# PROGRESS IN IDENTIFYING THE CAUSE OF RAMU STUNT DISEASE OF SUGARCANE

By

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**Abstract**

RAMU STUNT first appeared as a serious disease affecting plant growth and ratooning ability at Ramu Sugar Limited in Papua New Guinea during 1985–1986. The disease has not yet been found in Australia, but the insect vector occurs on many Torres Strait islands and at Bamaga and New Mapoon; therefore, Ramu stunt is a major biosecurity risk to the Australian sugar industry. The capability to monitor Ramu stunt disease and prevent or manage potential incursions in Australia would be greatly facilitated by identification of the causal agent, understanding the biology of the disease and the development of a robust diagnostic test. No evidence for a phytoplasma as the causal agent has been obtained. Several lines of evidence suggest that a virus is the causal agent of the disease. Disease-associated nucleic acids were cloned and a preliminary diagnostic test was developed based on cloned RNA sequences with homology to viral RNA-dependant RNA-polymerase. Although only used to screen limited numbers of samples, it is an important step towards the development of a more robust test and the exact identification of the causal agent.

## Introduction

Ramu stunt was unknown until 1985–1986 when poor yields, stool death and severe stunting became evident in commercial crops of *cv.* Ragnar sugarcane at Ramu Sugar Estate in Papua New Guinea (PNG) (Waller *et al.,* 1987). It is characterised by a marked reduction in growth rate, seen as reduced internode length, reduced root system and failure to ratoon (Suma and Jones, 2000).

Transmission between plants is rapid, and the sugarcane planthopper *Eumetopina flavipes* is the suspected vector (Kuniata *et al.,* 1994). Symptoms typical of Ramu stunt and *E. flavipes* are widespread in PNG (Magarey *et al*., 2002). The disease has not yet been found in Australia, although the insect vector occurs on many Torres Strait islands and at Bamaga and New Mapoon on Cape York Peninsula (Allsopp, 1991; Magarey *et al*., 2007).

Thus, Ramu stunt is an important biosecurity threat to the Australian sugar industry. The capability to monitor Ramu stunt disease and prevent or manage potential incursions in Australia would be greatly facilitated by identification of the causal agent, understanding the biology of the disease and the development of a robust diagnostic test.

A diagnostic test for Ramu stunt would also allow safer movement of sugarcane germplasm and identification of infected planthoppers. Currently, many sugarcane

organisations that import sugarcane germplasm are reluctant to import *Saccharum* species from PNG because of the risk of Ramu stunt.

Research to identify the causal agent of Ramu stunt has been supported by a project funded by the Australian Centre for International Agricultural Research (ACIAR) to assess pest and disease threats to *Saccharum* germplasm and sugar production in Papua New Guinea, Indonesia and Australia. The causal agent was suspected to be a virus (Jones *et al*., 1989) or a phytoplasma (Cronje *et al.*, 1999). Initial attempts to identify the causal agent focussed on double-stranded RNA extractions, but were unsuccessful (Magarey *et al.,* 2003). Recent work has concentrated on attempting to detect phytoplasmas or viruses, and good evidence for viral involvement in Ramu stunt-infected sugarcane has now been obtained. A preliminary diagnostic test has been developed.

## Materials and methods

Ramu stunt-infected and healthy sugarcane was supplied by Gou Rauka, Ramu Sugar Ltd, PNG. The highly susceptible cultivar Ragnar was used for most experiments.

Phytoplasma detection was based on the conditions for nested polymerase chain reaction (PCR) described by Suma and Jones (2000).

A small-scale virus purification method (miniprep) based on ultracentrifugation through two sequential sucrose cushions was developed from the method described in Lane (1986). The pelleted proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by silver staining. Peptides derived from excised protein bands were sequenced after a 16-hour tryptic digest and separation on a Capillary LC system (Australian Proteome Analysis Facility).

Nucleic acids associated with Ramu stunt were generated by reverse transcriptase PCR (RT-PCR) cloning. RNA purified from healthy and Ramu stunt-infected Ragnar viral minipreps was used as the template. The RT-PCR products were cloned, sequenced and the putative identity of the sequences was determined from BLAST searches of databases. The programs BLASTX (translated query vs protein database) and BLASTN (nucleotide query vs nucleotide database) were used (Altschul *et al*., 1997).

The diagnostic RT-PCR test for Ramu stunt is based on the forward and reverse primers, KB20Cfwd (5'GAG CTG ATA TTT AGA CTT AGG GTT GC3') and KB36Arev (5'GTT TTA CTG ATA TGG ACA ACT C3'), selected from two sequences generated during the RT-PCR cloning experiment (KB20 and KB36). These sequences were found to have high homology to the RNA-dependant RNA-polymerase from Rice stripe tenuivirus (Genbank accession AY186788) and Rice grassy stunt tenuivirus (Genbank accession AB009656). Initial development used RNA purified from viral minipreps as the template. For diagnostic screening, total RNA extracted from sugarcane leaves using a QIAGEN RNeasy kit was used as the template. Amplified products were resolved on 1% agarose gels.

## Results

### Attempts to detect a phytoplasma in sugarcane infected with Ramu stunt

DNA extracted from healthy and Ramu stunt-infected leaves was amplified initially with the primers SN910601 and P6, followed by a nested amplification using primers R16F2n and R16R2, as described in Suma and Jones (2000).

The amplification products were then digested with the restriction enzymes *Rsa*I, *Alu*I or *Hae*III, separated on 2% agarose or 3% NuSeive gels and compared to the restriction patterns obtained from known phytoplasma standards including Cynodon white leaf (related to sugarcane white leaf) and *Whiteochloa* grassy shoot (related to sugarcane grassy shoot). PCR products could be amplified from many of the samples provided by Ramu Sugar, including healthy plants (data not shown), suggesting that non-specific amplification was

occurring. After digestion with the restriction enzymes, the PCR products did not give digest patterns that resembled the standards (data not shown), confirming that non-specific products were being amplified.

**Attempts to detect a virus in sugarcane infected with Ramu stunt**

Healthy and Ramu stunt-infected sugarcane leaves were homogenised and centrifuged through two sucrose cushions. The resuspended pellet was examined under a Hitachi H7000 transmission electron microscope at QDPI&F Horticulture and Forestry Science, and small isometric viral particles were observed in minipreps extracted from Ragnar infected with Ramu stunt (Figure 1). These particles were estimated to be 30 nm in diameter.

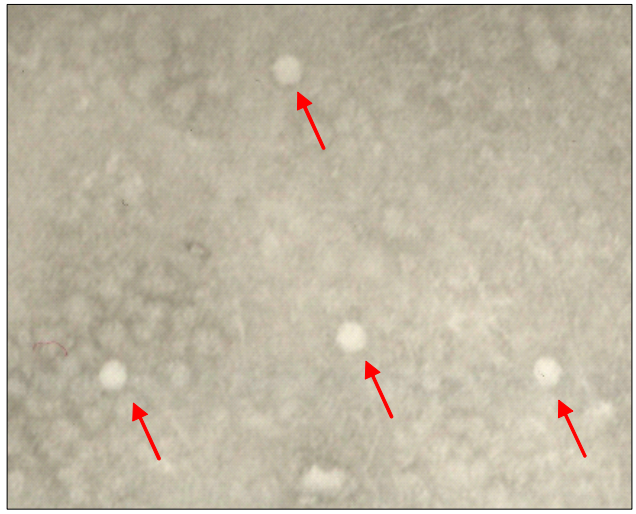

Fig. 1—Transmission electron micrograph of isometric viral particles observed in viral extracts from Ragnar infected with Ramu stunt.

The proteins within the miniprep pellet were analysed by separation on 4–12% gradient SDS-PAGE gels (Figure 2). A 36 kDa protein band was detected in plants showing Ramu stunt disease which was not detected in healthy Ragnar cane from PNG, Australian sugarcane, or sugarcane infected with Fiji disease virus or Sugarcane mosaic virus. Subsequently, this 36 kDa protein was detected in viral minipreps extracted from seven clones infected with Ramu stunt (Ragnar, Q125, N7, BJ-7013, PN93-18, PN93-38 and PN92-294), but not in minipreps from the corresponding healthy canes.

The protein band is very clear in good quality tissue but its presence is variable and degrades quickly if the leaves are in poor condition. A gel section containing the 36 kDa protein was excised from the SDS-PAGE gel, subjected to a tryptic digest, and analysed by internal sequencing. Three peptide sequences of 11, 15 and 20 amino acids in length were obtained; however, none of them had any significant homology with protein sequences contained within the non-redundant protein database using BLASTP (Altschul *et al.*, 1997).

**Characterisation of disease-associated nucleic acids**

***1. Primers based on the peptide sequences***

RT-PCR primers were designed to match each of the three peptides generated by sequencing the 36 kDa protein. RT-PCR products were generated only in samples infected with Ramu stunt, but all sequences generated had no significant matches using BLASTN or BLASTX.

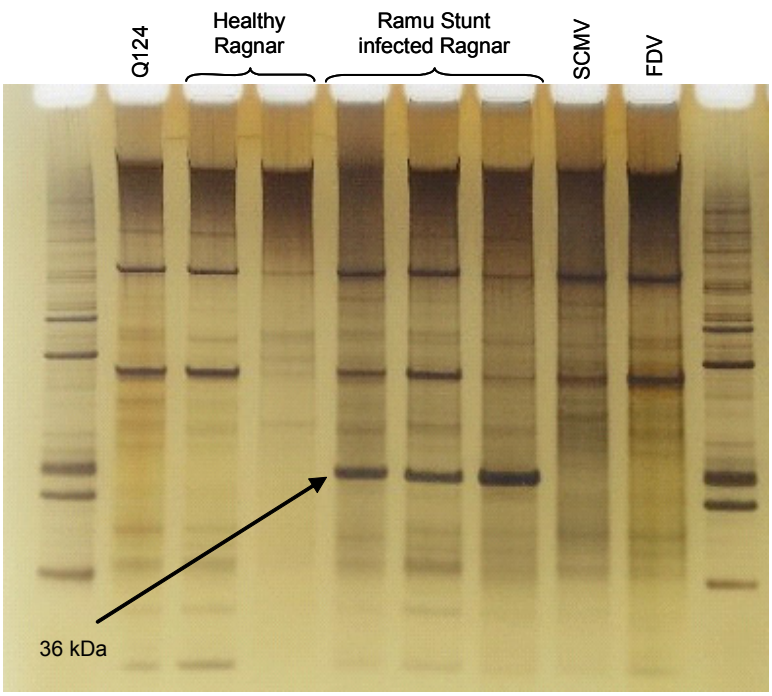


Fig. 2—A 36 kDa protein is observed in viral extracts from leaves infected with Ramu stunt, but not healthy leaves or leaves infected with Sugarcane mosaic virus (SCMV) or Fiji disease virus (FDV).

***2. Universal and random primers***

A random cloning approach was tried, using either a universal cDNA primer (Froussard, 1992) or random hexamers to prime the RT reactions. RNA purified from healthy and Ramu stunt-infected Ragnar viral minipreps was used as the template.

From this, sequences derived from only six RT-PCR products were homologous to viral protein sequences using the BLASTX algorithm. Of the six sequences, two had significant matches: sequence KB20 matched Rice stripe tenuivirus RNA-dependant RNA-polymerase with a score of 65.5 and E value of $8x10^{-10}$ and sequence KB36 matched Rice grassy stunt tenuivirus RNA-dependant RNA-polymerase with a score of 101 and E value of $1x10^{-20}$. However, this homology was only apparent at the amino acid level, and was not repeated when the sequences were subjected to homology searching using the non-redundant nucleic acid databases and BLASTN. The remaining four sequences had matches with Rice stripe tenuivirus and Maize yellow stripe tenuivirus, but with higher E values.

**Development of a diagnostic test**

PCR primers based on the two RNA polymerase sequences were designed and used to develop a diagnostic RT-PCR test. Using RNA purified from viral minipreps as the template, a 1 kb product was generated from Ragnar infected with Ramu stunt. This sequence was aligned with the original sequences to generate a 1358 bp contig. This sequence matched Rice stripe tenuivirus RNA polymerase with an E value of $5x10^{-43}$ using BLASTX. Rice grassy stunt tenuivirus was the second highest match, with an E value of $1x10^{-37}$.

Total RNA extracted from sugarcane leaves was used as the template to screen sugarcane samples. The RT-PCR product could be generated from the same canes that gave the 36 kDa protein (Figure 3). The RT-PCR products amplified from Ragnar, Q125, N7 and

PN93-18 were cloned and sequenced. The sequences amplified from total sugarcane RNA aligned closely with the 1358 bp contig cloned from viral RNA, with a maximum of three base changes at the nucleotide level. The sequences also had a high match to Rice stripe virus RNA polymerase at the amino acid level. For example, the sequence amplified from Ragnar sugarcane leaf RNA matched Rice stripe virus with an E value of $2x10^{-23}$ using BLASTX.

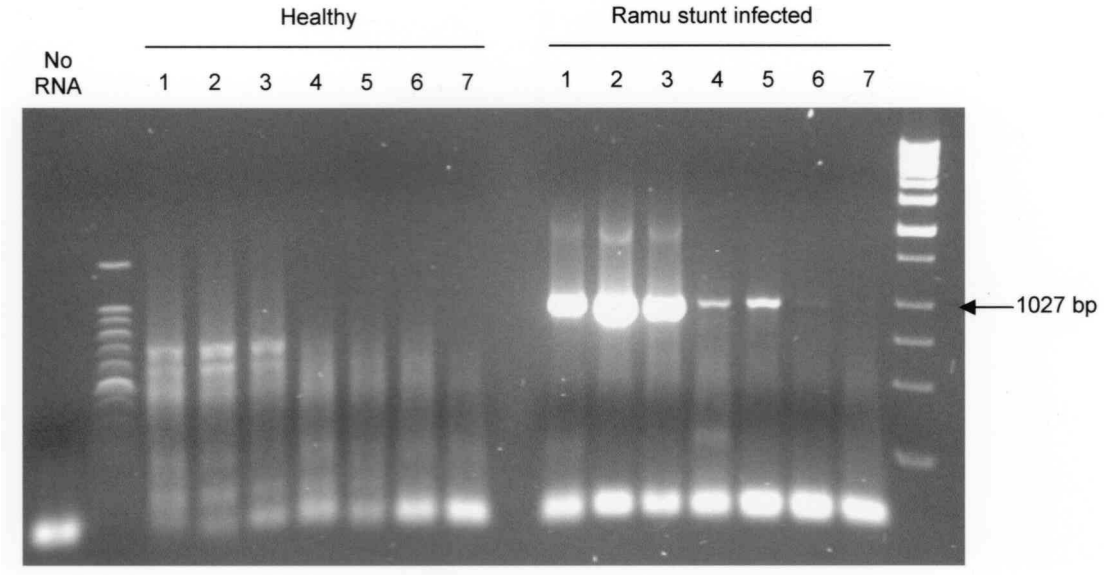


Fig. 3—Diagnostic test for Ramu stunt using RT-PCR. A 1027 bp product was amplified from RNA extracted from Ramu stunt infected, but not healthy leaves. Samples are from (1) Ragnar, (2) Q125, (3) N7, (4) BJ-7013, (5) PN93-18, (6) PN92-294 and (7) PN93-38.

## Discussion

The causal agent of Ramu stunt has been unknown since the disease appeared at Ramu Sugar in 1985. Accurate diagnosis is impossible without knowing the cause of the disease and without a reliable assay. The leaf mottling and striping symptoms and associated poor growth are not always diagnostic, because of symptom variation between cultivars. The molecular approaches described in this paper have advanced our understanding of the causal agent of Ramu stunt, and all evidence suggests that a virus is associated with the disease.

Cronje *et al.* (1999) claimed that a phytoplasma in the sugarcane white leaf group was associated with Ramu stunt, and a detailed nested-PCR protocol for detection of the phytoplasma was described in Suma and Jones (2000).

However, in our hands, this test generated non-specific PCR products from the samples sent to BSES Limited. At this stage, we have no evidence for the presence of a phytoplasma in Ramu stunt-infected sugarcane.

In contrast, there are now three lines of evidence implicating a virus as the causative agent of Ramu stunt in sugarcane:

(1) A 36 kDa protein was detected consistently in viral preparations of Ramu stunt;

(2) Isometric viral particles were observed in viral preparations from Ragnar infected with Ramu stunt; and

(3) Sequences of RNA with significant homology to tenuivirus proteins have been cloned from viral preparations of Ramu stunt.

The random RT-PCR cloning experiment produced six sequences with homology to Rice stripe virus, Rice grassy stunt virus and Maize yellow stripe virus, all members of the genus *Tenuivirus*. Tenuiviruses infect only members of the Poacae, including economically important crops such as rice, maize and wheat, cause symptoms such as fine stippling, stripes, chlorosis and white leaf, and are only transmitted by delphacid planthoppers. Thus, tenuivirus diseases share some characteristics with Ramu stunt. However, the virions of tenuiviruses comprise very long filaments, 3–10 nm in diameter, often forming a circular outline. The isometric viral particles seen under the electron microscope in this study do not match this morphology.

The three peptide sequences from the 36 kDa protein identified in viral preparations of Ramu stunt lacked homology to any known proteins. Most DNA sequences generated through random RT-PCR cloning also lacked homology to any known products. Only sequences from six clones were homologous to viral sequences using the program BLASTX, but not BLASTN. The inability to find homologues to Ramu stunt proteins and nucleic acids suggests that the virus is unique. Thus, it appears that the causal agent of Ramu stunt is not a tenuivirus, but may be a unique virus related to the tenuivirus group. This was observed with another sugarcane disease. Early sequence results suggested that the causal agent of sugarcane striate mosaic disease was a *Foveavirus* or *Carlavirus* (Choi *et al*., 1999). However, sequencing of the entire genome later revealed that Sugarcane striate mosaic associated virus (SCSMaV) was in neither genera, and probably represents a new plant virus taxon (Thompson and Randles, 2001).

Despite limited homology to known viral nucleic acid sequences, two cloned sequences were identified with good homology to the RNA-dependant RNA-polymerase. This is an essential protein for all RNA viruses and is involved in genome replication. The sequences were used to design a diagnostic test for Ramu stunt. The diagnostic test can detect a 1 kb RT-PCR product in a range of clones infected with Ramu stunt. Hence, while the causal agent is still unknown, a preliminary diagnostic test is available. The test can be used at Ramu Sugar as part of their disease management program, used by sugar industries wishing to import sugarcane germplasm from PNG, and used by both Australian and Indonesian sugar industries to confirm suspected disease outbreaks.

Current research is focussed on improving the viral purification process since pure viral preparation is necessary for cloning and sequencing of the entire viral genome, leading to identification of the virus. Research into vector transmission is also planned.

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