Papaya Ringspot Virus in Australia and the Development of Virus Resistant Plants

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**Abstract**

Papaya is a popular fruit crop in many tropical and subtropical regions. It grows quickly and is capable of bearing large crops. Because of extensive monoculture and a narrow gene-pool, papaya plants are susceptible to numerous diseases. Papaya ringspot virus, type P (PRSV-P), causes the most widespread of these diseases. Although it is not currently a major problem in Australia, PRSV-P dramatically reduces yield in many countries. *Vasconcellea quercifolia* is one of four wild *Carica* species that are known to be PRSV-P resistant. Hybrids of these species grow vigorously in the field. Males, females and hermaphrodites have been identified, and a few male hybrids exhibited some pollen fertility. A backcross generation has been produced. One plant was fertile and showed resistance to PRSV-P. Transgenic plants containing viral coat protein constructs have also shown resistance to PRSV-P in field plantings. In this paper, progress toward achieving PRSV-P resistance for papaya using both approaches is examined.

**INTRODUCTION**

Papaya or papaw (*Carica papaya* L.) is grown in tropical and subtropical regions worldwide. Papaya plants grow quickly, have a short juvenile period and bear large crops. However, because of extensive monoculture and a narrow gene-pool, the crop is susceptible to a number of seriously debilitating diseases that rapidly spread throughout plantations. By contrast, 21 related species in the genus *Vasconcellea* contain many useful genes for resistance, including Papaya Ringspot Virus (PRSV-P) resistance in *V. cauliflora*, *V. pubescens*, *V. quercifolia* and *V. stipulata* (Conover, 1964; Horovitz and Jiménez, 1967), Phytophthora resistance in *V. goudotiana* and papaw dieback resistance in *V. parviflora* (Drew et al., 1998).

In Australia, papaya is grown commercially in three distinct geographical regions, all of which are east of the Great Dividing Range: subtropical southeast Queensland, central Queensland and tropical north Queensland. The industry in north Queensland has expanded over the past 10 years, and now accounts for >80% of commercial production. There are 5 major diseases that hinder production of papaya in Australia: papaw dieback, yellow crinkle, *Phytophthora*, black spot and papaya ringspot virus.

Papaw dieback is consistently associated with phytoplasma infection (Gibb et al., 1996) and remains the major limiting factor to the papaya industry in southeast and central Queensland. In recent seasons, there have been serious outbreaks of the disease in tropical north Queensland. Early symptoms of dieback include twisting and curving of the apex, with rapid death from the apex downwards. The disease can be devastating and 90% of a plantation can be lost within weeks.

Yellow crinkle is caused by a phytoplasma distinct from that associated with dieback (Gibb et al., 1998). Major outbreaks of yellow crinkle usually occur following hot dry weather that favours the migration of leafhopper vectors from alternative hosts.
Plants develop a pronounced yellowing of leaves half-way up the canopy. Crown leaves become small, translucent and claw-like in appearance.

Root, trunk and fruit rot caused by *Phytophthora palmvora* and *P. parasitica* cause major losses in north Queensland, particularly after heavy monsoon rains.

Black spot is a relatively new disease, caused by *Asperisporium caricae*. The fungus is rapidly disseminated by air-borne spores and the disease is favoured by warm, humid conditions. Extensive leaf loss and fruit spotting occurs in the absence of thorough fungicide applications and plantation hygiene. Black spot now infects papayas growing in all districts on the east coast of Queensland.

PRSV-P was first reported in southeast Queensland in 1991 (Thomas and Dodman, 1993), and remains a major threat to the commercial papaya industry. Currently PRSV-P is the least important of the five diseases, as it has been contained in southeast Queensland for 10 years by strict quarantine measures, including restriction on movement of papaya and cucurbit planting material, and eradication of infected plants when outbreaks occur.

This paper reviews research projects that are being undertaken to produce plants resistant to PRSV-P. Resistant interspecific hybrids have been produced between Australian genotypes of *C. papaya* and *V. quercifolia* (Drew et al., 1998). The interspecific hybrids have been backcrossed to papaya and a fertile PRSV-P resistant plant has been produced. Australian genotypes of *C. papaya* have been transformed using microprojectile bombardment with a construct containing an untranslatable PRSV-P coat protein coding region (Mahon et al., 1996; Lines et al., 2002) and resistant plants have been grown to maturity in the field.

**PROGRESS WITH INTERSPECIFIC HYBRIDISATION**

*Papaya × cauliflora*

There have been numerous attempts to transfer PRSV-P resistance from *V. cauliflora* to *C. papaya* by hybridisation (Jiménez and Horovitz, 1958; Sawant, 1958; Horovitz and Jiménez, 1967; Khuspe et al., 1980; Litz and Conover, 1983; Manshardt and Wenslaff, 1989; Chen et al., 1991; Magdalita et al., 1996). Hybrids between *C. papaya* and *V. cauliflora* lack vigour, rarely survive to flowering and if they do, are infertile (Horovitz and Jiménez, 1967; Litz and Conover, 1983; Manshardt and Wenslaff, 1989).

In our research, a large-scale crossing program was made possible by the development of improved protocols for crossing, for in vitro culture and for plantlet production (Magdalita et al., 1996; Magdalita et al., 1998). Initially, 14 papaya genotypes were pollinated with *V. cauliflora* pollen. Many produced no zygotic embryos, however the southeast Queensland line 2.001 produced the best results in terms of numbers of embryos per fruit (Magdalita et al., 1998). Subsequently, a large-scale crossing program between *V. cauliflora* and *C. papaya* clone 2.001 yielded 2,099 embryos from 43,376 dissected seeds contained in 338 fruit. One thousand hybrid plants were established in the glasshouse, after being produced in vitro from the rescued embryos using procedures of Magdalita et al. (1996). All were resistant to PRSV-P. Only 3 survived to flowering in southeast Queensland and none were fertile. A few plants grew to maturity in the tropical climate of Los Banos in the Philippines, however attempts by Magdalita to backcross these to *C. papaya* were unsuccessful because their flowers were infertile (Magdalita pers comm).

In view of recent genetic analyses, it is unfortunate that so much effort has been directed to crosses between *C. papaya* and *V. cauliflora*. Analyses of isozyme and RAPD profiles, and comparison of DNA sequences from nuclear and mitochondrial genes all show that, of the known PRSV-P resistant varieties, *V. cauliflora* is genetically the most distant of the wild relatives from *C. papaya*, and that *V. quercifolia* and *V. pubescens* are the most similar to *C. papaya* (Jobin-Décor et al., 1996; Manshardt and Drew, 1998).
**Papaya × pubescens**

The efficient techniques that were developed by Magdalita et al. (1996, 1998) for crossing, embryo rescue and in vitro plantlet production of *C. papaya × V. cauliflora* hybrids were used to produce large numbers of *C. papaya × V. pubescens* hybrid plants using papaya clone 2.001 as the female parent (Drew et al., 1998). Three hundred hybrid plants of this cross were grown to maturity in the field. All were resistant to PRSV-P. Fifty percent of the plants produced female flowers on short peduncles, which were typical of female *C. papaya* or *V. pubescens* flowers. The other trees produced multiple flowers on long peduncles that were also all female but in morphology were typical of male *C. papaya* or *V. pubescens* flowers. Numerous attempts were made to pollinate these flowers with pollen from *C. papaya* plants, however none were successful.

**Papaya × quercifolia**

The techniques of Magdalita et al. (1996, 1998) were used to produce large numbers of hybrid plants from crosses between *C. papaya* and *V. quercifolia*. Papaya clone 2.001 was used as the female parent. Plants were manually inoculated with PRSV-P three times at intervals of two weeks. Seventy-five percent were resistant and 25% produced symptoms of the virus (Drew et al., 1998). Three hundred plants were established in a field planting and grew vigorously (Drew et al., 1998). All plants grew to maturity and flowered. Sex ratios of 2:49:49 of male:hermaphrodite:female were obtained. Of these, eleven were selected as being both resistant to the virus and fertile. Of the eleven F1 hybrids, 4 plants were hermaphrodite trees, the others were male. However males occasionally produced hermaphrodite flowers. Similarly, hermaphrodite trees sometimes produced male flowers. Fertility in these eleven was poor and only a few pollen grains were produced per flower. PRSV-P resistance was difficult to ascertain and after numerous inoculations of clones of the seven male lines (over a three year period), three of them produced some viral symptoms and were identified as having some susceptibility to the virus.

A back-crossing program commenced on the 29/11/99 in subtropical southeast Queensland. Plants of the line 2.001, which was the papaya parent used in the original F1 cross, were used as the female parent and the eleven interspecific hybrids were used as pollinators. Crosses were also attempted in north Queensland, to see if better results could be obtained in a tropical climate, however little success was obtained at this site.

No successful backcrosses were achieved using pollen from the hermaphrodite trees. All successful backcrosses resulted from pollination from five of the seven male trees. As three of the seven were eventually identified as being susceptible to PRSV-P, 4 male hybrids remained as useful pollinators for further backcross experiments.

In total, 1426 flowers of papaya clone 2.001 were pollinated with pollen from F1 hybrids. From these, 912 fruit were harvested for embryo rescue. The other crosses had been unsuccessful and small fruit abscised from the trees. From the 912 fruit, 63 embryos were rescued and cultured in vitro using the protocols of Magdalita et al. (1996). Some died and others grew slowly over two months, sometimes longer for weaker plants. Of the embryos that were obtained, only 50 grew to be strong plants and these were micropropagated in vitro.

In addition, eight female lines of the F1 cross *C. papaya × V. quercifolia* were planted in the field in southeast Queensland. Two hundred and fifty-five crosses were attempted with pollen from a male relative of 2.001. Fruit were harvested after 90 days, but no embryos were present in the seeds.

Twenty plants of each of the 50 micropropagated clones were established in an aphid-proof screenhouse. They were inoculated with PRSV-P three times at two-weekly intervals. One plant remained symptomless after the manual inoculations. Another 50 plants of this clone were inoculated three times in the screenhouse. One of these showed symptoms for a few weeks. The other 49 remained symptomless. Three were planted in the field and exposed to natural inoculation by aphid vectors from *C. papaya* plants having a high inoculum level. They were manually inoculated three times while growing.
in the field. After six inoculations and growth for nine months in the presence of PRSV-P infected plants, they showed no symptoms and were negative in ELISA. Two plants died of root rot after 9 months and the third developed symptoms of PRSV-P after 12 months in the field. PRSV-P infection was subsequently confirmed by ELISA. All three plants flowered, were male, and showed 90% pollen viability when pollen was cultured on Brewbaker and Kwak (1963) medium.

A second male plant and a female plant also showed no symptoms when 20 plant clones were inoculated three times in the screenhouse. However they developed symptoms of PRSV-P after 3 months in the field. As with the F1 plants, some appeared resistant to PRSV-P after multiple inoculations with the virus. The mechanism of defense of \textit{V. quercifolia} appears such that some hybrid and backcross plants are difficult to infect by manual inoculation, but do become infected after long exposure to inoculation by viruliferous aphids.

**PROGRESS WITH GENETICALLY ENGINEERED PLANTS**

Transgenic resistance based on virus-derived transgenes has been shown to be effective against many plant viruses, including PRSV-P (Gonsalves, 1998). The significant sequence variability in the coat protein gene of PRSV-P has necessitated individual transformation programs using local isolates of the virus (Bateson et al., 1994; Gonsalves, 1998).

Transgenic resistance was generated in Australian papaya cultivars using a transformation and regeneration protocol developed for a dioecious Australian cultivar based on microprojectile bombardment of secondary somatic embryos (Mahon et al., 1996). Somatic embryos of two Australian commercial papaya cultivars were bombarded using a particle inflow gun, with plasmid DNA containing the PRSV-P coat protein (CP) coding region and the \textit{nptII} gene encoding kanamycin resistance. Plantlets were selected and germinated on media containing kanamycin. Regenerated plants were characterised using Southern hybridisations with a probe to the PRSV-P CP gene to confirm the presence of the transgene and to determine the transgene copy number. Plants were acclimatised in a glasshouse and were manually inoculated with a field isolate of PRSV-P. All plants were inoculated three times at weekly intervals. Plants were assayed for virus concentration by ELISA two weeks after the final inoculation. A selection of plants were also analysed by reverse transcriptase PCR (RT-PCR) to confirm the ELISA results (Lines et al., 2002).

Transgenic plants and appropriate transgenic and non-transgenic control plants were exposed to natural inoculation by aphid vectors in a field situation having a high inoculum level of PRSV-P. Two transgenic lines, one from each papaya cultivar, consistently remained free of PRSV-P symptoms. Immunity of the two transformed lines was confirmed by failure to detect virus by ELISA and RT-PCR following repeated inoculations in a glasshouse and exposure to field infection for 18 months. The immune lines were shown to have up to four copies of the transgene. Both lines were male and produced viable pollen. Northern hybridisation showed that the coat protein transcript in the immune lines was degraded, therefore the mechanism of resistance appears to be post transcriptional gene silencing via RNA degradation (Lines et al., 2002).

The two Australian papaya lines produced in this work demonstrated immunity to Australian PRSV-P which was stable under high inoculum pressure in the glasshouse and field. The isolate of PRSV-P from which the coat protein coding region was originally derived, was collected from a small commercial plantation in southeast Queensland and was the site of one of the earliest reports of PRSV-P in Australia. To facilitate access to virus-infected material, the site was not cleared and was subsequently used for field trials. The sequence of this isolate was representative of the population of PRSV in Australia, which was shown to have less than 2% nucleotide sequence variation between the most different isolates (Bateson et al., 1994).
CONCLUSIONS

PRSV-P was first reported in Australia in 1991, and is therefore a relatively new pathogen for the local industry. As a result of the implementation of strict quarantine measures, it has been confined to southeast Queensland, its original outbreak location. Tropical north Queensland, the area of Australia’s primary production of papaya, remains virus free. However, in many countries PRSV-P has spread despite quarantine and traditional control measures, causing devastating losses in those countries (Gonsalves, 1998).

Four wild species related to *C. papaya* have been known for 40 years to exhibit broad-spectrum resistance to a wide range of PRSV-P strains. Access to these genes for resistance has been limited by the difficulties in crossing *C. papaya* with the related species. Recent DNA marker and sequencing studies on the genetic diversity of these species has elucidated the problem. *C. papaya* was shown to be quite different from its relatives (Jobin-Decor et al., 1996; Manshardt and Drew, 1998; Kim et al., 2002) and Badillo (2000) has recommended that the genus *Carica* retain only the species *papaya*, and that the remaining 21 former *Carica* species be placed in a separate genus, *Vasconcella*. Intergeneric, rather than interspecific crosses are difficult to achieve. Because *V. cauliflora* is genetically the most distant of the wild relatives from *C. papaya*, the results described in this paper indicate there has been an overemphasis on hybridization between *C. papaya* and *V. cauliflora*. Success is more likely if future efforts are applied to crosses between *C. papaya* and *V. quercifolia*.

The gene transfer strategy in Australia has involved determining the level of variation that existed in the virus population prior to selection of the transgene, developing transformation protocols for Australian papaya cultivars and developing genetically engineered resistance before the spread of the virus to the major growing areas. Stable resistance has been achieved in two Australian transformed cultivars. This resistance should be effective against other strains that may be derived from Australian PRSV-P, given the close similarity of all isolates tested so far in terms of nucleotide sequence homology. Long-term stability of transgenic resistance to PRSV-P in papaya is yet to be assessed, however it shows promise in many countries and is being used commercially in Hawaii (Ferreira et al., 2002).

Both approaches to solving the PRSV-P problem offer advantages and disadvantages. Using gene transfer, PRSV-P resistance can be added to a papaya genotype without altering the remainder of the genome. By contrast, interspecific hybridization requires a long backcrossing program. However, gene transfer requires embryogenic callus and this can only be obtained from zygotic embryos. Consequently, it is not possible to apply gene transfer protocols directly to an elite female or hermaphrodite cultivar, only to its progeny. In addition, gene transfer is hindered by patent obligations, legal and environmental restrictions on field trials and by consumer resistance to genetically modified food crops. Interspecific hybridization is not affected by these complications. However, both techniques provide valuable sources of virus resistance and continued investigation of both is warranted.

Literature Cited


