Arch Virol (2006) 151: 1447–1456
DOI 10.1007/s00705-005-0710-y

Archives of Virology
Printed in Austria

Variants of *Coconut cadang-cadang viroid* isolated from an African oil palm (*Elaies guineensis* Jacq.) in Malaysia

Brief Report

G. Vadamalai[1,3], **D. Hanold**[1], **M. A. Rezaian**[2], and **J. W. Randles**[1]

[1]Plant and Pest Science, School of Agriculture and Wine, University of Adelaide, Adelaide, South Australia, Australia
[2]Horticulture Unit, Commonwealth Scientific and Industrial Research Organization, Plant Industry, Glen Osmond, South Australia, Australia
[3]Department of Plant Protection, University Putra Malaysia, Serdang, Selangor, Malaysia

Received September 21, 2005; accepted November 22, 2005
Published online February 10, 2006

Summary. Variants of *Coconut cadang-cadang viroid* have been identified in a plantation oil palm growing in Malaysia. Three size classes are described, comprising 297, 293, and 270 nt. Compared with the 296-nt form of coconut cadang-cadang viroid (CCCVd), all variants substituted $C^{31} \rightarrow U$ in the pathogenicity domain and $A^{175} \rightarrow C$ in the right-hand terminus. Other mutations and deletions accounted for the different sizes. These are the first sequences reported for variants of *Coconut cadang-cadang viroid* in a species other than coconut palm, and this is the first evidence that variants closely related to CCCVd occur outside the Philippines.

*

Coconut cadang-cadang viroid (family *Pospiviroidae*, genus *Cocadviroid*) causes the lethal cadang-cadang disease of coconut palms (*Cocos nucifera* L.) in the Philippines. Total losses exceed 40 million palms [16]. Another cocadviroid, coconut tinangaja viroid (CTiVd), is associated with the decline of coconut production in Guam [27]. These viroids spread naturally but their epidemiology is poorly understood and no direct control measures are available [16]. Whereas

The GenBank accession numbers for the sequences reported in this paper are DQ097183–DQ097185.

CCCVd and CTiVd have only been characterised from coconut palm, a number of other species of palm and other monocotyledons in the Pacific region and South East Asia have been shown by molecular hybridisation to contain CCCVd-like RNAs [6, 9, 15, 18]. In commercially grown African oil palm in South East Asia and the South Pacific region such CCCVd-like molecules were frequently associated with an orange spotting disorder [6, 7] previously described as "genetic" orange spotting (GOS) [2, 5, 17, 22, 23, 26]. Since GOS occurs widely, and CCCVd is a quarantinable viroid, it was considered necessary to determine the degree of similarity between the CCCVd-like molecules and CCCVd. Because previous work [6] had shown that these CCCVd-like molecules were generally present at much lower concentrations than for CCCVd in coconut or oil palm [15], one oil palm with the highest detectable level of CCCVd-like RNA was selected for this study. We used a method for isolating and cloning circular molecules closely related by nucleotide sequence to CCCVd and describe the relationships at the molecular level between the variant CCCVd RNA sequences recovered from the oil palm and CCCVd.

Leaf samples were obtained from a four-year-old oil palm growing in a commercial plantation in Malaysia which had been shown by Northern blot hybridization [6] to contain CCCVd-like RNA (data not shown). Nucleic acids were extracted from leaflets of fronds 3 (third youngest), 10, and 20 using the polyethylene glycol (PEG) extraction method [6]. They were fractionated by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [21] in which the first dimension comprised a non-denaturing 5% polyacrylamide gel and the second dimension comprised a denaturing 5% polyacrylamide gel containing 8 M urea. Zones to which circular viroid molecules and their corresponding linear molecules were expected to migrate were identified by comparison with a parallel gel containing CCCVd. These zones were excised and eluted [20]. The RNA was collected by ethanol precipitation. Two sets of forward and reverse primers were designed from the $CCCVd_{246}$ sequence (GenBank accession number: J02050) for the reverse transcription-PCR (RT-PCR) amplification of CCCVd-related molecules; GVR4 (5′-TGT ATC CAC CGG GTA GTC TC-3′) and GV4 (5′-ACT CAC GCG GCT CTT ACC-3′) from nucleotides 191–172 and 192–209, respectively; GVR5 (5′-AGG TTT CCC CGG GGA TCC CTC AAG CGG CCT C-3′), and GV5 (5′-CGA ATC TGG GAA GGG AGC GTA CCT GGG TCG-3′) from nucleotides 71–41 and 76–105, respectively.

First-strand cDNA was synthesised with AMV reverse transcriptase (Promega) using the GVR4 and GVR5 reverse primers. This was amplified by PCR using a PCR master mix (Promega) containing 200 μM dNTPs, 1.5 mM $MgCl_2$, and 25 units/ml *Taq* DNA polymerase. PCR amplification products with primers GVR5/GV5 [10] or with primers GVR4/GV4 [18] were analysed by 5% PAGE or 2% agarose gel electrophoresis. Amplicons similar in size to that of CCCVd RT-PCR products were extracted from excised gel fragments using a MinElute Gel Extraction Kit (QIAGEN). They were cloned using a PCR Cloning Kit (QIAGEN) by ligation into the pDrive cloning vector (QIAGEN) and transformed into *E. coli* XL1-Blue competent cells (Stratagene) by electroporation (Bio-Rad

Gene Pulser) with settings of 1.8 kV, 25 μFD, and 200 Ω. Plasmids were prepared from transformed bacterial colonies and inserts excised and sized by PAGE. Seventeen clones were selected, and plasmids were sequenced at the Australian Genome Research Facility (AGRF) (Brisbane). Nucleotide sequences were compared by BLAST with available sequences on the NCBI database. Sequences were aligned using CLUSTAL W [1] after adjustments were made to maximize similarity using the GENEDOC program [11]. Secondary structure was predicted by Mfold version 3.1 [28].

The RT-PCR products from all three fronds of the oil palm were single amplicons ca. 300 bp in size, but they were detectable by silver staining only. To produce sufficient PCR product for cloning, the RT-PCR products were eluted after fractionation by 5% PAGE and re-amplified by PCR using the same primer set. Ethidium-bromide-stained amplicons were obtained with both primer sets (Fig. 1). As a control for this procedure, it was shown that no amplicons of viroid size were obtained from 2D-PAGE gel samples collected from zones outside the expected viroid region (Fig. 1).

Seventeen clones were obtained from the oil palm using both sets of primers. Twelve were 297 nt in size (OP_{297}), four were 293 nt (OP_{293}), and one was 270 nt (OP_{270}). OP_{297} and OP_{293} were obtained from all three fronds using both primer sets, and OP_{270} was obtained from frond 10 using GVR4/GV4 primers. All showed high sequence similarity with $CCCVd_{296}$, but none were identical to previously described forms of CCCVd [18, 19].

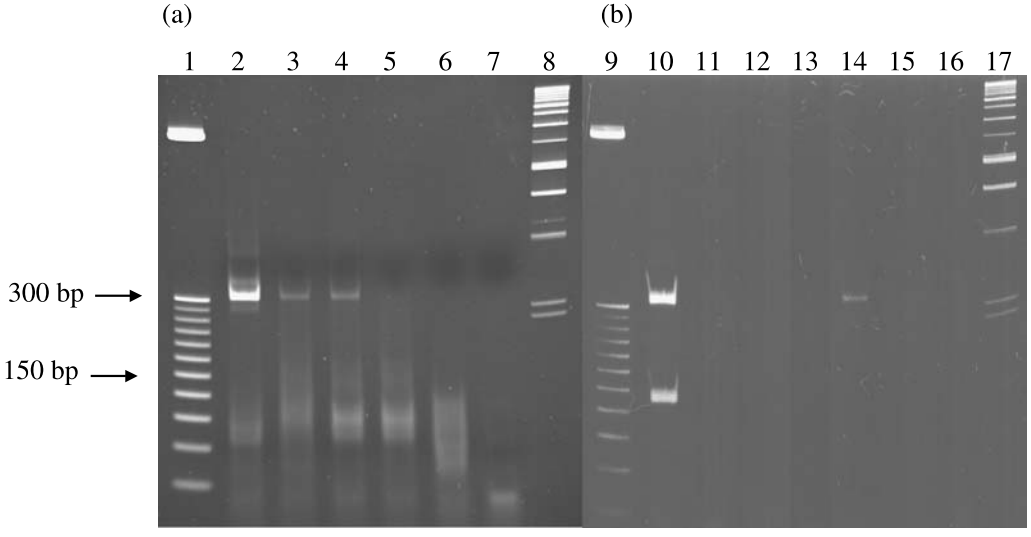

Fig. 1. PCR amplification of RT-PCR products from the circular viroid region of 2D-PAGE fractionated oil palm nucleic acids (*3, 4, 14*) compared with gel samples from the non-viroid region (*5, 6, 7, 11, 12, 13, 15*). **a** Primers GVR5/GV5, **b** primers GVR4/GV4. Size markers are 25 bp DNA ladder (*1, 9*), 1 kbp DNA ladder (*8, 17*) and the PCR product from the circular viroid region of CCCVd (*2, 10*) prepared the same way. *16* is a negative control (sterile distilled water as PCR template). This result was obtained from frond 10 of the viroid positive oil palm

CCCVd comprises two monomeric 'fast' ($CCCVd_{246}$ and $CCCVd_{247}$) and 'slow' ($CCCVd_{296}$ and $CCCVd_{297}$) electrophoretic forms as well as dimeric forms of each monomer [14]. $CCCVd_{247}$ differs from $CCCVd_{246}$, and $CCCVd_{297}$ from $CCCVd_{296}$, by the insertion of a cytosine at position nt 197 (see $*$, Fig. 2a). The slow RNAs are derived directly from the corresponding 'fast' forms by a partial duplication of the right-hand terminus (TR) of proposed rod-like secondary structure [8].

Of the 12 OP_{297} clones, four were identical and were defined in this study as the consensus sequence of OP_{297}. This sequence had 98% sequence similarity with $CCCVd_{296}$ and had the same duplication in the TR as described for $CCCVd_{296}$ (Fig. 2b). It differed by the insertion of a G in the TR and four single base substitutions (Fig. 2c), which were distributed across the pathogenicity domain (P), the central domain (C), and the TR. The remaining eight clones had one to five additional base substitutions in their sequence compared with the consensus sequence, and these were distributed across all five domains of the secondary structure (Fig. 2c). The possibility that the base changes were generated by PCR errors, including PCR-derived recombination [3] has been considered. However, the observations that some base changes were consistent across all variants and that the number of base changes observed greatly exceeds the reported error rate of $1–2 \times 10^{-4}$ for *Taq* polymerase [25] suggests that most are not due to random errors introduced by PCR amplification of these variants.

The consensus sequence of OP_{293} was derived from two identical clones of the four clones sequenced. As with OP_{297}, OP_{293} had a duplicated TR (Fig. 2d). It had 97% sequence similarity with $CCCVd_{296}$, differing by the deletion of four nucleotides, one insertion and four substitutions (Fig. 2d). Three of the deletions and one substitution occurred in the P domain. One deletion was in the C domain, and the other changes were in the TR. The other two clones had three and five additional base substitutions, respectively, which occurred in the variable (V), TR, and P domains (Fig. 2d).

OP_{270} had 90% sequence similarity with $CCCVd_{296}$. It showed no duplication of the top strand of the TR (Fig. 2e-indicated by grey bar) which, compared with OP_{297} and OP_{293}, represented a deletion of 27 nt. However, the lower strand of the TR was duplicated (Fig. 2e-indicated by black bar) except that the two base substitutions ($A^{149} \rightarrow C$ and $U^{151} \rightarrow C$) made this duplication imperfect. In addition, there were two more single base substitutions $C^{31} \rightarrow U$ in the P domain and $C^{221} \rightarrow U$ in the C domain. OP_{270} was predicted to have a branched secondary structure to accommodate the duplication of the lower TR domain alone (Fig. 2f). Absence of duplications of either the upper or lower strand of the TR domain has been previously reported in citrus exocortis viroid [24].

Because an arbitrary level of 90% sequence similarity and distinct biological properties are accepted as separating viroid species from variants [4], OP_{297}, OP_{293}, and OP_{270} can be considered as variants of CCCVd, and not a new species of cocadviroid. Each contain the minimal infectious $CCCVd_{246}$ sequence, and we propose that they be named as oil palm variants of CCCVd. These names should remain tentative until infectivity has been demonstrated. The provisional names

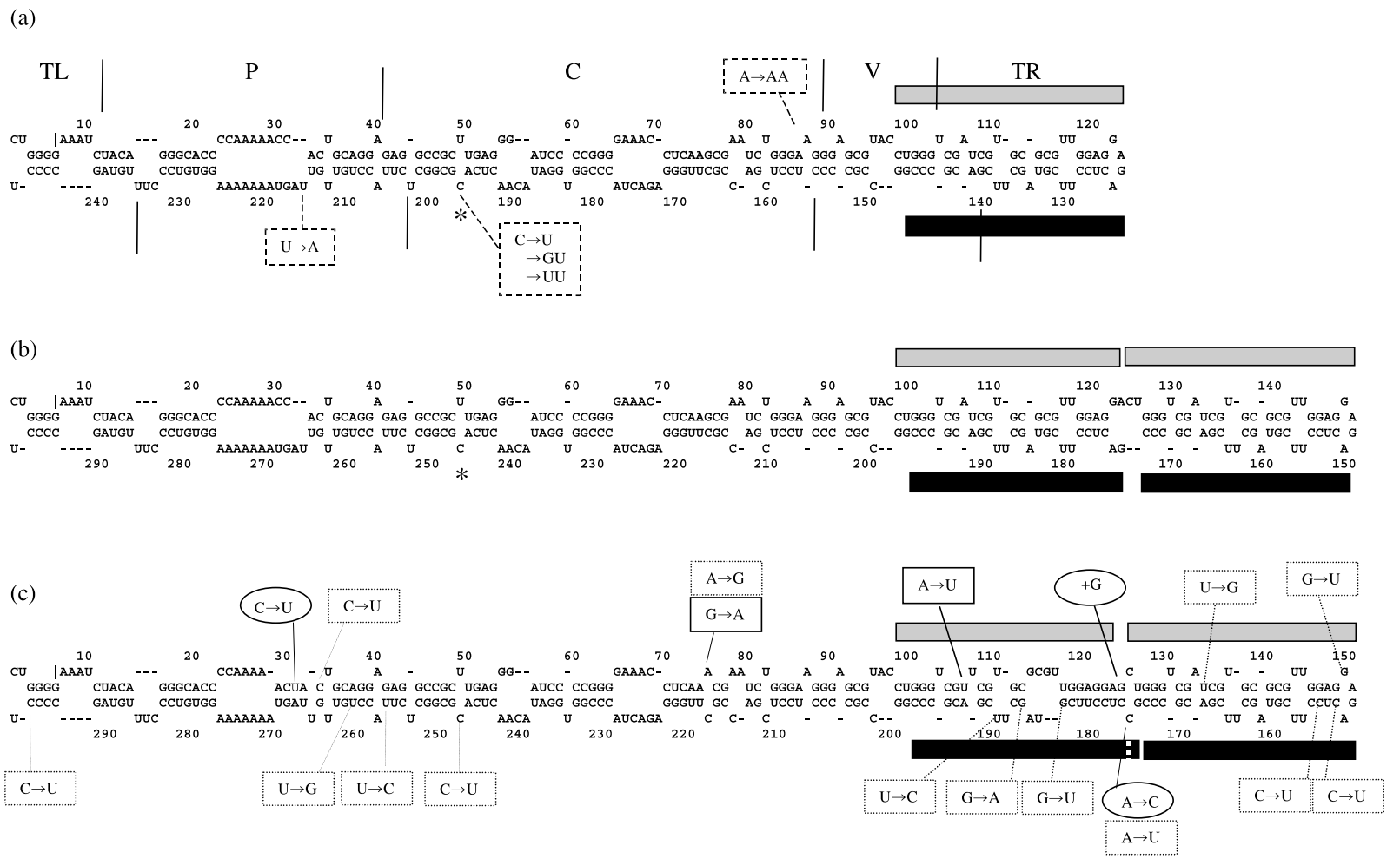

Fig. 2 (*continued*)

(d)
-A
C→U
-GU
U→C
-A
A→U
A→U
U→G
G→U
+G
A→C
G→U
G→A
G→C
C→G
(e)
C→U
C→U
A→C
U→C
(f)
5'
3'

are coconut cadang-cadang viroid (oil palm variant 297), coconut cadang-cadang viroid (oil palm variant 293) and coconut cadang-cadang viroid (oil palm variant 270). This suggestion provides for designation on the basis of size and can be revised as more data become available.

These oil palm variants were present at a much lower concentration than described for CCCVd. For example, after concentration by the PEG extraction procedure they were detectable only by Northern blot analysis of polyacrylamide gels (as previously described [6]) or by silver staining of RT-PCR amplicons (but not by the less sensitive ethidium bromide staining). Previous studies [10] showed that both CCCVd and CTiVd were detectable by ethidium bromide staining after one round of RT-PCR amplification of total nucleic acid extracts from coconut palm. They were also detectable in these extracts by ethidium bromide staining after agarose gel electrophoresis. CCCVd has been isolated from infected oil palms in the Philippines at levels similar to that in coconut palms [15], and it therefore seems possible that the low concentration of these oil palm viroids is a function of their variation in sequence from CCCVd rather than the species in which they occur. Mutations have been found to regulate replication and symptom expression of potato spindle tuber viroid variants [12, 13].

The base changes in the oil palm viroids differ from previously reported mutations in CCCVd [8, 19]. This is the first report that mutants of CCCVd occur in species other than coconut palm and outside the Philippines region. Mutation of viroids can give rise to a range of disease phenotypes, and for CCCVd, it has already been found that mutations in the P and C domain are associated with a severe form of coconut cadang-cadang disease known as 'brooming' (Fig. 2a) [19]. Now that the clones of the oil palm sequences are available, infectivity assays can be done to test their pathogenicity, to compare their levels of accumulation in tissues, and to see whether they are associated with phenotypes such as GOS.

Although more than one variant was recovered from a single oil palm, the majority of clones (71%) were of the 297-nt variant, suggesting that this was dominant in the nucleic acid sample and present in all three fronds. No variants of the 'fast' $CCCVd_{246}$ form were obtained in this study. This agrees with a previous report [6] that the CCCVd-like RNA from a GOS-affected oil palm in

Fig. 2. Secondary structures with a minimum computed folding energy at 37 °C of **a** $CCCVd_{246}$ (−112.5 kcal/mol), **b** $CCCVd_{296}$ (−137.3 kcal/mol), **c** consensus OP_{297} (−138.2 kcal/mol), **d** consensus OP_{293} (−143.4 kcal/mol), and **e** OP_{270} (−119.0 kcal/mol). **f** Shows the branched predicted secondary structure of OP_{270}. The sites where mutations occurred compared with $CCCVd_{296}$ are indicated. Those found in all three oil palm variants are indicated in solid oval text boxes, whereas those specific to one variant are shown in solid rectangular text boxes. Additional mutation sites found in single clones of OP_{297} and OP_{293} are indicated in dotted text boxes. Duplicated sequences are indicated with the same shade. White dots in the duplicated sequences indicate the site of a base substitution which makes the duplication imperfect. The base substitutions indicated in dashed text boxes in $CCCVd_{246}$ are the mutation points of the 'brooming' mutant [19]. TL, P, C, V, and TR indicate the five domains of CCCVd; terminal left, pathogenicity, central, variable, and terminal right, respectively

the Solomon Islands was about 296 nt in size, and suggested that the 'slow' viroid form is dominant in oil palm. In addition to the three size classes, there were minor and apparently sporadic variations among the individual variants. This appears to be a further example of viroids propagating in their hosts as populations of closely related sequence variants (quasi-species), with one or more predominating in the population [4].

The primers selected for RT-PCR were not designed to target sequences in other cocadviroids or pospiviroids, so this study does not exclude the possibility that viroids other than CCCVd, or distant variants of CCCVd, may be present in oil palm. Of immediate concern is the close similarity of the variants reported here to CCCVd. This prompts speculation that oil palm may be an important reservoir of CCCVd and a vehicle for the international distribution of a core sequence which has the potential to mutate to a pathogenic form.

In conclusion, the identification of these CCCVd variants in a commercially grown oil palm shows that viroid molecules closely related to the lethal CCCVd are present in oil palm plantations in a region outside the Philippines, the country where CCCVd is thought to be contained. The observation that they occur at a low concentration suggests that CCCVd variants may exist below the threshold of detection in some plant reservoirs. Such subliminal infections may provide hitherto unrecognised sources of viroid sequences which could mutate into pathogenic variants, with the potential to cause an economically important disease. Further work should include a study of the infectivity of this range of CCCVd variants in oil palm, their effects on symptom development and commercial yield, and their geographical distribution.

Acknowledgements

We gratefully acknowledge Consolidated Plantations Bhd (formerly Sime Darby), Malaysia for providing the leaf material for this study and Mr. Chung Gait Fee and Mr. Shaji Thomas of Consolidated Plantations for their help in collection of the oil palm leaf samples. We also thank Dr. Satish Dogra for helpful discussions and advice on cloning.

References

1. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31: 3497–3500
2. Coulter JK, Rosenquist EA (1955) Mineral nutrition of the oil palm. A study of the chemical composition of the frond in relation to chlorosis and yield. Malay Agricult J 38: 214–236
3. Fernandez-Delmond I, Pierrugues O, de Wispelaerae M, Guilbaud L, Gaubert S, Diveki Z, Godon C, Tepfer M, Jacquemond M (2004) A novel strategy for creating recombinant infectious RNA virus genomes. J Virol Methods 121: 247–257
4. Flores R, Hernandez C, Martinez de Alba AE, Daros JA, Di Serio F (2005) Viroids and viroid-host interactions. Annu Rev Phytopathol 43: 117–139
5. Forde SCM, Leyritz MJP (1968) A study of confluent orange spotting of the oil palm in Nigeria. J Niger Inst Oil Palm Res 4: 371–380

6. Hanold D, Randles JW (1991) Detection of coconut cadang-cadang viroid-like sequences in oil and coconut palm and other monocotyledons in the south-west Pacific. Ann Appl Biol 118: 139–151
7. Hanold D, Randles JW (2003) CCCVd-related molecules in oil palms, coconut palms and other monocotyledons outside the Philippines. In: Hadidi A, Flores R, Randles JW, Semancik JS (eds), Viroids, CSIRO Publishing, Australia, pp 336–344
8. Haseloff J, Mohamed NA, Symons RH (1982) Viroid RNAs of cadang-cadang disease of coconuts. Nature 299: 316–321
9. Hodgson RAJ, Randles JW (1999) Detection of coconut cadang-cadang viroid-like sequences. In: Oropeza C, Verdeil JL, Ashburner GR, Cardena R, Santamaria JM (eds), Current plant science and biotechnology in agriculture 35: Current advances in coconut biotechnology, Kluwer Academic Publishers, London, pp 227–246
10. Hodgson RAJ, Wall GC, Randles JW (1998) Specific identification of coconut tinangaja viroid for differential field diagnosis of viroids in coconut palms. Phytopathology 88: 774–781
11. Nicholas KB, Nicholas HB Jr, Deerfield DW II (1997) GeneDoc: Analysis and visualization of genetic variation. EMBNEW.NEWS 4: 14
12. Owens RA, Chen W, Hu Y, Hsu Y-H (1995) Suppression of *Potato spindle tuber viroid* replication and symptom expression by mutations which stabilize the pathogenicity region. Virology 208: 554–564
13. Qi Y, Ding B (2002) Replication of *Potato spindle tuber viroid* in cultured cells of tobacco and *Nicotiana benthamiana*: The role of specific nucleotides in determining replication levels for host adaptation. Virology 302: 445–456
14. Randles JW (1985) Coconut cadang-cadang viroid. In: Maramorosch K, McKelvey JJ Jr (eds), Subviral pathogens of plants and animals: viroids and prions. Academic Press Inc, Orlando, Florida, pp 39–74
15. Randles JW, Boccardo G, Imperial JS (1980) Detection of the cadang-cadang RNA in African oil palm and buri palm. Phytopathology 70: 185–189
16. Randles JW, Rodriguez MJB (2003) Coconut cadang-cadang viroid. In: Hadidi A, Flores R, Randles JW, Semancik JS (eds), Viroids. CSIRO Publishing, Australia, pp 233–241
17. Robertson JS, Prendergast AG, Sly JMA (1968) Diseases and disorders of the oil palm (*Elaeis guineensis*) in West Africa. J Niger Inst Oil Palm Res 4: 381–409
18. Rodriguez MJB (1993) Molecular variation in coconut cadang-cadang viroid (CCCVd). Ph.D thesis. The University of Adelaide
19. Rodriguez MJB, Randles JW (1993) Coconut cadang-cadang viroid (CCCVd) mutants associated with severe disease vary in both the pathogenicity domain and the central conserved region. Nucleic Acids Res 21: 2771
20. Sambrook J, Russell DW (2001) Molecular cloning: A laboratory manual, 3rd edn, CSH Laboratory Press, Cold Spring Harbor, New York
21. Schumacher J, Randles JW, Reisner D (1983) A two-dimensional electrophoretic technique for the detection of circular viroids and virusoids. Anal Biochem 135: 288–295
22. Smilde KW (1962) 10th Annual Report of the West African Institute for Oil Palm Research, West African Institute for Oil Palm Research, pp 71–78
23. Smilde KW (1963) 11th Annual Report of the West African Institute for Oil Palm Research, West African Institute for Oil Palm Research, pp 67–76
24. Szychowski JA, Vidalakis G, Semancik JS (2005) Host-directed processing of Citrus exocortis viroid. J Gen Virol 86: 473–477
25. Tindall KR, Kunkel TA (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 27: 6008–6013

26. Turner PD (1981) Oil palm diseases and disorders. Oxford University Press, Kuala Lumpur
27. Wall GC, Randles JW (2003) Coconut tinangaja viroid. In: Hadidi A, Flores R, Randles JW, Semancik JS (eds), Viroids, CSIRO Publishing, Australia, pp 242–245
28. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31: 3406–3415

Author's address: Dr. John W. Randles, Plant and Pest Science, School of Agriculture and Wine, University of Adelaide, Adelaide, South Australia, Australia; e-mail: john.randles@adelaide.edu.au