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Screening *Carica papaya* × *C. cauliflora* hybrids for resistance to papaya ringspot virus-type P

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Carica papaya, *C. cauliflora* and interspecific hybrids of these species were screened for resistance to two Australian isolates (338, 445) of papaya ringspot virus-type P (PRSV-P). Plants were manually inoculated with PRSV-P in the glasshouse and the reaction assessed 30 days later by back-inoculation to susceptible *Cucurbita pepo* and by a plate-trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA). Both parents and interspecific hybrids were also planted adjacent to infected *C. papaya* and 30 days later tested for PRSV-P by PTA-ELISA. All interspecific hybrid and *C. cauliflora* plants manually inoculated in the glasshouse or planted in the field failed to become infected, whereas *C. papaya* plants, in both situations, were infected by PRSV-P. In addition, the surviving interspecific hybrid and *C. cauliflora* plants tested negative, while all *C. papaya* plants were positive for PRSV-P in both the back-inoculation and PTA-ELISA tests. Thus, the interspecific hybrid and *C. cauliflora* plants were resistant to the Australian PRSV-P isolates.

INTRODUCTION

The papaya (*Carica papaya*) is grown for its popular melon-like fruit, commonly used as a dessert. In addition, the unripe fruit is a good source of papain, an enzyme used in the brewing of beer, as an ingredient in the manufacture of drugs and cosmetics, as an agent for degumming natural silk and as a shrink-resistance treatment for wool. The annual production of *C. papaya* worldwide is approximately 5.7 m tonnes (Food and Agriculture Organization of the United Nations, 1993). In Australia, the total area planted to *C. papaya* is approximately 500 ha, with an annual production of 7000 tonnes of fruit valued at $A 10–12 million (National Farmers Federation, 1993).

The most destructive disease of *C. papaya* worldwide is papaya ringspot caused by papaya ringspot virus-type P (PRSV-P, Litz, 1984; Manshardt, 1992), a definitive potyvirus species in the *Potyviridae* (Shukla *et al.*, 1994). Typical symptoms of PRSV-P on *C. papaya* include ringspots on the fruit, water-soaked lesions on the petioles and stem and chlorosis or mottle on the leaves. Infected seedlings seldom reach maturity, while severely infected adult plants decline rapidly and fail to produce fruit (Manshardt, 1992). The poor growth and low fruit yield are often the result of decreased photosynthesis and increased respiration induced by the virus (Marler *et al.*, 1993). Papaya ringspot was first detected in Australia in 1991 and, although currently confined to parts of south-east Queensland, it is a major threat to the Queensland industry (Thomas & Dodman, 1993).

Control measures used against PRSV-P include cultural practices, cross-protection and the planting of tolerant cultivars (Gonsalves, 1994). None of these has been very successful and the development of virus resistant cultivars is the most reliable solution for long-term control. None of the *C. papaya* cultivars tested has natural resistance (or immunity) to PRSV-P (Cook & Zettler, 1970; Magdalita *et al.*, 1988). However, several wild *Carica* species such as *C. cauliflora*, *C. pubescens* and *C. quercifolia* are resistant to PRSV-P (Horovitz & Jiménez, 1967). Conventional interspecific hybridization of *C. papaya* with these species has been difficult because of interspecific reproductive barriers (Manshardt & Wenslaff,

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1989). In Australia, interspecific hybrids between *C. papaya* and *C. cauliflora* were produced *via* interspecific hybridization and embryo rescue (Magdalita *et al.*, 1996). It was confirmed that all plants were hybrids of these two species by the use of morphological, random amplified polymorphic DNA and cytological markers (Magdalita *et al.*, 1997). However, it is unknown if these hybrids have PRSV-P resistance and their resistance status is critical to their future role in the breeding programme.

The enzyme-linked immunosorbent assay (ELISA), a powerful immunological test (Clark & Adams, 1977), is extensively used for detecting, identifying and quantifying viruses in many plant species (Clark, 1994). This test could be developed as a component of a reliable method for screening *C. papaya* × *C. cauliflora* hybrid plants for PRSV-P resistance. In this study, the resistance of the hybrid and parents was assessed by serological and biological screening following manual inoculation with two Australian isolates of PRSV-P and also exposure to natural field infection.

MATERIALS AND METHODS

Plant material and screening sites

C. papaya × *C. cauliflora* hybrid seedlings produced via hybridization and embryo rescue (Magdalita *et al.*, 1996) and true-to-type seedlings of *C. papaya* and *C. cauliflora* were used. The 120-day-old hybrid and parent seedlings were grown in a glasshouse at Redlands Research Station, Cleveland, Queensland and then transferred to a glasshouse in the Plant Protection Unit, Indooroopilly, Queensland for PRSV-P screening. Seven-day-old zucchini squash (*Cucurbita pepo*) seedlings were used for back-inoculation tests. Zucchini squash is highly susceptible to PRSV-P (Gonsalves & Ishii, 1980) and has been identified as a good host for virus isolation and propagation (Persley & Thomas, 1995). Field screening was undertaken at Bridgeman Downs, Queensland. All the screening sites were located around Brisbane, Queensland, Australia (27°S 28′, 153°E 01′).

Glasshouse and laboratory screening

The interspecific hybrid and parent plants were screened for PRSV-P resistance by manual inoculation. In total, 114 interspecific hybrids, three *C. papaya* and three *C. cauliflora* plants in a completely randomized design were inoculated with PRSV-P isolates 338 and 445 (Persley & Thomas, 1995) that had been maintained in zucchini squash cv. 'Green Ruffles'. The inoculum was prepared by homogenising infected squash leaf tissue in 0.1 M potassium phosphate, pH 7.0, containing sodium sulphite (1% w/v) and applied by gentle rubbing onto carborundum-dusted leaves. All plants were inoculated twice, with a 7-day interval between inoculations. Since PRSV-P symptoms develop within 25 days after inoculation under Queensland conditions (Thomas & Dodman, 1993), assessment of symptoms was made for up to 30 days. A randomly selected plant of the interspecific hybrid, *C. papaya* or *C. cauliflora* was maintained as a noninoculated control.

The interspecific hybrid and parent plants were screened for the presence or absence of PRSV-P by observing whether viral symptoms could be reproduced in a susceptible host (zucchini squash) after back-inoculation. Leaf samples from each of the 22 hybrid, 6 parent and control plants were homogenized in potassium phosphate solution (described above) and inoculated to squash seedlings, at least 10 seedlings for each sample. Inoculated seedlings were maintained in a glasshouse and assessed over 30 days for symptom development.

All plants were tested for PRSV-P 30 days after initial inoculation using a plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA, Mowat & Dawson, 1987) previously standardized for *C. papaya* (Thomas & Dodman, 1993). Leaf samples (0.1 g fresh weight) from each plant were homogenised (2 mL) in a carbonate coating buffer (2:5 v/v; 50 mM Na_2CO_3, 50 mM $NaHCO_3$ at pH 9.6) using a mortar and pestle. The samples (100 TL) were loaded individually into wells in the inside 6 rows and 10 columns of an ELISA microtitre plate (Dynatech Immulon 3 plates; Dynatech Laboratories Inc., Chantily, VA, USA). The plate was then covered with a plastic lid, placed in a box lined with moist paper towel and incubated at 5°C for 12 h. Two replicate wells were used for each sample. Duplicate sap extracts from noninoculated plants were also included as relevant controls. The two antisera used were PRSV-P 338 prepared in Queensland (Persley & Thomas, 1995) and PRSV-P HA kindly provided by Professor S. D. Yeh (National Chung-Hsing University, Taiwan). The antiserum was diluted (1:1000) with sap from noninoculated *C. papaya* and the hybrid that had been homogenised in a potassium phosphate saline solution (1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl plus 0.05% Tween 20, v/v; pH 7.4). This mixture was loaded on each well (100 TL) of the ELISA microtitre plate and incubated for 3 h at room temperature.

Table 1 The number of plants that were inoculated, that survived and that had visible symptoms of papaya ringspot virus-type P (PRSV-P). Reactions of the survivors to back-inoculation (BI) and to a plate-trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) are also shown (absorbance units at 410 nm). A positive (+) reaction indicates presence of the virus and a negative (−) reaction its absence

Treatment	Genotype	Number inoculated	Number surviving	Plants with symptoms	BI	PTA-ELISA (absorbance units)
Inoculated	Hybrid	114	22	0	−	0.054 ± 0.006
	C. papaya	3	3	3	+	0.603 ± 0.008
	C. cauliflora	3	3	0	−	0.020 ± 0.020
Non-inoculated	Hybrid	3	3	0	−	0.073 ± 0.020
	C. papaya	3	3	0	−	0.030 ± 0.001
	C. cauliflora	3	3	0	−	0.010 ± 0.005

Each well was washed 3 times between each step with potassium phosphate saline solution. The wells were then coated with a goat antirabbit alkaline phosphatase IgG conjugate (Sigma Chemical Co., St Louis, MI, USA) and incubated again for 3 h at room temperature. A *p*-nitrophenyl phosphate substrate (1:1, w/v in diethanolamine, pH 9.8) was added (100 TL) into the wells and incubated further for 30 min at laboratory temperature. Finally, the reaction absorbance (at 410 nm) was read using an ELISA plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantily, VA, USA). Samples were considered positive for PRSV-P infection when the mean absorbance (at 410 nm) exceeded twice the mean absorbance (at 410 nm) of the noninoculated controls (Thomas & Dodman, 1993).

Field screening

Twenty hybrids, three *C. papaya* and three *C. cauliflora* plants were manually inoculated with PRSV-P three days prior to planting in the field. The plants were 1.5 m apart in a field plot (37.5 m long, 1 m wide) aligned in a north–south direction. This plot was located in an area close to 78 other plants (approximately 1–2 years old) comprising various *Carica* species, other *Carica* interspecific hybrids and *C. papaya* cultivars infected with PRSV-P. PRSV-P symptoms on the survivors were assessed visually as described in the glasshouse experiment. The plants were grown using the standard cultural and management practices (O'Hare, 1993) for *C. papaya* in south-east Queensland.

All field-grown plants were tested 30 days later for the presence of PRSV-P using a PTA-ELISA. Leaf samples from these field-grown plants were collected and stored on ice. Control samples were from single, noninoculated plants of the hybrid, *C. papaya* and *C. cauliflora* grown in the glasshouse. All samples had replicate wells for the PTA-ELISA.

RESULTS

Glasshouse and laboratory screening

No PRSV-P symptoms were seen on any of the surviving hybrids 30 days after inoculation (Table 1). All other hybrids died, possibly as a result of hybrid breakdown, before symptoms could be assessed. No virus symptoms were seen on *C. cauliflora*, but the *C. papaya* plants developed typical PRSV-P symptoms, including leaf mottling and chlorosis, and water-soaked lesions on the petioles. The noninoculated control plants did not develop PRSV-P symptoms.

All the surviving hybrids and the *C. cauliflora* plants were negative for PRSV-P in the back-inoculation test (Table 1). The *C. papaya* plants all tested positive, symptoms on the back-inoculated squash plants being typical for PRSV-P and including leaf mottling and chlorosis. All noninoculated hybrid, *C. papaya* and *C. cauliflora* plants tested negative.

In the PTA-ELISA test (Table 1), the mean absorbance values (at 410 nm) for all hybrid and *C. cauliflora* plant samples were less than twice those of their noninoculated controls, indicating the absence of PRSV-P. Values for inoculated *C. papaya* plants were many times higher than those of the noninoculated controls, indicating the presence of PRSV-P.

Field screening

None of the surviving hybrid plants developed PRSV-P symptoms when observed 33 days after

Table 2 The number of plants that were inoculated or field-planted, that survived and that had symptoms of papaya ringspot virus-type P (PRSV-P). Reactions of the survivors to a plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) are also shown (absorbance units at 410 nm)

Treatment	Genotype	Number inoculated or field-planted	Number surviving	Plants with symptoms	PTA-ELISA (absorbance units)
Inoculated	Hybrid	20	12	0	0.087 ± 0.002
	C. papaya	3	3	3	0.257 ± 0.001
	C. cauliflora	3	3	0	0.034 ± 0.005
Non-inoculated	Hybrid	1	1	0	0.055 ± 0.002
	C. papaya	1	1	0	0.025 ± 0.002
	C. cauliflora	1	1	0	0.032 ± 0.002

inoculation or 30 days after, when planted in the field where PRSV-P was present (Table 2). The death of 8 plants was possibly due to hybrid breakdown. All the *C. papaya* plants, but none of *C. cauliflora*, developed PRSV-P symptoms. The noninoculated hybrid, *C. papaya* and *C. cauliflora* plants remained free from symptoms.

All hybrid survivors and *C. cauliflora* plants tested negative while *C. papaya* plants tested positive for PRSV-P as shown by PTA-ELISA (Table 2). The noninoculated hybrid, *C. papaya* and *C. cauliflora* plants tested negative.

DISCUSSION

Three tests (manual or back-inoculation, PTA-ELISA and field-planting in an area with high PRSV-P inoculum) were used in combination to determine if hybrid plants were resistant to the Australian PRSV-P strain. When the interspecific hybrid and *C. cauliflora* plants were examined, all were found to be resistant while all *C. papaya* plants were susceptible (Tables 1 and 2). This suggests that the hybrids had inherited resistance to PRSV-P from *C. cauliflora*. This observation confirms an earlier report of PRSV-P resistance in *C. cauliflora* × *C. papaya* hybrids (Manshardt, 1992).

The failure of PRSV-P symptoms to develop on the manually inoculated or field-grown hybrid plants indicates that these plants were resistant to both Australian PRSV-P isolates used. It is not known whether resistance is monogenic or polygenic. However, it appears that resistance is dominant as there was no variation in the level of resistance observed among the surviving F_1 plants. It is not known why some hybrid plants died. The possibility that the nonsurviving hybrids were susceptible F_1 progenies can not be discounted but it is considered more likely that they died because of hybrid weakness. The death of hundreds of plants that were not inoculated with PRSV-P (Magdalita *et al.*, 1997) indicates that hybrid weakness is a problem with this cross. Furthermore, the hybrid plants were highly sterile; one surviving plant did not flower for almost two years. These are typical characteristics of aneuploidy. In an earlier study, many of these hybrids were shown to be aneuploid mosaics ($2n = 16-18$; Magdalita *et al.*, 1997). Nevertheless, some reasonably vigorous, PRSV-P resistant hybrid plants were produced and they are now being maintained by *in vitro* micropropagation. In the future, it may be possible to treat these hybrids with chemicals that induce flowering (e.g. gibberellins), or chemicals that inhibit spindle fibre formation (e.g. colchicine) to produce fertile tetraploid plants that can be used for backcrossing to *C. papaya*.

Resistance to PRSV-P in papaya is also being sought by a transformation approach using pathogen-derived resistance (Fitch *et al.*, 1992). However, this approach is very strain specific, requiring transformation programs using PRSV-P isolates from each area where protection is required (Tennant *et al.*, 1994). Despite genetic incompatibility problems encountered with interspecific hybridization, it is worth pursuing as *C. cauliflora* and other wild species are resistant to many isolates of PRSV-P worldwide, in Hawaii, Australia, Philippines, South America (Alvizo & Rojkind, 1987; Magdalita *et al.*, 1988) and so offer a potentially durable source of resistance against a widespread and damaging disease. Therefore, both approaches could be exploited to introduce PRSV-P resistance into papaya. An ELISA test has been used previously to identify PRSV-P infected *C. papaya* (Gonsalves & Ishii, 1980; Thomas & Dodman, 1993). This test is more sensitive and convenient than back-inoculation tests when large

numbers of plants have to be screened (Miller & Martin, 1988). In addition, the accuracy of a PTA-ELISA is not influenced by the environmental conditions under which the plants are grown, as can be the case with back-inoculation tests (Magdalita *et al.*, 1988). Therefore the PTA-ELISA is a rapid and reliable diagnostic test available to confirm the resistance status of plants.

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