Variation in the Viroid-like RNA Associated with Cadang-Cadang Disease: Evidence for an Increase in Molecular Weight with Disease Progress

By JULITA S. IMPERIAL, Ma. JUDITH B. RODRIGUEZ AND J. W. RANDLES1*

Philippine Coconut Authority, Albay Research Centre, Guinobatan 4908, Philippines and 1 Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064

(Accepted 6 March 1981)

SUMMARY

The two cadang-cadang-associated RNAs (ccRNA-1 and ccRNA-2) occur as fast (f) and slow (s) electrophoretic variants. The detection of these variants is not affected by leaf storage or the nucleic acid extraction method. A systematic investigation has shown that their occurrence is related to the stage of disease development in coconut palms. Fast forms of both ccRNA-1 and ccRNA-2 predominated at the early stage of the disease, while only the (s) form was found at late stages. In some palms at the early stage a transition from the (f) to the (s) form was observed in progressively more recently developing infected fronds. ccRNA-1 (f) and cc-RNA-1 (s) are both circular, they differ in mol. wt., and they have nucleotide sequences in common. Preliminary evidence is presented that inoculum containing the (f) variant is more infectious than that containing the (s) variant.

INTRODUCTION

Two related RNA species (ccRNA-1 and ccRNA-2) with a unique nucleotide sequence (Randles & Palukaitis, 1979), and a predominantly circular structure (Randles & Hatta, 1979), are associated with the cadang-cadang disease of coconut. Their mol. wt. have been estimated by electron microscopy to be approx. 1 × 10^5 and 1.5 × 10^5 respectively (Randles & Hatta, 1979). ccRNA-1, which is present in larger amounts, has been shown to have viroid-like properties (Randles, 1975; Randles et al., 1976, 1977). Furthermore, the correlation of maximum infectivity with sucrose density-gradient fractions containing the highest concentrations of both ccRNA-1 and ccRNA-2 (J. W. Randles et al., unpublished results) suggests a viroid aetiology for the disease.

Cadang-cadang differs in two main respects from other diseases known to be caused by viroids. The first, mentioned above, is the association of the two unique RNA species with the disease. The second is the electrophoretic variation observed in these RNAs. This is shown either by the occasional detection of double bands (Randles et al., 1976), by variation in mobility of RNA extracted at different times from the same coconut palm (G. Boccardo, unpublished result), or by variation between palms collected at different localities (Randles & Salabao, 1978).

This paper describes an investigation of the nature of this electrophoretic variation, and in particular that of ccRNA-1. In this study we have taken advantage of the simple age-related frond-positioning of the coconut palm. With the production of new fronds at intervals of about 1 month (Randles et al., 1977), and the retention of fronds for up to 2 years after they...
first unfold, it is possible to study the time-course of changes in ccRNA-1 in fronds. We report here that a major change in the electrophoretic mobility and mol. wt. of ccRNA-1 occurs during development of the disease and that it is related to the length of time for which palms have been infected.

**METHODS**

**Source of palm material.** Mature diseased palms used in this study were from a number of sites in the Philippines. They were classified as being in the early, mid- or late stage of cadang-cadang (Nagaraj *et al.*, 1965; Price, 1971; Randles *et al.*, 1977). The duration of each stage varies, with estimated mean periods for 22-year-old palms of 2.9, 1.8 and 2.8 years respectively (Zelazny & Niven, 1980). Sampling, which was representative of all parts of fronds, was done by taking every 4th or 5th leaflet on both sides of the frond, discarding midribs, cutting the lamina into small pieces, mixing, and subsampling.

Juvenile palms used were those successfully inoculated at 2 to 3 months post-emergence in a number of mechanical transmission trials (Randles *et al.*, 1977; J. W. Randles *et al.*, unpublished results). The time between inoculation and assay was known for this population.

**Nucleic acid methods**

Nucleic acids were extracted from chopped leaflets by one of the following methods, depending on the analysis to be made.

**Method 1.** Fifty g samples were blended in 3 vol. (w/v) 0.1 M-Na₂SO₄. The extract was strained through nylon mesh, clarified by centrifugation and mixed with 5% polyethylene glycol (PEG) 6000 (Randles, 1975). The resulting precipitate was mixed and extracted with phenol–SDS and the aqueous phase was shaken with 0.5 vol. chloroform for 20 min. The nucleic acids were recovered by ethanol precipitation, dried, dissolved and mixed with 2 M-LiCl, left at 0 °C for 16 h, and the LiCl-soluble RNA recovered by ethanol precipitation.

**Method 2.** A solution containing 0.1 M-Na₂HPO₄, 0.1% sodium thioglycollate and 0.01 M-sodium diethyldithiocarbamate (Randles, 1975) replaced the 0.1 M-Na₂SO₄ used in Method 1. Otherwise all steps were as in Method 1.

**Method 3.** The nucleic acids were recovered by ethanol precipitation from the aqueous phase obtained after the phenol–SDS step in Method 1 and subsequently treated with Pronase and phenol–SDS (Randles, 1975).

Analytical gel electrophoresis under non-denaturing conditions was done in 5% polyacrylamide slab gels containing tris–borate–EDTA (TBE) buffer (Peacock & Dingman, 1968). Gels measured 140 × 170 × 0.75 mm and electrophoresis was at 12.5 mA/slab for about 2 h. For gel electrophoresis under denaturing conditions, samples were preheated in TBE-buffered 8 M-urea at 60 °C for 1 min, and gels were modified by the incorporation of 8 M-urea (Air *et al.*, 1976). Electrophoresis was at 15 mA for 3.25 h, either at room temperature (25 °C) or 60 °C. Gels were stained with 0.05% toluidine blue in 5% acetic acid.

Preparative gel electrophoresis was at 30 mA in non-denaturing 2.5% polyacrylamide slab gels 3 mm thick. Samples were denatured as above before loading, and nucleic acid components were eluted from the bottom of the gel at 1 ml/min using the preparative adaptor for the Biorad Model 220 gel apparatus. The eluate was monitored at A₂₆₀ and RNA in fractions was precipitated with ethanol in the presence of 0.2 M-sodium acetate. The composition of fractions was determined on analytical gels. ccRNA-1 prepared this way was free of ccRNA-2 detectable by staining.

**Inoculation.** Dried nucleic acid extracts were dissolved in SSC (0.15 M-NaCl–0.015 M-sodium citrate) and inoculated into the base of the spear leaf of coconut seedlings by high-pressure injection and slashing (Randles *et al.*, 1977).
Fast and slow variants of cadang-cadang RNA

Electron microscopy. The method of Randles & Hatta (1979) was used for examining the structure of denatured ccRNA-1 spread on formamide.

Molecular hybridization analysis. A $[^3H]c$DNA probe, complementary to ccRNA-1 (s) from a palm at the mid-stage of disease, was synthesized according to Randles & Palukaitis (1979). This cDNA was annealed with ccRNA-1 isolated by preparative gel electrophoresis from palms containing either ccRNA-1 (f) or (s), to a $R_0t$ between 0.36 and 0.55 mol. s/l. This $R_0t$ was about 200-fold greater than the $R_0t_4$ of homologous hybridization, so as to reach the maximum hybridization value, but was low enough to minimize any effects that minor contamination with ccRNA-2 might have on the result. Detection of hybrids by $S_1$ nuclease assay was done according to Randles & Palukaitis (1979).

RESULTS

Electrophoretic variation in ccRNA

Variation in the electrophoretic mobility of the cadang-cadang-associated RNAs was observed in gels containing between 3.3 and 20% polyacrylamide. Fig. 1 shows the major fast (f) and slow (s) forms separated on a 5% gel. These forms can occur alone, or together.

The variation in the ccRNA-2 follows that of the ccRNA-1 such that ccRNA-2 (f) accompanies ccRNA-1 (f), and ccRNA-2 (s) accompanies ccRNA-1 (s). Our studies have concentrated upon ccRNA-1 because of its higher concentration.

The form of ccRNA-1 was not affected by the extraction procedure. Forms remained as (f) or (s) when the tissue was extracted by any of the methods described. The incorporation of 0.5%, 1% or 2% diethylpyrocarbonate as a nuclease inhibitor in the extraction buffer of Method 1 similarly did not result in a change in the form extracted. Furthermore, storage of leaf at 4 °C for intervals up to 9 days, or frozen for intervals up to 4 days before extraction by standard procedures again had no effect on the form of ccRNA-1 extracted, even though other RNAs of host origin were no longer detectable as discrete bands after the longer storage times.

The relative electrophoretic mobilities of either the (f) or (s) forms from many palms were essentially the same, indicating that the (f) and (s) classification could be widely employed. They were recognized by the inclusion of marker samples in each gel slab. Although some extracts showed additional minor bands, these have not been investigated in this study.

In denaturing gels run at room temperature or at 60 °C, ccRNA-1 (f) and (s) migrated as single (f) or (s) bands respectively with an electrophoretic mobility characteristic of the circular form of ccRNA-1 (data not shown).

Structure of ccRNA-1 variants

cRNA-1 (f) and (s) from different palms were spread under denaturing conditions and examined by electron microscopy. Both preparations contained circular molecules (Fig. 2). ccRNA-1 (f) from one palm had a mean contour length of 107 nm (SE of mean = ±2 nm, n = 57) and ccRNA-1 (s) from another palm was 123 nm long (SE of mean = ±2 nm, n = 67). Thus the variation in electrophoretic mobility of (f) and (s) ccRNA-1 was not due to a difference in secondary structure, or to them occurring as linear or circular forms, but is due to a difference in mol. wt.

Comparisons of the nucleotide sequence of variants

cRNA-1 prepared from palms with either the (f) or (s) variant showed similar percentage hybridization values with cDNA prepared from ccRNA-1 (s) (Table 1). The sensitivity of molecular hybridization analysis for detecting differences between isolates of ccRNA-1 is limited by the relatively low maximum homologous hybridization values attainable with this
Fig. 1. Separation of ccRNA-1 (cc-1) and ccRNA-2 (cc-2) variants on 5% polyacrylamide gels. Nucleic acids are shown from palms containing; (1), fast (f) forms only; (2), slow (s) forms only; (3), fast + slow, where fast exceeds slow in quantity; (4), fast + slow, where slow exceeds fast.

Fig. 2. Electron micrographs showing circular molecules in preparations of (a) ccRNA-1 (f), and (b) ccRNA-1 (s). RNA in 90% formamide containing 10 mM-tris–HCl pH 8.5 and 1 mM-EDTA was heated at 60 °C, mixed with cytochrome c (34 µg/ml), and spread on 60% formamide containing 1.5 mM-tris–HCl pH 8.5 and 0.15 mM EDTA at 60 °C. Bar markers represent 100 nm.

system (Table 1; Randles & Palukaitis, 1979) so that minor differences in sequence may not be detectable. Nevertheless, the data show that there are no major differences between the nucleotide sequences of ccRNA-1 (f) and (s).
Fast and slow variants of cadang-cadang RNA

Fig. 3. Relative amounts of the fast and slow forms of ccRNA in sequentially sampled fronds from an early-stage palm which contained both forms. Fronds are numbered from the youngest on the left (second fully expanded frond) to the oldest. The oldest fronds contain (f) only, but (s) appears in fronds developing later. Also note (f) diminishes as (s) increases. Analysis was in non-denaturing gels.

Table 1. Percentage hybridization between [3H]cDNA [ccRNA-1(s)] and ccRNA-1(f) and (s) from other palms

<table>
<thead>
<tr>
<th>Palm</th>
<th>Variant of ccRNA-1</th>
<th>% Hybridization*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homologous</td>
<td>(s)</td>
<td>46</td>
</tr>
<tr>
<td>Heterologous</td>
<td>(s)</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>(f)</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>(f)</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>(f)</td>
<td>57</td>
</tr>
</tbody>
</table>

* Means of duplicate assays; values are corrected for self-hybridization of cDNA.

Table 2. Incidence of ccRNA-1 (f) and (s) in cadang-cadang-infected palms at different stages of disease*

<table>
<thead>
<tr>
<th>Variant of ccRNA-1</th>
<th>Early (39%)</th>
<th>Mid (8%)</th>
<th>Late (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(f)</td>
<td>33</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(f) + (s)</td>
<td>37</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>(s)</td>
<td>14</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

No. of palms at each stage

<table>
<thead>
<tr>
<th></th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>37</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

* Frond 3 or 6 was sampled from naturally infected palms.
Fig. 4. The assay of ccRNA from another early-stage palm in which fronds numbered left to right in order of increasing age were sampled. This figure is complementary to Fig. 3, and shows a situation where (f) is seen only in the oldest fronds, in combination with (s), and it is completely replaced by (s) in newly developing fronds. Analysis was in non-denaturing gels.

Relationship between variants and stage of disease

Of 154 mature diseased palms of different ages which were classified according to the stage of disease development and the ccRNA variant isolated from them (Table 2), ccRNA-1 (f) was found only in palms at the early and mid-stages of the disease, and the frequency was much lower at the mid-stage. ccRNA-1 (s) was found with increasing frequency as the stage of the disease advanced, and all late stage palms contained only ccRNA-1 (s). The frequency of palms with both forms was highest in the early stage.

A group of 35 diseased palms was selected in which the date of appearance of the first early symptoms was known. Assays done of the 5th youngest open frond within 30 months of the appearance of symptoms showed that 20 (57%) contained ccRNA-1 (f) only, 7 (20%) contained both (f) and (s), and 8 (23%) contained (s) only.
Fast and slow variants of cadang-cadang RNA

Table 3. Incidence of ccRNA-1 (f) and (s) in seedlings inoculated with unfractionated combined inocula at various intervals before first assay

<table>
<thead>
<tr>
<th>Time between inoculation and assay (years)</th>
<th>Variant of ccRNA-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(f)</td>
<td></td>
</tr>
<tr>
<td>&lt; 2</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>2-4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(f) + (s)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4. Results of a transmission trial using unfractionated inoculum containing either ccRNA-1 (f) or ccRNA-1 (s)

<table>
<thead>
<tr>
<th>Variant in inoculum</th>
<th>No. of seedlings with ccRNA at 11 months</th>
<th>16 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>(f)</td>
<td>5/10</td>
<td>10/10</td>
</tr>
<tr>
<td>(s)</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Uninoculated controls</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Relationship between variants and duration of infection in fronds of diseased palms

In a diseased palm, because of the age-related positioning of fronds, the oldest frond with ccRNA will be the first frond infected, and successively younger fronds will have been exposed to systemic infection later in disease development. Thus, each diseased palm consists of fronds in which the oldest and youngest infected expanded fronds can differ in age by up to 2 years.

Analyses were made of the ccRNA form in every frond, or in each alternate frond, of a series of palms at the early stage of infection. In some palms, either the (s) or (f) variants were observed in all fronds. On the other hand, some palms showed the patterns in Fig. 3 and 4, where there is a clear transition from the (f) variant in the oldest fronds through an intermediate stage where both (f) and (s) occur to the (s) form in the youngest, most recently infected fronds. We conclude from these observations that at the earliest stages of disease fronds were infected with the (f) variant. A transition phase then occurred during which the newly developing fronds became invaded with the (s) variant. As these fronds developed and the first infected fronds senesced, the (s) form became the only form present in fronds.

Relative infectivity of ccRNA from palms with (f) or (s) variants

The mature palms studied above were naturally infected. We therefore determined whether the (f) form also predominated in palms inoculated mechanically. Seedlings which had been successfully inoculated in a number of trials, using unfractionated inoculum combined from several diseased palms without regard to the form of ccRNA present, were classified according to the time between inoculation and the first assay for ccRNA, and the form of ccRNA isolated. The results (Table 3) showed that, overall, the (f) form was found more frequently than (s) or (f + s), but that (s) was detectable in a few seedlings at the earliest times tested.

Inoculum was then prepared by Method 3 from one palm with form (f) and another with form (s). As shown in Table 4, the infectivity of the inoculum from the palm containing form (f) was very high while none was detected with inoculum containing form (s). The detection of ccRNA (all with form (f)) 11 months after inoculation is the shortest interval from inoculation to ccRNA detection so far recorded, and the 100% transmission detected at 16
months is the highest value obtained in any transmission trial (Randles et al., 1977; J. W. Randles et al., unpublished results).

DISCUSSION

This study describes some aspects of the nature of the fast (f) and slow (s) variants of ccRNA and the relationship between them. Preparations of both variants of ccRNA-1 contain circular molecules, and so their different electrophoretic mobilities in denaturing gels arise from differences in their mol. wt., and not because they exist as circular or linear forms. Contour length measurements show that the (s) form is longer than the (f) form. The similar percentage homologies between the variants from several different palms show that a major part of (f) and (s) ccRNA-1 has the same nucleotide sequence.

The parallel changes in the electrophoretic mobilities of ccRNA-1 and ccRNA-2 suggests that ccRNA-2 increases in mol. wt. in the same manner as ccRNA-1. It therefore seems likely that the function and replication of these two RNAs is linked.

The results in Table 2 show a clear trend for a change from the (f) to the (s) variant as disease progresses. Furthermore, analysis of all fronds in several early stage diseased palms, selected because they contained (f) in the older fronds, showed how the pattern apparent in Table 2 arose. In these palms, the first fronds invaded by ccRNA would have contained the (f) form, and as the disease progressed, fronds which were produced later contained both (f) and (s), and then only (s). Thus, if (f) is present, it is replaced by (s) as disease progresses. The difficulty of recognizing very early stages of disease does not yet allow the conclusion to be drawn that all naturally infected palms first show only (f). Further observations on palms at the very early stage will be necessary to demonstrate whether the low percentage of (s) in early palms (Table 2) is due to the difficulty of identifying the early stage of disease, or to a low percentage of palms containing (s) in the first infected frond. The experimental transmission data in Table 3 show that (f) predominates in mechanically inoculated seedling palms up to 7 years after inoculation, but that (s) also occurs alone in a few seedlings very soon after ccRNA was first detectable by gel electrophoretic assay. Although Table 4 showed that inoculum containing (f) was more infectious than that containing (s), and that the ccRNA diagnostic of infection was detectable in the shortest period after inoculation hitherto observed, further studies on the comparative infectivities of the two forms purified from several sources are required.

The variation previously observed in the electrophoretic mobility of ccRNA-1 (Randles et al., 1976; Randles & Salabao, 1978) would now appear to be largely attributable to differences in the stage of disease in the palms sampled. Further investigations on the variation between geographical isolates will need to take account of the variation related to the stage of disease development.

We thank Dr T. Hatta for assistance with electron microscopy, Dr G. Boccardo and Dr N. A. Mohamed for helpful discussions, and Mr C. Davies and Mr A. Namia for excellent technical assistance. Part of this work was done under UNDP/FAO assistance in the Philippines. Support was also provided by the Australian Research Grants Committee.

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*(Received 16 December 1980)*