (15). These results indicated that both mutants might have great potential for controlling PRV by cross protection in Taiwan.

Field cross-protection trials were started in three locations in Taiwan in the fall of 1983 (15). In the Feng-Shan plot, protected papaya seedlings were mixed with unprotected papaya in a randomized complete block design; in the Kao-Shu plot, protected and unprotected seedlings were planted in alternate rows. These two plots were situated close or adjacent to papaya orchards that were severely infected with PRV. Nearly all of the unprotected plants had severe symptoms within 4 months after transplanting, and most of the protected plants had severe symptoms within 6 months—a delay in symptoms of only 1 or 2 months. No effort was made to rogue severely infected plants during the course of the experiment. Under these conditions, cross protection did not give economic benefit because breakdown occurred before fruit-set (15). In a test at Ta-Liao (Fig. 7), protected and unprotected plants were established in solid blocks and the plot was not situated adjacent to diseased papaya orchards. Furthermore, disease pressure within the test orchard was minimized by roguing severely infected plants once every 10 days up to the flowering stage. The data showed that protected trees had 82% greater fruit yield than unprotected plants, resulting in a 111% increase in income because of a much higher yield of good-quality fruit from protected trees than from the controls (15).

The failure of cross protection at the Kao-Shu and Feng-Shan sites could be attributed to several factors. Infected papaya orchards were close to the test orchards, the cross-protected and unprotected plants were interplanted, and severely diseased plants were not rogued. All these produced a great challenge pressure to the protected trees. Consequently, most protected trees became severely infected before the flowering stage, resulting in poor fruit production. It is unlikely, however, that farmers would grow their protected papaya plants mixed with unprotected ones. The solid-block test at Ta-Liao was closer to actual growing conditions, and its success brought hope for use of cross protection to control the devastating PRV in Taiwan.

Large-scale Application

The government proceeded with large-scale field trials in the spring (44,000 protected plants on 22 ha) and fall (200,000 protected plants on 100 ha) of 1984. All test orchards were solidly planted with protected or unprotected plants. End-of-the-year ratings of the spring plantings showed an average

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disease incidence of 31.1% in protected orchards and 82% in unprotected ones. The average fruit yield was 17.9 kg per protected tree and 7.3 kg per unprotected one. The income from protected fields was 109% more than that from unprotected ones. Ratings of the 1984 fall plantings at the end of 1985 showed a disease incidence of 45.5–68.5% in the protected orchards and 100% in the unprotected ones. Average yield ranged from 7.2 to 29.2 kg per protected tree and 0 to 12.5 kg for unprotected ones.

In general, the large-scale field trials during 1984–1985 reflected the results of the Ta-Liao test, and the cross-protection method was welcomed by the growers. Because of the success of the spring and fall plantings of 1984 (Fig. 8) and in order to fulfill the demand of the growers, the government expanded the large-scale plantings to 220 ha. More than 610,000 papaya seedlings were inoculated with PRV HA 5-1 or 6-1, then planted in the fields in the fall of 1985. Unfortunately, two severe typhoons ruined most of the test fields before harvest, and final yield data were not available. The much lower disease incidence before the typhoons drew much attention from the papaya growers, however.

In the fall of 1986, the Council of Agriculture of the Republic of China transferred the inoculation responsibilities to two private and several government nurseries and expanded the project to 550 ha. More than a million papaya seedlings were inoculated with HA 5-1. The government maintained stringent surveillance over nursery operations. National Chung Hsing University at Taichung preserved and checked all inocula of mild mutants to ensure their mildness on papaya. The original well-indexed inocula were kept as dry tissue stored at 4 C over dehydrated $CaCl_2$. The certified inocula were then mass-propagated in *C. metuliferus*, the best propagative host of PRV, and infected tissues were sent to three nursery centers owned by the government—Feng-Shan Tropical Horticultural Experiment Station, Chia-I Agricultural Experiment Station, and Taiwan Banana Research Institute—and to two private nurseries, Kuonung and Taitung. The inoculated papaya seedlings were monitored for infection by testing 1% of the seedlings with DAS ELISA 3–4 weeks after inoculation. Growers and planting areas were selected by the Department of Agriculture of Taiwan Provincial Government, which was also responsible for coordinating the demonstration and extension work on the control method.

The inoculations and seedling distribution went smoothly because of well-organized teamwork, strong support by the central and local governments, and the growers' great appreciation. By May 1987, the average disease incidence in the randomly selected orchards from 11 counties was 29%, compared with 85% for the unprotected controls. The fruit has been harvested since September 1987; data on the average yield of protected trees are not yet available, however.

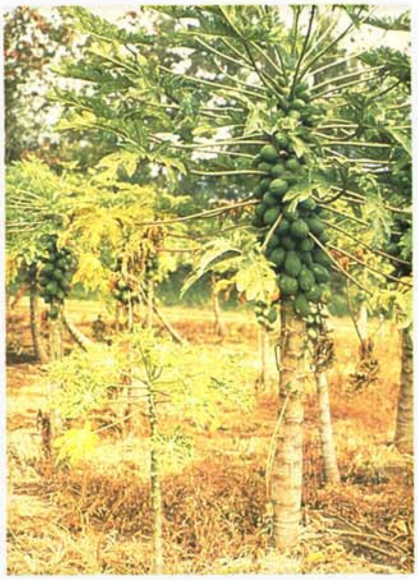

Fig. 6. In a field trial in Hawaii, unprotected plant infected with PRV shows severe mosaic and stunting (left) and protected plant shows normal growth and fruit-set (right).

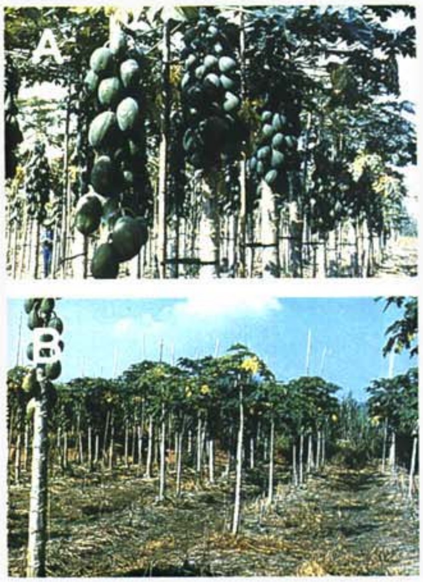

Fig. 7. In a field trial in Ta-Liao area of Taiwan in 1983, a tremendous difference in fruit yield is apparent between (A) papaya plants protected with PRV mild strain and (B) unprotected controls in the same orchard.

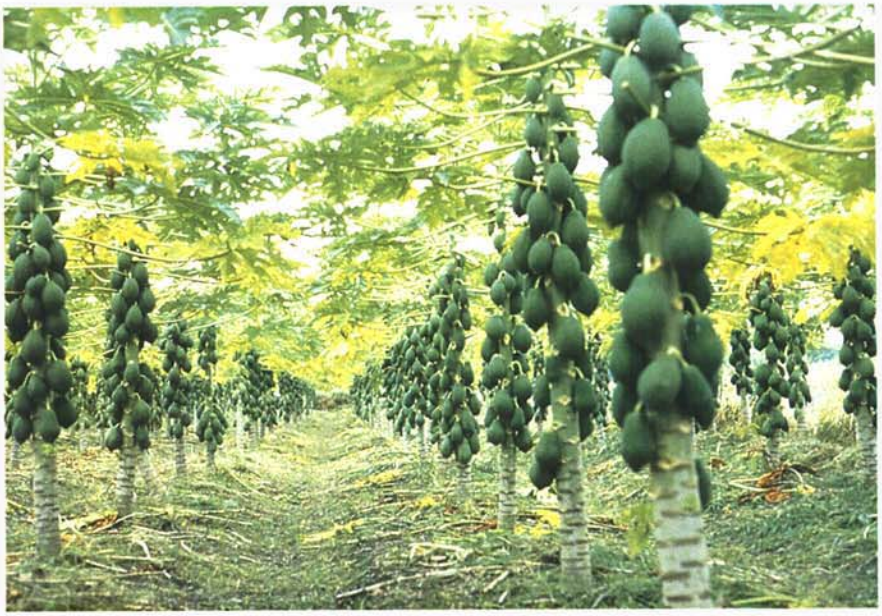

Fig. 8. Abundance of fruit in August 1985 on papaya planted in a large-scale test field at Hui-Lien in the fall of 1984.

Worldwide Interest

Because PRV is the major limiting factor for growing papaya in the tropics and subtropics, the development of useful mild strains has drawn much attention worldwide. Cross protection trials with PRV HA 5-1 and 6-1 strains are being conducted in Mexico, Florida, Hawaii, Thailand, and Israel; results are not yet available.

Observations from the Taiwan trials and from greenhouse studies indicated that cross-protection effectiveness of PRV HA 5-1 and 6-1 was influenced by strains of the challenge virus. Careful studies showed that PRV HA 5-1 gave better protection against two severe strains from Hawaii, PRV HA and HB, than against two severe strains from Taiwan (S.-D. Yeh, *unpublished*).

Similar results were observed in Thailand (D. Gonsalves, *unpublished*). Thus, the cross-protection effectiveness of PRV HA 5-1 and 6-1 will probably vary in different areas of the world. In Taiwan, several mild and stable mutants have been obtained recently by nitrous acid treatment of local severe strains, and cross-protection tests under greenhouse conditions against severe strains of Taiwan indicate that these newly induced mutants are superior to HA 5-1 and 6-1 (S.-D. Yeh, *unpublished*). The same course of action may be necessary in other countries where PRV HA 5-1 and 6-1 do not provide clear economic benefits in field trials of cross-protection.

Present and Future Efforts

Although cross protection is a general phenomenon of plant viruses, not all plant diseases caused by viruses can be controlled by using a mild virus strain for preimmunization. Careful attention to the selection of the best protective isolates of virus and to their introduction into the crop to be protected is essential (4).

For practical application of cross protection, the mild protective virus strain should: 1) not cause severe damage to the protected plants, 2) be stable for a long period, 3) protect plants against the effects of severe strains, 4) be suitable for infecting large number of plants, 5) not affect other crops in the vicinity of the crop protected, and 6) have no synergistic reactions with other viruses. The mild mutants of PRV, HA 5-1 and 6-1, satisfy all these criteria. Evaluation of their cross-protection effectiveness both in the greenhouse and in the field showed the mutants to be valuable mild virus strains for control of PRV by cross protection in Taiwan.

Under normal conditions, the papaya inoculated with the mild mutants grew well and did not show conspicuous symptoms. When temperature dropped below 20 C after rain, however, chlorotic spots appeared on leaves. Also, fruits set at this period showed small ring spots on the surface but were not deformed or smaller than usual. New leaves did not develop chlorotic spots when temperature rose above 25 C, and ring spots on fruits also became less apparent when the fruits were ripe. These mild effects of the mutants did not adversely affect plant growth and fruit production.

The pressure spray is a very efficient method for inoculating seedlings with mild strains. The additional cost calculated by private nurseries is only $0.014 (U.S.) for each seedling, or $28 for 1 ha (2,000 seedlings), whereas the economic benefit is several hundred times the additional cost. This explains the strong demand by growers for treated seedlings.

Severe symptoms caused by possible revertants were not seen in tests of manual and spray inoculations, indicating that HA 5-1 and 6-1 are stable. Precautions must always be taken to index and evaluate the inocula before large-scale application, however.

The mild virus strains systemically infected treated seedlings and persisted throughout the plants' lifetimes, so protected plants did not require reinoculation. Normally, protected trees began to produce fruit 8–10 months after transplantation and continued to yield fruit for another 5–6 months. The orchards were then abandoned because of unfavorable winter conditions and high incidences of breakdown. Control of PRV in Taiwan by cross protection has not restored papaya plants to perennial status yet, but growers no longer risk losing the whole crop to PRV.

Under certain conditions in the greenhouse and field, cross protection only delayed expression of severe symptoms, and superinfection by severe strains occurred. When cross protection broke down before flowering, no economic benefit was obtained. Breakdown may occur when: 1) the challenge virus is introduced into the nonexpanded young leaves around the apex, 2) severe challenge pressure surrounds the protected plants, 3) severe virus strains different from the parental virus of the mild mutants exist, and 4) inoculated seedlings escape infection with the mild strain.

More studies are needed to monitor the mild mutants in the field, to compare their ability to protect against different severe strains, to correlate the breakdown in cross protection to the population density of alate aphids and the inoculum density of severe strains, and, most important, to develop a bank of mild strains from local severe strains. All these efforts will help minimize the incidence of breakdown of cross protection.

The use of induced virus mutants to preimmunize papaya seedlings for control of PRV has become a routine practice in Taiwan. At present, roguing of diseased plants in the protected orchard, protecting seedlings with a plastic bag after transplanting, and intercropping with a high stem barrier crop such as corn are recommended to reduce the challenge pressure. Supplementation with suitable fertilizers to enhance the vigor of protected plants is also recommended. Recently, a tolerant variety introduced from Florida (kindly given to R.-J. Chiu by the late R. A. Conover) has been released in the field. When protected with HA 5-1, this variety significantly increases fruit yield, but poorer fruit quality and diecious sexuality make it less acceptable than other commercial varieties (S.-D. Yeh and R.-J. Chiu, *unpublished*). The integration of cross protection with agricultural control measures and the tolerant varieties of papaya brings hope for restoring the normal production of papaya in Taiwan.

Acknowledgments

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