

## SHORT COMMUNICATION

RT-PCR DETECTION AND PROTEIN-PROTEIN INTERACTION OF VIRAL COMPONENTS OF *PINEAPPLE MEALYBUG WILT-ASSOCIATED VIRUS 2* IN CUBA

E.G. Borroto-Fernández, J.A. Torres-Acosta and M. Laimer

*Plant Biotechnology Unit, Institute of Applied Microbiology BOKU, Nussdorfer Lände 11, A-1190 Vienna, Austria*

## SUMMARY

During a search for the causal agent of symptoms of mealybug wilt in Cuban pineapple plants, *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) was detected. A reverse transcription polymerase chain reaction (RT-PCR) test was developed to amplify seven ORFs using primers designed on the RNA sequence of a Hawaiian isolate of PMWaV-2. Cuban and the Hawaiian PMWaV-2 isolates shared high sequence homology. Yeast two-hybrid assays showed the homodimeric nature of the 20 kDa protein (p20) and the 22 kDa protein (p22), but not of the 34 kDa coat protein (CP). This is the first report of protein-protein interactions between isolated PMWaV-2 proteins.

*Key words:* *Ampelovirus*, electron microscopy, *Pineapple mealybug wilt-associated virus-2*, yeast two-hybrid system.

Mealybug wilt of pineapple (MWP) was first described in Hawaii and since then has been reported as a severe disease of pineapple crops worldwide (Carter, 1942; Rohrbach *et al.*, 1988; Hu *et al.*, 1993; Borroto *et al.*, 1998; Hughes and Samita, 1998). Leaves of MWP-affected plants turn bronze-red in colour, loose turgidity and show severe tip necrosis (Rohrbach *et al.*, 1988); symptoms are cultivar-dependent and in some cases plants recover (Sether and Hu, 2002). The metabolic changes induced in pineapple (cv. Smooth Cayenne) with strong MWP symptoms include the appearance of high levels of abscisic acid, soluble proteins, free proline and phenols, coupled with rises in peroxidase and acid invertase activities (Nieves *et al.*, 1996).

Although the involvement of viral agents and insect vectors is now accepted, the etiology of MWP has been controversial. The association of ampelovirus particles and mealybug species with the disease is long known (Hu *et al.*, 1996; Sether and Hu, 2002). Gunashinge and German (1989) identified long, flexuous, rod-shaped,

virus-like particles and isolated double-stranded RNA from MWP-diseased plants. Members of the *Closteroviridae* family have been associated with MWP in Hawaii, Australia and Cuba (Gunashinge and German, 1989; Hu *et al.*, 1993; Wakman *et al.*, 1995; Borroto *et al.*, 1998). In accessions around the world, at least three species of PMWaVs, (genus *Ampelovirus*, family *Closteroviridae*) exist (Melzer *et al.*, 2001; Martelli *et al.*, 2005; Sether *et al.*, 2005). As partially characterized, genome sizes were reported of 10.7 kb for PMWaV-1 (seven ORFs; accession no. AF414119) and 14.8 kb for PMWaV-2 (ten ORFs; accession no. AF283103), respectively (Melzer *et al.*, 2001). Among ampeloviruses, PMWaV-2 shares the highest sequence identity with *Grapevine leafroll-associated virus-3* (Melzer *et al.*, 2001).

In Cuba, MWP disease is an important economic problem for pineapple production, causing crop losses of up to 40% (Anonymous, 1989). Initial work undertaken to develop efficient detection tools confirmed the presence of closterovirus-like particles in MWP-affected pineapple plants (*Ananas comosus* cv. (L) Merr.) of cv Smooth Cayenne by electron microscopy (EM) (Borroto *et al.*, 1998).

The molecular analysis of Cuban isolates of pineapple ampeloviruses was done in order to further characterize the presence of PMWaV-2 in Cuba. Virus purification and EM were used to provide information about the size and shape of virus particles as well as to select infected plants for subsequent molecular characterization. Thus, RT-PCR in combination with sequencing was used to identify genomic sequences of PMWaV-2 and protein-protein interactions of some viral components were studied to gain a better insight of fundamental mechanisms of virus functions.

Pineapple plants of cv Smooth Cayenne originating from tissue culture propagation were cultivated in open field conditions at the "Tomás Roig" Experimental station at Ciego de Avila, Cuba. Leaf tissue of mealybug wilt-affected plants was macerated in a mortar in the presence of extraction buffer (0.5 M Tris-HCl, pH 8.4 containing 4% (v/v) Triton X-100 and 0.2% 2-mercaptoethanol) at a 1:2 (w/v) ratio. The stirring period for the slurry was 1.5 h and the filtrate was clarified by centrifugation at 8 000 rpm for 15 min in a RPR 16-8 rotor

(Hitachi, Tokyo, Japan). The supernatant was collected and layered over 5 ml of 20% (w/v) sucrose in TM buffer (100 mM Tris-HCl pH 8.5, 10 mM MgCl<sub>2</sub>) and centrifuged at 30 000 rpm for 2 h and 35 min in a Ti 70 rotor (Beckman, Palo Alto, CA, USA). The pellet was dissolved in 500 µl TM buffer for every 100 g of starting material. The suspension was stirred overnight at 4°C, then centrifuged at 10 000 rpm for 5 min. Samples were negatively stained with 2% aqueous uranyl acetate and observed with a JEOL (JEM-200 EX) electron microscope.

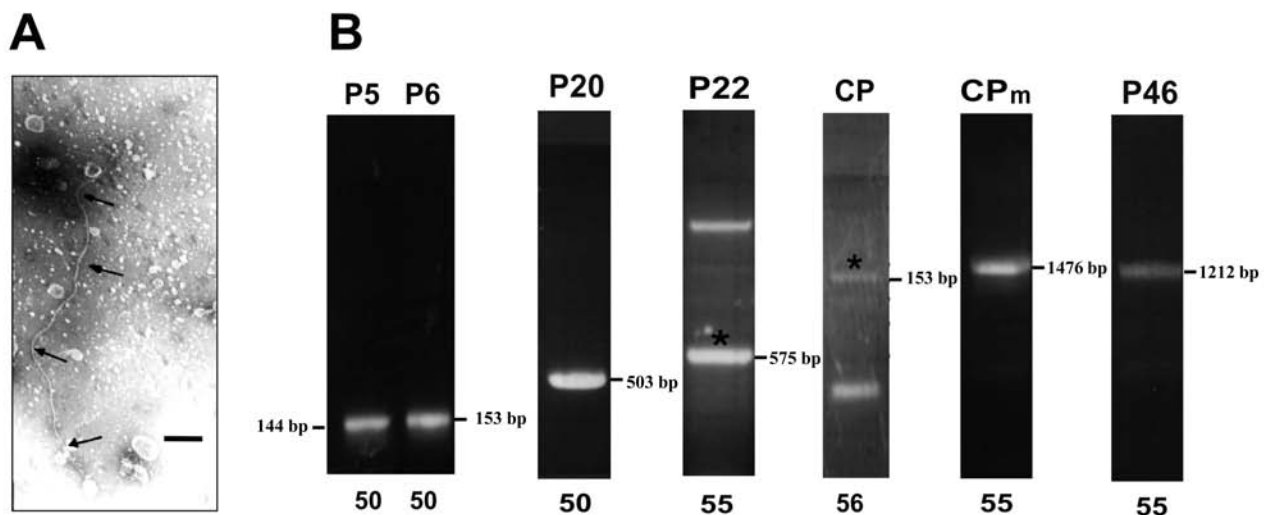
In order to process large amounts of MWP-affected pineapple leaves and to increase the virus titre, minor modifications to the purification protocol described by Gunashinge and German (1989) were necessary. Highest yields were obtained by increasing the stirring period of the first supernatant, by reducing the time of differential centrifugation and by prolonging density gradient centrifugation.

Electron microscopy of purified virus showed the presence of long, flexuous, rod-shaped virus particles strongly resembling those of PMWaV (Fig. 1A). No such particles were seen in symptomless greenhouse-grown plants derived from plants sanitized by meristem tip culture. Purified preparations of PMWaV from Hawaii and Australia were reported to have modal length of 1200 nm and 1200-1600 nm in purified preparations, respectively (Gunashinge and German, 1989; Wakman *et al.*, 1995). However in Australia, particles of 1700-1900 nm were also trapped from the sap of affected plants (Wakman *et al.*, 1995). The Cuban PMWaVs were isolated from 16 MWP-affected pineapple plants and 75% (36 out of 48) of the particles measured had an estimated length of 1200-1450 nm and a diameter of

12 nm (Fig. 1A). These values are similar to those described from Hawaii (Gunashinge and German, 1989), but different from those reported from Australia (Wakman *et al.*, 1995). However, the possibility of particle fragmentation during purification cannot be ruled out.

The presence of putative PMWaVs in Cuban pineapple crops prompted us to test the potential association between MWP and the presence of these viruses. At the time of our analysis a nearly full-length sequence of the PMWaV-2 genome was available (Melzer *et al.*, 2001; accession no. AF283103). Using this information, combined with our previous electron microscope (EM) observations for diagnosis of PMWaV, specific oligonucleotide primers were designed for use in RT-PCR to confirm the presence of this virus in Cuban MWP-affected pineapple crops (Table 1). Total RNA was isolated from 90 mg leaf tissue of three individual, EM-positive plants, using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quality and concentration were determined by gel electrophoresis and spectrophotometry, respectively. Five micrograms of total RNA was used as a template for reverse transcription using the Superscript RT II kit (Invitrogen, Carlsbad, CA, USA). Two microliters of cDNA were used as PCR template for optimizing the use of different primer sets (Fig. 1B). Proofreading polymerase (*pfu*, Stratagene, La Jolla, CA, USA) was used to ensure sequence fidelity. PCR amplicons corresponding to the predicted product sizes were cloned into pGEM-T (Promega, Madison, WI, USA) and five clones of each were sequenced and analyzed.

The resulting DNA sequences were almost identical (99.4% identity for CP and p20 and 97.5% identity for p22) to those of seven ORFs predicted from the se-



**Fig. 1.** Molecular identification of virus particles and RT-PCR analysis of PMWaV-2 in pineapple (Smooth Cayenne) tissue from Cuba. (A) Electron micrograph of PMWaV particle. Arrows indicate a virus-like particle (Bar = 200 nm); (B) Agarose gel electrophoretic analysis of RT-PCR products. Dashes indicate the correct band of the expected sizes. Numbers below each lane represent the annealing temperature used in the PCR program. Asterisk mark the correct band when more than one product was present.

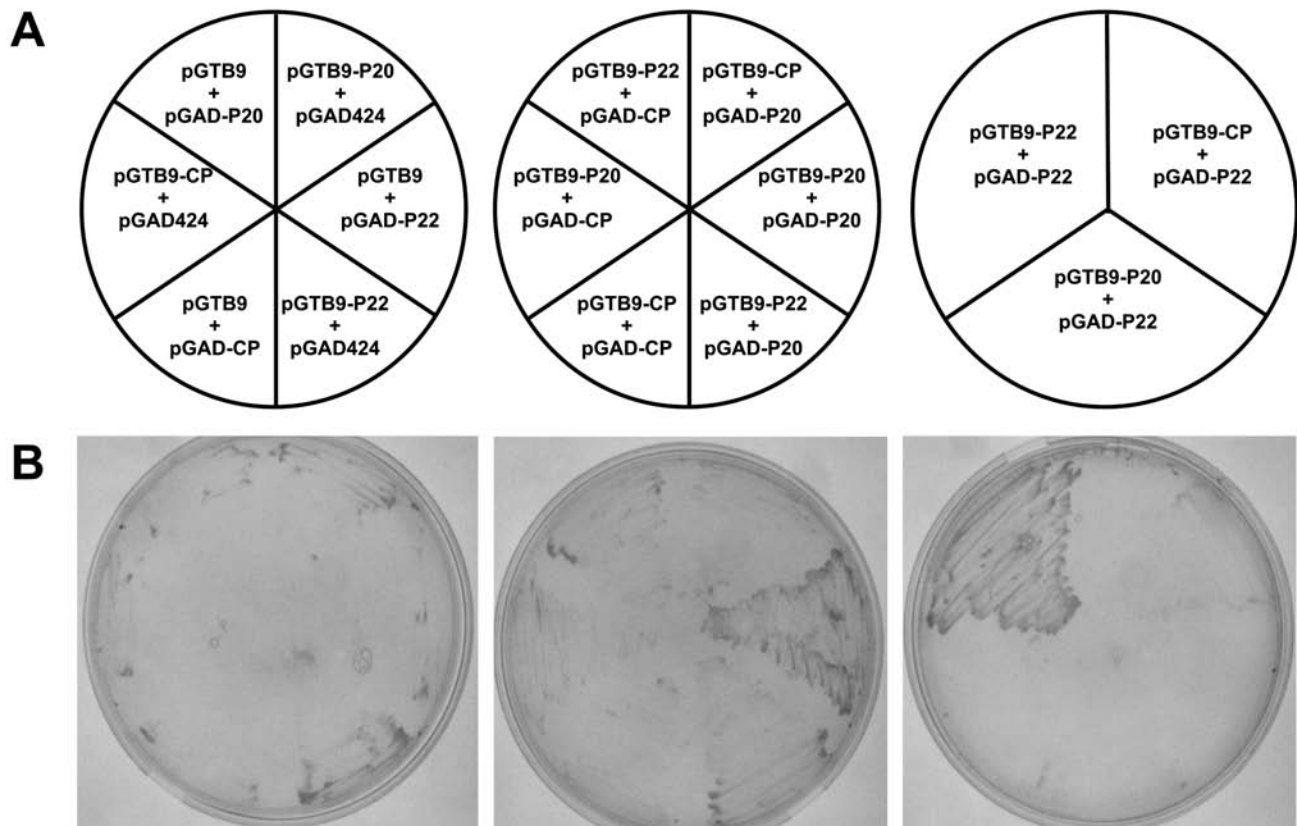
quence of PMWaV-2 and corresponded to: ORF 2 (p5, 144 bp), ORF 4 (p46, 1209 bp), ORF 5 (CP, 906 bp; accession no. DQ225114), ORF 6 (CPm, 1473 bp), ORF 7 (p20, 503 bp; accession no. DQ228819), ORF8 (p22: 575 bp; accession no. DQ228818) and ORF 9 (p6, 150 bp). This procedure allowed specific detection of nucleotide fragments of PMWaV-2 by RT-PCR within 8 h and conclusively confirmed the presence of PMWaV-2 in Cuban pineapples.

The extracts observed under the EM contained PMWaV-2 particles but dual infection with PMWaV-1 or Pineapple mealybug wilt-associated virus 3 (PMWaV-3), as observed in pineapple plants from Hawaii (Melzer *et al.*, 2001; Sether *et al.*, 2005), cannot be excluded.

The genome organization of PMWaV-2 resembles that of members of the *Closteroviridae* family (Martelli *et al.*, 2005). The 21-kDa protein (p21) of *Beet yellows virus* (BYV) is a suppressor of RNA silencing and it is known that proteins from other viruses of the genus *Closterovirus* also possess similar suppressor activity (Reed *et al.*, 2003; Chiba *et al.*, 2006). *Citrus tristeza virus* (CTV) is known to have evolved a strategy involving three different silencing suppressors: p20 and CP, which interfere with the systemic spread of silencing, and p23, which suppresses local silencing (Lu *et al.*, 2004).

The CP gene of PMWaV-2 has a 42.2 % amino acid identity with GRLaV-3 CP and a moderate homology to CPs of other closteroviruses (Melzer *et al.*, 2001). The same authors proposed that p20 or p22 in PMWaV-2 might have a similar activity, but with a different mode of action. Qu and Morris (2005) mentioned that the majority of characterized plant virus suppressors do not share any obvious sequence or structural similarity across viral families and groups.

Yeast two-hybrid technology has been successfully applied to investigate protein-protein interactions in closteroviruses (Gowda *et al.*, 2000). To investigate protein-protein interaction of PMWaV-2 components potentially involved in the regulation of virus pathogenesis, p20, p22 and CP were cloned into yeast two-hybrid vectors and co-transformed into yeast cells (Fig. 2). The DNA fragments of p20, p22 and CP genes were obtained by digestion of the pGEM-p20, pGEM-p22 and pGEM-CP plasmids with *EcoRI* and *BamHI* and subcloned in pGBT9 and pGAD424 (Clontech, San Jose, CA, USA). The yeast HF7c reporter strain (Clontech, San Jose, CA, USA) was co-transformed with pGAD-p20, pGAD-p22, pGAD-CP and empty pGAD424 against the pGTB9-p20, pGTB9-p22, pGTB9-CP and empty pGTB9 using the lithium acetate method (Gietz *et al.*, 1992). After incubation for two days on medium



**Fig. 2.** Yeast two-hybrid assays for the protein-protein interaction analysis of three PMWaV-2 proteins. (A) Diagram of arrangement of yeast strains on each set of plates; (B) Growth of yeast on His-lacking SC medium.

**Table 1.** Primer sequences used for the detection of seven ORFs of PMWaV-2 in pineapple.

ORF	Primers (5' to 3')
p5 (5 kDa protein)	<u>CCGAATTC</u> CATGTTAGACGCTTTCACAGCC GGGGATCCTTACGCCGCTCCATACGATCGC
p46 (46 kDa protein)	<u>CCGAATTC</u> CATGCATCGCGAGTCCGCCTTGAC CCGGATCCTTAAGTATTTCGAACCATACTCTCCGCC
CP (34 kDa coat protein)	GGGAATTCATGGCTCAGAATTACGTAGCCG GGGGATCCTACCCTGAAACAGCTCCCTGG
dCP (56 kDa diverged coat protein)	GGGAATTCCTATGTGGCTTTAAGCTTAATCG <u>CCGAATTC</u> CATGGAATTTTCAGCGGATACCTGC
p20 (20 kDa protein)	<u>CCGAATTC</u> CATGGAGTTTAGACCGATAGAAG GGGGATCCTTGGGTAACAGAATAGTTGCC
p22 (22 kDa protein)	<u>CCGAATTC</u> CATGAGTGAGGAGATCCTGAAGTCGGC GGGGATCCCGACAGTTTCGGGTATATAACTC
p6 (6 kDa protein)	<u>CCGAATTC</u> CATGAACACGAATGCTAAAAAATATC GGGGATCCTTAATATTCATTTATATCTTTTATTATC

*Underlined sequences correspond to enzyme restriction sites overhang (BamHI and EcoRI).*

without Leu and Trp at 30°C, colonies were plated on His-lacking SC (Synthetic Complete) medium. Interestingly, the components did not interact with each other. Homo-dimerization was shown by p20 and p22, as indicated by the ability to grow in autotrophic medium of the co-transformed yeast with both genes in both orientations (prey and bait). This result suggests that p20 and p22 play a preferential role *in vivo* as homodimeric rather than as heterodimeric complexes (Fig. 2) and suggests that these three proteins (p20, p22 and CP) apparently do not have any overlapping functions. Analysis of additional viral components will yield more information on their mechanisms and functions.

Our analyses confirm the presence of PMWaV-2 in Cuban pineapple plants. Results obtained from the yeast two-hybrid assays contribute to the knowledge of ampelovirus proteins with as yet unknown functions. Constructs involving the genes coding for p20 and p22 will be considered as candidates for the production of transgenic pineapple plants to study their possible protection against PMWaV-2, an approach that has been successfully used for potyviruses and closteroviruses (Di Nicola-Negri *et al.*, 2005; Fagoaga *et al.*, 2006).

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