Small Circular Single-stranded DNA Associated with Foliar Decay Disease of Coconut Palm in Vanuatu

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SUMMARY

The single-stranded DNA previously reported to be associated with foliar decay disease of coconut palm appears to be predominantly circular, on the basis of its behaviour in two-dimensional polyacrylamide gel electrophoresis, electron microscopy, and its resistance to end-labelling following treatment with alkaline phosphatase and polynucleotide kinase. The DNA sedimented at between 12S and 15S and had a contour length for the circular molecules consistent with the predominant DNA having a molecular weight of about 0.43 × 10^6, and comprising approximately 1300 nucleotides. These properties differ from the genomic DNAs of known plant viruses.

INTRODUCTION

Foliar decay is a serious disease of unknown aetiology which affects introduced varieties of coconut palm in Vanuatu (formerly New Hebrides) (Calvez et al., 1980). Foliar decay disease (FDD) is transmitted by Myndus taffini (Homoptera, Cixiidae), a species which has only been found in Vanuatu and New Caledonia (Julia, 1982; Bonfils, 1982; Julia et al., 1985; J. F. Julia, unpublished results). FDD is usually lethal in Malayan red dwarf palms within 1 to 2 years of the appearance of symptoms. Yellowing first appears in several leaflets of a frond between 7 and 11 positions down from the unopened spear leaf, and this is followed by general yellowing, necrosis and death of this frond, spread of the yellowing and necrosis to adjacent fronds, and reduction and death of the crown. Remission of symptoms and escape from disease occur infrequently in this cultivar. Cultivars and hybrids have been assessed for their tolerance to FDD and are recommended for planting to minimize the effect of FDD on new commercial plantations. However, some of the potentially most productive lines are susceptible (Calvez et al., 1985), and less-productive but tolerant lines must therefore be recommended.

Detection of a specific ssDNA in diseased palms (FDD-DNA) suggests that the disease has a viral aetiology but virus-like small spheres occasionally observed in preparations have so far not been specifically associated with the disease (Randles et al., 1986). This paper describes further characteristics of this DNA, and discusses these in relation to the possibility that FDD is caused by a virus.

METHODS

Extraction of FDD-associated DNA. FDD-DNA was extracted from coconut leaves by a modification of previously described methods (Randles et al., 1986). Leaf tissue (10 g) was chopped and blended with 120 ml 0.1 M-phosphate buffer pH 7, containing 10 mM-EDTA and 0.5 % monothioglycerol. The extract was strained through muslin, Triton X-100 was added to 2% and dispersed by shaking for 1 min. Bentonite (1 g) was added, dispersed by shaking, and the extract was centrifuged at 10000 g for 10 min. The supernatant fluid was mixed with polyethylene glycol (PEG 6000) to a final concentration of 8% (w/v) and insoluble material was collected after 1 h at 4 °C by centrifugation at 10000 g for 10 min. A resuspension of the pellet in 2 ml 0.5 M-sodium acetate pH 6.0, 10 mM-MgCl₂, 20% ethanol, 3% SDS was then shaken for 10 min with 0.5 vol. 90% phenol containing 0.1% 8-hydroxyquinoline and 0.5 vol. chloroform. The aqueous phase was recovered after centrifugation at 10000 g for 5 min, and nucleic acid was recovered by precipitation with 3 vol. ethanol in the presence of 0.1 M-sodium acetate.
Samples were sedimented, drained and dissolved in PAGE buffer containing 50% glycerol and xylene cyanol FF marker dye.

**Purification of FDD-DNA.** Leaf was extracted in 10 g lots up to and including precipitation with PEG 6000. The precipitates from each lot were combined and resuspended in 0.1 mM-phosphate buffer pH 7.6 (20 ml/100 g of leaf extracted) for 1 h. After clarification by centrifugation at 10000 g for 10 min, the mixture was treated a second time with 8% PEG 6000 for 1 h at 4 °C. The precipitate was collected by centrifugation at 10000 g for 10 min, resuspended in 6 ml in the 10 mM-phosphate buffer, then clarified by centrifugation at 10000 g for 10 min. The resuspension was centrifuged at 20000 g for 2 h in a R65 rotor (Beckman) and the pellet was suspended in 0.5 ml 2% SDS and 0.5 ml phenol, and shaken for 45 min at 25 °C. After low-speed centrifugation the supernatant fluid was re-extracted with 0.5 vol. phenol and 0.5 vol. chloroform for 1 min, centrifuged briefly, and nucleic acids in the aqueous phase were precipitated by the addition of ethanol.

The precipitate was dissolved in sterile double-distilled water and sedimented in a 10 to 40% linear sucrose density gradient for 4 h at 30000 g in a SW65 Ti rotor (Beckman). Fractions were mixed with 3 vol. ethanol in 0.1 M-sodium acetate and those containing FDD-DNA were identified by electrophoresis of the ethanol precipitates in polyacrylamide gels. Nucleic acids from pooled fractions containing FDD-DNA were centrifuged in 4 ml CsCl isopycnic density gradients (buffered in 9 mM-Tris, 9 mM-boric acid, 0.3 mM-EDTA, pH 8.3) with a starting density of 1.69 g/ml. Gradients were formed by centrifugation at 33000 r.p.m. for 64 h in a SW50 L rotor (Beckman), at 20 °C. Fractions were collected dropwise from the bottoms of the tubes, their densities were determined, and each was dialysed against unbuffered 0.2 M-sodium acetate for 3 h at 4 °C before precipitation with ethanol. Fractions containing FDD-DNA were identified by PAGE.

**PAGE.** Nucleic acid extracts from diseased palms were assayed for the presence of FDD-DNA in vertical slab 5% polyacrylamide gels containing 8 M-urea (Randles et al., 1986) and buffered with 90 mM-Tris, 90 mM-boric acid, 5 mM-EDTA, pH 8.3. Non-denaturing gels were similar except that no urea was used.

**Two-dimensional gel electrophoresis of FDD-DNA.** Purified FDD-DNA, and nucleic acid prepared in an identical manner from healthy palm, were precipitated with ethanol, dried, dissolved in deionized 50% formamide buffer in 10 mM-Tris–HCl pH 8.5, containing 1 mM-EDTA, and heated at 65 °C for 5 min. Filtered cytochrome c solution was added to give a concentration of 50 µg/ml, and the mixture was spread immediately from a glass slide onto a hypophase of 1.5 mM-Tris-HCl pH 8.5, containing 0.15 mM-EDTA in a 6 cm diam. plastic Petri dish. Grids, freshly coated with parlodion, were inverted on the hypophase, drained, stained for 20 s in 0.005% uranyl acetate in 90% ethanol, drained, dipped in petroleum spirit, drained, dried and rotary-shadowed in vacuo with Pt–Pd at about 6° (Randles & Hatta, 1979). Magnification was verified with a carbon grating replica.

**Alkaline phosphatase and polynucleotide kinase treatment of FDD-DNA.** Nucleic acid preparations were treated with alkaline phosphatase and polynucleotide kinase (Maniatis et al., 1982). Reaction mixtures of 10 µl contained nucleic acid, 50 mM-Tris–HCl pH 9.0, 0.5 mM-MgCl₂, 0.1 mM-ZnCl₂, 1 mM-spermidine and 0.02 units alkaline phosphatase (Sigma Type III-N). After incubation at 37 °C for 30 min, another 0.02 units of enzyme was added, and the incubation was continued for 30 min. The mixture was diluted with 40 µl water, and extracted twice with phenol–chloroform and twice with chloroform. Nucleic acids were precipitated with ethanol in the presence of sodium acetate, sedimented and dried. The pellet was resuspended in 50 mM-Tris–HCl pH 7.6, 10 mM-MgCl₂, 5 mM-dithiothreitol, 0.1 mM-spermidine, 0.1 mM-EDTA. Four units polynucleotide kinase (New England Biolabs) and 15 µCi [γ-32P]ATP (BRESA, Adelaide, Australia) were added and the mixture was incubated for 40 min at 37 °C. The reaction was stopped with 5 µl 0.2 M-EDTA, an equal volume of sample loading buffer was added and the reaction mixture was analysed in a denaturing 5% polyacrylamide gel. Labelled molecules were detected by autoradiography, and the FDD-DNA marker was detected by silver staining.

**RESULTS**

**Preparation of FDD-DNA**

Sucrose density gradient fractionation of the FDD-DNA extracted from the high-speed centrifugation pellet showed that it had a sedimentation rate between 12S and 15S (Fig. 1). It sedimented ahead of the 366 nucleotide circular ssRNA-2 of velvet tobacco mottle virus (VTMoV; Randles et al., 1981; Haseloff & Symons, 1982). Assuming that the mol. wt. of marker RNAs were 4S, 0.026 × 10⁶; 9S (circular VTMoV RNA-2), 0.120 × 10⁶; 16S, 0.56 x 10⁶; 18S, 0.70 × 10⁶; 23S, 1.1 × 10⁶; 25S, 1.3 × 10⁶, interpolation would place FDD-DNA in the molecular weight range 2.1 × 10⁵ to 4.8 × 10⁵. Other plant nucleic acids co-sedimented with
Foliar decay disease-associated circular DNA

Fig. 1. Relative sedimentation rate (S) of FDD-DNA (enclosed by dashed lines) in a linear sucrose density gradient. The relationship between S value and distance from the top of the gradient (mm) was determined by the use of marker 4S, 16S, 18S, 23S and 25S RNAs from *Nicotiana clevelandii*, and the circular ssRNA from VTMoV (9S).

Fig. 2. Distribution of FDD-DNA in a CsCl isopycnic density gradient, relative to that of plant DNA. The density range of the gradient is shown by the solid line.

FDD-DNA, as shown by PAGE analysis, necessitating further purification. In CsCl gradients (Fig. 2) FDD-DNA was distributed more broadly (density range 1.66 to 1.71 g/ml) than the contaminating plant DNA (range 1.69 to 1.70 g/ml) which was identified as a broad staining zone in PAGE. Consequently, for studies where relatively pure FDD-DNA was required, fractions were selected which had negligible plant DNA content.

**PAGE analysis of FDD-DNA**

In denaturing 5% gels, FDD-DNA migrated as a single band (Fig. 3) of low relative electrophoretic mobility but in both 3.3% and 5% non-denaturing gels it migrated as two or three separate bands (Fig. 3, 4).

**Two-dimensional PAGE analysis**

Two-dimensional PAGE is useful for identifying viroids and other circular RNA molecules which have different conformations and electrophoretic mobilities in non-denaturing and denaturing conditions. Extracts from diseased, but not from healthy palms, showed a diffuse band below the diagonal front in the position expected for a circular molecule. When FDD-DNA was further purified by a CsCl gradient step it separated into two bands in the first (non-denaturing) dimension and both of these bands showed retarded mobility in the second (denaturing) dimension characteristic of a circular molecule (Fig. 4). Both migrated about the same distance in the second dimension, indicating that they were essentially the same size when denatured.

**Circularity of molecules in preparations of FDD-DNA**

A number of circular molecules were observed in preparations of FDD-DNA. Those in the major class (range 200 to 800 nm) had a mean contour length of 466 \( \pm \) 14 nm (n = 59) (Fig. 5a, 6). Fewer circular structures of longer contour length were also observed (Fig. 6) as well as short (approx. 450 nm) and longer (900 to 1600 nm) (Fig. 5b) linear structures which were frequently...
Fig. 3. Electrophoresis in non-denaturing (n) and denaturing (d) 5% polyacrylamide gels of nucleic acids from healthy (H) and FDD-affected (F) palms. (a) Example of a sample with a double band (arrowed) in the non-denaturing gel, which migrates as a single band in the denaturing gel. (b) Example of a sample with three FDD-specific bands which denature to a single band. These bands are absent from extracts of healthy palms.

looped or twisted. No such structures were seen in preparations from healthy leaves. Large clumps of strands were also seen but were too dense to be measured and these were also seen in preparations from healthy leaf material. The small circles and linear molecules of corresponding length were almost exclusively present in the preparation analysed in Fig. 4, which by PAGE analysis appeared to contain FDD-DNA almost exclusively.

Reliable length measurements could only be obtained for the circular molecules. Assuming a linear density of $0.92 \times 10^6$ daltons/µm for ssDNA (Randles & Hatta, 1979), the mean molecular weight of a 466 nm circular molecule was estimated to be $4.3 \times 10^5$. Assuming a nucleotide density of 2827 nucleotides/µm for ssDNA spread under the conditions used here (Randles &
Fig. 4. Two-dimensional gel electrophoresis of purified FDD-DNA. The first dimension was in a 3.3% polyacrylamide gel under non-denaturing conditions; the second dimension was in a 3.3% gel under denaturing conditions. The first dimension showing the two silver-stained FDD-DNAs is in the lower panel, and their position following electrophoresis in the second dimension is arrowed. The position of the diagonal front is shown with a double-headed arrow.

Fig. 5. Rotary shadowed (a) circular and (b) long linear molecules in purified preparations of FDD-DNA. Bar markers represent 100 nm.
Hatta, 1979) the circles would comprise about 1300 nucleotides. The minor molecular component about 950 nm long (Fig. 6) would comprise about 2700 nucleotides and that of 1150 nm (Fig. 6) would comprise about 3300 nucleotides.

**Attempt to identify free ends by labelling with polynucleotide kinase**

If FDD-DNA is circular, procedures for labelling the 5' end of linear nucleic acids should fail to label it. The purified nucleic acids from healthy and diseased leaves, which were used for electron microscopy, were subjected to the end-labelling procedure and fractionated by PAGE, as described above. No specific labelling of FDD-DNA was observed. The autoradiographs showed labelling in the position of the plant DNA which was present in all samples. General light background labelling also occurred throughout the lane as would be expected for a continuum of nucleic acid molecules contaminating the preparation of FDD-DNA. This background was also visible after silver staining but was not strong enough to obscure the FDD-DNA. Thus, the absence of a radioactive band at the FDD-DNA position is consistent with it having no free ends. It is not known whether the few linear molecules observed by electron microscopy formed distinct bands in the gel, and some of the background labelling could have been due to these forms.

**DISCUSSION**

Determination of the aetiology of FDD is particularly important so that diagnostic procedures may be developed for application to epidemiology and control methods. Previous work demonstrated that a ssDNA of low electrophoretic mobility in 5% polyacrylamide gels was specifically associated with diseased Malayan red dwarf coconut palms. This FDD-DNA was extractable in low amounts only. This study was done to characterize further the FDD-DNA, and so to determine how it may be used as the basis of a diagnostic assay, and to predict the type of virus particle in which it may be encapsidated.

The results presented here show that preparations of the ssFDD-DNA (Randles et al., 1986) contain circular molecules of mean mol. wt. approx. $4.3 \times 10^5$. Circularity has been demonstrated by electron microscopy, two-dimensional PAGE and an inability to dephosphorylate and label the molecule by the polynucleotide kinase technique used to label the 5' end of linear polynucleotides. Linear molecules which were also seen in FDD-DNA preparations by electron microscopy and were of similar length to the circles, may be derived from the circles.
The circularity of FDD-DNA would explain the failure of end-labelling in earlier attempts to purify FDD-DNA.

The estimate of the molecular weight range of FDD-DNA from its S value in relation to a mixture of single-stranded linear and circular marker RNAs may not be accurate due to its unknown conformation under non-denaturing conditions. Nevertheless, the range obtained is consistent with the estimates obtained from electron microscopy, and the S value (12S to 15S) is consistent with the FDD-DNA having a molecular weight below that of geminivirus DNA ($s_{20,.w} = 16.0S$ at pH 7.0; Goodman & Bird, 1978).

FDD-DNA has a low electrophoretic mobility relative to its molecular weight in denaturing gels, and this would have been responsible for an erroneous previous estimate of $2 \times 10^6$ to $3 \times 10^6$ for its molecular weight (Randles et al., 1986) when linear DNA markers were used.

The detection of two or three bands in non-denaturing gels which have the same electrophoretic mobility in the second denaturing dimension suggests that under 'native' conditions the FDD-DNA occurs as two or three conformers which differ in secondary structure. Variations in secondary structure may explain the broad distribution of FDD-DNA in both zonal and isopycnic density gradient centrifugation.

All known plant viruses with ssDNA are placed in the geminivirus group. Geminiviruses contain either one or two circular ssDNA molecules of mol. wt. approx. $7 \times 10^5$ to $8 \times 10^5$ and comprising about 2700 nucleotides (Harrison, 1985). FDD-DNA is significantly smaller than geminivirus DNA, but is close to the size of the DNA of porcine circo virus (Tischer et al., 1982) which sediments at 12.7S, has a contour length of 633 µm, 1.76 kilobases, and a mol. wt. of $3.8 \times 10^5$. Nevertheless, as a few larger circles of about 2700 nucleotides have been observed in preparations by electron microscopy we cannot exclude the possibility that a geminivirus is implicated in FDD, and that the small circular DNA described here could represent the 'defective' circular ssDNA of about 1200 nucleotides observed in geminivirus infections (Harrison, 1985; Stanley, 1985). However, no geminivirus particles have been found in extracts from infected plants.

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