STUDIES ON THE ULTRASTRUCTURAL LOCALISATION OF VIROIDS AND OTHER PLANT PATHOGENS

Thesis submitted for the degree of
Doctorate of Philosophy
at the University of Adelaide

by

Roderick Bonfiglioli (B.Sc., Hons)
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SUMMARY

Viroids are the smallest known pathogens in nature. They are all pathogens of higher plants and there are 27 known viroids so far, with all but two of them being restricted to dicotyledonous plants. The remaining two are only found in monocotyledonous plants. Viroids are comprised only of naked, single stranded circular RNA molecules, they range in size from 246 to 463 nucleotides in length. They do not have a DNA phase in their existence and they are not known to code for any proteins. As they do not code for their own proteins, they must use host encoded enzymes for all stages of their replication and for all other biological functions where any other factors are required.

This work was designed to localise viroids at the histological and sub-cellular level and to determine with which cellular compartments the different viroids are associated. The localisation of viroids to specific sub-cellular compartments provides information that can be correlated to what we already know about the replication of viroids, and adds evidence to support the theories concerning the replication of the different viroids.

Taxonomically viroids are classified into two main groups, the avocado sunblotch viroid (ASBV) group with only 3 known members, and the potato spindle tuber viroid (PSTV) group with the remaining 24 members.

The ASBV group of viroids is characterised by the presence of a known and characterised self cleavage mechanism whereby the linear multimeric viroid molecules transcribed by host encoded polymerase enzymes are processed into circular monomeric viroid molecules. The PSTV group of viroids is characterised by the similarity of sequences seen between the different members of this group. The PSTV group is further divided up into three sub-groups, again based on sequence similarities.

Research into viroid replication has shown that a host encoded enzyme, the nuclear RNA polymerase II, is most likely to be responsible for the replication of viroids in the PSTV group, but is not involved in the replication of ASBV.

The majority of the work in this study, in both the viroid and the phytoplasma studies involved the development and trial of many different techniques and methods. Many different techniques for preparation of material for *in situ* hybridisation experiments were explored, and many different methods of *in situ* hybridisation were tried. Each different plant and particular type of plant material presented its own unique problems,

and only the successful techniques and methods are reported here. Two papers in a major international journal have resulted from this work, and a third is now in preparation.

In this work the ultrastructural location of three viroids was determined by high resolution *in situ* hybridisation in conjunction with transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). The three viroids studied in this work were ASBV, the type member of the ASBV group, and two viroids from the PSTV sub-group of the main PSTV group, namely citrus exocortis viroid (CEV) and coconut cadang-cadang viroid (CCCV).

ASBV was localised to the thylakoid membranes of the chloroplast, while CEV and CCCV were localised to the nucleoplasm and the nucleolus respectively. The results from these localisation studies is correlated to what we know about the replication of these viroids, and the different proposed models of viroid replication are re-examined in the light of the findings from this study.

The localisation of ASBV to the chloroplast is in keeping with earlier and subsequent works, and further underlines the differences between the ASBV group of viroids and the PSTV group of viroids. The localisation of ASBV to the chloroplasts suggests that the enzyme responsible for the replication of ASBV is therefore most likely to be the chloroplastic RNA polymerase, and this finding has interesting implications for the biology and evolution of the ASBV viroids.

CEV was localised to the nucleoplasm, which is in keeping with what we already know about the replication of CEV by the nucleoplasmic enzyme, RNA polymerase II, but the localisation of CCCV, which is also thought to be replicated by RNA polymerase II, to the nucleolus, raises some interesting questions. The questions that are raised focus on the possible replication of CCCV by the enzyme RNA polymerase I which is located in the nucleolus, and the possible post-replication translocation of the viroid from the nucleoplasm to the nucleolus. Within the scope of this study, it was not possible to answer these questions directly as the experiments did not actually determine the site of synthesis, rather they simply located the viroids to the specific sub-cellular compartment, which may only be a site of accumulation. The results did, however, provide fresh information which enabled us to look more critically at the proposed models of viroid replication.

A further aspect of this work focused upon developing an *in situ* hybridisation method to specifically identify and localise particular phytoplasmas in plant and insect cells. Phytoplasmas are important

pathogens of many agricultural and horticultural crops, and they present particular problems for *in situ* diagnostic work in that the only phytoplasma specific sequence of DNA or RNA that can be used so far as an *in situ* probe is derived from the highly conserved 16S rDNA sequence. The optimal probe length for in situ hybridisation work is thought to be around 250 to 600 bases in length, and the use of a probe of such length aimed at the 16S sequence would be expected to cross-hybridise with most of the prokaryotic kingdom.

The results of this *in situ* work with phytoplasmas showed that it is possible, given the correct hybridisation and stringency conditions, to use short oligonucleotides that were originally designed as PCR primers as probes for the identification of specific phytoplasma in *in situ* hybridisation experiments. This finding is most gratifying, as one of the main difficulties in performing *in situ* hybridisation experiments is experienced in obtaining a suitable probe, an exercise which normally involves a significant amount of sequencing and cloning. We gained specific hybridisation in high resolution *in situ* hybridisation experiments using transmission electron microscopy with a 22 base oligonucleotide labelled with digoxygenin.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference has been made in the text.

I consent to this thesis being made available for photocopying and loan.

Roderick Bonfiglioli

December 1997

STATEMENT OF CO-OPERATION IN RESEARCH

The Sections 2.3. to 2.3.5., 3.3. to 3.3.5. and 4.3. to 4.3.5. where confocal laser scanning microscopy was used were done in co-operation with Mr Daryl Webb, a graduate student in our laboratory. At the time of this co-operation, Mr Webb was in the early stages of his own PhD work and I was showing him the principles and techniques of *in situ* hybridisation. At all times, with respect to work reported in this thesis, the experimental designs and planning were my own, but Mr Webb contributed with supervised benchwork and operation of the confocal laser scanning microscope. The fruitful co-operation with Mr Webb continues to this day, we have one joint-authorship paper published, and another is in preparation. Mr Webb is now working on related topics for his thesis where he is designing and running his own experiments, as is mentioned in Chapter 5.

The work in Chapter 5 was part of a collaborative research project done in co-operation with Dr Jeannine Lherminier of the Centre for Electron microscopy at the INRA Phytoplasma Research Centre in Dijon, France, in the laboratory of Dr Elisabeth Boudon-Padieux. I was working on material provided by this laboratory and, as in the above mentioned work with Mr Webb, I was involved in training Jeannine Lherminier in the methods of *in situ* hybridisation. As before, the experimental design and planning were my own, and co-operation from Jeannine Lherminier was done with my supervision in an atmosphere of training and co-operation.

Publications arising from this work

Bonfiglioli, R.G., McFadden, G.I. and and Symons, R.H. (1994) In situ hybridisation localises avocado sunblotch viroid on chloroplast thylakoid membranes and coconut cadang cadang viroid in the nucleolus. Plant J. **6**, 99-104.

Bonfiglioli, R.G., Webb, D.R. and and Symons, R.H. (1996) Tissue and intra-cellular distribution of coconut cadang-cadang viroid and citrus exocortis viroid determined by *in situ* hybridisation and confocal laser scanning and transmission electron microscopy. The Plant Journal, **9**, 457-465.

(one of the figures from this paper was used as the front cover photograph for this issue of The Plant Journal)

Acknowledgements

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Rene Sforza, Xavier Daire, Denise,
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to name but a few

My apologies to anyone I have forgotten to mention.

Chapter One
Introduction to viroids

This is a general introduction to the viroids to provide the background to the development of the work in this thesis. To avoid needless repetition, the specific details of the individual viroids will be dealt with independently in the relevant chapters allocated to the individual viroids. The topics discussed in this introduction are more general and relate to the history of viroid research, as is relevant to this thesis.

Viroids are intriguing pathogens. They are the smallest known pathogens and they are composed of only single stranded circular RNA molecules. They vary in length from 246 to 463 nucleotides in length and they have so far only been found in higher plants. There are 27 known viroids so far, and all but two of them infect dicotyledonous plants and the remaining two infect monocotyledonous plants.

The diseases caused by viroids vary in severity and presentation, some may produce severe symptoms and ultimately lead to the death of the host plant, as is the case for Coconut cadang-cadang viroid (CCCV), or they may produce deformations or markings on fruit, as is seen for Potato spindle tuber viroid (PSTV) and Avocado sunblotch viroid (ASBV), or they may produce a range of symptoms from none, to a mild dwarfing or to a severe stunting, as is seen in Citrus exocortis viroid (CEV). Some viroid

infections may not give rise to any symptoms in some host plants, but may cause symptoms in other plants.

While these diseases caused by viroids have been known about for many years, determining the nature of the disease agents presented significant difficulties. Potato spindle tuber disease (now known to be caused by PSTV) was first described in 1922 (Martin, 1922) and exocortis disease, (also known as bark shelling or scaly butt) of citrus (now known to be caused by CEV) was first described in 1948 (Fawcett and Klotz, 1948).

The characterisation of the molecular nature of viroids began in the late 1960s (Diener and Raymer, 1967) with a description of the causative agent of Potato spindle tuber disease. In this report it was noted that the causative agent found in the sap from diseased potato plants had a low sedimentation rate, was not sensitive to phenol treatment but was sensitive to ribonucleases, and was therefore free RNA. In 1971, Diener (1971) further concluded, after noting the high mobility in polyacrylamide gels and density gradients of the RNA extracted from potato spindle tuber disease infected plants, that the causative agent was a low molecular weight RNA and also that no helper virus was involved, and proposed the term "Viroid" to describe the causative agent of potato spindle tuber disease and other pathogenic nucleic acids with similar properties. No protein bearing any resemblance to a putative coat protein was found for PSTV despite specific research on this topic (Zaitlin and Hariharasubramanian, 1970).

In 1968 Semancik and Weathers (1968) also noted that the causative agent for exocortis of citrus had a low sedimentation rate, was similarly sensitive to ribonuclease and that no virus-like particles could be found to be associated. It was then concluded by Semancik and Weathers in a further report (Semancik and Weathers 1972) that the causative agent of exocortis disease of citrus was therefore an infectious, low molecular weight RNA. More reports were subsequently made by different authors on the viroid-like nature of the causative agents of a number of other diseases now known to be caused by viroids, including chrysanthemum stunt disease (CSV) (Diener and Lawson 1972), coconut cadang cadang disease (CCCV) (Randles 1975), cucumber pale fruit disease (CSV) (Sänger et al., 1976), hop stunt disease (Sasaki and Shikata 1977) and avocado sunblotch disease (ASBV) (Palukaitis et al. 1979).

By this time then, viroids were known to be the causative agents of a number of important diseases and it was also known that viroids were not associated with any helper viruses, they had no coat proteins, were not encapsidated and were composed solely of free, low molecular weight closed circular RNA molecules. The full nucleic acid sequence of the first viroid to be sequenced was that for PSTV in 1978 by Gross et al. (1978) and more viroids were to be sequenced in the following years.

In 1973, Sogo et al.(1973) reported the first visualisation and physical parameter measurements of a viroid (PSTV) in plant extracts by transmission electron microscopy. In this report they showed that the PSTV viroid could be visualised in specially prepared fractions of plant extracts and the viroid was seen to be a thin linear structure no more than

50 nm in length, and they calculated its mass as 8.9 x 10^4 Daltons. The small size and shape of viroids (Sogo et al., 1973) makes the direct visualisation of viroids *in situ* virtually impossible, and there are no such reports in the literature.

The *in situ* localisation of viroids was first shown in 1989 (Harders et al., 1989) where PSTV infected tomato leaf material was subjected to *in situ* hybridisation with biotinylated cRNA probes complimentary to sections of PSTV. In this report by Harders et al. (1989) the resulting hybrids were subsequently detected by an anti-biotin antibody labelled with a fluorochrome and viewed by confocal laser scanning microscopy.

In this thesis this technique of *in situ* hybridisation is further developed and is used to localise three more viroids (CEV, CCCV and ASBV) at the cellular level by confocal laser scanning microscopy, and then also at the ultrastructural, or sub-cellular level, by transmission electron microscopy.

Taxonomically, viroids are divided into two main groups, the PSTV group and the ASBV group as shown in Figure 1a. The taxonomy of viroids is based on similarity of sequence data, as is the case for the PSTV group, or on the known method of self processing during replication, as in the ASBV group. The ASBV group is only comprised of three viroids, and all the rest are in the PSTV group. The viroids in the ASBV group are dissimilar to the viroids in the PSTV group, and are taxonomically clustered together because they possess a unique and well characterised ribozymal method of self processing called the hammerhead reaction (Hutchins et al., 1986). The PSTV group is itself divided into three

subgroups, the PSTV sub-group, the Apple scar skin viroid (ASSV) sub-group and the Coleus blumei viroid (CbV) subgroup (see Figure 1a). The taxonomy of these three sub-groups is based on similarities of sequence in the central stretch of nucleotides described as the central conserved region.

Despite much research, there is no evidence to show that viroids code for any proteins (Conejero and Semancik, 1977; Flores et al., 1978; Henriquez and Sänger, 1982). Attempts at *in vitro* translation of viroid RNAs has not resulted in any protein products (Semancik et al., 1977) and sequence analysis has shown poor conservation of any potential open reading frames between closely related viroid species (Haseloff and Symons, 1981; Visvader et al., 1982; Visvader and Symons, 1985).

As viroids are not known to code for any proteins, they must use host encoded proteins for all their replicative processes. During their replication they are known to exist as linear multimeric molecules (Grill and Semancik, 1978; Branch et al., 1981; Branch and Robertson, 1984), and these must be processed into monomers and then ligated into circular forms. The characterisation of their methods of replication has received much attention (Breuning et al., 1982; Owens and Diener, 1982; Branch and Robertson, 1984) and is one of a few areas of viroid research about which some significant knowledge has been achieved. Nothing is really known about how viroids interact at the molecular level with the biology of their host plants to cause disease, and their apparent simplicity in form is proving deceptive.

All the viroids in the PSTV group are considered to be folded into rod-like secondary structures held together by intra-molecular base pairing (Sänger et al., 1976) and they also share many similarities in sequences. Sequence homologies studied within the PSTV sub-group of viroids showed that there are five principal regions of sequence that are common to many viroids and these observations have lead to the development of a domain model of viroid structure (Keese and Symons, 1985). The domains determined are shown in Figure 1b and are applicable to all members of the PSTV group, but not to the ASBV group. Subsequent sequence studies using point mutational analysis in PSTV (Keese et al., 1988), sequence variation in CEV (Visvader and Symons, 1985) and swapping of domains by cDNA cloning techniques between severe and mild symptom isolates of CEV (Visvader and Symons, 1986) has since verified the function of the P or pathogenic domain of the PSTV sub-group of viroids by showing that sequence changes in this P domain does lead to changes in symptom expression.

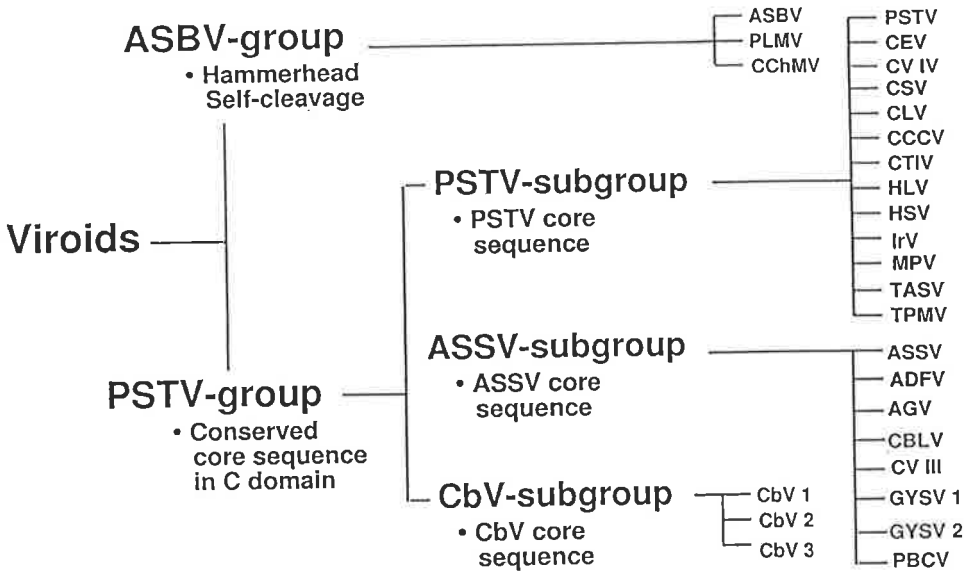
Despite the advances in viroid research mentioned above, very little information is available about the biology of viroid pathogenesis, we still do not know how they cause disease and we know very little about their interactions at the molecular level with the host cell biology. There are some proposed models of viroid pathogenesis that have been published (Dickson et al., 1981; Jakab et al., 1986; Haas et al., 1988; Hidinga et al., 1988; Vera and Conejero, 1990; Diener et al., 1993) and, while there is no substantial experimental evidence to support any of them, the relative merits of these will be addressed in the final discussion.

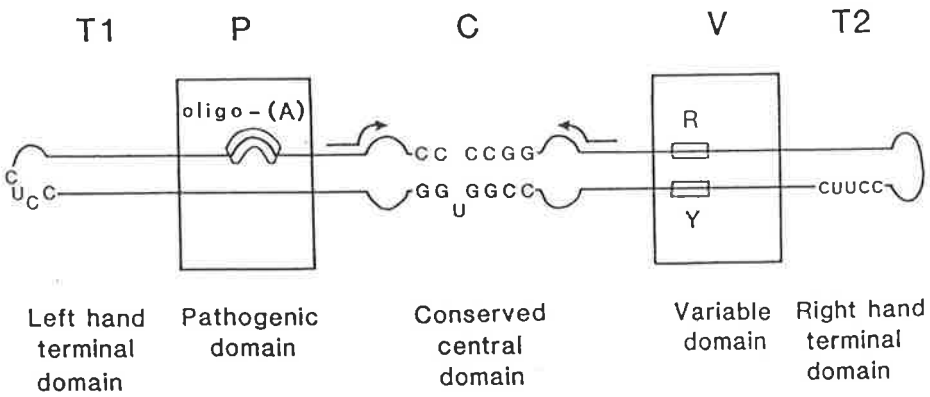
The *in situ* studies in this thesis were designed to improve our understanding of the biology of viroids by localising the viroids to specific sub-cellular compartments so that their presence there can be better related to what we know of the biological functions of those specific ultrastructural sites. What was needed was to find out if the ultrastructural localisations observed by *in situ* hybridisation experiments were consistent with what we already know about the replication of viroids, and if it was not consistent, to try and arrive at a suitable explanation as to why not.

1.2
Figures

1a. Taxonomic divisions of viroid groups.

1b. Domain model of viroid structures (after Keese and Symons, 1985).


ASBV-group
• Hammerhead Self-cleavage
ASBV
PLMV
CChMV
Viroids
PSTV-group
• Conserved core sequence in C domain
PSTV-subgroup
• PSTV core sequence
PSTV
CEV
CV IV
CSV
CLV
CCCV
CTIV
HLV
HSV
IrV
MPV
TASV
TPMV
ASSV-subgroup
• ASSV core sequence
ASSV
ADFV
AGV
CBLV
CV III
GYSV 1
GYSV 2
PBCV
CbV-subgroup
• CbV core sequence
CbV 1
CbV 2
CbV 3


T1
P
C
V
T2
oligo-(A)
R
Y
Left hand terminal domain
Pathogenic domain
Conserved central domain
Variable domain
Right hand terminal domain

Chapter Two.
In situ hybridisation of Citrus exocortis viroid (CEV).

2.1.1.
Taxonomic grouping of CEV

CEV is a member of the PSTV sub-group of viroids and shares varying amounts of sequence homology and common structural domains with other members of this viroid grouping. It has 60% sequence homology with PSTV and only 44% homolgy with CCCV (Keese et al. 1988), both of which viroids are also members of the PSTV sub-group. The PSTV sub-group of viroids is characterised by the presence of a common central conserved region of nucleotide sequence and a similar overall structure and arrangement of functional domains. CEV and ASBV, which are in different viroid groups only share a 29% sequence homology, a value that is in the range that can be expected from any two random sequences (Keese et al. 1988).

2.1.2.
Disease characetrisics

CEV is the causative agent of exocortis disease in citrus, a persistent disease, which was first described by Fawcett and Klotz (1948) and is characterised by scaling, shelling and splitting of the bark near the base of the trunk on susceptible varieties of citrus. While many citrus trees are

apparently symptomless, and possibly carriers of the viroid, more symptoms are usually seen on varieties used as rootstocks (Weathers and Calavan, 1961: Calavan et al., 1964). Leaf epinasty and severe stunting with or without reduction in yields may also be seen in susceptible plants. In tomato, which is used as an experimental indicator plant, the disease symptoms are characterised by severe stunting and epinasty.

2.1.3.
Host range and transmission

CEV is able to infect many members of the Rutaceae, some species of the Solanaceae, Cucurbitaceae, Leguminosae, Umbelliferae and Compositae (Runia and Peters, 1980). There is no known natural vector but "natural" spread is referred to by some workers without an explanation as to how it was achieved (Salibe and Moreira, 1965). It is presumed that most infections occur through the use of grafting tools, in conjunction with infected but symptomless varieties (Garnsey and Jones, 1967).

2.1.4.
Molecular characteristics

The molecular nature of the disease agent of CEV was first described by Semancik and Weathers (1972) and the sequence of the Australian isolate of the viroid (CEV-A) was determined and published by Visvader et al, (1982), and that of the Californian isolate of the viroid (CEV-C) by Gross et al. (1982). The viroid has a number of sequence variants that often correlate to variations in the disease presentation, such as the severity of disease symptoms and the presence of dwarfing properties. The isolate used in this work is CEV-A, it is 371 nucleotides in length and 67% of

those nucleotides are base paired, with 58% of those pairs being G:C pairs. It has a Tm of 52°C and has 58.6% GC and 41.4% AU base content. It is the largest monomeric viroid known so far. Evidence shows that the viroid is transcribed from a circular template by a host RNA polymerase in a continuos "rolling circle" model (Branch and Robertson 1984) that results in linear minus sense (-) multimeric copies of the viroid which are then processed into circular monomeric viroid molecules. The method of the processing from linear multimeric molecules to circular monomers is not known, but has been the subject of considerable (and still ongoing) research.

The replication of CEV has been studied by the use of specific inhibitors of the candidate host nuclear RNA polymerases, RNA polymerase I, II and III. The results of these studies which rely on the sensitivity of RNA polymerases I and II to high and low levels of α-amanitin respectively, and also for the sensitivity of RNA polymerase I to actinomycin-D, show that for PSTV (Spiesmacher et al. 1985; Schindler and Mühlbach, 1992) and CEV (Rivera-Bustamante and Semancik, 1989), the most likely candidate RNA polymerase is RNA polymerase II, but that the contribution of RNA polymerase I in the replication of at least CEV can not be fully excluded.

2.1.5.

Cytopathology and localisation studies

After PSTV, CEV is one of the most well studied viroids, and the studies on CEV have included the examination of viroid infected plant material by transmission electron microscopy in order to determine cytological changes due to the infection with CEV (Marton et al, 1982, Semancik and

Vanderwoude, 1976) and other viroids. These studies have identified disruptions to the internal membrane systems of infected cells, and malformations of the cell walls in CEV infected plant cells. These findings are also noted for ASBV (DaGraca, 1979: Desjardins and Drake, 1981: DaGraca and Martin, 1981) and PSTV (Semancik and Vanderwoude, 1976: Gruner and Santore, 1991). The exact relevance of the findings of these studies has been re-evaluated in a study by Gruner and Santore (1991) who found similar structures in the cells of plant cells that had been artificially stunted or subjected to stress. The incidence of these structures was, however, seen to be higher in viroid infected cells.

The implications of these findings, as discused by Gruner and Santore (1991), was that these membraneous structures were secondary responses to a variety of stress-inducing factors, including water stress, injury and virus or viroid infections, and that they do not therefore provide any diagnostic information as to the specific nature of a viroid infection, and they certainly do not yield any information as to the ultrastructural localisation of viroids.

A study by Gafny et al. (1995) used dot blot hybridisation techniques to follow the movement of the viroid through infected citrus seedlings, and showed that the viroid was eventually found throughout the whole plant, but with a higher concentration in the roots. This study, in which our host plants are tomatoes, has only found a lower level of the viroid in the roots.

2.2.

Ultrastructural localisation of CEV by *in situ* hybridisation and transmission electron microscopy

2.2.1. Materials and methods

In situ hybridisation experiments using plus and minus sense viroid specific biotinylated cRNA probes were used to localise CEV *in situ* using transmission electron microscopy on ultra thin sections of CEV infected tomato tissue. Initial localisation was done on early symptoms, and a time course study designed to determine if there was any change in the localisation of the viroid over a ten week study period was also done. Prior to any *in situ* hybridisation experiments, selected material was tested positive by dot blot hybridisation as described in McInnes et al. (1989).

2.2.2. Plant material

CEV extracted from infected tomato seedlings (kindly supplied by David Warrilow) was used to infect tomato seedlings by manual inoculation of the cotyledons of one week old plants. The tomato seedlings were grown in a growth chamber at 28°C with a 14 hour daylight cycle.

Samples of the leaves, petioles and roots of these plants and also of mock inoculated healthy control plants were taken at about 5 to 6 weeks of age

when the symptoms of growth stunting, epinasty, leaf yellowing and necrosis were fully evident on the infected plants.

A further series of samples were collected from CEV infected and mock inoculated healthy tomato plants at weekly intervals from 1 week post infection (plants were infected at 10 days of age) until 11 to 12 weeks of age when the plants were mostly dying. The purpose of this time course study was to test the possibility that the viroids accumulate at a site different to that of their synthesis.

2.2.3.
Sample fixation and preparation

Samples were cut while submerged in cold fixative to approximately 1 x 2 mm. The samples were fixed in 2.5% glutaraldehyde with 0.5% paraformaldehyde in 50 mM PIPES buffer, pH 7.6, at 4°C overnight on a rotator. Following fixation, the samples were washed three times in the buffer without the fixative, and then dehydrated through graded ethanols prior to embedding in L.R. Gold resin (London Resin Company), as per the manufacturer's recommended protocol. The samples were polymerised in capped 1 ml plastic sample tubes, which were kept on ice during the polymerisation phase to minimise any heat build-up. The samples were then kept protected from light and refrigerated until used for experimentation.

For sectioning, all equipment involved was kept as clean as possible, and DEPC treated water was used to float the sections in the boat of the

diamond knife. Standard ultra-thin sections (pale gold to silver sections, about 70 nm) were cut from the blocks and several of each such sections each were collected on pioloform (Agar Scientific) coated G200 gold or nickel grids. Pioloform coated grids were prepared by casting films made on glass microscope slides onto distilled water.

2.2.4.
Preparation of biotinylated cRNA probes

A full length Pst1 monomeric clone of CEV in pGem 2 (Promega) (kindly supplied by Dr Jim McInnes) was sub-cloned into the Pst1 site of Bluescript ~~pbsk+~~ pBSSK+ (Stratagene) and different orientations were used to transcribe full length plus and minus sense transcripts of the viroid. Transcription was done using T7 RNA polymerase with Biotin-11-UTP (Sigma Chemical Co) substituting for 60% of the unlabelled UTP in the transcription mixture, as described in McInnes et al. (1989). The prepared transcripts to be used as probes were purified by passing through a Sephadex G200 column to remove the un-incorporated nucleotides and then followed by ethanol precipitation.

The incorporation of the biotin-11-UTP into the transcripts was checked by taking 1µl of the transcription product, making a dilution series from it of 1:10 to 1:10000 and spotting the dilutions onto a nylon membrane and air drying them onto it. The membrane was then incubated in 5 ml of a strepavidin-alkaline phosphatase conjugate (Gibco) at 1 in 1000 dilution for 30 minutes and then the blot was developed with the coloured dye-enzyme

substrate system NBT/BCIP. The probe was still detectable at a dilution of 1:1000 within 10 minutes by the NBT/BCIP.

2.2.5.
In situ hybridisation (TEM)

The protocol for *in situ* hybridisation was basically as described by McFadden (1991), the hybridisation was done at 60°C overnight and the post hybridisation washes were also as described by McFadden, with the last wash being for 30 minutes at 60°C.

Hybrids were detected by using 10nm gold bead-labelled monoclonal anti-biotin antibodies (British Biocell).

2.2.6.
Results

Figures 2a and 2b are of sections of cells from the mesophyll tissues from CEV infected tomato plant leaves approximately 3 - 4 weeks post infection that have been probed with the minus-sense strand sequence of the CEV viroid by *in situ* hybridisation. Examination of these electron micrographs shows that the gold grains from the *in situ* hybridisation experiments are located across the nucleus, and that no concentration is seen in any other organelle within the cell. Examination of the nuclei from these micrographs shows that the viroid is distributed over the whole nuclear structure, and there is no noticeable concentration of the viroid in the nucleolus, although there do appear to be occasional concentrations of the viroid in patches throughout the nucleoplasm. Such concentrations in the

nucleoplasm are more evident in the CLSM image in Figures 2e and 2f. This pattern of sub-nuclear distribution was the same in all sections examined. The same distribution, but with significantly lower numbers of gold grains, was seen when the minus-sense strand CEV probe was used (data not shown).

Time course studies (with samples taken weekly in duplicate for 11 weeks, and with approximately 100 nuclei examined at each sampling), utilising *in situ* hybridisation and electron microscopy designed to follow the progress of CEV infection in tomato consistently showed that the viroid is distributed across the entire nucleus, with a gradual increase in the total amount, but with no change in the distribution of the viroid signal within the nuclear structure (see figures 2c and 2d).

2.3.
Histological localisation studies of CEV using Confocal Laser Scanning Microscopy

2.3.1.
Plant material, fixation and preparation

The same plant material that was used for the electron microscopy experiments was used in these experiments, except that the specimen size was increased to 2 to 3 mm x 2 to 3 cm. The specimens were fixed and washed in the same manner, and following washing were dehydrated to 70% ethanol (in four steps from 25%) and placed in an automated tissue

processor (Shandon Citadel Tissue processor) for paraffin wax infiltration and embedding.

2.3.2.
Preparation of sections for *in situ* hybridisation

Sections of leaf material infected with CEV and sections of uninfected leaf material of 5 to 8 microns in thickness were cut and floated on a dish of warm (40-45°C) DEPC treated water and collected onto silane (3-aminopropylsilane) coated slides (Sigma Chemical Co), allowed to air dry and stored in a refrigerator until used for experimentation. Each individual slide contained sections of both infected and uninfected material. The slides were air dried and dewaxed by immersion in xylene for 5 min, and the xylene was then removed by immersion in 100% ethanol for a further 5 min. The slides were taken to 70% ethanol for 5 min, and then to water. The sections were digested with pepsin at 1 mg per ml in 50 mM glycine buffer (pH 3) for 12 min at room temperature, washed twice in 50 mM tris-HCl, pH 7.4, 5 mM EDTA, dip washed in water, air dried, and then treated with RNase-free DNase (Boehringer) at 10 U per 100 µl in 50 mM tris-HCl buffer (pH 7.4) overnight at 37°C; a coverslip was applied and the slides were placed in a humid chamber to prevent evaporation. Following over-night DNase digestion, the slides were washed twice in 50 mM tris HCl buffer (pH 7.4) containing 5 mM EDTA and then subjected to *in situ* hybridisation.

2.3.3.

In situ hybridisation

This was done by applying 100 µl of *in situ* hybridisation buffer containing a range of dilutions of the cRNA transcript probes onto different slides. Optimal dilutions of the probe were in the range of 1:250 to 1:500 in *in situ* hybridisation buffer. The sections were then covered with a coverslip and the edges were liberally sealed with paraffin oil and the slide was placed on a "slide griddle" (MJ Research) which was placed on top of the heating block of and connected to a Thermal Cycler (MJ Research), the slides were incubated at 65°C for 4 hours to overnight. Following this incubation, the slides were cleaned by immersion in xylene for 5 min which removed the oil and also floated off the coverslip, then washed in 100% ethanol for 5 min, followed by 70% ethanol for 3 min and were then washed in 2 X SSC and 1 X SSC for 45 min each at 42°C and then incubated in 0.2 X SSC at 42°C for 30 min to remove any improperly hybridised or non-specifically bound probe. The slides were washed in water at room temperature for 3 min to remove salt and then air dried. Control experiments included the omission of the probe and omission of the fluorochrome as well as the use of healthy plant material. Omission of the probe is used to test for the presence of endogenous biotin as well as any other false signals.

2.3.4.
Detection of biotinylated cRNA probes by confocal laser scanning microscopy

The biotinylated probes were localised by applying to the washed slides approximately 1 ml of 1:1000 dilution in PBS (phosphate buffered saline) of a stock solution (1 mg/ml) of the fluorescent dye Cy5 conjugated to strepavidin (Jackson Immunochemicals) and incubating for 1 hour at room temperature. Cy5 is a far red fluorochrome and was used because the fixed plant material in all specimens examined had high levels of autofluorescence in both the red and green channels. In the far red channel, only very minimal low-level autofluoresence was observed. The slides were then washed in PBS for 30 minutes followed by several washes in water and then counterstained by dipping in a solution of ethidium bromide (500 ng/ml) for 10 min. The ethidium bromide stains any undigested DNA as well as staining specifically for lignin in xylem skeletal elements (O'Brien and McCully, 1981). The data were collected in the green channel to show the auto-fluorescence used to show some structural detail, and in the far red channel to show the Cy5 probe signal. The confocal microscope used was a Biorad MRC 1000 confocal laser scanning microscope.

2.3.5.
Results

In situ hybridisation experiments with minus sense viroid probes in conjunction with confocal microscopy showed that CEV was found in the vascular tissues. Figure 2g shows leaf vascular tissue from CEV infected tomato and the viroid signal is clearly seen in the vascular tissue. At higher magnification (not shown) it was possible to distinguish between the ribbed xylem vessels and the phloem cells, and the viroid signal was seen to be mostly confined to the phloem. Examination of multiple frames from each

of 12 sections of infected leaf material showed that the viroid concentration in the leaves is consistently higher than in any other part of the plant examined. The healthy control sample of leaf vascular tissue shown in Figure 2h has no viroid signal. Figure 2i shows vascular tissue in a section from a root sample of CEV infected tomato, and the viroid signal can be seen to be confined to a single line of vascular cells. Other than vascular tissues, the only other tissue type that was seen to contain the CEV viroid was the leaf mesophyll cells. Figure 2e shows the viroid located in the nucleus of a leaf mesophyll cell, the only cellular organelle to which we were able to locate the viroid in these cells.

Figure 2f is an enlargement of the nucleus seen in the CLSM image of Figure 2e but with the viroid signal presented as a false-colour intensity gradient image; the viroid signal is, as in the electron micrograph Figures 2a and 2b not specifically concentrated in the nucleolus, but is across the entire nuclear structure with localised concentrations in the nucleoplasm.

2.4. Discussion

The histological experiments clearly showed that CEV is located mostly in the vascular system. The phloem cells and the associated cells are the principal site of the viroid location, and it can be seen to be present in mesophyll cells close to the vascular tissues. The viroid is presumably moving out of the vascular tissue into the mesophyll cells and passive transport by the vascular tissue is the likely method of spread throughout the plant. This would certainly be in keeping with the finding by Gafny et

al. (1995) who found that the viroid spread progressively through the plant from the site of inoculation and eventually was present throughout the entire plant. This study did not find any viroid signal located in any mesophyll tissues where there was not a nearby vascular trace that also contained the viroid signal.

That the viroid seems to be located in the phloem cells and not in the xylem is not clearly understood. It is not known if there are any biological processes relating to the viroids functioning that are provided by the phloem cells and are not available in the xylem. The phloem sieve tube elements are basically an enucleate syncitium and as viroids do require nuclear enzymes to replicate, it would be reasonable to suggest that the phloem location is most likely one of simple convenience, rather than necessity. This reasoning is most likely explained by the idea that the viroid will only be transported through the plant after it has replicated to sufficient levels, and that the afferent transport system is the phloem, and not the xylem. It is possible that the viroid replicates in the nuclei of photosynthetically active cells, such as the vascular mesophyll and vascular parenchyma cells and is then carried out into the phloem system with the photsynthetic products for distribution to the rest of the plant, including the carbohydrate reserve tissues, such as the roots. This theory is certainly in keeping with the study by Gafny et al. (1995) who found a higher level of the viroid in the roots after completion of their time series studies.

The clear localisation of CEV to the nucleus is in concordance with other studies that show that the nuclear enzyme, RNA polymerase II is indeed responsible for the replication of CEV in the nucleus (Mühlbach and

Sänger, 1979). The electron micrographs showed that the viroid was located across the entire nuclear structure with no noticeable concentration in the nucleolus. This would indicate that RNA polymerase II is the nuclear enzyme most likely to be responsible for the viroid replication. The viroid signal is seen to have occasional concentrations or "hot spots" in the nucleoplasm (as shown in figure 2f) and this is in keeping with the known distribution of RNA polymerase II which is known to occur in patches, or discrete domains within the nucleoplasm (Bregman et al., 1995). That no change in the localisation of the viroid was seen in the time course experiments further indicates that there is apparently not any specific nucleolar involvement in the replication of CEV in the nucleus.

Other studies on the localisation of viroids in the PSTV group (Harders et al, 1989: this study on CCCV, see Chapter 4) have found that the viroids are apparently concentrated in the nucleolus, and the reason for this is not clear if the replicating enzyme is indeed RNA polymerase II as is thought to be the case for the viroids in the PSTV group (Mühlbach and Sänger, 1979). For viroids which are found in the nucleolus at a higher local concentration than they are in the nucleoplasm, it is possible that either RNA polymerase I is principally or secondarily involved in their replication, or that there is some form of active transport that is causing the viroids to become concentrated in the nucleolus against a concentration gradient.

The differences seen between the localisation of CEV (this study) and those seen for PSTV (Harders et al. 1989) and CCCV (this study, see Chapter 3) are intriguing, and no experimental evidence is yet available to explain this phenomenon properly. The observed differences would seem

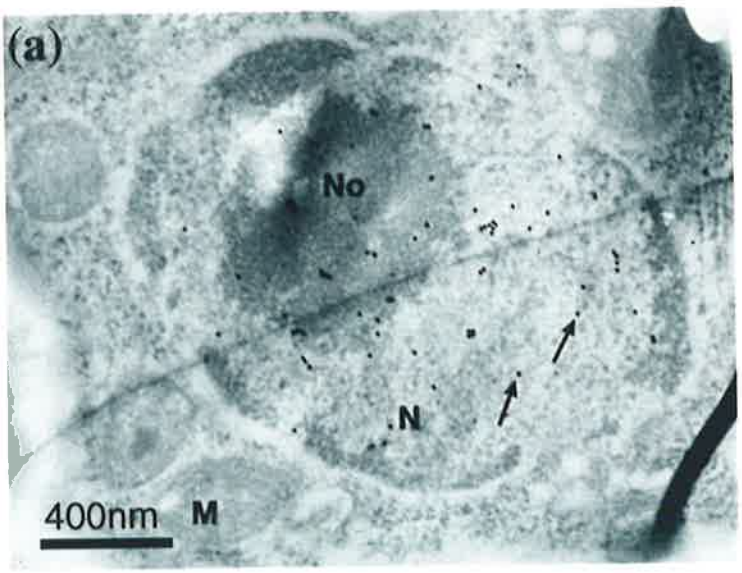
to indicate differences in the biological mechanisms involved for the different viroids, which is not expected as the viroids within the PSTV group all share similar features and it would be reasonable to expect that their biological processes would also be similar, especially as *in vitro* studies on the replication processes have certainly not shown any significant differences (Mühlbach and Sänger, 1989: Rivera-Bustamante and Semancik, 1989: Schindler and Mühlbach, 1992) between the utilisation of enzymes for replication by the different viroids in this group.

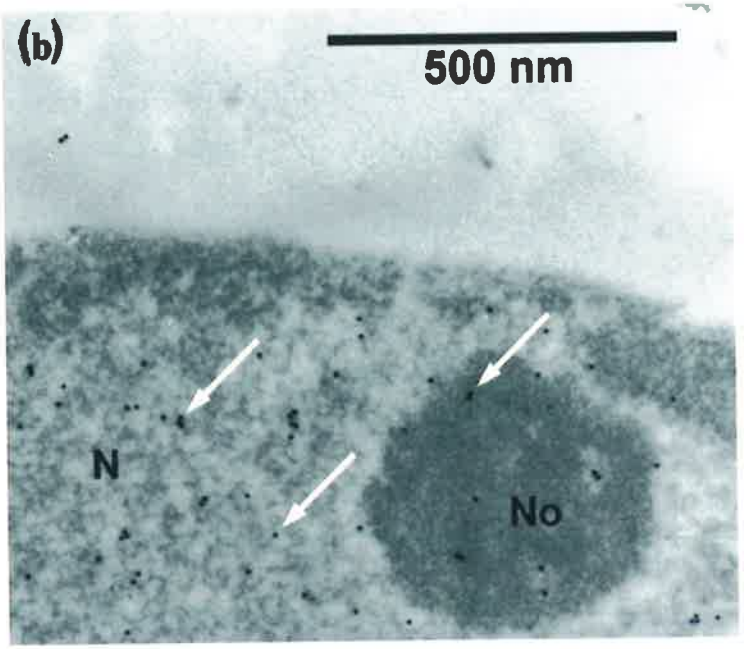
2.5.

Figures

***In situ* hybridisation experiments with CEV.**

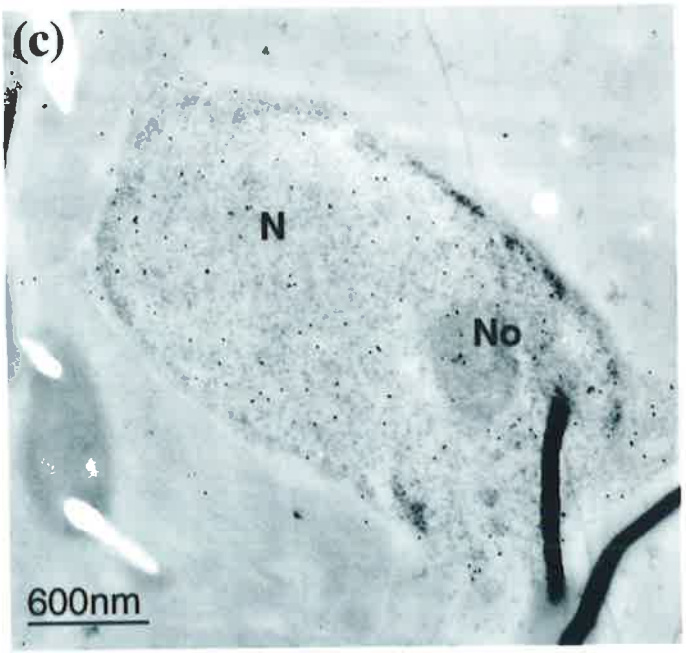
2a. and **2b**. TEM *in situ* hybridisation experiment. CEV infected mesophyll cells, 3-4 weeks post infection, probed with biotin labelled minus sense strand probe, labelled with 10 nm gold bead-labelled anti-biotin monoclonal antibodies (arrows). Note beads across whole nuclear structure. N = Nucleus, No = nucleolus, M = mitochondrion.

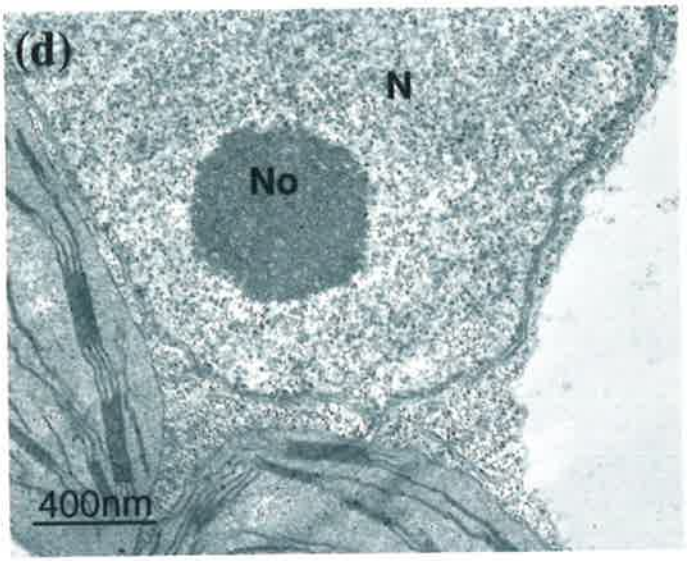

(a)
No
N
400nm
M


(b)
500 nm
N
No

2c. As for 2a and 2b, but at end of time course experiment, 10 weeks.

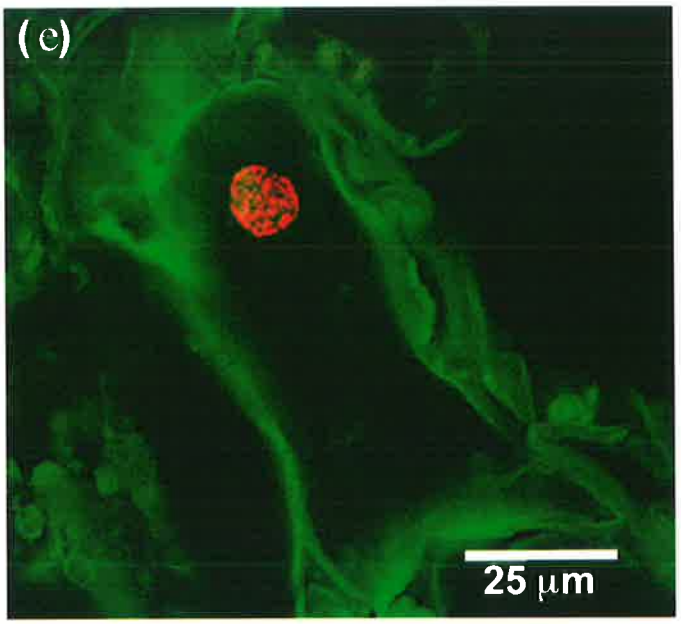
2d. Healthy control.

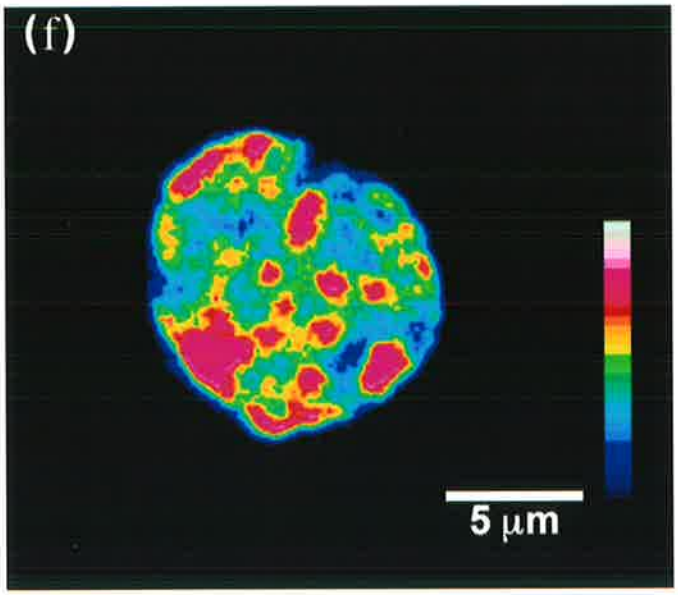

(c)
N
No
600nm


(d)
N
No
400nm

2e. Confocal laser scanning microscope image, in situ hybridisation of CEV infected mesophyll cells with minus sense probe, labelled with Cy5 conjugated strepavidin (red signal) (Jackson Immunochemicals). Note nuclear location of signal.
the green signal is cellular autofluorescence.

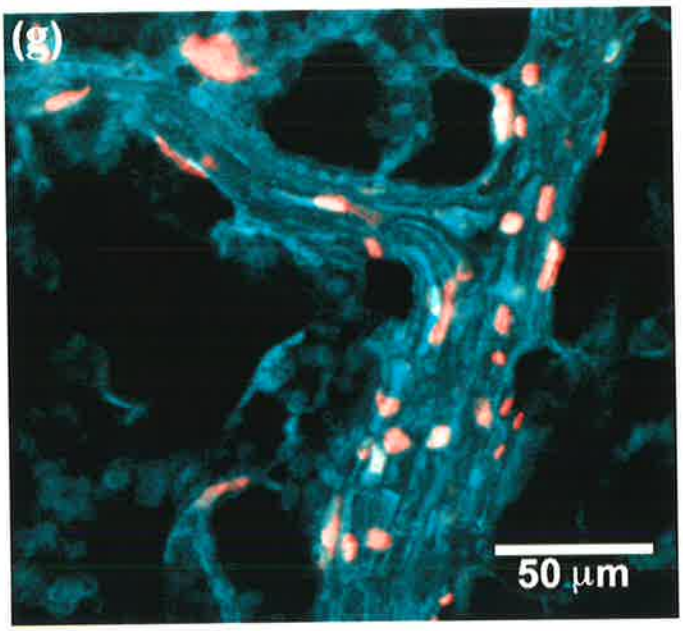
2f. False colour image of nucleus from 2e. Shows distribution of viroid signal across nucleus structure with concentrations in nucleoplasm.

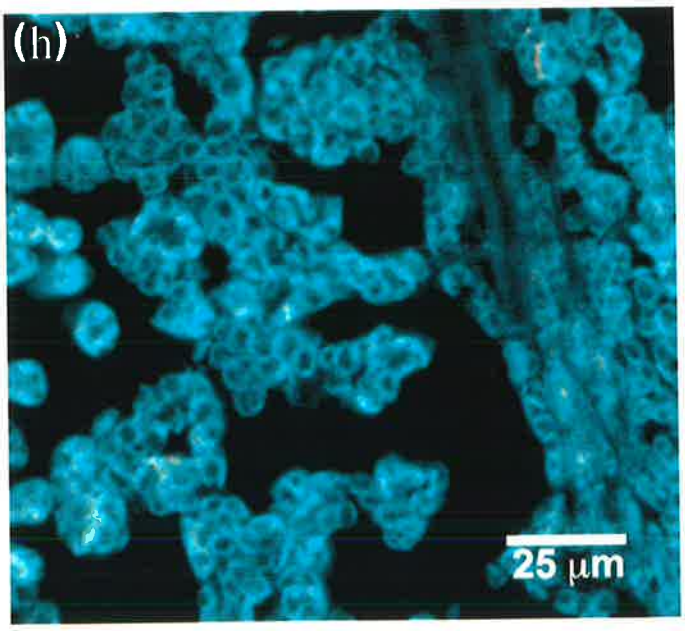

(e)
25 μm


(f)
5 μm

2g. CLSM, as in 2e, but shows signal in the vascular bundles in mesophyll tissue.

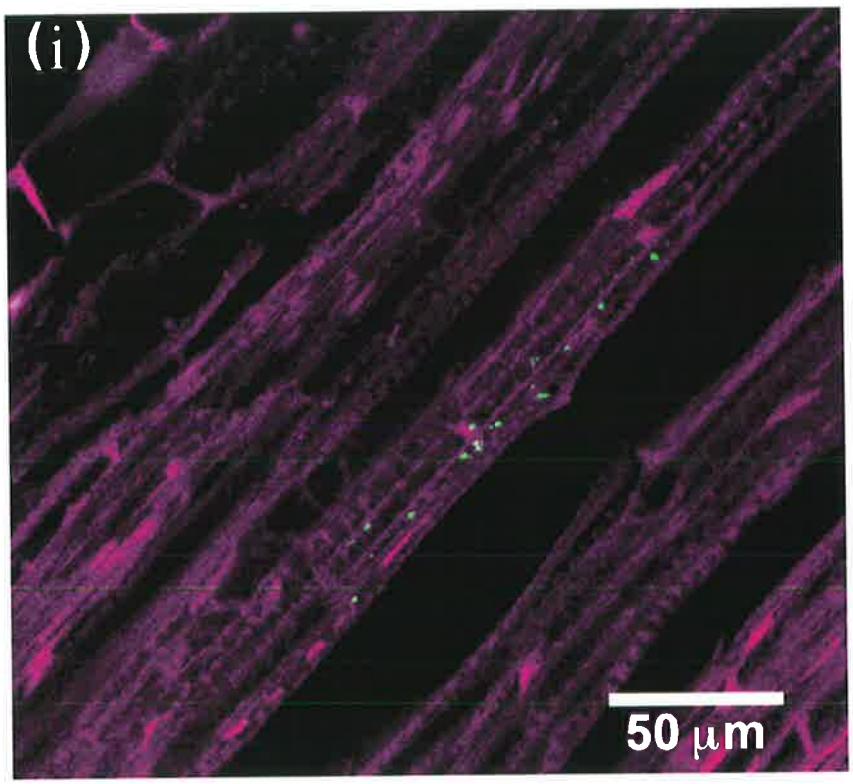
2h. CLSM, as in 2e, but healthy tissue. Note lack of signal.


(g)
50 μm


(h)
25 μm

2i. CLSM, as in 2e, but shows vascular tissue in root section.

Blue is ~~other~~ the viroid hybridisation signal, purple is cellular autofluorescence.


(i)
50 μm

Chapter Three
In situ hybridisation of Coconut cadang cadang viroid (CCCV)

3.1.1.
Taxonomic grouping of CCCV

Coconut cadang cadang viroid is a member of the PSTV sub-group of viroids. It is the smallest viroid characterised so far in the PSTV group, and it shows a 47% sequence homology with the type member of the group, PSTV, and only a 44% homology with CEV. CCCV has only a 26% sequence homology with ASBV which, as discussed for the homology between CEV and ASBV, is a homology that could be expected between any two random sequences (Keese et al. 1988). CCCV shows a 64% homology with coconut tinangaja viroid (CTiV), which is the closest related viroid to CCCV and is also restricted to monocots.

3.1.2.
Disease characteristics

CCCV is the causative agent of cadang cadang disease, a lethal decline disease of coconut palms seen in the central Philippines that causes the death of coconut palms over a period of approximately eight to sixteen years. The palms gradually lose their fronds until only a "telegraph pole" trunk is left. The fronds of affected palms develop small chlorotic blotches that expand and coalesce to form a chlorotic mottle. During this decline

period, nut size decreases and production eventually ceases altogether (Randles, 1975,: Zelazny et al, 1982,: Hanold and Randles, 1991). Coconut palms are very important to the economy of the Philippines and CCCV therefore presents a major problem to farmers in the regions where it occurs. In terms of economic impact, CCCV is certainly the most economically important viroid.

3.1.3.
Host range and transmission

CCCV is apparently restricted to the genus Cocos in the arecoid group of the Palms and, together with coconut tinangaja viroid (CTiV), they are the only two viroids known to be found in monocotyledonous plants (Boccardo et al.1981; Keese et al. 1987), while all other known viroids are restricted to dicotyledonous plants. We used the oil palm (*Elaeis guineensis*) as the experimental host. The viroid may be mechanically transmitted by slashing and stabbing an inoculum of extracted nucleic acids from an infected palm into the trunks of young healthy seedlings or by high pressure injection of the inoculum into the base of healthy seedlings (Imperial et al. 1985).

The mechanical mode of transmission takes some two to three years before symptoms show (Hanold and Randles, 1991b). The method of natural transmission is not known, but coleopterous insects are suspected as being non-specific vectors, but it is also suspected that the methods of propagation may also be involved in what appears to be "natural" transmission. It has also been shown that some CCCV related conditions

are apparently spread via seeds and that CCCV itself can be isolated from pollen (Hanold and Randles, 1991a, 1991b). Coconut palms produce a large amount of pollen and flower regularly and this could also be a possible route of viroid transmission.

3.1.4.
Molecular characteristics

CCCV is possibly the smallest known pathogen. The molecular nature of the disease agent of CCCV was first described by Randles et al.(1976) and the sequence of the viroid was determined and published by Haseloff et al. (1982). The viroid has a number of sequence variants that were originally detected by the "speed" with which they ran on polyacrylamide gels (Randles 1975). These sequence variants correlate to monomeric and dimeric forms of the viroid with or without a series of sequence extensions to, and or repeats of, the right hand terminal (T2) domain of the viroid (Haseloff et al. 1982). There is also some evidence to suggest that these sequence variants are found at different stages in the progression of the cadang-cadang disease (Imperial and Rodriguez, 1983). The isolate used in this work is CCCV-DO, it is 246 nucleotides in length and 66% of those nucleotides are base paired. It has a Tm of 49°C and has 59.2% GC and 40.8% AU base content with 68% of its G:C bases paired. As for other members of the PSTV viroid group, CCCV is presumed to be transcribed from a circular template by a host RNA polymerase in a continuos "rolling circle" model (Branch and Robertson 1984; Hutchins et al., 1985) that results in linear multimeric copies of the viroid which are then processed into circular monomeric viroid molecules. However, specific studies on the

replication of CCCV have not yet produced conclusive results. Our earlier *in situ* localisation studies on CCCV have shown that the viroid is located in the nucleus (Bonfiglioli et al. 1994) and that it is also concentrated in the nucleolus (Bonfiglioli et al. 1996) so some concordance with other members of the PSTV viroid sub group with regard to replication strategies would not be surprising.

The fact that CCCV and CTiV are the only viroids that are known to infect monocotyledenous plants, and that they are restricted to monocots does raise the possibility that there may be some monocot specific factors in their replication cycle. Connelly et al. (1994) have identified a monocot specific element (MSE) for transcriptional regulation that is in fact present in CCCV and CTiV. This MSE is a short 6 base sequence having the sequence RGCCCR (where R = A or G) and may be present in either orientation in single or multiple copies. In monocot plants it is found -30 to -130 bases upstream of the Upstream transcriptional element (USE) and is required for efficient transcription in monocots. Deletion of this element can reduce transcription by up to 85% in monocots. This sequence is also present in the central conserved region of all PSTV viroids, but in CCCV and CTiV it is present partly on a protruding non base-paired bulge region on the lower section of the variable domain in (and only in) CCCV and CTiV. Because of the location of this MSE sequence partly on a non-base paired bulge region it may be more accessible for interaction with some host specific polymeric factors, such as RNA polymerases. That this MSE sequence is located in the variable domain raises the possibility that it may not be very permanent and in some sequence variants it may be altered and this could lower the possibilty of this being a significant sequence.

3.1.5.

Cytopathology and localisation studies

Some studies were carried out on cadang-cadang diseased palm frond tissue before the nature of the disease agent was determined (Randles 1975; Zelazny et al. 1982). Camera lucida and other light microscopy examinations were made of tissue from cadang-cadang diseased and healthy coconut palms and it was shown that diseased tissue showed the presence of spherical intracellular inclusion bodies (Protacio, 1062), that there was a marked phloem necrosis similar to that observed with Aster yellows (Arguelles-Rasa, 1968) and a generalised disorder of the normal structure of the pallisade cell layers associated with a disruption of the chloroplasts (Rillo et al. 1972). Electron microscopy examination of diseased tissue showed intracellular inclusion bodies that were seen to be intra-chloroplastic inclusion bodies and to consist of dense patches of granular material with a uniform particle size smaller than ribosomes. These granular inclusions were thought to be either viral or phytoferritinous in nature (Plavsic-Banjac et al. 1972).

3.2.

Ultrastructural localisation of CCCV by *in situ* hybridisation and transmission electron microscopy

3.2.1.

Materials and methods

In situ hybridisation using plus and minus sense viroid specific biotinylated cRNA probes were used to localise CCCV *in situ* using transmission electron microscopy on ultra thin sections of CCCV infected oil palm tissue.

3.2.2.
Plant material

Small pieces of (1 x 2mm) of CCCV infected material were cut from the youngest fronds of oil palm showing the small chlorotic blotches that are characteristic of the cadang-cadang disease. Comparible sections of similarly aged but healthy frond material were collected from healthy oil palms. The infected palms had been infected ten years previously by slashing and stabbing the innoculum into the base of the trunk while the palms were seedlings.

3.2.3.
Plant material, fixation and preparation

Small leaves or peices of leaves from newly mature leaf tissue were cut from both infected and uninfected plants and submerged in chilled fixative solution. Small samples (2 x 1 mm) were then cut from these peices (in the fixative solution) with a fresh scalpel blade and the samples were then fixed and prepared for electron microscopy according to McFadden (1991) with the following modifications; fixation was in 2.5% glutaraldehyde and 0.5%

paraformaldehyde for 24 hours at 4°C during which time the samples were gently and constantly rotated. The fixative solution also contained 1.5% nicotine (Sigma Chemical Co) to counteract the action of the enzyme polyphenol oxidase.

Following fixation, the samples were washed three times in the buffer without the fixative, and then dehydrated through graded ethanols prior to embedding in L.R. Gold resin (London Resin Company), as per the manufacturs recommended protocol. The samples were polymerised in capped 1 ml plastic sample tubes, which were kept on ice during the polymerisation phase to minimise any heat build-up. The samples were then kept protected from light and refrigerated until used for experimentation.

For sectioning, all equipment involved was kept as clean as possible, and DEPC treated water was used to float the sections in the boat of the diamond knife. Standard ultra-thin sections (pale gold to silver sections, about 70 nm) were cut from the blocks and several of each such sections each were collected on pioloform (Agar Scientific) coated G200 gold or nickel grids. Pioloform coated grids were prepared by casting films made on glass microscope slides onto distilled water.

3.2.4.
Preparation of biotinylated cRNA probes

Biotinylated cRNA transcripts of approximately 400 nucleotides corresponding to full-length sense and anti-sense monomeric copies of the

CCCV viroid sequence plus some flanking vector sequence were transcribed from a full length Eag1 monomeric CCCV cDNA clone (Kindly supplied by Dr J. McInnes) inserted into the polycloning site of the Bluescript transcription vector, pBSK+ (Stratagene). As with the ASBV clone, the cloned CCCV sequence was inserted into the vector at a site that avoided the incorporation of the GC rich Not1 site in the subsequent cRNA transcript (see previous chapter). The plasmid was linearised and transcription was done with RNA polymerase T3 or T7 to obtain sense and anti-sense transcripts respectively. The transcripts were labelled by the incorporation of biotin-11-UTP into the cRNA transcript. This was done by the substitution of 60% of the UTP in the transcription mixture with biotin-11-UTP (Sigma chemicals) (McInnes et al. 1989).

3.2.5.
In situ hybridisation

The protocol for *in situ* hybridisation was as described by McFadden (1991). Hybridisation was done overnight at a range of temperatures between 55°C and 65°C and the post hybridisation washes were also as described by McFadden (1991) at the same temperature. The biotinylated probes were localised on the sections with 5 nm diameter gold beads conjugated to a monoclonal anti-biotin antibody (Biocell) and the sections were stained with saturated uranyl acetate and viewed in a transmission electron microscope.

3.2.6.
Results

A total of ten sections from new leaves on two different CCCV infected oil palms were analysed, and all of these showed strong hybridisation signals. Eight sections from uninfected palms were also examined and no hybridisation signal above background levels was observed. In contrast to ASBV, the plus form of CCCV was localised in the nucleus of cells in tissue sections prepared from leaves of infected oil palm (Figure 3a). Twenty five cells showing hybridisation signals were selected and the distributions and numbers of gold grains was analysed. Each nuclei was found to contain between 90 to 190 gold grains giving a concentration of 8 to 10 gold grains per ~~mm2~~ μm^2 within the nucleus. A higher concentration of viroid signal was seen in the nucleoli of such nuclei (Figure 3b), with a concentration of the signal sometimes seen towards the inside edge of the nucleolus. In sections that showed a good cross section of the whole nucleolus which revealed the interior structure of the nucleolus, it could often be seen that the signal was concentrated in the fibrillar cortex of the nucleolus (see Figure 3c). Most of the nuclei containing the hybridisation signals for CCCV were located in the cells surrounding the vascular bundles (data not shown). Some minimal background labelling of less than 1 gold grain per mm2 was seen, mostly in the cytoplasm.

When the numbers of gold grains on the sections were counted and calculations made, it was estimated that there were at least five times the number of CCCV molecules in the nucleoplasm as compared to the nucleolus. The calculations were based on the relative radii of, and the relative signal strength between, the nuclei and the nucleoli in the tissue sections examined, and the assumption that the nuclei and nucleoli are spherical.

In probing with the plus transcript for the minus form of CCCV, a weak signal was detected throughout the whole nucleus (data not shown). Control experiments for CCCV were carried out as for ASBV and CEV with the same result that no signal was found in the uninfected control sections (Figure 3d).

3.3.
Histological localisation studies of CCCV using Confocal Laser Scanning Microscopy

3.3.1.
Plant material, Fixation and preparation

The same plant material that was used for the electron microscopy experiments was used in these experiments except that the specimen size was increased to 2 to 3 mm x 2 to 3 cm. Fixation and preparation was as described in chapter 2, and nicotine at 1.5% was added to the fixative as also described in the previous section.

3.3.2.
Preparation of sections for *in situ* hybridisation

Sections of oil palm rachis material infected with CCCV and sections of uninfected rachis material of 5 to 8 microns in thickness were cut and floated on a dish of warm (40-45°C) DEPC treated water and collected onto silane (3-aminopropylsilane) coated slides (Sigma Chemical Co),

allowed to air dry and stored in a refrigerator until used for experimentation. Each individual slide contained sections of both infected and uninfected material. The slides were air dried and then dewaxed by immersion in xylene for 5 min, and the xylene was then removed by immersion in 100% ethanol for a further 5 min. The slides were taken to 70% ethanol for 5 min, and then to water. The sections were digested with pepsin at 1 mg per ml in 50 mM glycine buffer (pH 3) for 12 min at room temperature, washed twice in 50 mM tris-HCl, pH 7.4, 5 mM EDTA, dip washed in water, air dried, and then treated with RNase-free DNase (Boehringer) at 10 U per 100 µl in 50 mM tris-HCl buffer (pH 7.4) overnight at 37 °C; a coverslip was applied and the slides were placed in a humid chamber to prevent evaporation. Following over-night DNase digestion, the slides were washed twice in 50 mM tris HCl buffer (pH 7.4) containing 5 mM EDTA and then subjected to *in situ* hybridisation.

3.3.3. *In situ* hybridisation

This was done by applying 100 µl of *in situ* hybridisation buffer containing a range of dilutions of the cRNA transcript probes onto different slides. Optimal dilutions of the probe were in the range of 1:250 to 1:500 in *in situ* hybridisation buffer. The sections were then covered with a coverslip and the edges were liberally sealed with paraffin oil and the slide was placed on a "slide griddle" (MJ Research) which was placed on top of and connected to a Thermal Cycler (MJ Research), the slides were incubated at 65°C for 4 hours to overnight. Following this incubation, the slides were cleaned by immersion in xylene for 5 min which removed the oil and also

floated off the coverslip, then washed in 100% ethanol for 5 min, followed by 70% ethanol for 3 min and then washed in 2 X SSC and 1 X SSC for 45 min each at 42°C and then incubated in 0.2 X SSC at 42°C for 30 min to remove any improperly hybridised or non-specifically bound probe. The slides were washed in water at room temperature for 3 min to remove salt and then air dried. Control experiments included the omission of the probe and omission of the fluorochrome as well as the use of healthy plant material. Omission of the probe is used to test for the presence of endogenous biotin as well as any other false signals.

3.3.4.
Detection of biotinylated cRNA probes by confocal laser scanning microscopy

The biotinylated probes were localised by applying to the washed slides approximately 1 ml of 1:1000 dilution in PBS (phosphate buffered saline) of a stock solution (1 mg/ml) of the fluorescent dye Cy5 conjugated to strepavidin (Jackson Immunochemicals) and incubating for 1 hour at room temperature. Cy5 is a far red fluorochrome and was used because the fixed plant material in all specimens examined had high levels of autofluorescence in both the red and green channels. In the far red channel, only very minimal low-level autofluoresence was observed. The slides were then washed in PBS for 30 min followed by several washes in water and then counterstained by dipping in a solution of ethidium bromide (500 ng/ml) for 10 min. The ethidium bromide stains any undigested DNA as well as staining specifically for lignin in xylem skeletal elements (O'Brien and McCully, 1981). The data were collected in the green channel to show

the auto-fluorescence used to show some structural detail, and in the far red channel to show the Cy5 probe signal. The confocal microscope used was a Biorad MRC 1000 confocal laser scanning microscope.

3.3.5.
Results

CCCV was located in the vascular system of CCCV infected oil palm by CLSM and *in situ* hybridisation, as can be seen from Figures 3e and 3f. Figure 3e shows that the viroid signal was at times seen in the cells on the periphery of the vascular bundle, while Figure 3f shows a more central location of the viroid within the middle of the vascular bundle. Figure 3g shows CCCV in the mesophyll cells with single foci of signal apparently in the nuclei. The healthy control samples, as for example in Figure 3h, showed no signal for CCCV in any tissues. The observations shown in these figures are typical observations from multiple frames from 12 sections of infected and healthy material. As for CEV, CCCV was not seen in other types of tissues, such as the palisade cells, but was very occasionally seen in the epidermal cells (not shown).

3.4.
Discussion

The confocal microscopy experiments clearly localise the CCCV viroid to the vascular tissue, which presumably reflects how the viroid spreads in the infected trees. That the viroid is found in the edge of the vascular bundles is probably because it is moving out of the vascular bundles and replicating

to higher (perhaps more detectable) levels in the vascular mesophyll and other cells surrounding the bundles.

The localisation of CCCV to the nucleus and the nucleolus is initially in concordance with what is already known about the sub-cellular location of viroids in the PSTV group (Harders et al. 1989), ~~and the information.~~ Whether the higher actual concentration of CCCV in the nucleolus is due to the nucleolus being the site of CCCV replication, or whether the viroid accumulates there after replication elsewhere, such as in the nucleoplasm, is not known. The overall larger amount of viroid in the nucleoplasm is consistent with what could be expected if CCCV is replicated by RNA polymerase II. RNA polymerase II inhibition studies have indicated that it is the nucleoplasmic RNA polymerase II that is principally involved in the replication of viroids in the PSTV group, at least for CEV (Rivera-Bustamante and Semancik, 1989) and for CEV and PSTV (Spiesmacher et al. 1985; Tabler and Tsagris, 1990; Schindler and Mühlbach, 1992).

The higher actual concentration of viroid in the nucleolus would mean that if the viroid was accumulating in the nucleolus after replication in the nucleoplasm, then it would have to move against a concentration gradient that would presumably require some form of active transport. In the absence of any form of active transport going against concentration gradients, it would be expected that the viroid would be moving from an area of higher concentration into an area of lower concentration, in this case, that is from the nucleolus to the nucleoplasm. Such a site of origin would implicate RNA polymerase I as the replicating enzyme.

The pattern of distribution of the viroid in the nucleolus, especially the location of it within the region of the fibrillar cortex, as shown in Figure (4c) is very similar to the patterns of rDNA gene transcription by RNA polymerase I as discussed in Highett et al. (1993a) and Shaw et al. (1995). Shaw et al. (1995) also noted that the central nucleolar vacuole, and the perinucleolar knobs are transcriptionally inactive zones. Both these structures are visible in the nucleolus shown in Figure (4c), and they do not contain any significant amount of viroid signal. Shaw et al. (1995) concluded that the area of the nucleolus where they demonstrated transcription of the rDNA genes, which appears to be the same zone in which we found CCCV, and which is comprised of multiple fibrillar centres in a granular matrix which surround a central vacuole, is a zone where they located "nascent and newly completed transcripts attached to and surrounding active genes". The concentration of CCCV in this area could be interpreted as circumstantial evidence for the involvement of RNA polymerase I in the replication of CCCV. However, whether these patterns of viroid distribution, also seen in other tissue sections, are related to transcriptional or spliceosomal processes within the nucleolus, or are the result of viroids binding to other molecules that are involved in nucleolar transcription or splicing events, or even perhaps just to an accumulation of viroids at specific sites, is not known. Some proposed models of viroid pathogenesis and how they might relate to the results of this work will be discussed in the final discussion.

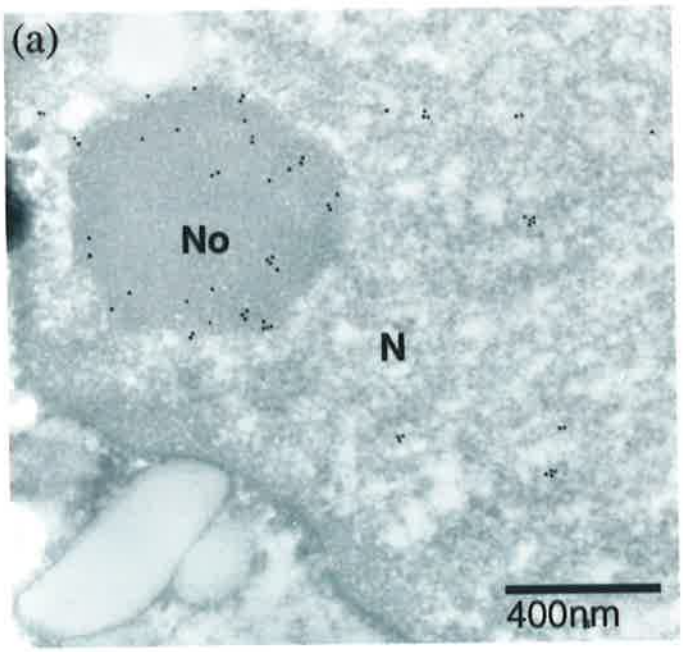
3.5.

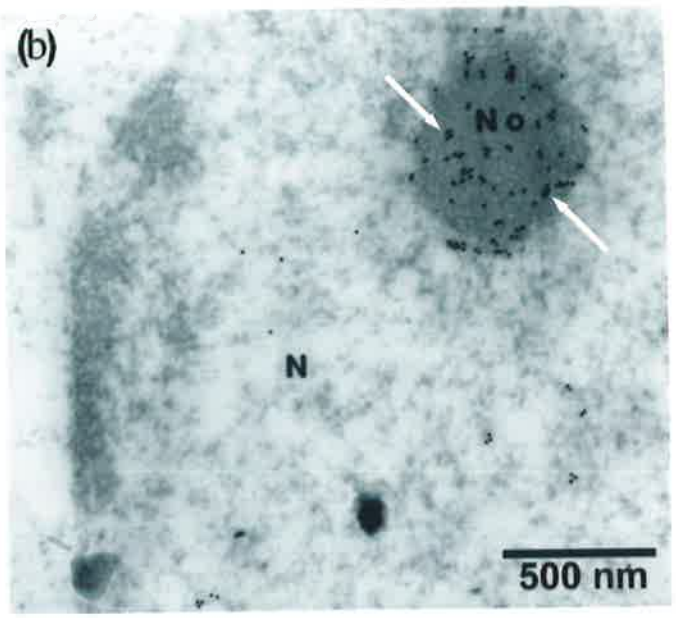
Figures

In situ hybridisation experiments on CCCV

3a. In situ hybridisation experiment on CCCV infected oil palm tissue. Nucleus (N) with nucleolus (No). Minus sense biotinylated probe labelled with 10 nm gold beads conjugated to anti-biotin monoclonal antibodies (British Biocell). Note concentration of gold beads in the cortical area of the nucleolus and not in the centre.

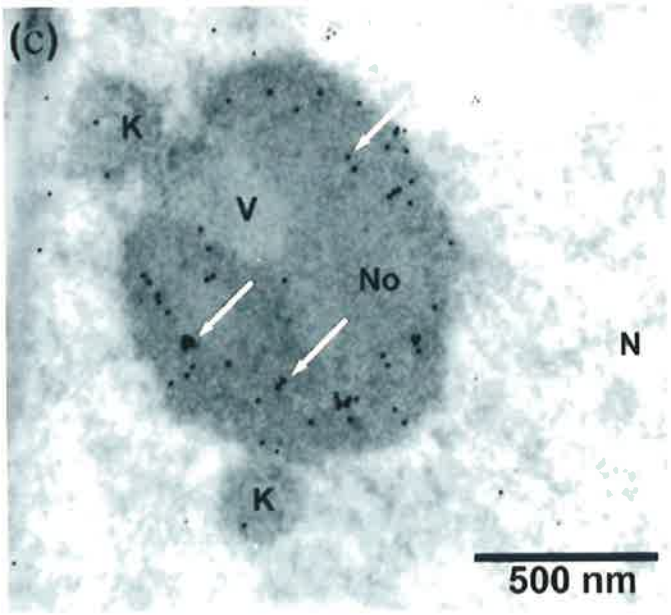
3b. As for 3a, note concentration of gold beads (arrows) in nucleolus. N = Nucleus, No = nucleolus.

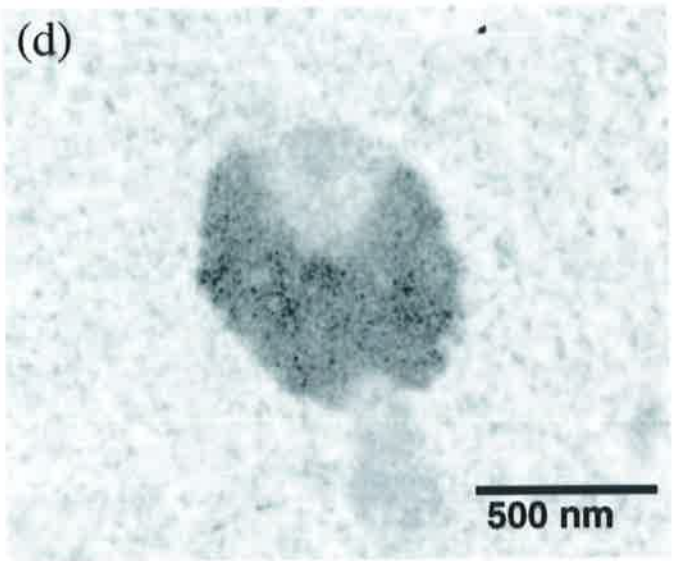

(a)
No
N
400nm


(b)
No
N
500 nm

3c. As for 3a, but note structure of nucleolus (No). Gold beads (arrows) are located in the fibrillar cortex towards the inner edge of the nucleolus, and are not seen in the central nucleolar vacuole (v), or in the perinucleolar knob (k).

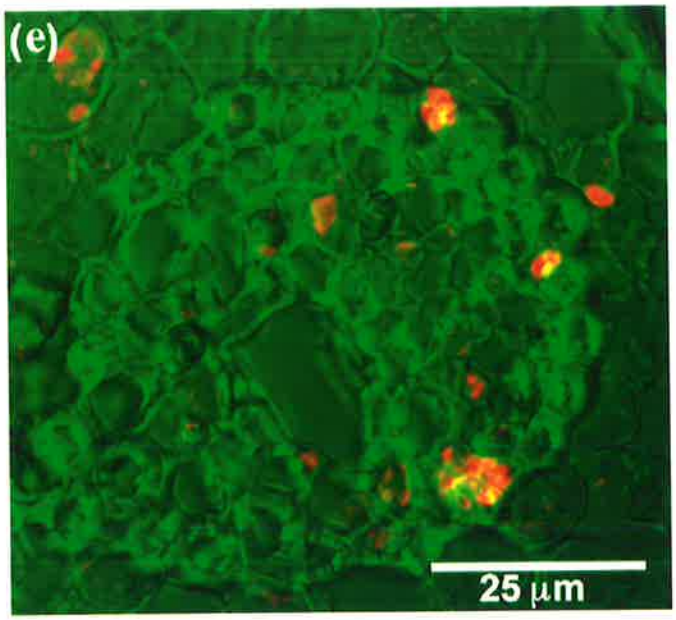
3d. As for 3a, nucleus with nucleolus, but healthy control. There is some very dark staining material in the nucleolar cortex, but it is in just fact granular material and not gold beads.

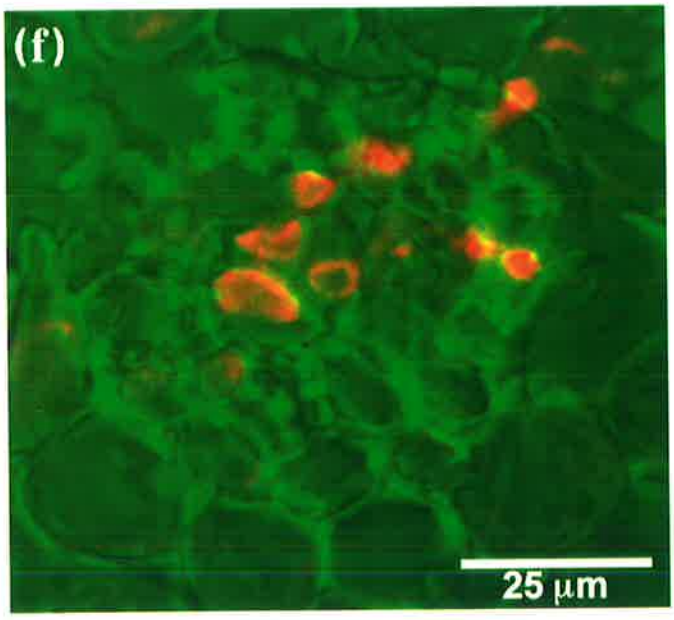

(c)
K
V
No
N
K
500 nm


(d)
500 nm

3e. Confocal laser scanning microscope image, CCCV infected oil palm mesophyll tissue. In situ hybridisation experiment, tissue probed with minus sense biotinylated probe, labelled with Cy5 conjugated to strepavidin (red signal) (Jackson Immunochemicals). Note the signal is showing in the cells on the periphery of the vascular tissue.

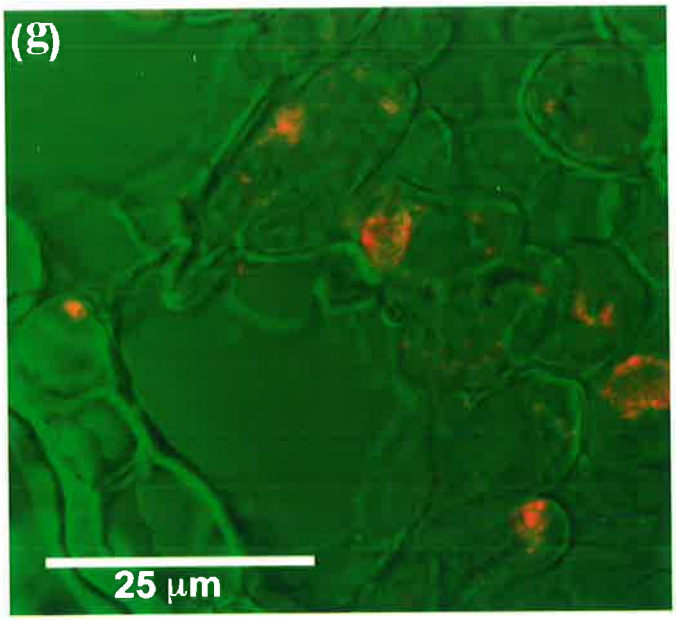
3f. As for 3e, but signal is showing in cells in the centre of the vascular bundle.

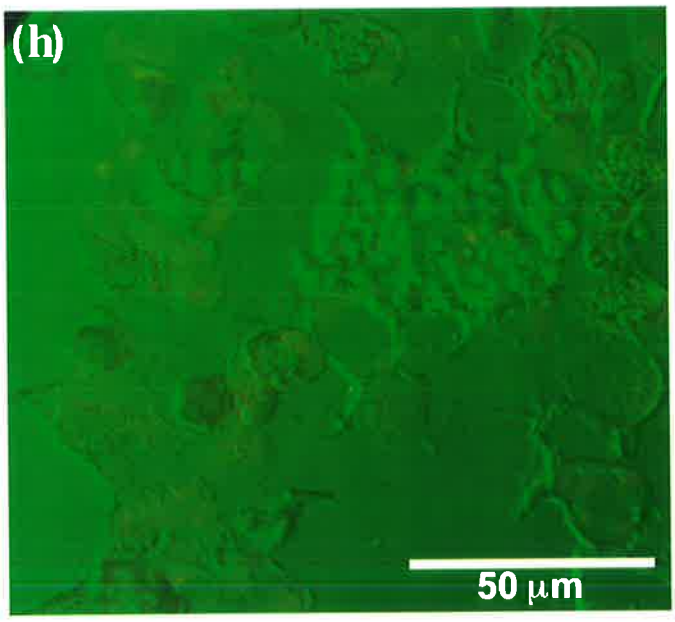

(e)
25 μm


(f)
25 μm

3g. As for 3e, but signal can be seen to be confined to nucleii.

3h. As for 3e, but healthy tissue.


(g)
25 μm


(h)
50 μm

Chapter Four
In situ hybridisation of Avocado Sunblotch Viroid (ASBV)

4.1.1.
Taxonomic grouping of ASBV

Avocado sunblotch viroid (ASBV) is the type member of the ASBV group of viroids. This group of viroids now contains three viroids, ASBV, Peach latent mosaic viroid (PLMV) and Chrysanthemum chlorotic mottle viroid (CChMV). This group of viroids is characterised by the method of processing, or the method by which the linear multimeric viroid transcripts are processed into single circular viroids. The method of processing for the viroids in this group is by a ribozyme-type self-cleavage mechanism, or auto-catalytic processing reaction (Forster et al., 1987). This reaction was first demonstrated *in vitro* for ASBV (Hutchins et al. 1986; Symons, 1991) and more recently for PLMV (Hernandez and Flores, 1992; Beaudry et al. 1995) and now also for CChMV (Navarro and Flores, 1997) and is described as the Hammerhead reaction (Forster et al., 1987). The name of the reaction is derived from the structure determined for the active part of the ribozyme self-cleaving sequence. While the three viroids in this group all share the common property of ribozyme mediated self-cleavage by the hammerhead structure, there are some important differences in the structure and also in the ribozyme self-cleavage reactions.

ASBV was the first viroid in this group to be sequenced and fully characterized (Symons,1981). Structural analysis determined by the simplest base-pairing combinations indicates that the most likely conformation of this viroid is a rod-like structure (Symons, 1981). In contrast, thermodynamic structural determinations based on the lowest free energy models for PLMV and CChMV, which are both larger than ASBV (336-339 and 398-399 nucleotides in length respectively, as compared to 247 for ASBV) have indicated that these two viroids are both most likely to be branched structures (Hernandez and Flores, 1992; Navarro and Flores, 1997).

There is a consistent pattern of similarities between PLMV and CChMV and a pattern of dissimilarities between those viroids and ASBV. This pattern of similarities has led to the proposal of a sub-grouping within the ASBV group with the PLMV and CChMV being seperated from ASBV as the "Pelamoviroids" (Flores, unpublished). ASBV has a much higher A + U content than the "Pelamoviroids" and it is also freely soluble in 2M LiCl, whereas the "Pelamoviroids" are not. *In vitro* ~~Studies~~ studies on the Hammerhead structure in the ASBV molecule suggest that the hammerhead structure needs to form a dimer or double hammerhead in order to be thermodynamically stable (Forster, et al. 1988) whereas both PLMV (Hernandez and Flores, 1992) and CChMV (Navarro and Flores, 1997) appear to be quite stable as single hammerhead structures

An interesting feature of the viroids in the ASBV group is that they seem to have a markedly narrow host range in comparison to most members of

the PSTV and ASSV groups (Navarro and Flores 1997). The significance of this restriction in host range is not understood at present.

4.1.2.
Disease characteristics

ASBV is the causative agent of sunblotch disease of avocado (*Persea americana*) which was first described by Horne and Parker (1931). This disease is persistent in avocado and is characterised by patchy variations in the green coloration of the leaves, yellowing of the leaf veins and interveinal areas, irregular striations and spots on the stems and petioles and yellow, orange or white necrotic areas, or blotches on the skin of the fruit that renders it unmarketable. The disease has a very variable presentation, and some trees that are assayed positive for the disease by dot-blot hybridisation or PCR may show only a few or none of the symptoms of the disease. The variation in the presentation of the symptoms of the disease has recently led to the disease being described as a syndrome with the variations in symptom presentation being partially linked to nucleotide sequence variations within the ASBV molecule (Semancik, 1995).

4.1.3.
Host range and transmission

The host range of ASBV is very narrow, with only some members of the *Lauraceae* being affected, including avocado and cinnamon (*Cinnamomum zeylanicum*), the only known methods of transmission are by graft

inoculation, manual transmission and seed transmission (De Graca and Van Vuuren, 1980).

4.1.4.
Molecular characteristics

ASBV is structurally and thermodynamically distinct from the PSTV viroids (Steger et al. 1984). It is a circular 247 nucleotide RNA molecule with a high AU to GC percentage (61.1% AU, 38.9% GC) that gives it a lower Tm of 38°C than the PSTV viroids (Symons, 1981) and it does not share any of the domain structures of the PSTV viroids, such as the common central conserved region. 67% of its sequence is base paired, with 34% of them being G:C base pairs. The viroid was first characterised by Palukaitis et al. (1979) and was sequenced by Symons (1981). In vitro studies on the replication of ASBV have shown that the replication of the viroid is not inhibited by α amanitin, and this is taken to indicate that the viroid is not dependent upon RNA polymerases II or III for its replication (Marcos and Flores, 1992). There have been no successfull *in situ* microscopy studies of ASBV prior to this study.

4.1.5.
Cytopathology and localisation studies

Some electron microscopy studies have been done on ASBV infected plant material with a view to determining the changes induced in the normal cellular structure by infection with ASBV. It has been reported that cells infected with ASBV show the development of protrusions of the

plasmamembrane which are described as "plasmalemmasomes" (DaGraca, 1979: Desjardins and Drake, 1981) and "paramural bodies" (Dagraca and Martin, 1981) and membrane whorls (Desjardins and Drake, 1981) have also been reported, as have vesicular structures in the nuclei (Desjardins and Drake 1981). Nearly all these reports mention that abnormalities, often gross, are seen in chloroplastic structures and that the thylakoid system is greatly disrupted. A report by Mohamed and Thomas (1980), where sub-cellular fractionation, followed by RNA extraction from each of the fractions was used showed that the ASBV viroid was mostly associated with the crude chloroplast fraction.

The common theme of the formation of abnormal cytological structures and the disruption of the normal chloroplastic structures is interesting from a pathological perspective and may eventually lead to a better understanding of the mechanisms of viroid pathogenesis. On their own, however, such results of infection by the viroid are not really useful as diagnostic indicators for ASBV infection. It has been demonstrated by Gruner and Santore (1991) that similar abnormal cellular structures can be induced by factors other than viroid infection that also induce stress in the plants, such as growth restriction and water stress. Similar chloroplastic disruptions and abnormal structures are also reported for a number of viral infections (see Francki et al, 1985 and references therein).

4.2.
Ultrastructural localisation of ASBV by *in situ* hybridisation and transmission electron microscopy

4.2.1.
Materials and methods

In situ hybridisation using plus and minus sense viroid specific biotinylated cRNA probes were used to localise ASBV *in situ* using transmission electron microscopy on ultra thin sections of ASBV infected avocado plant tissues.

4.2.2.
Plant material

Avocado seedlings (*Persea americana*) were inoculated by patch grafting with ASBV infected avocado tissue grafts about 10 years ago and have since been maintained in glasshouses.

4.2.3.
Plant material, fixation and preparation

Small leaves or pieces of leaves from newly mature leaf tissue were cut from both infected and uninfected plants and submerged in chilled fixative solution. Small samples (2 x 1 mm) were then cut from these pieces (in the fixative solution) with a fresh scalpel blade and the samples were then fixed and prepared for electron microscopy according to McFadden (1991) with the following modifications; fixation was in 2.5% glutaraldehyde and 0.5% paraformaldehyde for 24 hours at 4°C during which time the samples were gently and constantly rotated. Following fixation, the samples were washed three times in the buffer without the fixative, and then dehydrated

through graded ethanols prior to embedding in L.R. Gold resin (London Resin Company), as per the manufacturers recommended protocol. The samples were polymerised in capped 1 ml plastic sample tubes, which were kept on ice during the polymerisation phase to minimise any heat build-up. The samples were then kept protected from light and refrigerated until used for experimentation.

For sectioning, all equipment involved was kept as clean as possible, and DEPC treated water was used to float the sections in the boat of the diamond knife. Standard ultra-thin sections (pale gold to silver sections, about 70 nm) were cut from the blocks and several of each such sections each were collected on pioloform (ref) coated G200 gold or nickel grids. Pioloform coated grids were prepared by casting films formed on glass slides onto water.

4.2.4.
Preparation of biotinylated cRNA probes

Biotinylated cRNA transcripts of approximately 300 nucleotides corresponding to full-length sense and anti-sense monomeric copies of the ASBV viroid sequence plus some flanking vector sequence were transcribed from a full length Sau1 monomeric ASBV cDNA clone (kindly supplied by Candice Sheldon) inserted into the polycloning site of the Bluescript transcription vector, pBSK+ (Stratagene). The cloned ASBV sequence was inserted into the vector at a site that avoided the incorporation of the GC rich Not1 site in the subsequent cRNA transcript. The Not1 site contains sequence that is reported to hybridise with some

ribosomal sequences at hybridisations temperatures of 50°C or less (Witkiewicz et al. 1993). The plasmid was linearised and transcription was done with RNA polymerase T3 or T7 to obtain sense and anti-sense transcripts respectively. The transcripts were labelled by the incorporation of biotin-11-UTP into the cRNA transcript. This was done by the substitution of 60% of the UTP in the transcription mixture with biotin-11-UTP (Sigma chemicals) (McInnes et al. 1989).

4.2.5.
In situ hybridisation

The protocol for *in situ* hybridisation was basically as described by McFadden (1991). The sections were collected on G200 nickel grids, air dried and the grids were then floated on drops of Protienase K at a concentration of 5µg/ml for 10 minutes at room temperature. Following protienase treatment, the grids were washed three times in DEPC treated water and then air dried. Hybridisation was done overnight at a range of temperatures between 55°C and 65°C and the post hybridisation washes were also as described by McFadden (1991) with the final wash being for 30 minutes at the same temperature as the hybridisation. The biotinylated probes were localised on the sections with 15 nm diameter gold beads conjugated to a monoclonal anti-biotin antibody (Biocell) and the sections were stained with saturated uranyl acetate for 20 minutes and viewed in a transmission electron microscope.

4.2.6.
Results

A total of eleven sections from newly mature leaves from two different ASBV infected avocado plants were analysed and, of these, seven sections showed strong hybridisation signals. Eight sections from two different uninfected avocado plants were examined and none showed any hybridisation signals above background levels. Twenty five cells showing hybridisation signals were selected and the distribution and the numbers of gold grains were counted and their distribution analysed.

ASBV was found in the chloroplasts of mesophyll cells in the fixed sections of avocado leaves. Approximately 85% of the hybridisation signal (see Figure 4a) was associated with the thylakoid membranes, and this seemed to be mostly on the exterior of the thylakoid stacks. The remainder of the signal was distributed throughout the stroma (Figure 4a). Each chloroplast section was found to contain between 80 to 300 gold grains which gave a concentration of 10 to 18 gold grains per μm^2. ASBV was localised mostly in the chloroplasts of cells in the yellow symptomatic tissue of infected leaves. A common feature was that cells in this yellow tissue contained chloroplasts that were grossly enlarged with disordered thylakoid structures, as well as more normal looking chloroplasts. While both of these types of chloroplasts contained ASBV, the more normal looking chloroplasts contained higher levels of signal (data not shown). A much lower level of signal was found, however, in chloroplasts in the greener areas on the same leaves (data not shown). When probing for the minus form of ASBV, a weak signal was seen, (Figure 4b) also in the chloroplasts; this is consistent with the much lower concentrations of minus forms relative to the plus forms in infected plant nucleic acid extracts as

reported earlier (Bruening et al. 1982; Hutchins et al. 1985). Control experiments showed minimum background labelling only (of less than 1 gold grain per μm^2) in samples prepared from uninfected leaves (Figure 4c), or in infected samples that were probed with a vector only sequence or treated with RNaseA before hybridisation (Figure 4d).

4.3.
Histological localisation studies of ASBV using Confocal Laser Scanning Microscopy

4.3.1.
Plant material, fixation and preparation

The same plant material that was used for the electron microscopy experiments was used in these experiments, except that the specimen size was increased to 2 to 3 mm x 2 to 3 cm. The specimens were fixed and washed in the same manner, and following washing were dehydrated to 70% ethanol (in four steps from 25%) and placed in an automated tissue processor (Shandon Citadel Tissue processor) for paraffin wax infiltration and embedding.

4.3.2.
Preparation of sections for *in situ* hybridisation

Sections of wax embedded leaf material infected with ASBV and sections of uninfected leaf material of 5µm to 8µm were cut and floated on a dish of

warm (40-45°C) DEPC treated water and collected onto silane (3-aminopropylsilane) coated slides (Sigma Chemical Co), allowed to air dry and stored in a refrigerator until used for experimentation. Sections of both infected and uninfected material were collected on each individual slide. The slides were air dried and dewaxed by immersion in xylene for 5 min, and the xylene was then removed by immersion in 100% ethanol for a further 5 min. The slides were taken to 70% ethanol for 5 min, and then to water. The sections were digested with pepsin at 1 mg per ml in 50 mM glycine buffer (pH 3) for 12 min at room temperature, washed twice in 50 mM tris-HCl, pH 7.4, 5 mM EDTA, dip washed in water, air dried, and then treated with RNase-free DNase (Boehringer) at 10 U per 100 µl in 50 mM tris-HCl buffer (pH 7.4) overnight at 37 ^{0}C; a coverslip was applied and the slides were placed in a humid chamber to prevent evaporation. Following over-night DNase digestion, the slides were washed twice in 50 mM tris HCl buffer (pH 7.4) containing 5 mM EDTA and then subjected to *in situ* hybridisation.

4.3.3.
In situ hybridisation

This was done by applying 100 µl of *in situ* hybridisation buffer containing a range of dilutions of the cRNA transcript probes onto different slides. Optimal dilutions of the probe were in the range of 1:250 to 1:500 in *in situ* hybridisation buffer. The sections were then covered with a coverslip and the edges were liberally sealed with paraffin oil and the slide was placed on a "slide griddle" (MJ Research) which was placed on top of and connected to a Thermal Cycler (MJ Research), the slides were incubated at

65°C for 4 hours to overnight. Following this incubation, the slides were cleaned by immersion in xylene for 5 min which removed the oil and also floated off the coverslip, then washed in 100% ethanol for 5 min, followed by 70% ethanol for 3 min and then washed in 2 X SSC and 1 X SSC for 45 min each at 42°C and then incubated in 0.2 X SSC at 42°C for 30 min to remove any improperly hybridised or non-specifically bound probe. The slides were washed in water at room temperature for 3 min to remove salt and then air dried. Control experiments included the omission of the probe and omission of the fluorochrome as well as the use of healthy plant material. Omission of the probe is used to test for the presence of endogenous biotin as well as any other false signals.

4.3.4.
Detection of biotinylated cRNA probes by confocal laser scanning microscopy

The biotinylated probes were localised by applying to the washed slides approximately 1 ml of 1:1000 dilution in PBS (phosphate buffered saline) of a stock solution (1 mg/ml) of the fluorescent dye Cy5 conjugated to strepavidin (Jackson Immunochemicals) and incubating for 1 hour at room temperature. Cy5 is a far red fluorochrome and was used because the fixed plant material in all specimens examined had high levels of autofluorescence in both the red and green channels. In the far red channel, only very minimal low-level autofluoresence was observed. The slides were then washed in PBS for 30 min followed by several washes in water and then counterstained by dipping in a solution of ethidium bromide (500 ng/ml) for 10 min. The ethidium bromide stains any undigested DNA as

well as staining specifically for lignin in xylem skeletal elements (O'Brien and McCully, 1981). The data were collected in the green channel to show the auto-fluorescence used to show some structural detail, and in the far red channel to show the Cy5 probe signal. The confocal microscope used was a Biorad MRC 1000 confocal laser scanning microscope.

4.3.5.
Results

ASBV was located within the vascular system of ASBV infected avocado leaf tissue (Figure 4e, 4f) using CLSM but, while ASBV was previously localised at the ultrastructural level to the thylakoid membranes of the chloroplast using electron microscopy (Bonfiglioli et al. 1994), it was not possible to reliably localise ASBV to the subcellular level using CLSM. This is possibly because the chloroplasts, which are effectively a compartment within the main cellular compartment, may not be as easily permeabilised by proteinase digestion as is the nucleus. Unlike the nucleus, chloroplasts have no pores (Kirk and Tilney-Bassett, 1978; Schnell and Blobel, 1993) to further assist the entry of probes and fluorochromes to their interiors. ASBV was not reliably seen to be localised to any other cellular organelles or structures, and the healthy controls (Figure 4g) did not show any signal.

4.4.
Discussion

Viroids are currently classified into two principal taxonomic groupings, based upon nucleotide sequence and structural domains as in the PSTV group (Keese and Symons, 1985; Koltunow and Rezaian, 1989), or on the ability to self-cleave via the hammerhead mechanism, as in the ASBV group (Hutchins et al. 1986) which now also includes PLMV (Hernandez and Flores, 1992) and CChMV (Navarro and Flores, 1997). The difference in the ultrastructural locations between ASBV and the now-known ultrastructural locations of three of the viroids from the PSTV group (CCCV and CEV, this study; PSTV, Harders et al. 1989) implies that ASBV and the PSTV viroids utilise different RNA polymerases in their replication, and this establishes further distinctions between them. This implication is further strengthened by the study of Marcos and Flores (1992) which showed that the replication of ASBV was not inhibited by concentrations of α amanitin that would normally be expected to inhibit both the action of RNA polymerases II and III. Considering that ASBV replication is not inhibited by α amanitin, and that we found ASBV to be localised in the chloroplast, it is reasonable to consider whether the chloroplastic RNA polymerase is involved in the replication of ASBV. Recent work has also shown, however, that there is another nuclear-encoded but chloroplastic-located RNA polymerase that is in fact very similar to the T3 and T7 RNA polymerases from bacteriophages (Cermakian et al., 1996: Hedtke et al., 1997). This phage-type polymerase must also be considered as a candidate for ASBV transcription, especially as it is already known that a phage RNA replicase (Qβ) is able to transcribe PSTV *in vitro* (Owens and Diener, 1977).

While there are a few reports of virus associations with the chloroplasts, such as Lin et al., (1993) who found the RNA of Bamboo mosaic virus in the chloroplasts, the associations are not specific. The RNA of Bamboo mosaic virus is also found in almost every other cellular compartment, whereas the association between ASBV and the chloroplasts appears very specific. In the TEM *in situ* hybridisation studies ASBV was not seen in any other ultrastructural location. Both the plus and the minus forms of the viroid were found in the chloroplast, with the minus forms at the expected very low level. No signal above background levels was found for ASBV, in either the plus or minus forms, in any part of the nucleus, nucleolus or any other cellular compartment or organelle, so it therefore seems unlikely that the viroid is replicated in the nucleus (or elsewhere) and then transported to the chloroplast where it then accumulates.

While the localisation of the viroid at the histological level by confocal laser scanning microscopy did not seem to localise the viroid to the chloroplasts, it did localise it to the vascular tissue, this may reflect the way the viroid is transmitted throughout the plant. The wax embedded avocado tissue tissue was very difficult to work with, it is suspected that in the thick tissue sections inhibitory substances such as polyphenolics and starches may be retained and these may be interfering with hybridisation. The tissue is noted for going brown quickly on exposure to air.

Since the chloroplast RNA polymerase is prokaryotic in nature, whereas the nuclear RNA polymerases are eukaryotic (Rajasekhar et al. 1991), such fundamental differences may different pathways of evolutionary development between the ASBV and the PSTV groups of viroids. None of

the ASSV group of viroids have yet been localised at the sub-cellular level, although their similarity in sequence and structure to the PSTV group of viroids would suggest that they will most likely be found in the same organelles as those viroids.

Subsequent to our publication of this work (Bonfiglioli et al. 1994), Lima et al. (1994) have also published a paper using *in situ* hybridisation techniques similar to our own, where they detected ASBV in chloroplasts of avocado leaves. In this work they used cRNA probes labelled with digoxygenin instead of biotin, and acheived a similar result to our own, but with significantly lower signal strength. The lower signal strength (less gold beads) on their sections could well be due to their use of UV light to polymerase the resin, which would also be expected to cross-link the nucleic acid bases in the target viroid molecules.

Three separate studies have now shown that the ASBV viroid is associated with the chloroplasts. The work by Mohammed and Thomas (1980), where sub-cellular fractionation was used, this study where biotinylated probes were used, and the study by Lima et al. (1994) where digoxygenin labeled probes were used, all localised the ASBV viroid to the chloroplast or to the chloroplast rich fraction. While the study by Mohammed and Thomas (1980) showed that the majority of the viroid was localised in the chloroplast fraction in sub-cellular fractionation experiments, there was also a small but significant amount of label in the nucleus. It is probable that the relatively crude centrifugational techniques used to separate the different cellular fractions did not produce pure nuclear fractions and that the nuclear signal seen in this study could have resulted from some

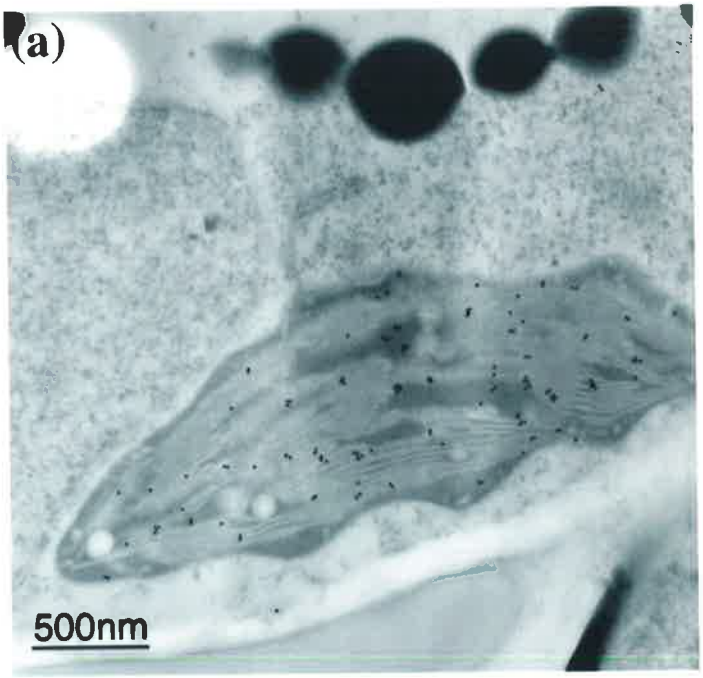
chloroplastic contamination of the nuclear fraction. That the nuclear localisation was not found in either the study by Lima et al. (1994) or in this study supports this possibility.

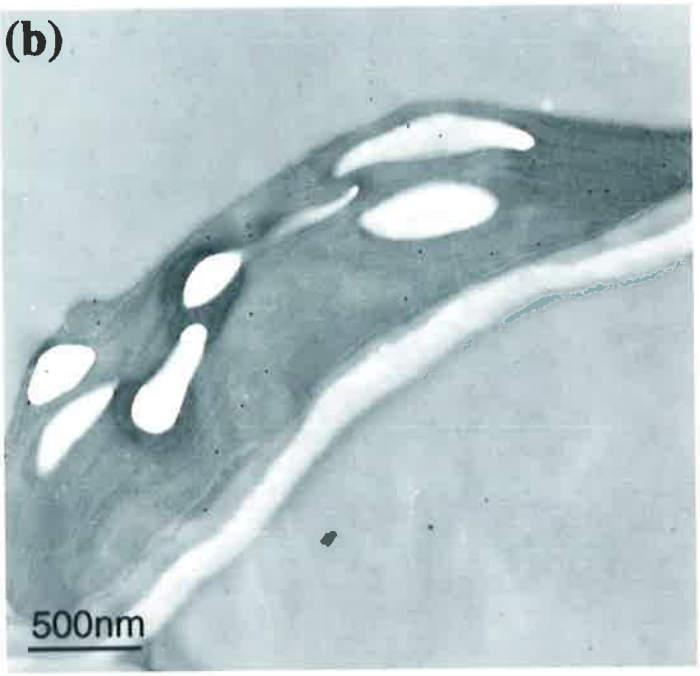
4.5.
Figures

In situ hybridisation of ASBV

4a. Transmission electron micrograph of a chloroplast in an ASBV infected avocado leaf mesophyll cell hybridised *in situ* with a biotinylated minus sense ASBV Sau1 monomeric probe. Note the distribution of the gold beads, they are located mainly (approximately 85%) on the outside surface of the thylakoid membrane stacks. The hybrids are localised with a monoclonal anti-biotin antibody (British Biocell) labeled with 15nm gold beads (British Biocell).

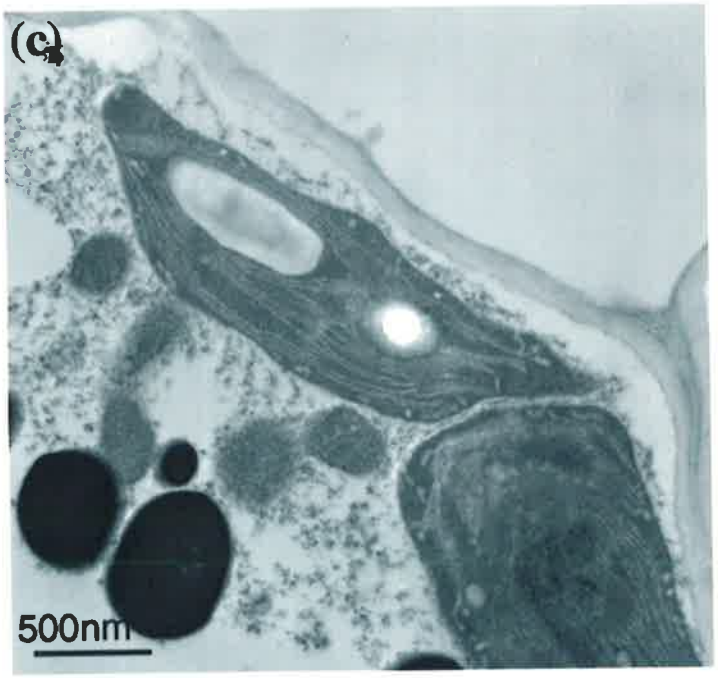
4b. As for 4a, but ASBV infected leaf mesophyll tissue hybridised with a biotinylated plus sense ASBV Sau1 monomeric probe. The detection of the hybrids is the same as for figure 4a, note the low number of beads compared to Figure 4a.

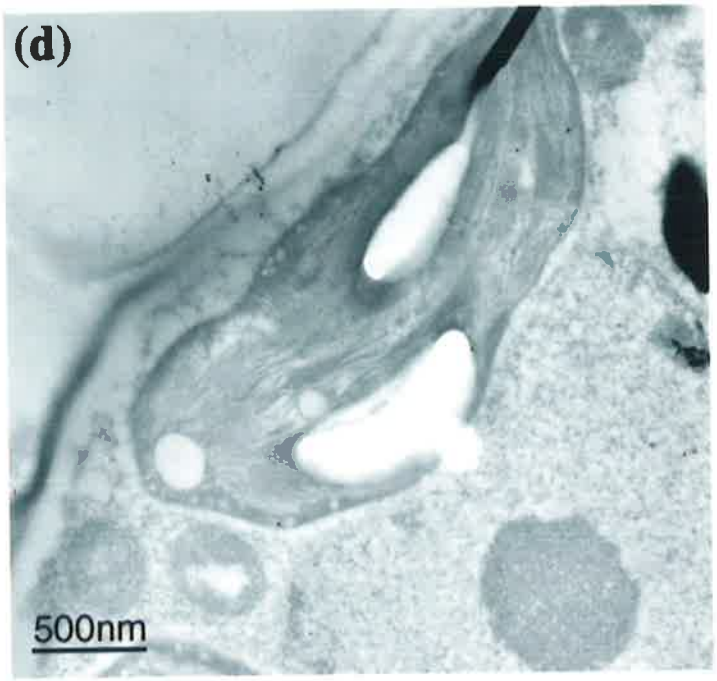

(a)
500nm


(b)
500nm

4c. Healthy avocado leaf mesphyll tissue hybridised with a biotinylated minus sense ASBV Sau1 monomeric probe. The detection of the hybrids is the same as for figure 4a,

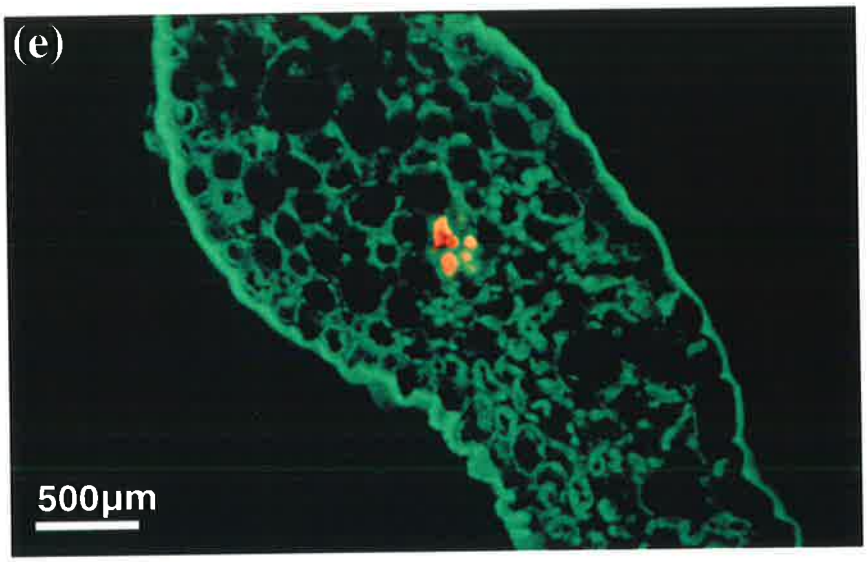
4d. ASBV infected leaf mesophyll tissue treated with RNAseA before hybridisation with the biotinylated ASBV Sau1 monomeric probe. The detection of the hybrids is the same as for figure 4a,

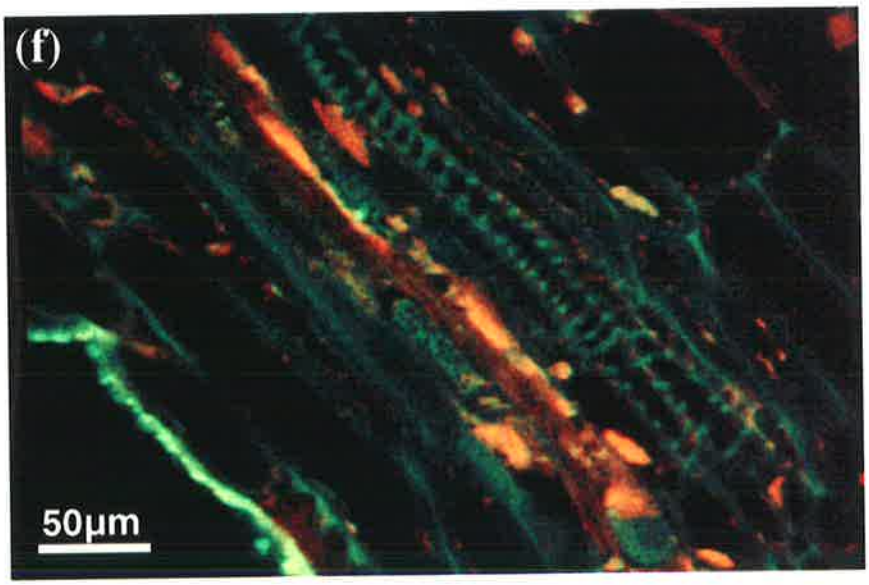

(c)
500nm


(d)
500nm

4e. Confocal laser scanning microscope image of ASBV infected avocado leaf tissue hybridised with a biotinylated minus sense ASBV Sau1 monomeric probe. The bright red/orange signal is the fluorochrome Cy5 which is conjugated to strepavidin (Jackson immunochemicals) and can be seen to be localised in the vascular system in the centre of the leaf tissue.

4f. As for 4e, but cross section of vascular tissue at higher magnification.


(e)
500μm


(f)
50μm

Chapter Five
Oligodeoxynucleotides as Probes for *In Situ* Hybridisation with Transmission Electron Microscopy to localise Specific Phytoplasmas in Plant cells

5.1.1. Introduction

Phytoplasmas are prokaryotic single celled pathogens of a wide range of higher plants. Taxonomically they are in the class mollicutes and they have some characteristics of bacteria, yeast and fungi. When they were first discovered (Doi et al. 1967) their visual similarity in transmission electron microscopy studies to mycoplasmas lead them to be described as mycoplasma-like organisms, or MLOs, a name now no longer used. The term phytoplasmas denotes the fact that they are in fact taxonomically very closely related to mycoplasmas (they are in the same family), but are found in plants.

In their plant hosts, phytoplasmas are obligate intracellular parasites where they are limited to the phloem cells of the vascular system. They are vectored by insects, mostly leafhoppers, in which they are also intracellular parasites. They are able to replicate in both their insect vectors and in their host plants, but it is not clear if they have plants or insects as their primary

hosts. It has been speculated that they are basically parasites of insects that have "hitched a ride" on plants (Barbara Sears, unpublished). If this theory is correct and insects are their primary hosts, then the plants would in fact be their vectors, as there is no evidence to show that the infection can be transmitted directly between insects. In the insect vectors, after initial acquisition of the phytoplasmas, the phytoplasmas can eventually be found in most internal organs, including the salivary glands (Lherminier et al., 1990) but there are no reports in the literature of any transmission of any phytoplasma between insects.

In grapevines phytoplasmas are the presumptive causative agents of conditions commonly described as "Grapevine Yellows". Despite the regular association between the disease symptoms of the various types of Grapevine yellows and the presence of phytoplasmas in the phloem cells of affected plants, Kochs postulates have never been fully satisfied for the pathogenic properties of phytoplasmas. This is because they are not yet culturable *in vitro*, and this obstacle presents significant problems for the progress of phytoplasma research.

Grapevine yellows (GY) diseases associated with the presence of phytoplasmas occur in most viticultural regions of the world. In spite of the similarity of the symptoms seen in affected grapevines, specific GY diseases have been shown to be associated with specific, diverse phytoplasmas. The taxonomy of phytoplasmas has so far been determined mainly by the nucleic acid sequence of the 16S-23S rRNA gene operon, which is so far the only stretch of DNA to be sequenced for nearly all known phytoplasmas. In Europe, two taxonomically distinct groups of

phytoplasmas, the Elm yellows group (EY) and the Aster yellows group (AY) have so far been identified in GY infected grapevines (Daire et al., 1997). The phytoplasma which is associated with Flavesence dorée (FD, or in English, Golden yellows) belongs to the EY group and those of Bois noir (BN, named after the black pustules that appear on the shoots of affected grapevines) and Vergilbungskrankheit (VK) belong to the stolbur subgroup of the AY group (Seemüller *et al.*, 1994) group of phytoplasmas. They are vectored to grapevines by two different insect species. In addition, two other phytoplasmas have been characterised from grapevines in Australia (Australian grapevine yellows, AGY) (Padovan et al., 1996) and in the USA (Leaf curl and berry shrivel, LCBS) (Prince et al., 1993). In Europe, all cultivars of grapevine seem to be susceptible, but some cultivar based variation in response is seen.

Methods to specifically localise and to distinguish between different phytoplasmas *in situ* are thus necessary for the development of *in situ* studies aimed at determining the replication strategies and movement of phytoplasmas within their plant hosts and also within their insect vectors. However, woody host plants, such as grapevines and fruit trees, are known to be difficult subjects in which to detect phytoplasmas by any method due to the very low titre of the phytoplasmas and because of the phenolic and other compounds present in the plant tissues and cells.

Polyclonal and monoclonal antibodies (Lherminier et al., 1994), DNA probes and DNA primers for PCR reactions (Lee et al., 1994), together with the relevant extraction protocols, have been successfully developed for specific diagnosis and detection of phytoplasmas in *in vitro* assays

(Lherminier et al., 1990; Lherminier and Boudon-Padieux, 1995). Polyclonal antibodies have been successfully used for *in situ* immuno-localisation in green herbaceous plants and in insect vectors where the titres of the phytoplasmas are much higher, but only one source of polyclonal antisera has ever been successfully used to localise phytoplasmas (FD) in *in situ* studies of grapevines (Personal communication, Boudon-Padieu).

In situ species specific detection of phytoplasmas is therefore a problematic task. The only DNA sequence that has been sequenced for most phytoplasmas is the 16S RNA gene. This sequence is widely conserved across most of the prokaryotic kingdom and it has been a challenge with many phytoplasmas to find stretches of unique sequence that are even long enough to work as species specific PCR primers. *In situ* hybridisation traditionally uses longer sequences (at least 200 bases in length) as probes, but a probe of this length targeted against the 16S RNA gene could be expected to hybridise to most prokaryotic organisms and could not therefore even be used as a general phytoplasma specific probe.

The use of shorter sequences has drawbacks in the lessening of control over the stringency of the hybridisation reactions caused by the reduction in the length of the probe. In this study it was decided, however, to try to develop the use of oligodeoxynucleotides as species specific probes for *in situ* hybridisation reactions in conjunction with Transmission Electron Microscopy (TEM). Similar techniques have been developed for confocal microscopy (Amann et al., 1995, and references therein) and the development of such techniques for TEM is a logical extension.

5.1.2.
Plant material, Phytoplasmas, fixation and preparation.

Periwinkles (*Catharanthus roseus* L.) were infected via graft transmission with grafts from periwinkles infected the FD and the BN stolbur phytoplasmas and raised under greenhouse conditions. Sections of plant material were taken when the periwinkles became symptomatic for phytoplasma infection, within about 6-8 weeks of graft.

Samples of plant vascular tissue from infected and healthy periwinkles were fixed in cold PIPES buffer (pH 7.8) containing 4% paraformaldehyde and 0.5% glutaraldehyde for a few hours and then infiltrated with either LRWhite resin or LRGold resin (London Resin Company). Infiltration with LRWhite resin was done at minus 18°C and polymerisation was by UV light at the same temperature. Processing in LRGold resin was at room temperature and polymerisation was initiated with the accelerator benzoyl peroxide. Nicotine was not used in the fixative solutions.

5.1.3.
Preparation of oligodeoxynucleotide probes.

Oligodeoxynucleotides (22 mers), which were originally designed as PCR primers for amplification of specific phytoplasma DNA (Lee et al., 1994; Maixner et al., 1995) were end labelled with digoxygenin or biotin by amine linkage during synthesis and were purified by HPLC to remove any

unlabelled oligodeoxynucleotides or unincorporated nucleotides. Oligodeoxynucleotides R16(V)F1 (Lee *et al.,* 1994) and fStol (Maixner *et al.,* 1995) were selected on the basis of their specificity for FD and BN, respectively. The minus (-) forms were used as they should be able to hybridise to both the gene sequences and the rRNA products. R16(V)F1 (Lee et al., 1994) is a unique sequence in the 16S gene of the EY group of phytoplasmas (to which FD belongs) and should therefore differentiate between BN and FD. The fStol oligo (Maixner et al., 1995) is a unique sequence in the 16S gene of the stolbur phytoplasma sub-group of the AY group of phytoplasmas which include BN.

5.1.4.
In situ hybridisation

The blocks containing the tissue samples were cut to give silver or pale gold sections which were mounted on pioloform coated G200 Nickel grids. Prior to *in situ* hybridisation, the sections were digested with a range of concentrations of pepsin (5 to 500 µg /ml, in 50 mM glycine buffer pH 3) for 10 minutes and then washed three times in water. The grids were then submerged in 100 µl of hybridisation buffer containing 20 nmoles of either, or in some cases, both oligoprobes in the base of a closed Eppendorf tube and immersed in boiling water for 45 seconds to denature the target nucleic acid. The Eppendorfs were then immediately transferred to a 39° C incubator overnight to allow the probes and the target sequences in the sections to hybridise. Instead of immersion in boiling water, some of the samples were subjected to four 10 second treatments of microwave

irradiation (with a pause of ten seconds between each treatment) at full power in a standard 650 Watt domestic microwave oven.

The hybridisation buffer contained 40 mM PIPES buffer, 0.1% PVP (10K), 0.1% Ficoll 4000, 0.02% SDS, 0.14 M NaCl and a range of concentrations of formamide in 5% increments from 10 to 45% and was adjusted to pH 7.8. The hybridisation buffer was adapted from methods described for similar procedures developed for use with confocal laser scanning microscopy in references from Amann *et al.,* (1995).

5.1.5.
Detection and visualisation of hybridised probes

Following hybridisation and washing, the detection of the hybridised probes was performed in a one step procedure by incubation with gold labelled anti-biotin or anti-digoxygenin monoclonal antibodies (Boehringer Manhiem) and then stained with 3% uranyl acetate for 10 minutes and examined with a Hitachi H600 electron microscope at 75kV.

Control experiments included (i) experiments without adding probe in the hybridisation mixture, (ii) use of an unrelated probe, (iii) use of the sense (+) probe and (iv) digestion of the tissue sections with RNAse A prior to hybridisation.

5.1.6.

Results

Optimal hybridisation signal for the fStol (-) oligoprobe (Maixner et al., 1995) (for the Bois Noir phytoplasma) which was labelled with digoxygenin was obtained with the use of a pre-hybridisation pepsin digestion of the tissue sections of 10 μg/ml for 10 minutes and with the formamide concentration of the hybridisation buffer between 20 and 30%. Immersing the samples in Eppendorf tubes into boiling water for 45 seconds prior to hybridisation significantly increased the hybridisation signal. The use of microwave treament of the sections prior to hybridisation resulted in an increase of the signal strength, but the specificity of the signal was significantly reduced.

The gold (10 nm) beads of the labelled anti-digoxygenin antibodies (British Biocell) were almost exclusively labelling groups of phytoplasmas and were not seen on any other cellular structures or organelles (see Figures 5a, 5b, and 5c). Mature forms of phytoplasmas that are present in lower densities in phloem cells, as shown in Figure 5a, were well decorated with gold particles, as were the senescent forms, also in phloem cells, as shown in Figure 5b. The hybridisation signal was greatly reduced when sections were digested with RNase A prior to hybridisation, as shown in Figure 5d. A very light hybridisation signal was seen when the sense (+) probe was used and no hybridisation signal was seen with an unrelated oligoprobe or with longer non-ribosomal phytoplasma transcripts. Labelling was equally successful with the material which was embedded at low temperature in LRWhite resin and that which was embedded at room temperature in LRGold resin (London Resin Company) (results not shown).

The fStol (-) oligoprobe hybridised only with BN infected tissues and did not hybridise with any FD infected tissues. We were not able to achieve any specific hybridisation signal with the R16S(V)R1(-) oligoprobe (Lee et al., 1994) (for the FD phytoplasma) in any of the tissue sections with any of the experimental conditions which were investigated. The gold beads (20 nm) of the labelled anti-biotin antibodies were present only in very low numbers and were only seen on the cell walls of the plant cells, a common place for non-specific labelling to be found. The gold beads were not located on any other cellular structures or organelles, nor were they present on any individual phytoplasmas or groups of phytoplasmas.

The literature describing similar techniques for the detection of other prokaryotic organisms by confocal laser scanning microscopy often report (Amann et al., 1995, and references therein) that selected oligodeoxynucleotides that had functioned efficiently as species specific PCR primers did not always work as *in situ* hybridisation probes and that a number of oligodeoxynucleotides needed to be tried before one was found that worked as a probe. The reason for this is not known, but is perhaps related to the proposed secondary structure of the target sequences. Hence further experimentation with other oligodeoxynucleotides specific for the FD phytoplasma will be done.

5.2.
Discussion

To the best of our knowledge this work is the first report of a successful transmission electron microscopy study using oligodeoxynucleotides as probes for post embedding *in situ* hybridisation in plant cells. The results obtained for the BN phytoplasma show that it is possible to find stringency conditions for the hybridisation reaction which avoid non-specific hybridisation with such oligoprobes.

The initial good results with the hybridisation experiments with the BN phytoplasma shows that this technique has great promise for use in transmission electron microscopy. One of the principal limitations to the technique of *in situ* hybridisation is the availability of an appropriate probe. Synthesis of the probe normally requires a lengthy process of cloning, screening, sequence determination, sub-cloning and *in vitro* transcription of the appropriate sequence. The ability to use oligodeoxynucleotides as probes removes or diminishes these obstacles thus making the technique more available and faster. With some further development, this technique has the potential to make a very valuable contribution to *in situ* electron microscopy studies.

The aim of this work is to develop this species-specific *in situ* diagnostic technique for use on candidate insect vectors of specific phytoplasma diseases. The insect vectors determined so far for phytoplasma diseases are leafhoppers (Lherminier et al., 1990: Maixner et al., 1995) or psyllids (Davies et al., 1992). Specific insects will vector specific phytoplasmas and the relationship between the insect vector and the phytoplasma is usually very specific. *In situ* studies have shown that the phytoplasma can be found within specialised cells of the basement membrane of the mid-gut, in

the haemolymph, in the malphigian tubules, in the brain and most importantly in particular acinar cells of the salivary glands (Lherminier et al., 1990). The phytoplasma exists within the insect vector as an intracellular parasite and is only vectored once the phytoplasma reaches the acinar glands in the salivary glands. Only particular phytoplasmas will be able to cross the initial barrier (the basement membrane of the mid-gut) and actually leave the mid-gut. It is thought that there are at least two intracellular sites where the phytoplasmas may replicate to high levels and these are the specialised cells in the basement membrane of the mid-gut and in the acinar cells of the salivay glands (Lherminier et al., 1990).

A problem we have found is that when individual insects are tested by PCR to see if they contain phytoplasmas, the protocol involves grinding the insect in a buffer and extracting the DNA for use as base sample material in a PCR test. If the insect has recently fed on an infected plant, it is highly likely to contain the phytoplasma in its mid-gut, and because of this it is probably going to return a positive result in the PCR test and this may lead to erroneous conclusions about the nature of the relationship between the insect and the pathogen. *In situ* studies will clearly show the location of the phytoplasmas within the candidate insect vector and provide more accurate information as to the exact nature of the relationship between the phytoplasma and the insect. The purpose of these studies then is to accelerate the transmission studies of the phytoplasma by candidate insect vectors, a process that is normally very slow and laborious.

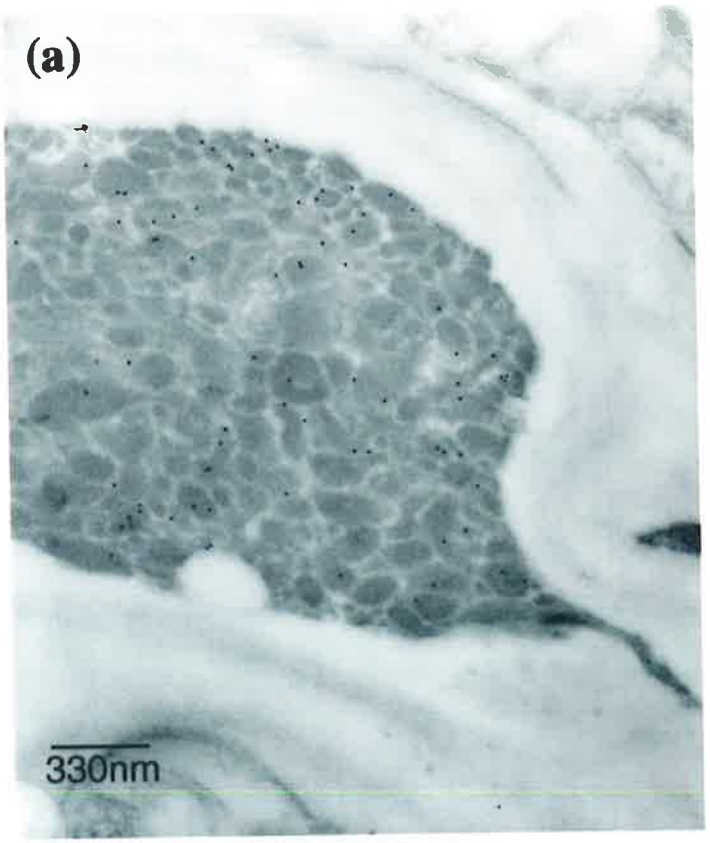
In a collaborative project with Mr Daryl Webb, a graduate student in our laboratory, we have already achieved some success in the use of

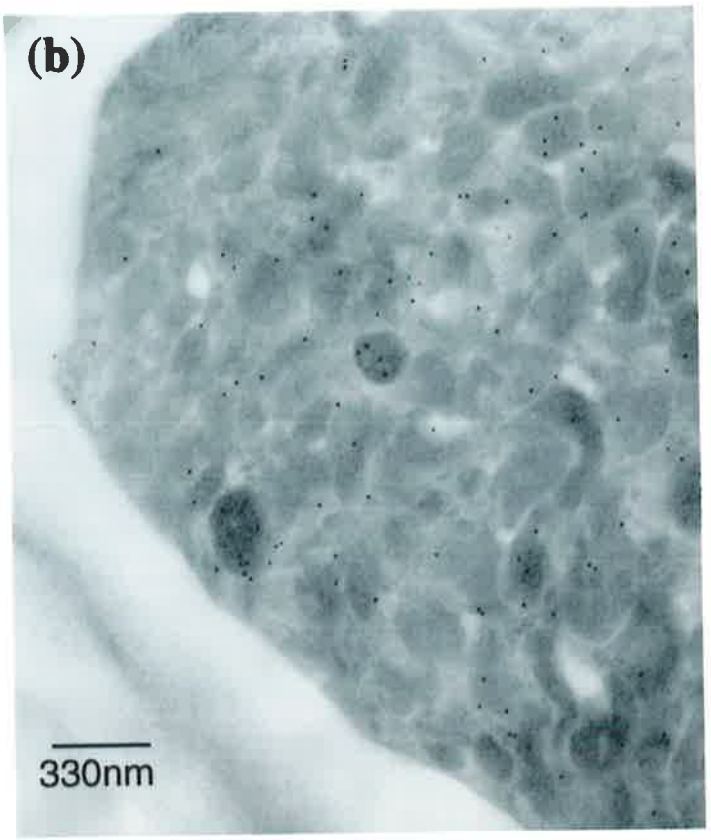
oligodeoxynucleotides for *in situ* detection and localisation of phytoplasmas within the different tissues of insect vectors. As the experimental work for this project has been mostly his responsibility, he will report on the results of this work in his thesis.

5.3.
Figures

Electron micrographs of *in situ* hybridisation of oligoprobes with BN phytoplasmas in BN infected periwinkle *(Catharanthus roseus)* phloem cells.

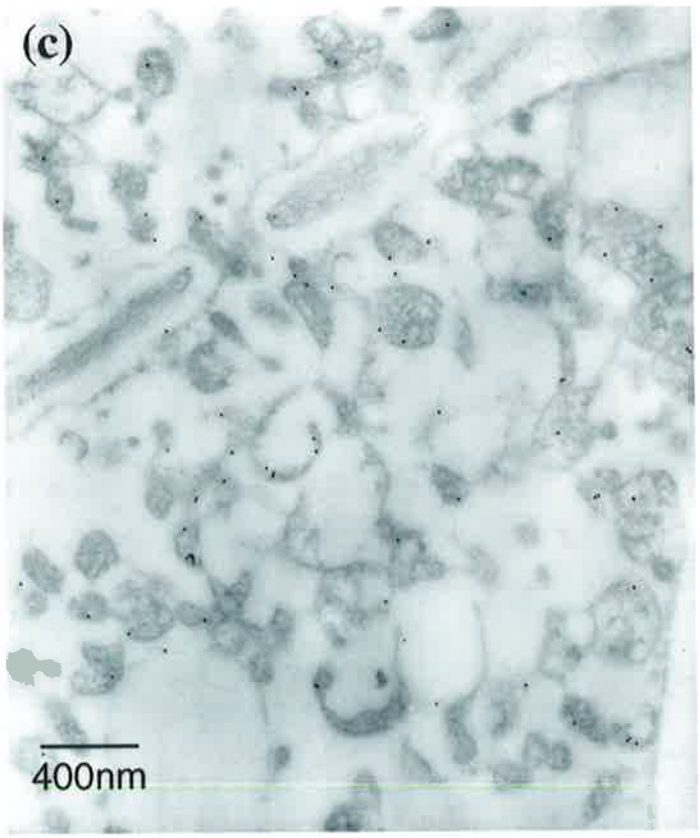
5a and **5b.** Electron micrographs of BN phytoplasmas in two different phloem sieve tube element cells. The micrographs show mature forms of the phytoplasma completely filling the phloem sieve tube elements. The oligoprobe used here for the *in situ* hybridisation was fStol (-)-digoxygenin and the antibodies used were 10 nm gold anti-digoxygenin. Note the absence of gold beads on the cell walls, the gold beads are labelling the ribosomes which are found in the interior of the phytoplasmas. Bars = 330 nm.

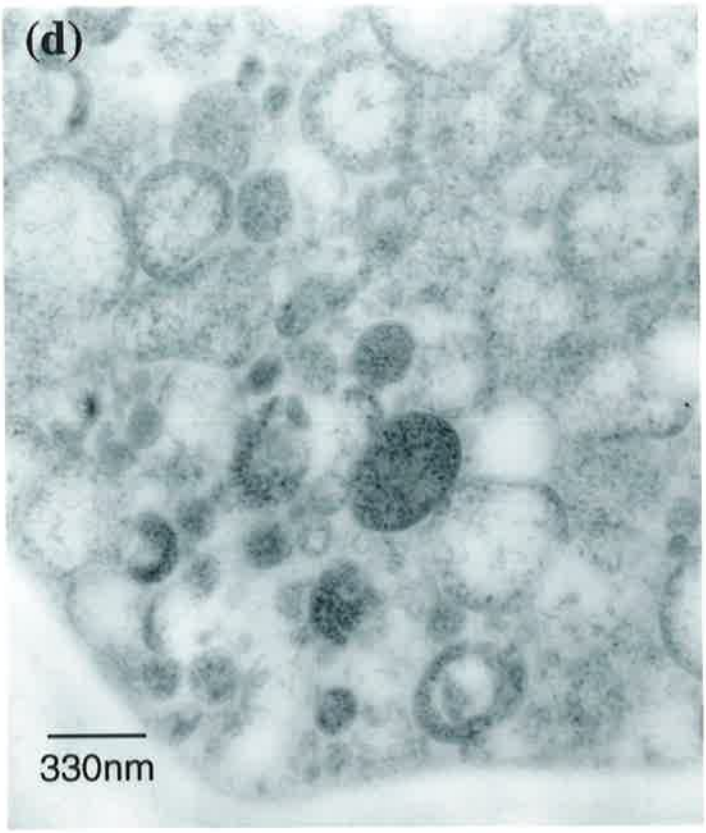

(a)
330nm


(b)
330nm

5c Electron micrograph of in situ hybridisation of the oligoprobe fStol (-)- digoxygenin with BN phytoplasmas using the same experimental material and methods as shown in Figures 5a and 5b. This picture shows mature forms of phytoplasmas moving through a phloem sieve plate. Some of the phytoplasmas are broken, possibly by osmotic shock during tissue sample preparation. Bar = 400 nm.

5d Electron micrograph of *in situ* hybridisation under the same experimental conditions as for Figures 5a, 5b and 5c but with RNaseA treatment of the tissue sections prior to in situ hybridisation. Note the absence of gold beads. Bar = 330 nm.


(c)
400nm


(d)
330nm

Chapter six
Discussion

The purpose of these *in situ* studies was to localise specific viroids at the ultrastructural level and to therefore increase our knowledge of where viroids are found and see if we can match the information we get to what we already understand about the replicative biology of viroids. While we are able to relate the results of this work to what we already know about the biology of viroids, a particular caveat that could not really be addressed in this work was that it is not known if the viroids are synthesised (replicated) at the sites where they are found, or whether these sites are simply sites of accumulation. The experiments done in this work were mainly designed to develop the methodology and address what questions are within the scope of these experiments. Experiments designed to show specific sites of replication will be a much more difficult task.

Prior to this work, the specific localisation of viroids by microscopy at the ultrastructural level has so far been limited to the study by Harders et al, (1989) where they used fluoresence in-situ hybridisation experiments with cRNA probes against PSTV done on preparations of isolated, purified nuclei from tomato tissue using confocal laser microscopy. The results they achieved showed that the viroid, PSTV, was concentrated in the nucleolus, and this finding is not strictly in keeping with what we know about the replication of viroids in the PSTV group.

All evidence so far does show that the nuclear RNA polymerase II is most likely responsible for the replication of PSTV (Mühlbach and Sänger, 1979: Spiesmacher et al., 1985: Schindler and Mühlbach, 1992), and this is a nucleoplasmic enzyme (Hendzel and Bazett-Jones, 1995: Bregman et al., 1995). If RNA polymerase II is responsible for the replication of PSTV then we need to understand why PSTV is found in the nucleolus in such concentration, and not mostly in the nucleus, where it appears it is synthesised.

The observed presence of CCCV in the nucleus is initially consistent with the study by Harders et al., (1989), however, the distribution of CCCV between the nucleus and the nucleolus as shown in figures 3a to 3c was not the same as that observed for PSTV. While PSTV was located principally in the nucleolus (Harders et al., 1989), CCCV was present in both the nucleoplasm and the nucleolus in a ratio of 5:1, but with a higher concentration in the smaller nucleolus. Regardless of the potential minor differences between the similar results for CCCV in this thesis and those for PSTV in the study by Harders et al. (1989), an explanation is required as to why the two viroids are concentrated in the nucleolus, where they are not likely to be synthesised.

The observed distribution for CEV is arguably more in keeping with what we already know about the replication of the PSTV group of viroids. CEV was found to be distributed across the nucleoplasm, as shown in figures 2a to 2f, and while it was sometimes observed in the nucleolus, there was not any obvious localised concentration in that organelle. Time course studies on CEV did not indicate any time related factor in the sub-nuclear

distribution of this viroid up to ten weeks post infection. The distribution of CEV was across the entire nucleus with scattered concentrations of viroid throughout the nucleoplasm and this finding is consistent with the localisation of the large sub-fragment of RNA polymerase II, as described in Bregman et al. (1995).

In related work (Bonfiglioli and Warrilow, unpublished), the murine monoclonal antibody, IgG2a 8WG16, (from Promega) targeted against the non phosphorylated hexapeptide repeat motif of the carboxy terminal of the largest sub-unit of RNA polymerase II (Thompson et al., 1990 : Warrilow, 1996) was used to immunolocalise the largest sub-unit of RNA polymerase II *in situ* in tomato tissue. We found that the signal in the *in situ* immunolocalisation experiments was very low, but this was expected as the enzyme is known to be present only in relatively small amounts in the nucleoplasm, but has a very high rate of activity (Warrilow, 1996). The immunolocalisation signal was only found in discrete clumps in the nucleoplasm, and not in the nucleolus (data not shown), which is certainly in keeping with the work discussed above by Bregman et al. (1995) and further confirms the nucleoplasmic localisation of this enzyme..

The localisation of ASBV in the chloroplast detailed in Chapter four is certainly in keeping with what we know about ASBV. The study by Mohammed and Thomas (1980) showed that the viroid was found in the chloroplast fraction, and the results from this study have since been further verified by the results from Lima et al., (1994) who have used a different labelling system and came up with a similar result.

Viroids from the ASBV group have very little in common with the rest of the viroids in the PSTV group, except that they are also comprised of circular single stranded RNA and are of comparable size. It is not surprising therefore that the biology should be different, and the clear localisation of ASBV, in both the plus and the minus forms, to the chloroplast is an interesting find for this project with implications for both the biology and evolution of ASBV group viroids. We await further developments in the ultrastructural localisation of the other two members of the ASBV group.

It is tempting to speculate that the differences in the ultrastructural locations observed between CEV and CCCV in our study might be due to differences in the host plants (monocots vs dicots), but the PSTV studies by Harders et al. (1989) where PSTV was found to be localised in the nucleolus were also done using tomato, which is the same host plant we used in our CEV studies where CEV was found to be spread across the entire nucleus. The PSTV experiments by Harders et al. (1989) used isolated purified nuclei and it is possible that the nucleolar concentration seen may be an artefact associated with the preparation of the nuclei. In view of our own results with CCCV, however, this distribution being purely artefactual is less likely, and some other explanation is therefore still required.

Examination of the pattern of the distribution of signal for CCCV in the nucleolus in Figures 3e and 3f shows that the viroid is sometimes seen to form short strings or clumps of signal, and is often seen to be concentrated towards the inner periphery of the nucleolar structure, as seen in Figure 3e.

The pattern of distribution of the viroid in Figure 3e is very similar to the patterns of rDNA gene transcription by RNA polymerase I as discussed in Highett et al. (1993a) and Shaw et al. (1995). Shaw et al. (1995) also noted that the central nucleolar vacuole, and the perinucleolar knobs are transcriptionally inactive zones. Both these structures are visible in the nucleolus shown in Figure 3c, and they do not contain any significant amount of viroid signal. Shaw et al. (1995) concluded that the area of the nucleolus where they demonstrated transcription of the rDNA genes, which appears to be the same zone in which we found CCCV, and which is comprised of multiple fibrillar centres in a granular matrix which surround a central vacuole, is a zone where they located "nascent and newly completed transcripts attached to and surrounding active genes". The concentration of CCCV in this area could be interpreted as circumstantial evidence for the involvement of RNA polymerase I in the replication of CCCV. However, whether these patterns of viroid distribution, also seen in other tissue sections, are related to transcriptional or splicing (or spliceosomal) processes within the nucleolus, or are the result of viroids binding to other molecules that are involved in nucleolar transcription or splicing events, or even perhaps just to an accumulation of viroids at specific sites, is not known.

The rRNA splicing events that take place in the nucleolus are well established and involve the transcription, by RNA polymerase I, of a 7.2 kb pre-ribosomal RNA transcription unit (TU). This TU is the precursor molecule which is then cut into the 28S, 18S and the 5.8S and is then complexed with ribosomal proteins and the 5S within the nucleolus (Wachtler et al., 1991: Highet et al., 1993a, 1993b). An interesting

experiment to test the theory of viroids interfering with rRNA splicing processes could be to assess the relative amounts of precursors (the 7.2kb TU) and products (28S, 18S and 5.8S) in respect to the concentration of viroids found in the same plant tissues. If the number of precursor products builds up as do the number of viroids, then the theory could be correct.

A further interesting possibility here is that the rRNA splicing mechanisms present in the nucleolus may also be the same mechanisms that process viroids from their linear multimeric forms to the circular monomeric forms. It is possible that linear multimeric viroid transcripts are imported into the nucleolus and then processed along with the rRNA precursor TUs , which could explain their presence in the nucleolus. How processing actually does occur for viroids in the main PSTV group is, however, still a mystery.

There are precedents for the movement of small RNAs between the sub-nuclear compartments and even out into the cytoplasm and back again into the nucleus (see Baserga et al., 1992 and Baserga and Stietz, 1993, and references therein). The small nuclear and nuceolar RNAs (snRNAs snoRNAs), especially the U RNAs which are complexed with proteins to become the snRNPs, or small ribonucleoproteins, are the nucleic acid components of the spliceosomes and are good examples of such mobile small RNAs. While some of these snRNAs are known to be synthesised in the nucleoplasm, their site of synthesis does not necessarily reflect their site of activity or known location (Baserga et al., 1992 and Baserga and Stietz, 1993). U8 RNA and U13 RNA are synthesised by RNA polymerase II in the nucleoplasm and are found in the nucleolus. The 5S and the 7S RNAs

are synthesised by RNA polymerase III in the nucleoplasm, but are found in the nucleolus (Highett et al., 1993b) and in the cytoplasm (Haas et al., 1988) respectively.

The other, and more simple explanation, however, is that CCCV may be replicated by RNA polymerase I, which is a nucleolar enzyme (Scheer and Rose, 1984), and that the viroids seen in the nucleoplasm have diffused out into the nucleoplasm after replication in the nucleolus. A problem with this idea though is that RNA polymerase I is known to be almost exclusively present in the dense fibrillar centres of the nucleoli (Raska et al., 1989: Scheer and Rose, 1984), and in this study the viroids were found in the fibrillar cortex region, but then this is also exactly the same place where the rRNA genes are found which are known to be transcribed in the nucleolus (Wachtler et al., 1991). There is, however, in vitro evidence that shows that the replication of viroids (Mühlbach and Sänger, 1979) including PSTV (Spiesmacher et al., 1985; Schindler and Mühlbach, 1992) and CEV (Rivera-Bustamante and Semancik, 1989) are both sensitive to a-amanitin thereby indicating that they are both most likely to be transcribed principally, if not exclusively, by RNA polymerase II. Doubts raised about the value of using actinomycin-D in assays for activity of RNA polymerase I, (Grill and Semancik, 1980; Rivera-Bustamante and Semancik, 1989) however, must mean that the question of the involvement of RNA polymerase I in viroid replication remains open.

The models published to-date on the possible mechanisms of viroid pathogenicity are rather diverse. There are three related models that all draw on viroids having sequence homology to various positions on

different small nuclear or nucleolar RNAs (snRNAs or snoRNAs) including the 5'end of U1 RNA from Novikoff hepatoma cells and some splice site junctions in pre-mRNA (Dickson, 1981), the internal transcribed sequence 2 (ITS2) in tomato, an intron-like sequence between the 5.8S and 28S rRNA (Jakab et al., 1986) and to the small domain of the 7S RNA in tomato (Haas et al., 1988). Some sequence homologies between viroids and U RNAs from rat Novikoff hepatoma cells have been noted (Kiss and Solomosy, 1982, 1983), but as viroids are unique to plants, the exact relevance of these studies is not known. In another model, viroids are seen to bind to a double-stranded RNA activated protein kinase and to alter the pattern of phosphorylation of this kinase protein (Hiddinga et al., 1988; Vera and Conejero, 1990; Diener et al., 1993). The common theme in all these papers is that the viroids (CEV and PSTV) are postulated to exert their pathogenic effects by binding to, and thereby interfering with, the proper functioning of these snRNAs, snoRNAs or proteins. In view of the varied models proposed in the papers discussed above, and the results described here in this study where viroids in the PSTV sub-group are found in different ultrastructural locations, we consider it possible that, if viroids do exert their pathogenic effects in this interfering manner, then the binding of viroids to target snRNAs, snoRNAs or proteins may be more varied than previously thought. Different viroids may preferentially bind to different target molecules, or to the same target molecules at different sites and cause different effects, and this may also be the basis of the diverse host response seen between the different viroids.

The results from this study are certainly useful for addressing the questions of viroid biology, perhaps not conclusive in any degree with regard to sites

of synthesis or accumulation, but still useful for addressing the questions. While we find both the plus and minus sense of ASBV located on the thylakoid membranes of the chloroplast, and we do not find it anywhere else, we can say that the evidence is increasing that that there is where it is probably synthesised, but the results do not say this for certain. The same applies to the other findings concerning the other viroids; the lack of signal at any other site is certainly interesting, but failure to find the viroids at any other sub-cellular location does not mean they are not there.

It is very pleasing to find that it is possible to use labelled PCR primers as *in situ* probes, as shown in Chapter six on the use of oligodeoxynucleotides as *in situ* probes for specific phytoplasmas in plant cells. The major stumbling block of this in situ methodology has been the construction of a probe, which usually requires significant sequencing and cloning before a useable sequence is found. The ability to simply label and use a PCR primer is a great advantage, especially if it retains the fine discrimination usually only seen with the use of larger probes.

In conclusion, it can be said that the work in this thesis certainly shows that this technique of *in situ* hybridisation has great promise for contributing to our understanding of complex biological problems, such as the replication strategies of viroids. Future work could usefully be directed towards designing experiments where viroids are labelled and remain (once labelled) at the site of synthesis and therefore address the problem of site of synthesis versus site of accumulation. The technique also has promise for developing better and faster studies for the transmission by insect vectors of pathogens such as phytoplasmas and other micro-organisms.

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