gained in developing methods for avocado breeding will be applied to other tree crop species.

RAPID INDEXING OF SUNBLOTCH VIROID IN AVOCADOS AND OF EXOCORTIS VIROID IN CITRUS

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Abstract. It appears feasible to replace the time-consuming biological indexing of the sunblotch disease of avocados and possibly also of the exocortis disease of citrus by a more rapid method which is highly specific and can be completed in several days This new method involves the sensitive detection of the avocado sunblotch viroid and the citrus exocortis viroid in partially purified nucleic extracts of candidate trees by a technique known as hybridization analysis Details of this new method are given together with a summary of the results so far obtained

INTRODUCTION

Viroids are the smallest pathogenic agents known and consist of single-strand, circular RNA molecules which are only 300-400 residues long (3). Unlike normal plant viruses, viroids are not protected by a protein coat and are spoken of as naked molecules. They infect a wide variety of plants and, in many, produce severe disease symptoms. Of the eight viroids so far described, at least five are of considerable agricultural importance; these are potato spindle tuber viroid, hop stunt viroid, cadang-cadang viroid of coconuts, avocado sunblotch viroid, and citrus exocortis viroid. Only the latter two are present in Australia.

A characteristic property of viroids is their slow rate of growth and the long time taken for symptom development as compared with many plant viruses. Indexing for the presence of viroids by symptom development in suitable indicator plants can take a minimum of two to three weeks for such viroids as potato spindle tuber viroid or two years and more for avocado sunblotch viroid, and especially cadang-cadang viroid. Hence, the development of more rapid procedures for the indexing of a least some viroid diseases is most important. This paper summarizes our results so far on the development and use of a very specific and rapid indexing procedure for avocado sunblotch viroid and citrus exocortis viroid.

AVOCADO SUNBLOTCH VIROID AND CITRUS EXOCORTIS VIROID

Avocado sunblotch, a serious disease affecting avocados (*Persea americana Mill*), was first described in California nearly

50 years ago and more recently in Australia (9). Its infectious nature was established by seed transmission, by grafting and more recently by pollen transmission (4). Indexing for the presence of sunblotch disease has been carried out by graft transmission to suitable indicator avocado seedlings with a requirement of 15 avocado seedlings for each avocado test sample and maintenance of these plants for a minimum of two years (1). This procedure obviously places severe limits on glasshouse space and restricts the number of avocado samples that can be indexed at any one time. Also, the long time taken for the assay can cause serious delays in the release of material for rootstocks and for grafting.

The purification and characterization of a viroid (avocado sunblotch viroid, ASBV) from sunblotch infected avocados (2,6,8) has allowed the development of a new rapid indexing procedure which is described below. Although ASBV has not yet been shown to be the causative agent of sunblotch disease, it is always found in infected but not in healthy avocados, and all evidence indicates that it is an essential component of the disease (see below).

Exocortis disease of citrus is normally indexed using Etrog citron *(Citrus medica* 'Etrog') or *Gynura aurantiaca.* Although the citrus industry is well established and there is a reasonable supply of CEV-indexed trees for use as a source of seed for rootstock or for budwood for scions, it is considered that there is still a need for rapid indexing of CEV (R. van Velsen, personal communication). This could possibly become more important in the future with the potential widespread use of the so-called dwarfing principle of citrus which is believed to be a strain of CEV

PRINCIPLE OF MOLECULAR HYBRIDIZATION ANALYSIS FOR INDEXING OF VIROIDS

Basically, the principle and practice of the use of hybridization analysis for the indexing of viroids are relatively simple and straightforward (Figure 1). The first requirement is for a rigorously purified viroid and we have developed methods which allow the facile purification of ASBV, CEV and chrysanthemum stunt viroid (7). Only 1 to 2 μg of this viroid is required to produce highly radioactive (^{32}P) complementary DNA (cDNA) to the viroid (6). This ^{32}P-cDNA probe is synthesized enzymatically and its nucleotide sequence is an exact complementary copy of the nucleotide sequence of the viroid. Under optimal experimental conditions of ionic strength and temperature, which are determined empirically, the single-strand ^{32}P-cDNA probe will combine (hybridize) with the single-strand viroid RNA such that the nucleotide residues in the DNA are exactly base-paired with the com-

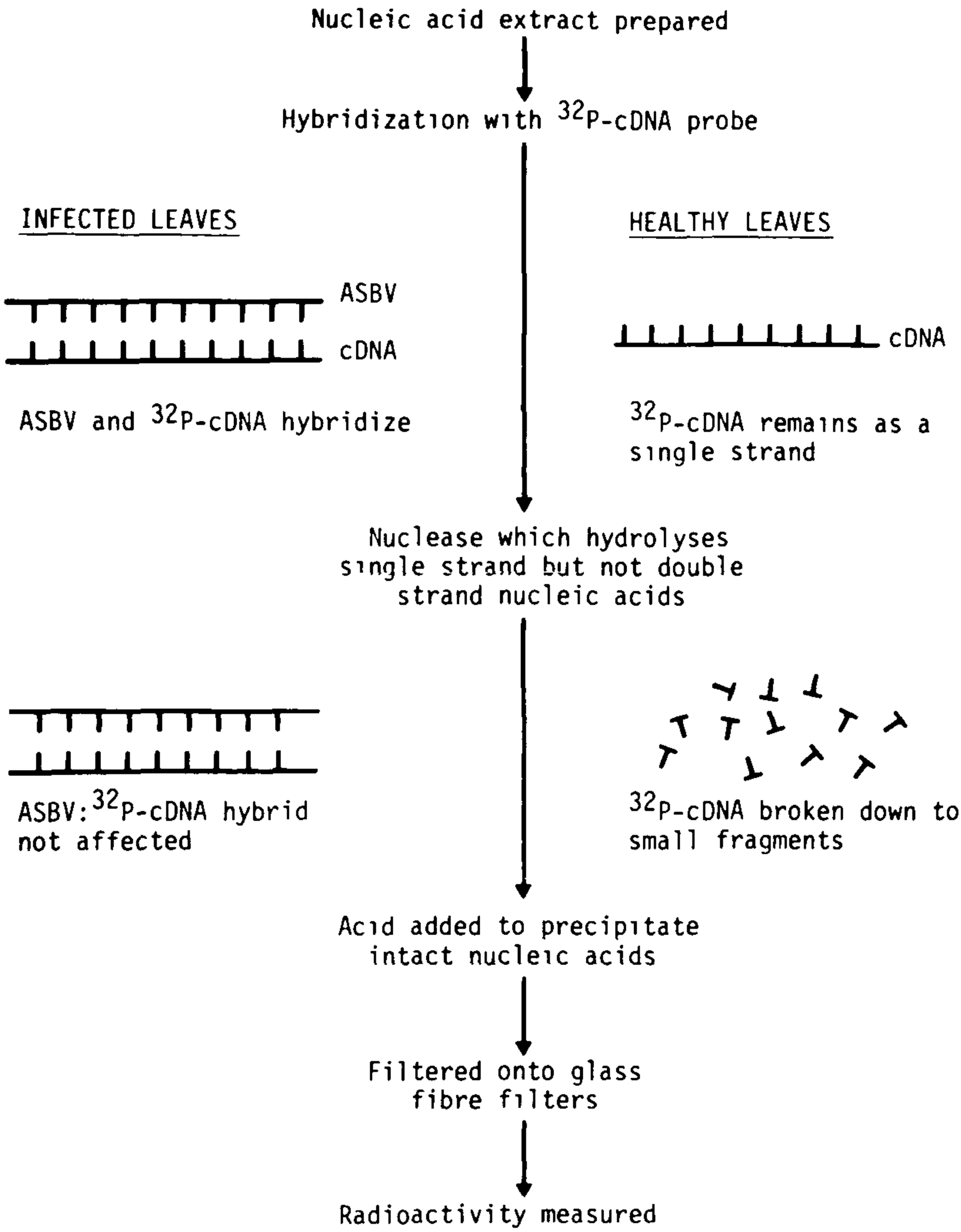

Figure 1. Summary of the procedure for the hybridization analysis of avocado sunblotch viroid in partially purified nucleic acid extracts of avocado leaves

plementary nucleotide residues in the RNA; i.e., the A, C, G and T residues in the cDNA will base-pair with the U, G, C and A residues in the RNA, respectively.

The ^{32}P-cDNA probe is then hybridized under defined conditions with the partially purified extract of RNA from the candidate tree, usually for three days. During this time, the ^{32}P-cDNA probe will hybridize with any viroid RNA sequences present in the extract to form a double-strand DNA:RNA hybrid; if there is no viroid present, then no double-strand hybrids will form as the hybridization reaction is extremely specific. The reaction mixture is then treated with an enzyme (nuclease) which specifically hydrolyses single-strand, but not double-strand, nucleic acid molecules. Hence, the double-strand cDNA:RNA hybrid remains intact while any ^{32}P-cDNA which has not hybridized is digested to very small fragments. The double-strand ^{32}P-cDNA:RNA hybrid molecules can be precipitated by the addition of strong acid and are collected by filtration on to a fine filter of glass fibres; only large fragments are precipitated in this way so that all small ^{32}P-cDNA fragments pass through the filter. The amount of radioactivity retained on the filter is then determined in a radioactive counter. If no radioactivity is found on the filter, then there are no viroid sequences present in the nucleic acid extract (see comments below on the lower level of sensitivity). If a high proportion (over 50%) of the radioactivity initially added in the ^{32}P-cDNAprobe is found on the filter, then viroid sequences are present in the nucleic acid extract.

In summary, therefore, a ^{32}P-cDNA probe is hybridized with a partially purified nucleic acid extract of the candidate tree and the proportion of the ^{32}P-cDNA which is converted to a nuclease resistant, acid-precipitable form is determined. If the leaf extraction is done on Monday, the hybridization reaction can be set up on Tuesday and assayed on Friday; the whole procedure can therefore be completed in five days.

APPLICATION OF THE HYBRIDIZATION ANALYSIS FOR INDEXING OF SUNBLOTCH VIROID

Since ASBV has yet to be shown to be the causative agent of sunblotch disease, it is important to show that ASBV is always present in trees indexed biologically as positive for sunblotch and absent in all trees indexed biologically as negative. Application of the hybridization assay to partially purified nucleic acid extracts of 12 avocado isolates indexed as positive for sunblotch showed that all isolates were also positive for ASBV by the hybridization assay. The results therefore show a 100% correlation between sunblotch disease and the presence of ASBV by the cDNA hybridization analysis.

By appropriate variation of the hybridization assay (6,7) it is possible to determine the concentration of ASBV in the partially purified nucleic acid extracts. In four separate avocado isolates, the concentration of ASBV varied 10,000-fold, from 0.2% to 2×10^{-5}% by weight. At the higher concentrations of ASBV (above 2×10^{-2}% by weight), the presence of the viroid in the partially purified nucleic acid extracts can be detected visually by electrophoresis of samples of the nucleic acid extracts on polyacrylamide gels followed by staining of the gels with dyes to show up the nucleic acid bands. This is certainly simpler and faster than the cDNA hybridization analysis, but this approach cannot be used for the routine indexing of sunblotch because of its low sensitivity.

The lower limit to the detection of ASBV sequences in partially purified nucleic acid extracts by the cDNA hybridization analysis is about 1×10^{-5}% by weight. Although it is technically difficult to further lower the sensitivity more than several-fold using the method described above, it is hoped that future work (see below) will simplify the overall procedure and increase the sensitivity at least 10-fold. Until many more estimates are made of the level of ASBV in sunblotch infected avocados, it will not be possible to determine the lowest level of ASBV that can produce the characteristic sunblotch symptoms.

APPLICATION OF THE HYBRIDIZATION ANALYSIS FOR INDEXING OF CITRUS EXOCORTIS VIROID

We have so far only carried out very preliminary work in this area. It has been possible to purify CEV, to prepare ^{32}P-cDNA to it and to use it in hybridization assays. However, we have not done routine indexing on a number of isolates nor have we determined the concentration of CEV in partially purified extracts of citrus isolates infected with CEV. However, all evidence so far indicates that we will be able to use the cDNA hybridization analysis for indexing of CEV in the same way as we have done for ASBV.

FUTURE DEVELOPMENTS

The method used so far for the preparation of the ^{32}P-cDNA probe for ASBV only provides low yields of the product. If much larger quantities of the cDNA probe can be prepared, then it becomes feasible to use a higher concentration of the cDNA probe for the hybridization assay in a way which would increase the sensitivity of the assay about 10-fold. Our future approach in this area is to make use of recombinant DNA technology to clone viroid sequences in the form of DNA in bacteria and this should allow the production of large quantities of the cDNA probe. It is

envisaged that a central laboratory could then be responsible for the regular preparation of the radioactive probe for use by regional laboratories for the routine indexing of sunblotch and exocortis diseases.

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