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Suppression of *Papaya ringspot virus* infection in *Carica papaya* with CAP-34, a systemic antiviral resistance inducing protein from *Clerodendrum aculeatum*

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Abstract CAP-34, a protein from *Clerodendrum aculeatum* inducing systemic antiviral resistance was evaluated for control of *Papaya ringspot virus* (PRSV) infection in *Carica papaya*. In control plants (treated with CAP-34 extraction buffer) systemic mosaic became visible around 20 days that intensified up to 30 days in 56% plants. During this period, CAP-34-treated papaya did not show any symptoms. Between 30 and 60 days, 95% control plants exhibited symptoms ranging from mosaic to filiformy. In the treated set during the same period, symptoms appeared in only 10% plants, but were restricted to mild mosaic. Presence of PRSV was determined in induced-resistant papaya at the respective observation times by bioassay, plate ELISA, immunoblot and RT-PCR. Back-inoculation with sap from inoculated resistant plants onto *Chenopodium quinoa* did not show presence of virus. The difference between control and treated sets was also evident in plate-ELISA and immunoblot using antiserum raised against PRSV. PRSV RNA was not detectable in treated plants that did not show symptoms by RT-PCR. Control plants at the same time showed a high intensity band similar to the positive control. We therefore suggest that the absence/delayed appearance of symptoms in treated plants could be due to suppressed virus replication.

Keywords Induced systemic resistance · Inhibition of virus replication · RT-PCR · Immunoblot · ELISA

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Papaya is an economically important plant and prone to several viral diseases (Verma and Prasad 1986). The disease caused by *Papaya ringspot potyvirus* with its symptoms of mosaic, severe distortion, filiformy and fruit malformation (Jensen 1949) is highly devastating and creates serious commercial problems for the papaya grower (Gonsalves 1998). Modern approaches to control viral infections in plants include induction of systemic resistance (Prasad and Srivastava 2001; Ritzenthaler 2005), and pathogen-derived resistance (Chen et al. 2001; Chiang et al. 2007; Tennant et al. 2005). In more recent times, gene silencing mechanisms and RNA interference have been considered worthy (Krubphachaya et al. 2007). Induced systemic resistance (ISR) leads to systemic resistance in susceptible plants, induced by rhizobacteria and higher plant proteins (Prasad and Srivastava 2001; Van Loon et al. 1998). Systemic resistance-inducing proteins from non-host plants such as *Clerodendrum inerme*, *C. aculeatum*, and

Boerhaavia diffusa have been characterised (Prasad et al. 1995; Verma and Awasthi 1979; Verma et al. 1996). In most cases, these proteins are basic and range between 29 and 34 kDa e.g. CIP-29 from *C. inerme*, BD-30 from *B. diffusa* and CAP-34 from *C. aculeatum* have molecular masses of 29, 30, and 34 kDa, respectively. While Verma et al. (1996) named the protein from *C. aculeatum* as CA-SRIP, we are calling it CAP-34 based on its molecular weight, in conformity with the others. In this study, we examined the potency of CAP-34 to protect papaya against PRSV following mechanical inoculation under glasshouse conditions. We demonstrate that CAP-34 is able to suppress systemic PRSV infection of papaya, and suggest through symptomatic, serological and RT-PCR evidence that the effect of CAP-34 could be through suppressing PRSV replication.

Experimental *Carica papaya* and *Chenopodium quinoa* plants were maintained in an insect-free glasshouse. *Carica papaya* is the systemic host for PRSV and *C. quinoa* is a local lesion host for PRSV. Two month-old papaya plants were taken for experimental work, whereas *C. quinoa* was used at the six-leaf stage for bioassay. CAP-34 was purified to electrophoretic homogeneity (Verma et al. 1996), and was used to induce resistance against PRSV in both hypersensitive and systemic hosts. Three basal leaves of *C. quinoa* plant were treated with CAP-34 at concentrations of 40, 80 and 120 μg ml^{-1}. The treated as well as non-treated leaves were challenge-inoculated with PRSV 24 h later. Dilution of PRSV inoculum was adjusted so as to give 22–25 lesions per leaf on *C. quinoa*. Lesions that appeared 7 days post-inoculation were recorded. Systemic anti-PRSV resistance was expressed as a decrease in lesion number at remote site. While no lesions appeared in plants treated with 120 μg ml^{-1} CAP-34, a few lesions did appear as the CAP-34 concentration was reduced; however, resistance was still induced to substantial levels with 40 μg ml^{-1} CAP-34 (Table 1).

Experiments with systemic infections were carried out on glasshouse-grown 2 month-old *C. papaya*. Susceptible papaya were treated with CAP-34 weekly for 3 weeks, at the end of which they were challenged with PRSV. Control sets of papaya were mock-treated weekly with buffer before being challenge-inoculated. Subsequent to PRSV challenge, the weekly treatments of CAP-34 were continued. Observations were recorded up to 2 months post-inoculation. Disease grade was rated using the following scale: 0: no symptoms, 5: mosaic, 7.5: mosaic and distortion, 10: mosaic, distortion and filiformy. Disease severity was calculated using the formula of Yang et al. (1996):

$$\text{Disease severity} = \frac{(\text{Disease grade} \times \text{number of symptomatic plants in each grade}) \times 100}{(\text{Total number of plants})\ (\text{Highest disease grade})}$$

Mosaic appeared 20 days after inoculation in control plants, whereas CAP-34-treated plants did not show any symptoms. Severity of symptoms increased from mosaic to filiformy 60 days post-inoculation in 95% control plants whereas only 10% treated plants showed symptoms after 60 days, but only mild mosaic (Table 2).

Detection of PRSV in induced-resistant (CAP-34 treated) and control-susceptible (buffer treated) papaya was made 30 and 60 days post-PRSV challenge through bioassay, plate-ELISA, immunoblot and RT-PCR. Leaves of induced-resistant and susceptible papaya challenged with PRSV were harvested 30 and 60 days post-inoculation, ground in 200 mM sodium acetate buffer pH 5.2 at a dilution of 1:1 (*w*/*v*) and the sap inoculated on *C. quinoa*. Lesions were counted 7 days after inoculation. Three replicates of five plants each were used.

Indirect plate-ELISA was carried out as described by Clark and Adam (1977) for detecting PRSV in plants of treated and control sets. Polyclonal antibodies raised against PRSV in rabbit were used (antiserum was a gift from Dr. R.K Jain, IARI, New Delhi). Both anti-PRSV (primary antibody) and goat anti-rabbit IgG-alkaline phosphatase conjugate (secondary antibody) were used at a dilution of 1:1,000 (*v*/*v*) in PBS-T. The reaction was developed by addition of substrate *p*-nitrophenyl phosphate (Bangalore Genei, India). Absorbance was read at 405 nm by an ELISA plate reader (BioRad Laboratories, USA).

Presence or absence of PRSV in susceptible and induced-resistant papaya was reconfirmed by

Table 1 Systemic resistance induction by CAP-34 in *Chenopodium quinoa* against *Papaya ringspot virus*[a]

Concentration of CAP-34 (μg ml^{-1})	Average number of local lesions± SEM[b]	
	Control plants	Treated plants (remote untreated site)
120		0.0±0.0
80	22±1.3	1.0±0.37
40		5±0.94

[a] Buffer/CAP-34 was applied on three basal leaves of a six-leaved *Chenopodium quinoa* plant. PRSV challenge was made 24 h post-treatment and lesions were counted 7 days post-inoculation. Three replicates with five plants per replicate were maintained. Lesion numbers shown are for the remote untreated leaf to demonstrate systemic induction.

[b] Standard error mean

immunoblot. Leaf sap of treated and control papaya was extracted as before, clarified and electrophoresced on SDS-PAGE (Laemmli 1970). Western transfer and immunoblot was carried out as described by Towbin et al. (1979). Both antiserum raised against PRSV (primary antibody) and goat anti-rabbit antibodies conjugated with alkaline phosphatase (secondary antibody; Sigma Chemical Co, USA) were diluted to 1:1,000 in TBS-T. Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co, USA) served as substrate.

To confirm the presence or absence of PRSV RNA in challenged papaya, total RNA was extracted from control and treated papaya after 30 and 60 days of PRSV challenge inoculation with the help of RNEasy plant RNA extraction kit (Qiagen, Germany). RT-PCR was carried out with primers specific to PRSV CP having the following sequence, forward 5′ GAATGAAGCTGTGGATGCTGGTTTGAA 3′ and reverse 5′ TCAGTTGCGCATACCCAGGAGAGTG CATG 3′, which were formulated from the conserved region in the CP gene of some Indian PRSV strains (Jain et al. 2004). Reverse transcription was carried out by incubating 3 μl RNA with 2 μl MMuLV reverse transcriptase along with the primers, and 2 μl 10 mM dNTPs each (Fermentas Inc., USA) at 50°C for 45 min; 2 μl *Taq* polymerase (Fermentas Inc., USA) was then added to the reaction mixture for PCR in an MJ PTC-150 thermal cycler (MJ Research, USA), programmed as follows: 92°C×30 s, 50°C×45 s, 72°C×45 s; 30 cycles were given with a final extension at 72°C for 10 min. The RT-PCR products were electrophoresced on a 1% agarose gel in Tris–borate–EDTA buffer, stained with ethidium bromide, visualised on a UV transilluminator, and photographed.

The data presented is at two specific points, 30 and 60 days post-PRSV challenge. The PRSV-challenged control plants showed symptoms about 18 to 20 days after challenge, while those treated with CAP-34 did not. The observation at 30 days showed considerable mosaic on the control plants, but nothing on treated papaya. The control plant leaves developed distortions, and acute filiformy 45 to 50 days after PRSV inoculation, presented in our data at 60 days. At 60 days, 10% CAP-34 treated plants showed mild mosaic, the majority remaining symptomless (Table 2). No filiformy was seen in these 10% plants (Fig. 1). Clearly, CAP-34 treatment had not allowed symptoms

Table 2 Systemic resistance induction by CAP-34 in papaya against *Papaya ringspot virus*[a]

Parameters		Observation days			
		Buffer-treated papaya		CAP-34-treated papaya	
		30 days p.i.[b]	60 days p.i.	30 days p.i.	60 days p.i.
Symptoms	Mosaic	+++	+++++	−	+
	Distortion	+	+++	−	−
	Filiformy	−	++	−	−
Disease grade		7.5	10	0	5
Calculated disease severity (%)		56	95	0	10

[a] Leaves of papaya plants were treated once a week with buffer/CAP-34 for 3 weeks. PRSV challenge was made 24 h after the third treatment. Observations were recorded 30 and 60 days post-inoculation. Three replicates with 20 plants in each were maintained. Assignation of disease grade and calculation of disease severity were as given in text.

[b] Post-inoculation

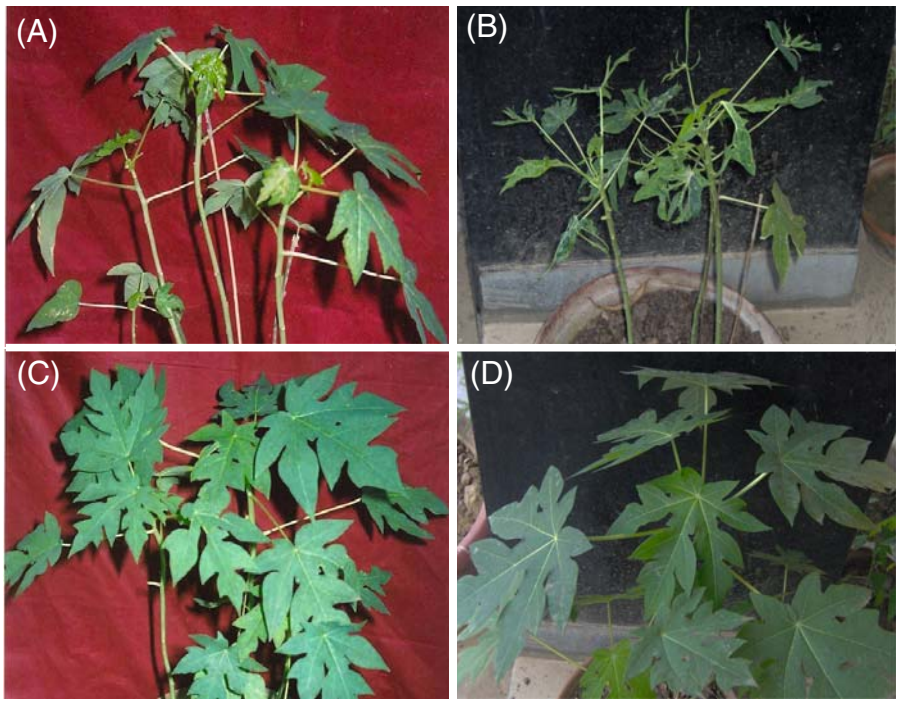

Fig. 1 Effect of PRSV infection on papaya plants treated with buffer and CAP-34. **a**, **b** show Control plants 30 and 60 days after PRSV challenge, while **c**, **d** show CAP-34-treated plants 30 and 60 days post-PRSV inoculation

to develop at all in the majority, and suppressed the infection/symptom appearance in the rest. Upon back-inoculation, lesions developed on *C. quinoa* leaves inoculated with sap from control papaya (10±0.84 for 30 days; 15±1.33 for 60 days), but no lesions were seen with sap from CAP-34-treated papaya in both 30 and 60 day samples (Fig. 2a). It was therefore not just a case of lack of symptoms, but actually an absence of PRSV itself. Results of plate-ELISA (Fig. 2b) showed that values of samples from control papaya challenge-inoculated with PRSV were higher than blank (0.352± 0.025) by more than 1.5 times in the 30-day samples (0.540±0.036), and about 2.5 times (0.805±0.07) that of blank 60 days post-inoculation. This demonstrated that PRSV was present and replicating in the control papaya. In CAP-34-treated papaya, samples taken 30 and 60 days after challenge, however, the ELISA readings (0.376±0.047 and 0.392±0.012, respectively) remained close to the blank values (Fig. 2b). Paired *t* test of the ELISA of 30 and 60 day samples between control and treated gave $P=0.1881$ and 0.0294 (significant), respectively. *P* values for samples from both these observation stages for back-inoculation lesions were 0.0003, which is statistically extremely significant.

We also searched for the PRSV CP by immunoblot. CP was clearly visualised in samples showing symptoms in non-induced control plants while it was not detected in 30 day-old induced-resistant papaya. Further, while the antigen–antibody recognition was pronounced in control plants 60 days post-PRSV challenge, there also appeared a faint recognition band in the resistant 60 days post-inoculation sample taken from the 10% plants with mild mosaic (Fig. 2c).

To bolster our argument that CAP-34 induced resistance operates through suppressing PRSV replication, RT-PCR was carried out using PRSV CP specific primers. When the amplification products were electrophoresced on an agarose gel, they showed intense bands of the correct size (approx. 900 bp) in the positive control, as well as in the 30 and 60 day post-PRSV challenged buffer-treated papaya (Fig. 2d). Nothing was visible in CAP-34-treated 30 day post-PRSV challenged papaya; however, a faint band corresponding to the PRSV CP gene size was detected in the 60 day samples from CAP-34-treated plants exhibiting mild mosaic (Fig. 2d). Also, RT-PCR was carried out with both RNA from treated plants that showed very mild symptoms, and RNA from treated plants that did not show any

symptoms at all. Those plants that did not show any symptoms were also devoid of PRSV RNA.

Plant viral disease control has remained elusive for want of a mechanism to check viral replication in the infected plants. Our evidence shows beyond doubt that in CAP-34-treated papaya, PRSV replication is indeed suppressed. Recently, Picard et al. (2005) showed that pokeweed antiviral protein (PAP), a ribosome-inactivating protein from *Phytolacca americana*, inhibited *Brome mosaic virus* replication in plant cells. The difference however, is that CAP-34 suppresses viral replication following induction of resistance in planta as against PAP that was incubated with BMV RNA3 before inoculation.

Several control measures have been tried to contain the damage caused by PRSV, and viruses infecting plants in general (Chen et al. 2001; Chiang et al. 2007; Krubphachaya et al. 2007). Recent use of transgenic technology has held out promise; however, pathogen-derived resistance comes with its own pitfalls (Tripathi et al. 2004). In the backdrop of the search for an efficient and viable viral disease control model in plants, our system of ISR clearly seems a good option, especially since we demonstrate

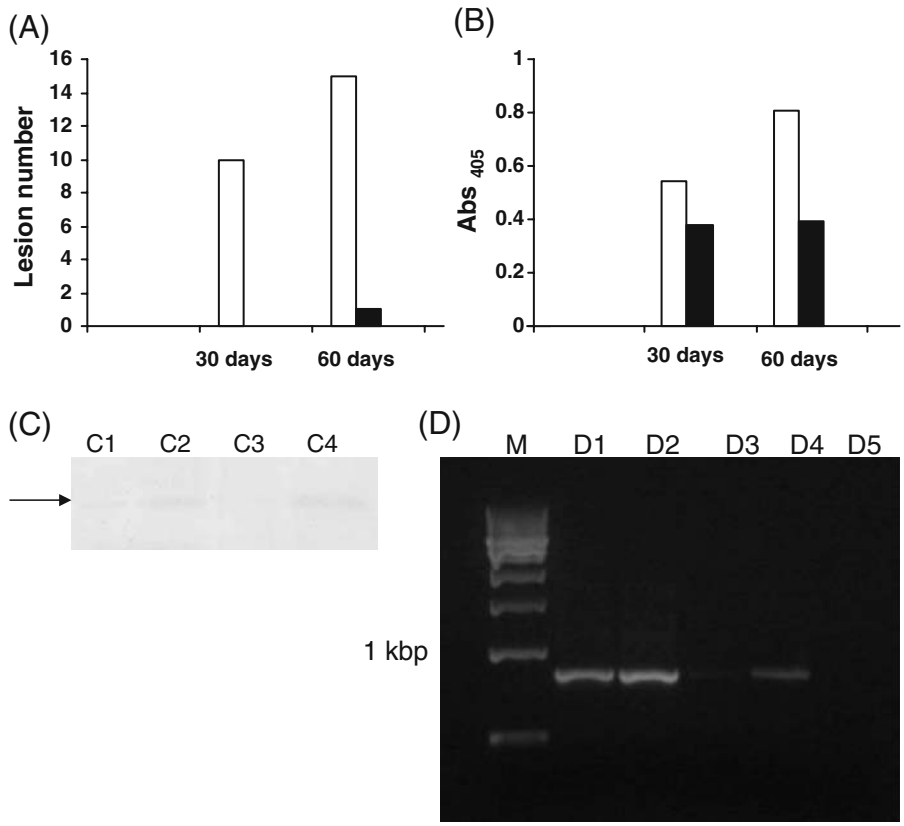

Fig. 2 Determination of presence of viral coat protein/RNA by back-inoculation (**a**) plate-ELISA (**b**) immunoblot (**c**) and RT-PCR (**d**) in papaya leaf after treatment with buffer and CAP-34 weekly for 3 weeks and then challenge-inoculation with PRSV. **a** demonstrates that back-inoculations from control papaya show presence and increase in lesions on *Chenopodium quinoa* (*white bars*) with time, while treated papaya sap shows occasional lesions from papaya with mild mosaic (*black bars*). ELISA results (**b**) show virtual absence of PRSV in treated papaya (*black bars*, data equivalent to blank) and presence as well as increase in PRSV CP at 30 and 60 days control (*white bars*). In **c**, lanes *C1* and *C2* show CP in 30 and 60 day control plants, lane *C3* with 30 day treated sample shows no CP while lane *C4* has sample from 60 day treated papaya showing mild mosaic. In **d**, RT-PCR shows PRSV RNA in 30 and 60 day control (lanes *D1* and *D2*) while no RNA is seen in 30 and 60 day treated symptomless papaya (lanes *D3* and *D5*). Lane *D4* shows presence of some virus in 60 day treated papaya with mild mosaic. Lane *M* carries 500 bp ladder

that CAP-34 treatment places a check on PRSV replication.

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