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STUDIES ON THE ULTRASTRUCTURAL LOCALISATION OF VIROIDS AND OTHER PLANT PATHOGENS

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at the University of Adelaide

by

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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.