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Occurrence of *Papaya ringspot virus* Infecting Papaya in Ivory Coast

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ABSTRACT

Papaya orchards located in different production areas in Ivory Coast as well as papaya plants growing in the district of Abidjan were surveyed in early February 2006. A wide range of leaf symptoms including mosaic, yellowing, chlorotic-line patterns, downward and upward curving of margins, and shoe-stringing was observed in the field. Ringspots were also observed on some stems and fruits. Sixty leaf samples of which fifty seven symptomatic samples were collected in the field for virus identification. In order to test for the presence of *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV), the double antibody sandwich-enzyme linked immuno-sorbent assay (DAS-ELISA) was conducted using leaf extracts made from dried samples. None of the samples was positive for PaMV. Nine samples out of the 60 tested, coming from three locations, were positive for PRSV. Transmission electron microscope observation of leaf-dip preparation of the ELISA-positive samples revealed flexuous particles characteristic of potyviruses. To confirm the identity of PRSV, a 676 bp RNA fragment representing part of the coat protein gene with the 3'-untranslated region, was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from one PRSV-positive sample (An 1) using appropriate primers. The nucleotide sequence of the PCR product (Genbank Accession Number DQ84023) confirmed the taxonomy of the virus as being PRSV (Diallo *et al*. 2007). The PRSV Anyama isolate (An1) of PRSV identified in this study shows 97 % identity with PRSV strain P (PAYP) (Genbank Accession Number D00595). There is a possibility of other papaya infecting viruses present in the country.

Keywords: detection, electron microscopy, ELISA, molecular characterisation, symptom

INTRODUCTION

Papaya (*Carica papaya*) is grown in Ivory Coast for the local and export markets. Varieties grown are Solo and Golden. Growing papaya was encouraged in the country as a strategy to diversify from traditional crops such as coffee, cocoa and banana. An increasing number of growers became involved with papaya growing. From three plantations registered in 1994, there were more than 80 plantations in 2002 (Lannuzel 2002). A few years later unfortunately, virus-like diseases appeared. Disease incidence reached 100% in some fields resulting in several growers abandoning papaya production. Some of them have abandoned their old plantations and moved to different areas to establish new ones. The aetiology of the disease is unknown.

Several virus genera are involved in papaya diseases: *Potyvirus*, *Rhabdovirus*, *Begomovirus*, *Potexvirus* and *Tospovirus* (Tennant *et al*. 2007). A number of virus species have been reported to infect papaya worldwide. They include *Papaya mosaic virus* (PapMV) (Brunt *et al*. 1996), *Papaya lethal yellowing virus* (PLYV) (Silva *et al*. 1997), *Papaya leaf distortion mosaic virus* (PLDMV) (Kawano and Yonaha 1992; Maoka *et al*. 1995; Chen *et al*. 2002), *Papaya leaf curl virus* (PLCV) (Saxena *et al*. 1998), *Tomato spotted wilt virus* (TSWV) (Gonsalves and Trujillo 1996), *Moroccan watermelon mosaic virus* (Arocha *et al*. 2007) and *Papaya ringspot virus* (PRSV). Among them, PRSV which was first described by Jensen in 1949 is the most widely distributed. Within the last ten years, the virus has been found in Iran (Pourrahim *et al*. 2003), the French Polynesia and the Cook Islands (Davis *et al*. 2005), and Bangladesh (Jain 2004). In Africa, PRSV has been reported in Togo, Sierra Leone, Kenya, Zambia and South Africa (Taylor 2004), in the Lake Victoria region (Ndunguru and Rajabu 2002) and in Ivory Coast (Diallo *et al*. 2007). *Papaya ringspot virus*, a member of the *Potyviridae* family, is the most studied virus of papaya. It is an aphid-transmitted virus, with flexuous filamentous particles of 780 nm x 12 nm containing a single-stranded positive sense RNA genome (Purcifull *et al*. 1984). There are two distinct types of PRSV: the type P (papaya) which infects papaya and the type W (watermelon) which infects only cucurbit species (Purcifull *et al*. 1984).

The objective of the present study was therefore to identify the main virus(es) found in different papaya production zones in Ivory Coast. We report in this paper on the identification of *Papaya ringspot virus* as a virus infecting papaya and a major threat for papaya producers in Ivory Coast.

MATERIALS AND METHODS

Sample collection and antisera

In February 2006, 57 leaf samples from infected plants and three samples from symptomless plants were collected from seven locations: Abidjan, Ahouakro, Anyama, Azaguié, Songon, Tiassalé and Yamoussoukro. After symptoms recording, each leaf sample was dried between newspaper sheets and transported to the UK and processed at Rothamsted Research (Hertfordshire, UK). All samples were kept at room temperature until used. Commercial kits containing the antisera to the following viruses were used: PRSV

Received: 5 August, 2008. Accepted: 10 September, 2008.

Original Research Paper

and *Watermelon mosaic virus*-2 (WMV-2) (Bioreba, Switzerland) and PapMV and PRSV (DSMZ, Germany).

Serology test - ELISA

The double-antibody sandwich (DAS) method of the enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (1977) was used in certified Nunc–Immuno plates Maxi Sorb F96 (Nunc, Roskilde, Denmark). Reaction volumes of 200 µL per well were used. Each IgG was diluted 1/1000 in the coating buffer (50 mM carbonate-bicarbonate buffer (pH 9.6) containing 0.02 % NaN_3) and 200 µL was added to each well. The ELISA plates were covered tightly, placed in a humid box and incubated at 4-6°C overnight. The wells were emptied and washed 3 times with the indicated washing buffer [10 mM phosphate buffer (pH 7.4) containing 140 mM NaCl, 3 mM KCl and 0.05 % Tween 20 (PBST)]. Any excess liquid was removed by blotting the plates on paper towels. Approximately 0.2 g of each dried leaf sample was ground in liquid nitrogen and homogenized in 2 mL of extraction buffer (20 mM Tris buffer (pH 7.4 at 25°C) containing 137 mM NaCl, 3 mM KCl, 2 % PVP 24 kD, 0.05% Tween 20 and 0.02% NaN_3). The plant extract (200 µL) was added to each well. The plate were covered and placed in a humid box for overnight incubation at 4-6°C. After washing, the specific enzyme-linked antibody diluted 1/1000 in the conjugate buffer (20 mM Tris buffer (pH 7.4 at 25°C) containing 137 mM NaCl, 3 mM KCl, 1 $MgCl_2$, 2% PVP 24 kD, 0.05% Tween 20, 0.2% BSA and 0.02 % NaN_3) was added in the wells. The plates were incubated at 30°C for 5 h in a humid box and washed as usual. The substrate buffer (1 M diethanolamine pH 9.8, containing 0.02 % NaN_3) in which *p*-nitrophenyl phosphate was dissolved (1 mg/mL) was added to the wells. The incubation was done at room temperature in the dark. The intensity of colouring was measured with an ELISA reader Multiskan EX (Thermo Electron Corporation, Shanghai, China) at 405 nm. Samples were considered positive when $A_{405\ nm}$ exceeded two times that of the uninfected controls.

With the DSMZ kit, ELISA tests were conducted according to the manufacturer's instructions. Except for the substrate buffer, the buffer used in all the different stages was PBST (pH 7.4). Each sample (approximately 0.2 g), ground in liquid nitrogen, was homogenized in 2 mL of PBST. The following antiserum dilutions were used: 1/500 for PRSV (AS-0805) and 1/1000 for PapMV (AS-0725).

Electron microscopy

Sap extracts of dried papaya leaves were prepared by grinding 0.5 g with 1.5 mL of distilled water. After 15 s spinning, 0.5 mL of the supernatant was transferred to a test tube and an equal volume of 2% phosphotungtic acid (PTA) was added. For negative staining electron microscopy, the virion preparations obtained were placed on formvar-coated grids and examined under a Jeol transmission electron microscope.

Molecular characterisation

For the confirmation of the identification of PRSV, one sample (isolate An1) that tested positive for PRSV by ELISA and showed *Potyvirus*-like particles in leaf-dip examination under the EM, was selected. RNA was extracted using a CTAB RNA method adapted from Lodhi *et al*. (1994).

Individual dried papaya leaf samples (0.5 g) were ground in a plastic bag with 5 mL of the CTAB buffer. For the RNA extraction, 1.5 mL of the mixture was used. The final pellet was resuspended in 100 µL of molecular grade water and stored at -20°C.

The one step RT-PCR kit called Reverse-iT reddy mix version (ABgene, Epsom, UK) was used.

The 50 µL PCR reaction contained: 25 µL of the 2X Master Mix; 0.8 µM of Oligo dT; 2 µM of the forward primer; 1uL of RT Blend and 9 µL of water. The forward primer used was Uni 3 primer 5′-ATG GTN TGG TGC ATT GAG AAT GG-3′. The PCR conditions were as follows: first cycle of 30 min at 48°C and 2 min at 94°C; 35 additional cycles of 30 sec at 94°C, 1 min at 50°C and 1 min at 72°C; and a final incubation at 72°C for 7 min. The PCR products were separated by 1% agarose gel electrophoresis buffered in 0.5X TBE (Sambrook *et al*. 1989) and stained with ethidium bromide. The PCR products were purified using Promega Wizard SV gel and PCR clean up system (Promega, Hamshire, UK). Direct sequencing was performed by Sequiserve (Vaterstetten, Germany) with the primers oligo dT and Uni3.

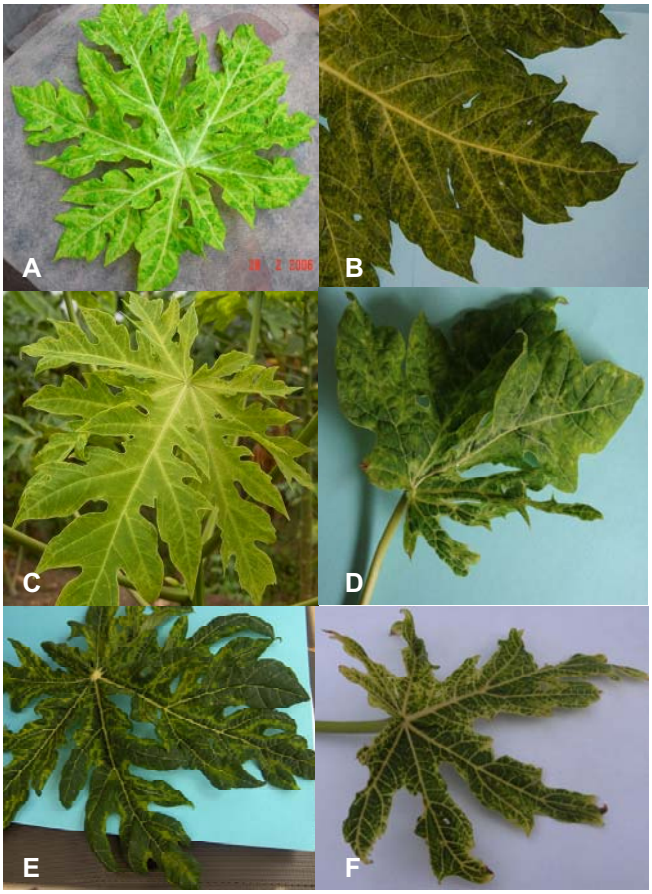

Fig. 1 Range of leaf symptoms on papaya found in the field. Mosaic (**A**); vein yellowing and dotting (**B**); yellowing (**C**); mosaic and shoestring (**D**); chlorotic line pattern (**E**); small rigid leaf (**F**). **A**, **B**, **C** and **D**: symptoms on PSRV-infected plants.

Fig. 2 Water-soaked rings on stem of an infected papaya plant.

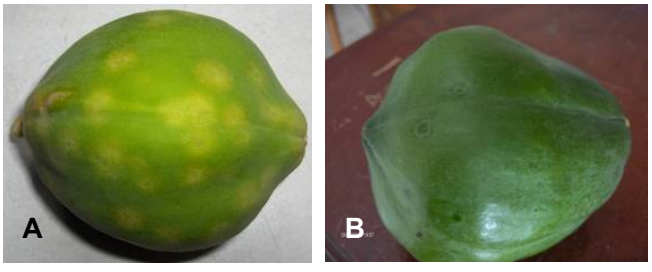

Fig. 3 Ring spots on ripening (A) and green fruit (B).

RESULTS AND DISCUSSION

A wide spectrum of symptoms was observed on the foliage of papaya in the field. They included mosaic (**Figs. 1A, 1D**), chlorotic dotting and vein yellowing (**Fig. 1B**), leaf yellowing (**Fig. 1C**), leaf distortion and shoe-stringing (**Fig. 1D**), chlorotic line patterns and downward leaf margins curling (**Fig. 1E**), leaf rigidity (**Fig. 1F**), and upward leaf curling. The leaf symptoms also varied in severity. Mosaic expressions ranged from mild to severe with puckering on the leaf. Water-soaked rings were also apparent on the stem of some plants (**Fig. 2**). Blemishes on ripe fruits were chlorotic with darker green centres (**Fig. 3A**). On green fruit of some plants, distinct darker green rings and arcs were visible (**Fig. 3B**). Even though these symptoms are typical of those caused by PRSV on papaya (Chang 1979; Purcifull *et al*. 1984; Gonsalves 1994), other viruses could also be present in single or mixed infections. For example, when an infection by *Papaya mosaic virus* alone causes mosaic, in mixed infections with PRSV, symptoms such as leaf distortion, mottling and mosaic are observed (Nao-Carrazana *et al*. 2006).

Papaya plants showing these virus-like disease symptoms have been observed in Ivory Coast for a number of years (HA Diallo, pers. comm.). Disease symptoms characteristic of viral infection are found to be widespread in both organized papaya orchards and in backyard stands throughout Abidjan. For this survey conducted in 2006, a wide range of symptoms was observed throughout the production zones (**Table 1**). Some symptoms were only found in specific plantations while others were more widely distributed. For example chlorotic line patterns were only found in some plantations in Yamoussoukro. Moreover, different symptoms were also found in the same plantation (**Table 1**). The wide distribution of the symptoms could be the result of the free transport of infected plant material from one zone to another in the country due to a lack of effective quarantine service in place. For example, young papaya plants from the nursery are sometimes moved from one area to another.

From the fifty seven symptomatic papaya leaf samples collected only nine were positive for PRSV detection in the ELISA test and none of the samples tested reacted positively to PaMV and WMV-2 antisera (**Table 2**). It has been shown that PRSV isolates from papaya sometimes react with WMV-2 antiserum. Indeed, Thomas and Dodman (1993) reported that even though the Australian isolates of PRSV-P reacted strongly in ELISA to antibodies to PRSV-P and PRSV-W, they also had weak reaction to WMV-2. Therefore, to determine if some of the Ivorian isolates of PRSV will also react to WMV-2 antiserum, this antiserum was included in the test. In this study, the number of sample positive for PRSV is very low. The tests were repeated 3 times and the same results were obtained in each assay. This could in part be explained by the fact that several papaya plantations where the plants showed characteristic PRSV symptoms have been abandoned and now have been replaced by other crops. These plantations were therefore not included in this study. For the plantations visited, some have been established in new areas with no history of papaya production. Since all the fifty seven samples showed some symptoms, it could be hypothesised that: 1) PRSV is present but at a concentration that is too low to be detected by the ELISA test and 2) other viruses or pathogens are infecting papaya plants in Côte d'Ivoire. There was therefore no relationship between PRSV symptom expression and the virus detected. The PRSV positive samples were found in three production zones (Abidjan, Anyama and Yamoussoukro) (**Table 2**). All five samples collected from Abidjan, showing symptoms typical of PRSV, were positive for PRSV in ELISA, where only three of the twelve samples from Anyama were positive. PRSV has a worldwide distribution and within a country, it can be found in different ecosystems. That is the case for example in Brazil (Lima *et al*. 2002).

Two ELISA kits were used to detect any possible reaction difference. Using the DSMZ ELISA kit, one additional sample (Ya15) was tested positive for PRSV in Yamoussoukro (**Table 4**). That sample was not positive when the

Table 1 Symptoms description of leaf samples collected in different plantations.

Sample	Zones	Plantations	Symptoms
Ya1	Yamoussoukro	Sg	severe M
Ya2			CA – LP
Ya3			CA
Ya4			mild M
Ya5			VC
Ya6		Kab	M + VC + HL
Ya7			VC + HL+ D
Ya8			VC + HL+ D
Ya9			Mt
Ya10			VC + HL + D
Ya1			M + VC
Ya12			VC + Y
Ya13		Kon	VC
Ya14			mild M
Ya15			M
Ya16		Bay	mild M + VC
Ya17			mild M + VC
Ya18		Ana	LP + dCu
Ya19			M
Ya20			LP + dCu
Ya21			M + P + VC
Ya22			M
Ya23			LP + dCu
Ya24			severe M
Ah1	Ahouakro	Ama	NS
Ah2			NS
An1	Anyama	SB	YD
An2			YD
An3			M + VC
An4			M + upCu
An5		SM	Mt
An6			Mt
An7			severe M+ VC
An8			severe M+VC+ D
An9			M + VC + P
An10			M + YD
An11			M + VC + D
An12			M + P
Ti1	Tiassalé	ST	M + VC
Ti2			VC
Ti3			YD
Ti4			M + VC + P
Ti5			M + VC + P
Az1	Azaguié	Ga	YD
Az2			YD
Az3			severe M
Az4			CA
So1	Songon	Yac	M
So2			Y
So3			Mt
So4			Mt
So5			Mt
So6			Y
So7			Y
Ab1	Abidjan	Riv	severe M + P
Ab2			severe M + P
Ab3			severe M + P
Ab4		AA	severe M
Ab5		Abo	NS
Ab6		Abo	severe M and SS

NS = no symptom, CA = chlorotic area, D = distortion, dCu = downward margin curling, HL = hard leaf, LP = line pattern, M = mosaic, Mt = mottle, P = puckering, upCu = upward leaf curling, SS = shoe-string, VC = vein clearing, Y = yellowing.

[a]Serological reactivity measured in DAS-ELISA as the absorbance at 405 nm (A_{405}). Plants were considered infected when A_{405} > 2 times the mean A_{405} of the healthy controls.

[b]Transmission electron microscopy observation of leaf-dip.

Table 2 Relationship between symptom expression, ELISA reactions and EM observation.

Sample	Symptoms	DAS-ELISA[a] (WMV-2)	DAS-ELISA[a] (PaMV)	DAS-ELISA[a] (PRSV)	Presence of potyvirus-like particles with TEM[b]
Ya1	severe M	-	-	-	-
Ya2	CA – LP	-	-	-	-
Ya3	CA	-	-	-	-
Ya4	mild M	-	-	-	-
Ya5	VC	-	-	-	-
Ya6	M + VC + HL	-	-	-	-
Ya7	VC + HL+ D	-	NT	-	-
Ya8	VC + HL+ D	-	NT	-	-
Ya9	Mt	-	NT	-	-
Ya10	VC + HL + D	-	NT	-	-
Ya11	M + VC	-	-	-	-
Ya12	VC + Y	-	-	-	-
Ya13	VC	-	NT	-	-
Ya14	mild M	-	NT	-	+
Ya15	M	-	-	+	+
Ya16	mild M + VC	-	-	-	-
Ya17	mild M + VC	-	-	-	-
Ya18	LP + dCu	-	-	-	-
Ya19	M	-	-	-	-
Ya20	LP + dCu	-	-	-	-
Ya21	M + P + VC	-	-	-	-
Ya22	M	-	-	-	-
Ya23	LP + dCu	-	-	-	-
Ya24	severe M	-	-	-	-
Ah1	NS	-	-	-	-
Ah2	NS	-	-	-	-
An1	YD	-	-	+	+
An2	YD	-	-	+	+
An3	M + VC	-	-	+	+
An4	M + upCu	-	-	-	-
An5	Mt	-	-	-	-
An6	Mt	-	-	-	-
An7	severe M+ VC	-	-	-	+
An8	severe M+VC+ D	-	-	-	-
An9	M + VC + P	-	-	-	-
An10	M + YD	-	-	-	-
An11	M + VC + D	-	-	-	-
An12	M + P	-	-	-	-
Ti1	M + VC	-	-	-	-
Ti2	VC	-	-	-	-
Ti3	YD	-	-	-	-
Ti4	M + VC + P	-	-	-	-
Ti5	M + VC + P	-	-	-	-
Az1	YD	-	-	-	-
Az2	YD	-	-	-	-
Az3	severe M	-	-	-	-
Az4	CA	-	-	-	-
So1	M	-	-	-	-
So2	Y	-	-	-	-
So3	Mt	-	-	-	-
So4	Mt	-	-	-	-
So5	Mt	-	-	-	-
So6	Y	-	-	-	-
So7	Y	-	-	-	-
Ab1	severe M + P	-	-	+	+
Ab2	severe M + P	-	-	+	+
Ab3	severe M + P	-	-	+	+
Ab4	severe M	-	-	+	+
Ab5	NS	-	-	-	-
Ab6	severe M and SS	-	-	+	+

NS = no symptom, CA = chlorotic area, D = distortion, dCu = downward margin curling, HL = hard leaf, LP = line pattern, M = mosaic, Mt = mottle, P = puckering, upCu = upward leaf curling, SS = shoe-string, VC = vein clearing, Y = yellowing. NT = not tested

[a]Serological reactivity measured in DAS-ELISA as the absorbance at 405 nm (A_{405}). Plants were considered infected when A_{405} > 2 times the mean A_{405} of the healthy controls.

[b]Transmission electron microscopy observation of leaf-dip.

Bioreba kit was used. The A_{405}nm values for PRSV varied according to the ELISA kit used. A higher value range was obtained with the Bioreba kit (**Tables 3-4**). This could be due to a faster reaction time of this kit or a better sensitivity.

Since PRSV was detected in only nine out of fifty seven leaf samples, all the samples were examined under the electron microscope to observe possible virus particles. All samples tested positive for PRSV (with Bioreba or DSMZ) contained flexuous rod-shaped particles (approximately 800 nm) (**Fig. 4**), characteristic of a *Potyvirus*. In the two sam-

Table 3 Absorbance values of PRSV-positive samples using the Bioreba kit.

Sample N°	Geographical location	*$A_{405\ nm}$
An1	Anyama	1.072
An2		0.647
An3		0.544
Ab1	Abidjan	1.066
Ab2		0.689
Ab3		0.998
Ab4		1.399
Ab6		0.825

Healthy control = 0.138
Positive control = 1.3646
*Values represent mean of duplicate wells

Table 4 Absorbance values of PRSV-positive samples using the DSMZ kit.

Sample N°	Geographical location	*$A_{405\ nm}$
Ya15	Yamoussoukro	0.224
An1	Anyama	0.271
Ab1	Abidjan	0.182
Ab4		0.305
Ab6		0.224

Healthy control = 0.073
Positive control = 0.793
*Values represent mean of duplicate wells

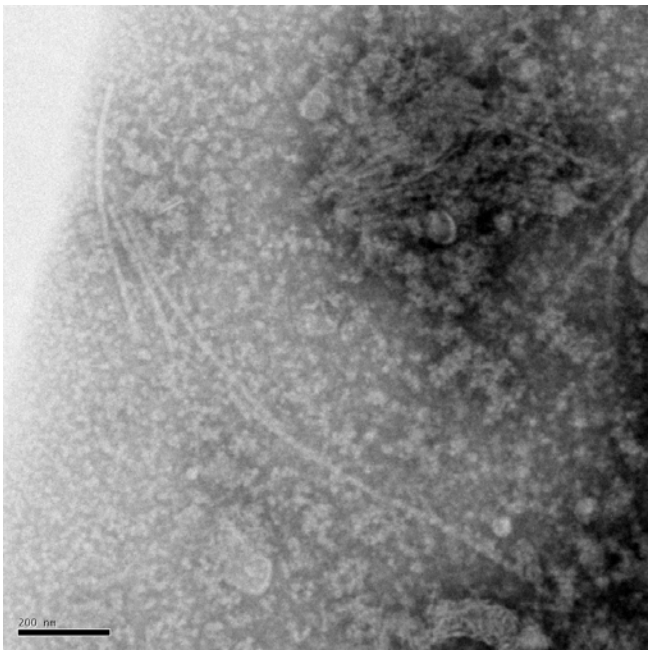

Fig. 4 PRSV particles observed by TEM of dried papaya leaves (leaf dip).

ples showing respectively mild mosaic and severe mosaic with vein clearing that did not react positively to PRSV antiserum, *Potyvirus*-like particles of about 800 nm long were observed under the electron microscope. Another virus tentatively classified as a *Potyvirus* and known to infect papaya is *Papaya leaf distortion mosaic virus* (PLDMV). The leaves of diseased plants show yellow mosaic patterns, deformation and shoestring while oil streaks are present on the stems and ringspots on the fruits (Kawano and Yonaha 1992). The disease caused by this virus was originally thought to be Papaya ringspot. However, PLDMV is in not serologically related to PRSV (Kawano and Yonaha 1992). Comparison of the *cp* sequences of PLDMV and PSRV indicated 49-59% sequence similarity at amino acid level (Maoka *et al*. 1996), confirming that these are two distinct viruses. This could explain the results obtained in our study, although PLDMV has not previously been described in Africa. Another possibility is an infection by the *Moroccan watermelon mosaic virus* (van der Meer and Garnett 1987), a tentative member of the *Potyvirus* genus. Indeed, this virus has been recently reported to infect papaya in the Democratic Republic of Congo in Central Africa (Arocha *et al*. 2007). In addition to the typical PRSV symptoms on the leaves and fruits, tumor-like growths were observed within the trunk of the papaya trees. However, in our study, these symptoms were not observed since no internal stem tissues observations were made. Additionally, in some samples (82%) showing virus-like disease symptoms, no *Potyvirus*-like particles were found. Instead, other virus-like particles were observed in some samples. They included *Rhabdovirus*-like particles as well as isometric particles (50 nm in diameter) (data not shown).

With the RT-PCR, a 676 bp fragment representing part of the coat protein gene and the 3′-untranslated region of one isolate of PRSV from Anyama (An1) was sequenced. Comparison of the nucleotide sequences of isolate An1 (Genbank accession n° DQ84023) and PRSV isolate PAYP from the USA (Genbank accession n° D00595) representing PRSV strain P gene for most of the nuclear inclusion (Nib) protein, coat protein and 3′-untranslated region) indicated 97% identity (**Fig. 5**). There were 16 nucleotides differences between the two sequences, with 2 nucleotides inserted at positions (463 and 486) and 2 gaps at positions (461 and 483) on the sequence of PRSV isolate Anyama. This result confirms the taxonomy of PRSV as being one of the viruses identified to infect papaya in Ivory Coast. As suggested in this paper, it is possible that other viruses are present in papaya orchards in the country. In depth investigations should therefore continue and include some electron microscopy aspects using fresh samples. In addition, more sensitive detection methods such as RT-PCR or PCR should be used for all samples to be tested. They will be tested for example for PRSV, MWMV and PLDMV as well as for other viruses. In order to provide efficient and adequate disease control measures, the first step is proper disease diagnosis. The aetiology of the disease must been known. This shows that a lot has to be done quite rapidly. This study is the beginning.

ACKNOWLEDGEMENTS

This work was funded through the African Fellow Programme managed by Rothamsted International, UK.

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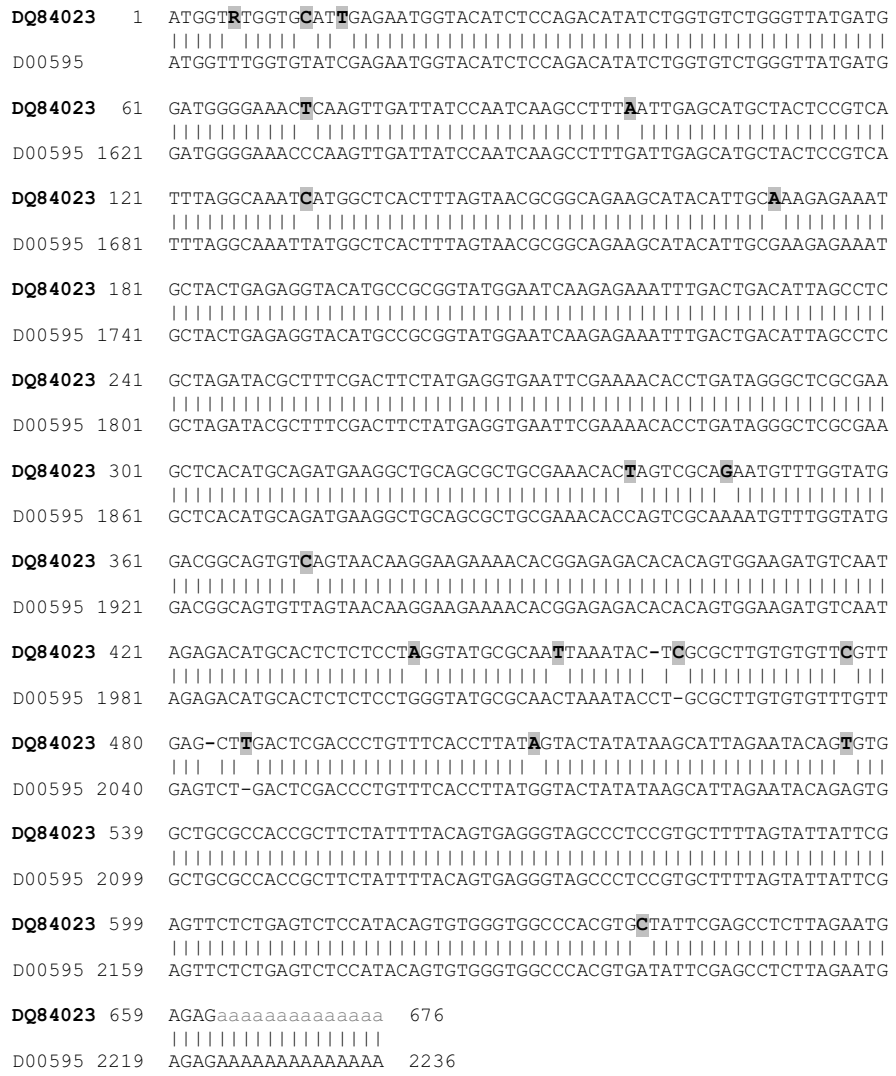

Fig. 5 Comparison of nucleotide sequence of the coat protein (CP) and 3′-untranslated region of a published data (Genbank accession no. D00595.1) and the Anyama isolate of PRSV (Genbank accession no. DQ84023). Gaps (-) have been introduced for maximum alignment.

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