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**CONTENTS**


| | |
|---|---|
| **PRACTICAL SECTION FOR GROWERS** | 4 |
| **Commercial benefits** | 4 |
| **Background and objectives** | 4 |
| **Specific targets** | 5 |
| **Summary of results** | 5 |
| **Action points for growers** | 8 |
| **Anticipated practical and financial benefits** | 8 |
| **MILESTONES** | 9 |
| **Year 1** | 9 |
| **Year 2** | 10 |
| **Year 3** | 11 |
| **Expenditure** | 13 |
| **SCIENCE SECTION** | 14 |
| **INTRODUCTION** | 14 |
| **MATERIALS AND METHODS** | 17 |
| **RESULTS** | 33 |
| **Objective 1 – Devise PCR-based assay for *Apple stem pitting virus*** | 33 |
| **Objective 2 - Comparison of assays for *Apple stem grooving virus*** | 40 |
| **Objective 3 – Expression of coat proteins of *Apple stem pitting virus* and *Apple stem grooving virus* in yeast for antiserum production** | 47 |
| **Objective 4 – Development of a PCR based method for the detection of *Strawberry crinkle virus*** | 56 |
| **CONCLUSIONS** | 66 |
| **INDUSTRIAL RELEVANCE AND EXPLOITATION** | 68 |
| **TECHNOLOGY TRANSFER** | 70 |
| **REFERENCES** | 72 |

## PRACTICAL SECTION FOR GROWERS

### Commercial benefits of the project

The project has provided a firm basis for the development of laboratory assays for two fruit viruses (one affecting apple and pear, the other affecting strawberry). The current tests for these viruses entail grafting to indicator plants and such tests are slow and expensive. The cost of such tests for tree fruit has led to severe cuts in the EMLA scheme for provision of virus free fruit trees. We have made a comparison of assays for one fruit tree virus and have demonstrated the reliability of laboratory tests for this virus (apple stem grooving) and the value, therefore, of continuing the development and comparison of such assays for other viruses. This will strengthen the machinery for providing healthy planting material at all levels from Nuclear Stock to Certified plants.

### Background and objectives

The importance of viruses to the quality of propagation material and to the establishment and performance of fruiting plantations has been recognised for many years; it was a major reason for developing schemes for producing healthy planting material of strawberry and fruit trees.

The success of such schemes depends upon reliable tests for viruses. These have, until recently, consisted of bioassays conducted in the glasshouse or the field. Bioassays are used as standard throughout the world for testing the highest quality planting material (Nuclear Stock or their equivalent) but such tests are not always reliable. They are also expensive and take a long time to provide results (months for strawberries and years for fruit trees).

There are clear advantages to using laboratory tests for detecting viruses but they have to be thoroughly tested with a range of isolates and compared against the best, established test. This has been done for apple stem grooving virus in this project.

The project has targeted two important viruses, for which routine laboratory tests have not previously been available, (apple stem pitting virus and strawberry crinkle virus) with the objective of developing laboratory assays for them. Apple stem pitting virus is associated with at least four diseases of apple and pear and several indicator species are required for field tests, making this a very expensive virus to test for. Strawberry crinkle virus occurs world-wide and so far the only means of assay is by leaf grafting. The virus is difficult to purify and the information did not exist at the start of this project for devising any kind of laboratory assay.

A fourth major objective was to investigate the possibility of cloning virus coat protein in yeast as a route to obtaining high quality immunogen for 'difficult' viruses (for serology tests such as ELISA). ELISA is a simple and well-tried procedure and is the assay of choice, providing the reagents are of sufficient quality to achieve good results. Success in this objective would improve the chances of attaining good results with a wider range of viruses than is possible at the moment.

**Specific targets for the project**

1. Devise a PCR-based laboratory assay for apple stem pitting virus.
2. Refine and validate the detection methods for apple stem grooving virus.
3. Express apple stem grooving virus and apple stem pitting virus coat protein genes in yeast for antiserum production.
4. Develop a PCR-based method for the detection of strawberry crinkle virus.

**Summary of results**

<u>Objective 1. Assay for apple stem pitting virus (ASPV)</u>

In the first two years of the project the effectiveness of a newly published protocol for detecting ASPV by immunocapture PCR, was assessed. It detected the virus in many of the isolates of ASPV in the reference collection at East Malling but not all of them. Modifications to the test improved its performance but results were inconsistent, particularly with a group of virus isolates from pear. These isolates were from trees with stony pit disease, which is associated with this virus and may be caused by it.

The tests showed, surprisingly, that in extracts from dormant wood it was easier to detect ASPV by a combination of electron microscopy and serology than by the potentially highly sensitive PCR. Antiserum to ASPV is not commercially available and in the final year efforts were directed at a) making antiserum to ASPV and b) obtaining sequence information of an isolate of ASPV from pear with stony pit disease.

These final year objectives were partially achieved. The coat protein gene of an isolate of ASPV was cloned into a plasmid vector and the protein was expressed in the bacterium *Escherichia coli*. Sufficient protein has been purified from this to prepare antiserum. A small amount of sequence was obtained of the ASPV isolate from pear, but this was insufficient for meaningful comparison with other isolates.

At the end of the project there is: 1) a viable PCR assay for many, but not all, strains of apple stem pitting virus; 2) the material to prepare antiserum to the virus and 3) the knowledge that serology may provide a better assay for this virus (applicable to a wider range of isolates) than the more sensitive PCR-based assay.

Objective 2. Refine and validate detection protocols for apple stem grooving virus (ASGV)

Four assays for this virus were compared for their effectiveness in detecting ASGV in a sample of 105 apple and oriental pear trees. Twenty two of these trees were infected and all infections were detected by the serology assay (ELISA) and by the nucleic acid test (immunocapture PCR). Only eighteen infections were detected by the standard field test, which takes three years to complete and involves double budding to the sensitive Virginia Crab indicator. Eighteen infections were also detected by a glasshouse bioassay that involves double budding to the indicator *Malus micromalus* and takes only three months to obtain a result. There were inconsistencies between the two bioassay results and three infections detected by *M. micromalus* were undetected by the Virginia Crab test and *vice versa*.

It was surprising that the field test was less reliable than the laboratory tests. Double budding tests are recognised internationally as being the best assays and the standard

by which all other tests are judged. These results strongly indicate that, for some viruses at least, this is not true. Provided that adequate comparisons are made, laboratory assays should be applied to a wide range of fruit viruses with confidence.

Objective 3 – Expression of coat proteins of apple stem pitting (ASPV) and apple stem grooving viruses in yeast for antiserum production.

This objective was only partially achieved. The coat protein gene of apple stem pitting virus was successfully cloned into plasmid vectors and expressed in the yeast *Pichia pastoris*. However, expression was inconsistent. This did not allow the conditions of expression to be optimised. Evidence was obtained that the problem could be resolved by using a different plasmid expression vector that is not sensitive to the methanol:cell density ratio.

By the end of the project the ASPV coat protein gene had been cloned into a yeast vector that is not sensitive to methanol and will allow continuous expression of the protein. The project ended before this could be done. However, the ASPV coat protein gene was cloned into the bacterium *E. coli* and sufficient protein has been expressed for antiserum production (see objective 1). The antiserum will be of value for furthering this research, for the immunocapture phase of PCR tests and for electron microscope detection of the virus but probably not for the simple and robust ELISA.

Objective 4 – Development of a PCR based method for the detection of strawberry crinkle virus (SCV)

During the first two years of the project attempts were made to obtain sequence of SCV by a subtractive hybridisation procedure. All clones obtained by this method were plant associated and not from the virus, although several were from genes coding for proteins known to be involved with plant defence.

A different, and novel, approach was taken at the end of year two. The gene sequences of seven viruses in the same taxonomic group as SCV were compared. The viruses were from groups infecting mammals, fish, insects and plants but despite their

diversity, similarities were found in the L (large) protein gene of these viruses. This information was used to design primers that successfully amplified part of the L protein gene of SCV. This is the first sequence to have been obtained of this virus.

Subsequent work obtained more of the L gene sequence and primers were designed that consistently detected SCV in the experimental host *Physalis pubescens*. They also detected the virus in the aphid vector but detection in strawberry was not always successful. A small amount of development work is now needed to provide, for the first time, a reliable laboratory diagnostic test for this damaging virus.

**Action points for growers**

The results from this project cannot be acted upon directly by growers. The aim of the work is to improve the monitoring of healthy planting material. Growers should always seek plants that are Certified and originate from virus tested material.

**Anticipated practical and financial benefits from the study**

The project has provided and validated a quicker, cheaper and more reliable assay for apple stem grooving virus than existed before the project began.
A small amount of development work should provide similar tests for two other viruses (apple stem pitting virus and strawberry crinkle virus).
The development and application of up to date methods for monitoring the health of planting material is vital to the success of the Nuclear Stock Association and the Plant Health Propagation Scheme for providing high quality plants to the fruit growing industry.

**MILESTONES**

**Milestones for year 1**

1a Transfer of six isolates of apple stem pitting virus from fruit trees to *Nicotiana occidentalis* for maintenance in the glasshouse

1b,c Design and assessment primers that will amplify products from six isolates of apple stem pitting virus

2a Bud rootstocks in the field with 100 sources of budwood for apple stem grooving virus field tests

2b Transfer six isolates of apple stem grooving virus from apple and/or oriental pear to *Chenopodium quinoa* for maintenance in the glasshouse

2c i) Obtain graftwood of *Malus micromalus* (indicator species) and graft to M26 for use as budwood

ii) Bud seedlings raised in 1997/98 for a preliminary glasshouse assay

3a Design primers from published sequence to amplify the entire coat protein region of apple stem grooving virus for cloning into a yeast expression vector

3b Devise a protocol for producing sufficient quantity and quality of RNA of apple stem pitting virus for amplification experiments

4a Establish six isolates of strawberry crinkle virus in *Physalis* by aphid and/or mechanical transfer from strawberry to use as working material. Isolates will be maintained in strawberry and (if possible) in *Physalis* throughout the project

4b Establish a protocol for obtaining extracts from *Physalis* enriched for strawberry crinkle virus RNA

These milestones were achieved with the following exceptions:

1a: six isolates of apple stem pitting were transferred to *N. occidentalis* but they were all lost during the summer. This had no adverse effect on the project as three isolates were re-established in *N. occidentalis,* and it was possible to use material directly from fruit trees for assessing the effectiveness of primers to amplify a wide range of isolates.

2c: the graftwood of *Malus micromalus* indicator was thin and insufficient buds took to provide material for the large-scale glasshouse assay for apple stem grooving virus that was planned for 1999. This was postponed for one year but completed during the project.

**Milestones for year 2**

1 b,c Primers designed in year 1 will have been tested against the panel of isolates in herbaceous plants and modified as necessary so that they will amplify all isolates consistently (use may also be made of information from the sequencing in objective 3)
Mch 99

1 d Obtain nucleotide sequence data for ASPV isolate 7/40 Aug 99

2 e i) Trial PCR and ELISA assays will have been conducted on material from herbaceous plants and on forced buds of apple and pear infected with ASGV to optimise assay conditions Feb 99
ii) PCR and ELISA assays will have been conducted on 100 cvs from Brogdale for comparison with bioassays Jun 99

3 a,b Sufficient sequence information will have been obtained for the coat protein gene of ASPV to have been cloned into one or more of the pPIC vectors
Mch 99

3 c i) Yeast will have been transformed with the *Pichia* vectors containing the coat protein region sequences of ASGV and ASPV Jun 99
ii) Recombinant yeasts that express the ASGV and ASPV coat proteins will have been selected Aug 99

4c i) A subtractive cDNA library will have been constructed from SCV template RNA Dec 98

ii) The library will have been screened by +/- differential hybridisation Mch 99

4d Up to four of the longest SCV clones will have been selected and partially sequenced to confirm their viral origin Aug 99

These milestones were achieved with the following exceptions.

1d Clones derived from material infected with ASPV isolate 7/40 have so far contained only plant and not virus-related cDNA. This milestone was therefore carried forward to year 3.

3 Two yeast cell lines were transformed, via homologous recombination, with both ASGV and ASPV coat protein containing expression vectors. However, recombinant protein expression was not detected in initial small-scale expression studies. This milestone was therefore carried forward to year 3.

4 The milestones in objective 4 (strawberry crinkle virus – SCV) depended upon a positive outcome to milestones 4c ii) Screening the library and 4d Partial sequencing of SCV clones to confirm their viral origin. By the end of year 2 only plant related cDNA had been obtained. The milestones for objective 4 were re-scheduled in year 3 to take account of this.

**Milestones for year 3**

1 d Obtain nucleotide sequence data for ASPV isolate 7/40 Apr 00

1 e i) The PCR protocol will be tested for its ability to amplify ASPV in extracts from woody plants in the autumn Nov 99

ii) The protocol will be adjusted as necessary for use with extracts from trees, using experimental material obtained in the autumn and herbaceous extracts spiked with frozen leaf and bark from apple Mch 00

iii) The final protocol will have been assessed with samples from trees from a) the East Malling collection, b) any positives from 2a and c) key isolates from overseas Jun 00

2a The standard double-budding field assay for ASGV in 100 apple/oriental pear cvs will have been completed Nov 99

2d The glasshouse assay for ASGV on the same 100 cvs as used in 2a will have been completed Aug 00

3c i) Expression of the coat proteins by the yeast recombinants will have been optimised and scaled up Oct 99

ii) Protocols for purifying the expressed proteins will have been devised and used to produce immunogens Dec 99

iii) ASPV coat protein will be expressed and purified from *E. coli* Aug 00

3d i) Antisera will have been prepared by immunising rabbits Mch 00

ii) Antisera will have been evaluated for the detection of ASPV and ASGV in extracts from herbaceous and woody plants Jun 00

4c ii) Continue screening the cDNA libraries until SCV clones are found Feb 00

4d Sequence up to four of the longest SCV clones to confirm their viral origin Apr 00

i) SCV clones will have been analysed to produce a partial map of the viral genome Jun 00

ii) Amplification primers will have been designed, a range of SCV isolates amplified and the primers modified accordingly, to provide primers that satisfactorily amplify the range of isolates in *Physalis* Jun 00

4e The primers will be assessed for their use in amplifying SCV in extracts from stawberry plants and a protocol for field use will have been developed Jul 00

These milestones were met with the following exceptions.

3 c) and 3 d) The objectives concerning expression of virus coat protein in yeast were not met due to problems with erratic expression, thus preventing optimisation of expression conditions and protein purification.

Medium scale production and native purification of the protein was achieved in *E. coli*. Sufficient protein has been purified for antiserum production but antiserum was not obtained before the project ended.

**Expenditure**

| | |
|---|---|
| Approved expenditure for year 3: | £123,707 |
| Approved expenditure for project: | £390,599 |
| | |
| Actual expenditure for year 3: | £125,837 |
| Actual expenditure for project: | £390,594 |

Staff effort (based on 223 working days per year)

| | |
|---|---|
| Band 4 | 20% |
| Band 6 | 100% |
| Band 7 | 15% |
| Band 8 | 15% |
| Band 5 | 2% |
| Student | 100% |

## SCIENCE SECTION

## INTRODUCTION

Viruses are important pathogens of fruit plants, affecting the quality of propagation material as well as the establishment and performance of fruiting plantations. This has been recognised for many years and was a major reason for founding schemes for the production of healthy planting material of strawberry (Harris, 1937) and fruit trees (Cutting & Montgomery, 1973).

Viruses were originally discovered in fruit plants because sensitive indicator species or cultivars were planted, usually unintentionally, and infection gave rise to severe symptoms. These coincidences gave rise to bioassays, which are recognised internationally as being the most reliable means of testing fruit planting material for viruses and are required by European Certification schemes (OEPP/EPPO, 1992) for the highest level of health status (Nuclear Stock).

Despite this requirement, bioassays can be misinterpreted as symptom expression is dependent on growing conditions, particularly with strawberry indicators. In addition, bioassays are time-consuming and take months (strawberries) or years (fruit trees) to complete. The time factor is particularly important with tree fruit and prevents the rapid introduction of desirable new clones of accredited health status into industry. The cost is also high for conducting bioassays and has led to the virtual collapse of the original EMLA scheme for producing healthy planting material.

Laboratory methods of virus assay have advantages over bioassays in speed, cost, the capability for large-scale testing and precise identification of virus strains or serotypes. The prospects of providing such methods for a significant spectrum of fruit viruses have not been good until recently. However, molecular methods provide new opportunities for characterising and diagnosing viruses and the object of this project is to apply such methods to two key virus pathogens of strawberry and tree fruit: apple stem pitting virus (ASPV) and strawberry crinkle virus (SCV).

Apple stem pitting virus is associated with at least four disease syndromes in apple and pear, each requiring a separate woody indicator species and it is possibly also

associated with stony pit in pear which requires a three year test in a fruiting tree for diagnosis (Leone *et al*., 1995). A strain of the virus was purified by Koganezawa & Yanase (1990) but the resulting antiserum was not suitable for ELISA (enzyme-linked immunosorbent assay) and other research groups have been unable to purify any other strains of the virus. Sequence data is available for two isolates of the virus (Jelkmann, 1994) and provided a starting point for designing primers for this project. Since the start of the project, methods and primers have been published for the detection of ASPV in extracts from the leaves of *Nicotiana occidentalis*, apple and pear (Jelkmann & Keim-Konrad, 1997) by ELISA and immunocapture PCR (IC-PCR). These methods have potential but need assessment with a wide range of virus isolates.

ELISA is a less demanding process compared to assays for nucleic acid and is preferable to them provided that sensitivity is adequate. One strategy for providing the immunogen to obtain sero-reagents is to clone the virus coat protein gene(s) for expression in a prokaryote. Antisera produced in this way have usually not been useful for detection of fruit plant viruses by ELISA although they have been satisfactory for other experimental uses e.g. serologically specific electron microscopy. Recently, antiserum to ASPV was obtained by expressing the coat protein gene in *E. coli* as a chimaeric protein lacking four amino acids at the N-terminus and possessing a tail of six histidine residues for affinity purification. This antiserum detected ASPV in extracts from the leaves of *N. occidentalis* and pear but not from apple. Also, the antiserum worked in 'plate-trapped' ELISA but not in the more reliable antibody-sandwich types of assay (Jelkmann & Keim-Konrad, 1997). An alternative to expression of the coat protein in *E. coli* is to use the yeast *Pichia pastoris* for protein expression. High levels of easily purified protein can be obtained which are likely to be structurally more similar to the native protein than that expressed in a bacterial system. This alternative is being investigated.

Strawberry crinkle virus (SCV) is one of the most damaging viruses of strawberry. There are numerous strains of varying pathogenicity and although it is often latent, the virus combines synergistically with other aphid-borne viruses to induce severe damage (Frazier *et al*., 1987). The standard leaf-grafting technique can often be unsuccessful in detecting virus, possibly because of erratic distribution in infected plants (Converse *et al*., 1988). The virus has been purified in small amounts only and

no antisera have been produced that are effective for detecting SCV in strawberry plants (Schoen & Leone, 1995; Hunter *et al*., 1990). The results suggest that a more successful approach might be to use molecular methods to obtain sufficient sequence information to develop a diagnostic test.

SCV is a plant rhabdovirus and a feature of the family *Rhabdoviridae* is the transcription of genes, from the negative sense virus RNA, via polyadenylated mRNAs (Murphy *et al*., 1995). This characteristic was utilised in the project to obtain RNA from infected plants for subtractive hybridisation and cloning.

Information on the sensitivity of laboratory assays and their ability to detect a wide range of virus strains is essential for any decision on the usefulness and reliability of the assay in comparison with the best method available. There has been sufficient progress with apple stem grooving virus (ASGV) for several laboratory methods to be compared with the standard field assay and a glasshouse assay (Howell *et al*., 1996) and this is an aim of the project.

The project has sought to exploit molecular methods to devise laboratory assays for two important viruses of fruit plants for which routine laboratory assays have not previously been possible (apple stem pitting and strawberry crinkle). It has investigated the possibility of cloning virus coat protein in yeast as a route to obtaining high quality immunogen for 'difficult' viruses. In addition, it has compared a range of field and laboratory assays for one virus (apple stem grooving) to provide a model for the acceptance of laboratory assays internationally to accredit the health status of high quality propagation material such as Nuclear Stock plants.

## MATERIALS AND METHODS

### Virus isolation and maintenance

Extracts were made by grinding the leaves or bark of fruit trees, infected with apple stem pitting or apple stem grooving virus, (about 0.1g/2ml of buffer) with a pestle and mortar. The buffer for initial extraction and for passage between herbaceous plants was: 0.025 M sodium/potassium phosphate pH 7.5 containing 0.2 g/litre sodium diethyl dithiocarbamate (DIECA), 0.2 g/litre sodium thioglycollate, 1.5 g/litre polyethylene glycol (mol. wt 6000). Herbaceous host plants were dusted with carborundum (300 mesh), the leaves wiped with the plant extract and the excess inoculum rinsed off the leaves. Herbaceous plants were kept in a glasshouse compartment with supplementary lighting during winter to provide 16 h daylength. *Apple stem grooving virus* was maintained in *Chenopodium quinoa* and *Apple stem pitting* virus in *Nicotiana occidentalis* (37B).

For *Strawberry crinkle virus* (SCV), the transfer from strawberry to the herbaceous hosts *Physalis pubescens* and *Nicotiana occidentalis* was made by aphids (*Chaetosiphon fragaefolii*). Uninfected aphids, reared on healthy plants of *Fragaria vesca* subsp. *semperflorens* or *Fragaria vesca* clone UC-5, were transferred to infected plants and reared for a minimum of 14 days. Aphids were then transferred in groups of 10-20 to the herbaceous test plants to allow transmission of the virus. Transmission between the herbaceous plants, for experimental and maintenance purposes, was by sap inoculation using an extract of 2-5 g plant material in 5-10 ml buffer (225 mM phosphate pH 7.8, containing 10 g/litre Celite and 5 g/litre sodium sulphite). Plants of *Physalis pubescens* were held in the dark for 24 h before inoculation to increase susceptibility.

Virus isolates were collected from commercial and experimental fields in the UK, mainly in Kent, but also in East Anglia (Table 1). Presence of SCV in these plants was confirmed by grafting onto UC-5. SCV isolates were maintained in the original host plants and/ or in UC-5 indicator plants in an insect-proofed tunnel.

**Table 1.** Summary of the SCV isolates.

| Isolate name | Place of collection | Date of collection |
|---|---|---|
| G90/11 | East Anglia | 1990 |
| G90/27 | East Anglia | 1990 |
| Y94/13 | Kent | 1994 |
| C97/1 | Kent | 1997 |
| C97/2 | Kent | 1997 |
| C98/1 | Kent | 1998 |

For library construction, plants were inoculated with SCV isolate G90/11 and control plants for providing RNA were inoculated at the same time with a mixture of buffer and water. The healthy and infected plants were grown in a randomised design in the glasshouse.

Confirmation of infection with SCV in commercial strawberry plants was by leaf grafting to *F. vesca* UC-5 and /or by symptoms in *P. pubescens.*

**Care of trees in glasshouse double-budding test**

MM106 rootstocks (7-9 mm) from F P Matthews Ltd were potted in forestry liners at the end of February. The liners were 250 mm deep, diameter 64 mm (650 ml), from Stuewe & Sons, 2290 SE Kiger Island Drive, Corvallis, Oregon, USA. The compost was 9/1 peat/loam containing 4 kg/$m^3$ Osmacote Plus. Rootstocks were budded in mid April and cut back to the top bud a month later. The pots were irrigated with drippers that were controlled with a timer to deliver 65 ml water twice daily. The plants were given Maxicrop Triple drenches in July and August and sprayed with Nimrod for mildew and an acaricide as needed. The plants were assessed for bud take and symptoms before discarding in September.

**Double-budding field assay for *Apple stem grooving virus* (ASGV)**

400 M26 were planted in double rows, in March, in soil treated with chloropicrin (200 litres per hectare), at a spacing of 1.0 m between rows and 0.5 m within rows. The rootstocks were budded in August, each with a test bud about 10 cm above soil level

and a bud of the Virginia Crab indicator 15 cm above the ground. The buds were placed on the north side of the rootstock. Buds from each tree under test were grafted to three replicate rootstocks. Five positive controls, grafted with buds from trees known to be infected with ASGV and five negative controls that lacked infector buds, were included after each run of 10 tests. Grafting tape was left in place for 6 wk and bud-take recorded one to two months later. The rootstocks were cut back to the top scion bud during the winter and allowed to grow for three seasons before being cut down for symptom assessment. The trunks were cut at about 30 cm above the graft union and 5 cm below it. The shoots were then autoclaved at 121 $^{o}$C for 15 min and plunged into warm water. The bark was then easily removed for observation of the condition of the wood and union.

**Enzyme-linked immunosorbent assay (ELISA)**

Assays for *Apple stem grooving virus* (ASGV) were conducted with antiserum provided by Loewe Phytodiagnostica. Reagents were diluted according to the supplier's recommendations and used in a 'Simultan-Cocktail-ELISA'. Samples were extracted (at a dilution of approximately 0.1 g plant material in 5 ml buffer) in phosphate-buffered saline containing 0.5 ml Tween 20, 20 g polyvinyl pyrrolidone, 2 g ovalbumin, per litre. 100 µl each of sample and alkaline phosphatase-labelled antibody was incubated in each well of a 96 well microtitre plate, overnight at 6 $^{o}$C. The plate was developed with 4-nitrophenyl phosphate substrate for 1 hour and the reaction monitored at 405 nm. Each sample was duplicated and the plates each included five negative samples (10 wells) and a positive control. Samples were considered infected if the ELISA values were greater than the negative mean value plus three times the standard deviation.

**Basic protocol for immunocapture-PCR (IC-PCR)**

Thermofast PCR plates (Advanced Biotechnologies) were treated with crude antiserum diluted 1:1000 in carbonate coating buffer (100 µl per well) and incubated for 5 h at 30 $^{o}$C. Plant tissue (0.1 g) was homogenised with 2 ml PBS/TPO (phosphate buffered saline containing 0.5 ml/litre Tween 20, 20 g/litre

polyvinylpyrrolidone (mol. wt 44 000), 2 g/litre ovalbumin). The homogenate was centrifuged at 13 000 rpm for 3 min in a minifuge. Antibody was removed from the plates by inversion and the plates were washed with 120 µl PBS/Tween per well. 100 µl of extract was placed in each well and incubated at 4 ºC overnight. Plates were washed with 120 µl PBS/Tween per well three times, using a pipette to transfer fluid.

PCR was performed as a one-tube protocol in a 25 µl volume containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 % Triton X-100, 0.25 mM each dNTP, 5 pmole each primer, 0.25 units AMV Reverse Transcriptase (Promega), 0.5 units DNA Polymerase (Red Hot Taq, Advanced Biotechnologies). Reactions were overlaid with 50 µl mineral oil and incubated at 42 ºC for 45 min and 94 ºC for 2 min followed by 40 cycles of 94 ºC for 1 min, 60 ºC for 1 min, 72 ºC for 2 min, then 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 5 min. 5 µl of each reaction was analysed by electrophoresis in a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and pGEM size markers (Promega).

**Table 2.** Primers for amplification of ASPV. The nucleotide positions of primers refer to ASPV isolate PA66 (accession no. D21829); primers are designed to regions in either the coat protein (C.P.) or the polymerase (RdRp) genes.

| Target | Primer | Primer sequence | Primer Position |
|---|---|---|---|
| Poly A tail | anchor | CGGGATCCGTCGACAAGCTTTTTTTTTTTTTTTT | |
| Adapter to anchor | adapter | CGGGATCCGTCGACAAGC | |
| Diagnostic C.P. | ASPV1 | ATAGCCGCCCCGGTTAGGTT | 9237-9256 |
| Diagnostic C.P. | ASPV3 | CTCTTGAACCAGCTGATGGC | 8993-9012 |
| Diagnostic C.P. | ASPV5 | ATGTCTGGAACCTCATGCTGC | 8873-8893 |
| Diagnostic RdRp | ASPV F1 | AGCGGTTGCCTATTTTTGCTCC | 3480-3501 |
| Diagnostic RdRp | ASPV R5 | GTGAGGTCAAAGATGCTGAAACC | 3748-3770 |
| Coat protein | ASPV7956 | ATGACTTCCAATGGATCCCA | 7930-7949 |
| C.P. (Pichia pPICZB) | SPFB | CGCGCTCGAGGATGGCTTCCAATGGATCCCAGCC | |
| C.P. (Pichia pPICZαA) | SPFα | GGCGAGCTCGAGAAAAGAACTTCCAATGGA | |
| C.P. (Pichia) | SPR | CAGATGGGACCTATGTACCGGACATCC | |
| C.P. (E.coli pQE70) | SP70F | CGTTCAAGCATGCCTTCCAATGGATCCC | |
| C.P. (E.coli pQE70) | SP70R | ATTAATAGATCTCTTCCTAATAGATAAGAC | |
| C.P. (E.coli pQE40) | SP40F | CGTTCAAGATCTACTTCCAATGGATCCC | |
| C.P. (E.coli pQE40) | SP40R | ATTAAAAGCTTACTTCCTAATAGATAAGAC | |

**Immunocapture-PCR for *Apple stem grooving virus* (ASGV)**

ThermoFast plates (Advanced Biotechnologies) were coated with 100 µl antiserum (Loewe) diluted 1:200 in carbonate coating buffer. Young unfurled leaf samples were ground in PBS/TPO buffer and the extract diluted to 1:400. They were incubated in the plates overnight at 4 $^0$C for immunocapture to occur. A two step RT-PCR, based on Jelkmann and Keim-Konrad (1997) was carried out using primers designed to regions in the coat protein gene.

Reverse transcription: 20 µl reaction containing 4 µl of 5x reaction buffer (Promega), 1.25 mM each dNTP, 35 U ribonuclease inhibitor (MBI), 20 pmoles oligo dT primer and 0.5 U AMV-RT (Promega). Reactions were overlaid with mineral oil and incubated at 37 $^0$C for 1 hour.

PCR: 50 µl reaction containing 3 µl of cDNA, 1x PCR buffer (Gibco BRL), 2 mM MgCl2, 0.4 mM each dNTP, 0.4 pmoles of each primer and 2 U Taq (Gibco BRL). Reactions were overlaid with mineral oil and cycled in an OmniGene PCR machine (Hybaid) 95 $^0$C for 90 s followed by 35 cycles of 95 $^0$C 30 s, 60 $^0$C for 30 s and 72 $^0$C for 60 s. 10 µl of each reaction was analysed by electrophoresis in a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide and 1 kb DNA ladder (Gibco BRL).

Primers SGTL4 and SGTL5 were used initially to amplify a 664bp region (nucleotides 5594-6257 isolate P209 [Yoshikawa *et al* 1992], accession number D14995) from the coat protein coding region of 11 different ASGV isolates. The resulting PCR fragments were sequenced directly so sequence data presented is of the most common variant. Despite the high degree of similarity over this coat protein region (96.6-99.6% at the nucleic acid level) the European apple isolates cluster together into a cluster that is distinct from P209, the Oriental pear and citrus tatter leaf isolates (data not shown). Diagnostic primers SGTL6 and SGTL9 were designed to areas of complete nucleic acid homology between the 11 isolates sequenced although there is one mismatch in each primer with respect to P209. An ASGV diagnostic product of 424bp is amplified by this primer pair (nucleotides 5762-6185 isolate P209).

**Table 3.** Primers for amplification of ASGV.

| Target | Primer | Primer sequence |
|---|---|---|
| Diagnostic | SGTL4 | GAGAGGATTTAGGTCCCTCT |
| Diagnostic | SGTL5 | CTCCTAACCCTCCAGTTCCA |
| Diagnostic C.P. | SGTL6 | AAGGTGAAAGCTTTGAAGGCA |
| Diagnostic C.P. | SGTL9 | TCAAAAGCTTTGGGCCATTTC |
| C.P. (Pichia pPICZB) | GVFB | GCCGCTCGAGGATGGGTTTGGAAGACGTGCTTCAAC |
| C.P. (Pichia pPICZαA) | GVFα | GGCGAGCTCGAGAAAAGAAGTTTGGAAGACGTGCTTCAAC |
| C.P. (Pichia) | GVR | GGCGAGCTCGAGAAAAGAAGTTTGGAAGACGTGCTTCAAC |

## Immunosorbent electron microscopy

Carbon-coated electron microscope grids were floated on drops of crude antiserum, diluted 1:500 in phosphate buffered saline pH 7.2, for 10 min to coat with antiserum. The grids were rinsed by floating on distilled water in a 5 ml plastic cup and were then floated on plant extract (0.1 g/2 ml) for 20 min. After rinsing, the grids were floated on antiserum diluted 1:50 to decorate virus particles. The grids were finally rinsed, stained with 1.5% aqueous ammonium molybdate and examined in a Jeol JEM 100S transmission electron microscope.

## Isolation of dsRNA from *N. occidentalis*

The dsRNA extraction procedure is basically as described by Valverde (1990). However, dsRNA was extracted from 7g of ASPV 7/40 infected *N.occidentalis* leaf material and only 1 cycle of cellulose chromatography was necessary. After extensive washing with 1xSTE containing 16% ethanol, to remove contaminating ssRNA, the retained dsRNA was eluted, collected and concentrated by ethanol precipitation.

**Construction of an immobilised cDNA library from dsRNA template**

200ng ASPV 7/40 dsRNA was captured onto Dynabeads oligo$(dT)_{25}$ (Dynal) after denaturing at 100°C for 5 min and incubating at 65°C for 2 min. First strand cDNA synthesis was carried out using AMV RT (Promega) according to the manufacturers instructions. The RNA is melted (94°C for 1 min) away from the cDNA leaving a reusable solid-phase cDNA library. Second strand synthesis requires a sequence specific primer and several ASPV primers were used (ASPV7956, ASPV F1, ASPV5, ASPV3 and random primers). This second strand cDNA was used as the template for PCR amplification (50 cycles: 94° for 45 sec, 50° for 45 sec and 72°C for 2 min).

***Pichia pastoris* expression system (Invitrogen)**

*Pichia pastoris* maintenance

*Pichia pastoris* strains and recombinant clones were streaked onto YPD slopes (1% yeast extract, 2% peptone, 2% dextrose + 2% agar), grown at 30°C for 2 days and stored at 4°C.

Construction of expression constructs and transformation of *Pichia pastoris*

*P.pastoris* cells (strains GS115 and KM71) were made competent using chemical treatment according to the Invitrogen EasyComp transformation kit instructions.

Transformation of the chemically competent *P.pastoris* cells was also carried out according to the Invitrogen EasyComp transformation kit. A no DNA control and the appropriate plasmid were transformed alongside the *Pme* I linearised Pichia expression vector DNA and plated onto selective media (YPD agar + 100μg/ml Zeocin) to allow the growth of Zeocin resistance *P.pastoris* transformants. Direct PCR screening of the *P.pastoris* clones was carried out to check for the presence of the viral coat protein according to Linder *et al*., 1996. All KM71 recombinant colonies have a $Mut^S$ phenotype but the GS115 transformants were screened for only the $Mut^+$ phenotype.

Expression studies

Small scale expression of recombinant *P.pastoris* clones was carried out using buffered media (GMGY & BMMH) according to the EasySelect Pichia Expression kit instructions. The culture conditions used were dependent on the *P.pastoris* Mut phenotype.

Extraction of yeast proteins

Two methods of protein extraction were investigated. However, the protocol recommended by Invitrogen proved more successful, although more complicated than using the Y-PER reagent (Pierce).

Polyacrylamide gel electrophoresis

An equal volume of cell lysate was added to 2x SDS PAGE buffer (186mM Tris, 6% SDS, 30% glycerol, 15% mercaptoethanol, 0.003% bromophenol blue), boiled for 5 min before loading onto two 12.5% polyacrylamide gel. One gel was Western blotted and the other Coomassie blue stained to check the protein extraction method.

Western Analysis

Semi-dry western blotting (BioRad) was carried out using a single buffer system, 1X Towbin transfer buffer (25mM Tris, 192mM Glycine, 20% methanol). The membrane was probed with commercially bought antisera according to the manufacturers instructions, using NBT/BCIP to detect the presence alkaline phosphatase.

## ***E.coli*** **expression system (Qiagen)**

*E.coli* maintenance

*E.coli* host strains, M15 and SG13009, containing the repressor plasmid pREP4 were maintained on LB agar (10g/litre tryptone, 5g/litre yeast extract , 10g/litre NaCl and 15g/litre agar) containing kanamycin.

Recombinant clones, *E.coli* containing both the expression (pQE) and the repressor (pREP4) plasmids, were maintained on LB agar containing 25μg/ml Kanamycin and 100μg/ml ampicillin.

ASPV-expression vector constructs

The ASPV coat protein gene was re-amplified from the yeast expression vector using primers (SP70F & R; SP40F & R) designed for direct cloning into the QIAexpress vectors pQE40 and pQE70 (Qiagen).

Expression studies

Small-scale expressions of recombinant clones were carried out following growth on LB medium and its modifications induced with 1mM IPTG over a 4-hour period. No striking differences between the level of expression in different media or *E.coli* host strain were observed

Large-scale expression studies were carried out using LB medium plus antibiotics with the ASPV-pQE70 expression construct in the *E.coli* strain SG13009. Cell lysates were prepared from 200ml of culture harvested (4000 xg for 20 min, 4°C) after 4 hours induction with 1mM IPTG. Cell pellets were resuspended in 4ml lysis buffer containing 1mg/ml lysozyme and incubated on ice for 30min. Cells were sonicated for six 10 second bursts with a 10 second cooling period between each burst. The lysate was centrifuged (10,000 xg for 20 min at 4°) and the supernatent removed for protein purification.

Protein purification

Small-scale production: Proteins were purified under both denaturing and native conditions using Ni-NTA spin columns (Qiagen).

Large scale production: Two methods of protein purification under native conditions were assessed; batch purification under native conditions using Ni-NTA matrix (Qiagen) and HisTrap columns (Pharmacia Biotec). To obtain optimum purity of the ASPV recombinant protein the HisTrap columns were used with a lysis buffer (10mM

Imidazole, 20mM phosphate, 0.5M NaCl). The recombinant protein was eluted with 5 ml of elution buffer (300mM Imidazole, 20mM phosphate, 0.5M NaCl).

Protein Assay

Protein assays were carried out using BSA standards following the Microassay procedure (Bio-Rad).

**Isolation of RNA for *Strawberry crinkle virus* work**

Plant material was collected and frozen in liquid nitrogen. RNA was extracted immediately or leaf material was stored at –80 ˚C. RNA was extracted by one of the methods described below or with the commercial RNeasy Plant Mini Kit (Qiagen Ltd., Crawley, West Sussex). Extreme care was taken not to contaminate the samples with ribonucleases (RNases). Gloves were worn throughout the extraction procedure and changed frequently. Glassware and metal spatulas were baked at 180 ˚C for at least 8 hours. Plastic ware was submerged in a 0.1 % diethyl pyrocarbonate (DEPC) solution and autoclaved at 121˚C for 15 min. To all solutions, except those containing Tris, 0.1% DEPC was added, the solution was shaken vigorously and autoclaved at 121˚C for 15 min. Tris solutions were made up from a uncontaminated pot of Tris crystals with DEPC-treated water. Adjustment of the pH was with the aid of pH measuring strips, pH range 4 to10 (Sigma-Aldrich Ltd., Poole, Dorset). Disposable tips, pipettes and centrifuge tubes were considered to be RNase free.

Martin method

This method for the extraction of RNA from plant material was based on Martin and Northcote (1981). Plant material was ground into a fine powder in liquid nitrogen using mortar and pestle. The powdered leaf was transferred to a 50 ml screw cap tube and taken up in twice its volume RNA extraction buffer (50 mM Tris-HCl, pH 9.0; 150 mM LiCl; 5mM EDTA; 5% lauryl sulphate (SDS)). An equal volume of phenol: chloroform: IAA (25: 24: 1) was added and the sample was mixed by vigorous vortexing for at least 3 min. The phases were separated by centrifugation at 5000 *g* for 10 min. The upper aqueous layer was transferred to a new 50 ml screw cap tube. Phenol: chloroform extraction was repeated once more, followed by a single extraction with an equal volume of chloroform. The aqueous layer was transferred to

a 30 ml Corex tube (Sorvall, Newtown, CT, USA). RNA was precipitated with a third volume of 8 M lithium chloride (LiCl) (Sigma) and incubated at –80 ˚C for at least 1 hour. RNA was spun down by centrifugation at 12,000 *g* at 4 ˚C for 30 min. The pellet was washed twice in 10 ml of 0.15 M NaCl/ 70 % ethanol. The pellet was recovered by centrifugation at 12,000 *g* at 4 ˚C for 15 min. The pellet was air-dried and re-suspended in an appropriate amount of nuclease-free water (Sigma). The quantity and purity of the isolated RNA was determined by absorbence at 320 to 240 nm.

Covey method

RNA was extracted from *P. pubescens* according to the method described by Covey and Hull (1981) with the following alterations: RNA and DNA fractions were not separated but total nucleic acid was precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubated at –80 °C for 1 hour. Nucleic acid was collected by centrifugation at 12,000 *g* at 4 ˚C for 30 min. The pellet was re-suspended in 0.5 ml DNase buffer (50 mM Tris-HCl, pH 7.9, 5mM $MgCl_2$) and incubated with 16.0 µg of RNase-free DNase (Sigma) for 15 min at 37 °C. Enzymes were removed by phenol: chloroform extraction and RNA was precipitated by addition of LiCl to a final concentration of 3M. The reaction was incubated at – 80 ˚C for at least 30 min, then centrifuged at 10,000 *g* for 20 min. The pellet was washed in 70 % ethanol, air dried and re-suspended in an appropriate volume of nuclease-free water.

MacKenzie method

RNA was extracted from tissue high in phenolic components and polysaccharides by an adaptation of the method described by MacKenzie *et al.* (1997). The homogenisation with 20% sarcosyl was omitted. After the RNA was eluted from the columns, it was precipitated with 0.4 volume of 8M LiCl (Sigma). RNA was recovered by centrifugation at 10,000 *g* for 20 min. The resulting pellet was washed in 70% ethanol and air-dried.

The RNA was re-suspended in 25 µl of nuclease-free water (Sigma) and either stored at –80 ºC or used directly in downstream applications.

Isolation of mRNA from total RNA

Poly-adenylated messenger RNA was isolated from total RNA using Dynabeads Oligo(dT) (Dynal, Oslo, Norway) according to the manufacturer's instructions. Messenger RNA was either eluted from the beads, for library construction and hybridisation techniques, or beads were re-suspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) for RT-PCR and related techniques.

**Methods for obtaining *Strawberry crinkle virus* L gene sequence**

Comparison of rhabdovirus sequences, design of degenerate primers and RT-PCR

Degenerate primers were designed to the conserved regions in rhabdovirus large protein gene. Conserved regions were identified through multiple sequence alignment of the amino acid sequences of the large proteins of seven different rhabdoviruses, in four genera, that infect mammals, fish, insects and plants. These were: *Rabies virus* (RV) (Acc. no. AB009663), *Vesicular stomatitis virus* (VSV) (Acc. no. J02428), *Snakehead rhabdovirus* (SHV) (Acc. no. AF147498), *Infectious haematopoietic necrosis virus* (IHNV) (Acc. no. L40883), *Viral haemorrhagic septicaemia virus* (VHSV) (Acc. no. Z93414), *Sonchus yellow net virus* (SYNV) (Acc. no. L32603) and *Rice transitory yellows virus* (RTYV) (Acc. no. AB011257). Multiple sequence alignments were carried out by the Clustal Method using the MegAlign programme of the DNAStar software (Lasergene). Degenerate primers were designed to positive-sense RNA (mRNA or positive-sense genomic intermediate) and primer sequences are given in Table 4.

**Table 4.** Primers for amplification of *Strawberry crinkle virus*

| Target | Primer | Primer sequence | Primer Size |
|---|---|---|---|
| Rhabdovirus L protein | LPROT1 | GAYAAAGCWATATCMMYGACAAGRTCAGAG | 29 |
| | LPROT3 | AAAAATCTKGCYWTRRTCTTYAWYTCTCKYTCYTTGGG | 38 |
| | LPROT5 | CCAKSCYTTTTGYCTWAKRCCTTC | 24 |
| 3' RACE – Poly-A Tail | RACE SYN | $CGGGATCCGTCGACAAGC(T)_{18}$ | 36 |
| 3' RACE – PCR | ADAPTER | CGGGATCCGTCGACAAGC | 18 |
| 3' RACE – SCV L gene | LSP1 | GGATTCAGTGTAGTATCTTCCAGCCAC | 27 |
| | LSP2 | CAGATGGGACCTATGTACCGGACATCC | 27 |

| | | | |
|---|---|---|---|
| Detection – SCV L gene | SCD1FW | ACTGTAATGTCACCAGAGAAG | 21 |
| | SCD1RV | TTCTGACACTAGTAGATCTCC | 21 |
| | SCD2FW | ACTGTAATGTCACCAGAGAAGAGAAGAGG | 29 |
| | SCD5RV | TATACATAGGCTGTTCATGAGG | 22 |
| Strawberry ADH | FVADFW | AACGAAACAATGTCTCTTGG | 20 |
| | FVADRV | TGAAATGGACATAATTCAGGC | 21 |

RT-PCR with degenerate primers was carried out as follows: 1 µg of total RNA, extracted from healthy or SCV-infected *P. pubescens* leaves, was mixed with 20 pmoles of degenerate reverse primer, LPROT3 or LPROT5, and 10 nmoles of each dNTP (Promega). The mixture was heated at 65 ºC for 5 min, then chilled on ice. The cDNA synthesis reaction was carried out in a total volume of 20 µl in 1 x M-MLV buffer (Promega) using 200 U of M-MLV reverse transcriptase (Promega) and 40 U of RNase Inhibitor (LifeTechnologies). The reaction was incubated at 37 ºC for 60 min, followed by 15 min at 70 ˚C to inactivate the reverse transcriptase. The amplification reaction was carried out with 5 µl of the cDNA in a total volume of 50 µl. The reaction mixture contained 1 x PCR buffer (Advanced Biotechnology), 75 nmoles of $MgCl_2$, 20 pmoles forward primer LPROT 1 (Table 4), 20 pmoles reverse primer LPROT 3 or LPROT 5, 10 nmoles of each dNTP and 1 U of Red Hot *Taq* polymerase (Advanced Biotechnology). The PCR was carried out in a Techne Genius thermal cycler and an initial incubation at 94 ºC for 2 min was followed by 5 cycles at 94 ºC for 30 sec, 37 ºC for 30 sec, 72 ºC for 2 min, 35 cycles at 94 ºC for 30 sec, 50 ºC for 30 sec, 72 ºC for 2 min and a final incubation at 72 ºC for 10 min.

Rapid amplification of cDNA ends to extend the L gene sequence

Rapid amplification of cDNA ends (RACE) was used to extend the known sequence of the SCV large protein mRNA towards the 3' end. For 3' RACE, first strand cDNA was synthesised from mRNA extracted from SCV-infected *P. pubescens* leaves using an oligo (dT)-adapter primer, the 3' RACE synthesis primer (see Table 4). The reaction mixture contained 0.5 µg mRNA, 1 x First Strand Buffer (LifeTechnologies), 200 nmoles of DTT, 20 pmoles of primer, 10 nmoles of each dNTP, 40 U of RNase Inhibitor (Helena Bioscience) and 200 U of SuperScript II (LifeTechnologies). The reaction was incubated at 45 ºC for 50 min, then at 75 ºC for 15 min. For PCR amplification, 5 µl of the first strand cDNA was used in total reaction volume of 50

µl. The reaction mixture contained 10 nmoles of each dATP, dCTP, dGTP and dTTP, 20 pmoles of gene specific primer, LSP1 (Table 4), 20 pmoles of adapter primer (Table 4), 0.4 x buffer A (LifeTechnologies), 0.6 x buffer B (Life Technologies), 1 x eLONGase polymerase mix (LifeTechnologies). The PCR was carried out in a Techne Genius thermal cycler, using an initial incubation at 94 ºC for 1 min, followed by 35 cycles at 94 ºC for 30 sec, 60 ºC for 30 sec, 68 ºC for 4 ½ min and a final incubation at 72 ºC for 10 min. A second, hemi-nested PCR was carried out with 1 µl of 10 x diluted PCR product in the same manner as the first PCR. The primers used for the hemi-nested PCR were the adapter primer and a second gene specific primer, LSP2 (Table 4).

**RT-PCR for SCV detection in extracts from Physalis and strawberry**

SCV mRNA and genomic viral RNA were detected by two-step RT-PCR or nested RT-PCR as described below. For the two-step RT-PCR, the cDNA synthesis mixture contained 100 ng RNA, extracted from healthy or infected strawberry or *P. pubescens*, 1 x M-MLV buffer (Promega), 10 nmoles of each dNTP, 20 pmoles of reverse primer, SCD1RV or SCD5RV (see Table 4), 40 U of RNase Inhibitor (LifeTechnologies) and 200 U of M-MLV-RT (Promega). The first strand cDNA synthesis reaction was incubated at 37 ºC for 60 min. The PCR reaction mixture contained 5 µl of first strand cDNA, 1 x PCR buffer (Sigma), 10 nmoles of each dNTP (Promega), 20 pmoles of each forward (SCD1FW) and reverse primer (SCD1RV or SCD5RV), 2.5 U of *Taq* polymerase (Sigma) in a total volume of 50 µl. Amplification was carried out in a thermal cycler (Techne Genius) with and initial incubation at 94 ºC for 2 min, followed by 35 cycles at 94 ºC for 30 sec, 54 ºC for 30 sec, 72 ºC for 1 min and terminated with an incubation at 72 ºC for 10 min. For the detection of the viral negative sense genome, the forward primer was used to synthesise first strand cDNA. The conditions for the first strand cDNA synthesis and the PCR amplification were the same as for the amplification from positive-sense virus RNA.

Nested RT-PCR was carried out on RNA extracted from infected strawberry plants. First strand cDNA was synthesised as described above, from the SCD5RV primer. The first amplification step was carried out with primers SCD1FW and SCD5RV. The PCR amplification was carried out in a thermal cycler using an initial incubation at

94 ˚C for 2 min, followed by 30 cycles at 94 ˚C for 30 sec, 54 ˚C for 30 sec, 72 ˚C for 1 min and terminated by 72 ˚C for 10 min. Nested PCR was carried out using the primer combinations SCD2FW + SCD1RV. Nested PCR used 1.0 µl of the first amplification reaction in a total volume of 50 µl. The PCR conditions were the same as for the first amplification reaction.

**Table 5.** Expected product sizes for SCV L gene primers

| Forward primer | Reverse primer | Product bp |
|---|---|---|
| SCD1FW | SCD1RV | 574 |
| SCD1FW | SCD5RV | 1050 |
| SCD2FW | SCD1RV | 574 |

The quality of the RNA extraction from strawberry was tested by RT-PCR using primers designed to the strawberry alcohol dehydrogenase gene. First strand cDNA synthesis was carried out as described above, using primer FvAdRV (Table 4). Amplification by PCR was carried out as described for RT-PCR from total RNA.

Single-tube RT-PCR was carried out with total RNA extracted from healthy and infected *P. pubescens* and strawberry leaves. RNA (100 ng) was combined with 20 pmoles of forward primer, 20 pmoles of reverse primer and 20 nmoles of each dNTP (Promega) and heated at 65 ˚C for 10 min, then chilled on ice. To this mixture 5.0 µl 10 x PCR-buffer (Amersham), 1.5 µl 50 mM $MgCl_2$, 40 U of RNasin RNase inhibitor (LifeTechnologies), 200 U M-MLV reverse transcriptase, 0.5 U of *Taq* polymerase (Amersham) and water to a total volume of 50 µl were added. Amplification was carried out in a thermal cycler (Techne Genius) using an incubation at 37 ˚C for 60 min (reverse-transcription), 70 ˚C for 5 min, 94˚C for 2 min followed by 30 cycles at 94 ˚C for 30 sec, 54 ˚C for 30 sec, 72 ˚C for 2 min, and a final incubation at 72 ˚C for 10 min. For the detection of SCV from RNA extracted from strawberry plants, a second, nested PCR was carried out in the same manner as described for nested PCR.

Analysis of amplified product

Aliquots (8 µl) of the RT-PCR or nested RT-PCR products were analysed by electrophoresis on a 1.5% agarose gel in 0.5 x TBE buffer (45 mM Tris-borate, 1 mM

EDTA), containing 0.5 g/ml ethidium bromide at 150 V for 45 min. The amplified DNA fragments were visualised using an UV transilluminator and photographed with a GelDoc 1000 imaging system (BioRad). Sizes of the PCR products were determined by comparison with 1Kb DNA molecular weight marker (LifeTechnologies)

Cloning and sequencing amplified products

PCR products were cloned into the pTOPO vector (Invitrogen) according to the manufacturer's instructions. Clones were tested for inserts by blue/white colony screening and colony PCR. Plasmid inserts were sequenced by SequiServe (Germany). Sequences were analysed through similarity search by basic local alignment search tool (BLAST) (Altschul et al., 1997) and multiple alignments with other rhabdovirus proteins by the Clustal Method using DNAStar software (Lasergene).

## RESULTS

### Objective 1 – Devise PCR-based assay for *Apple stem pitting virus* (ASPV)

Designing and testing primers for a PCR-based assay for ASPV

Apple stem pitting virus infection was confirmed by ISEM in bark extracts from 25 apple trees and three pears, affected by ASPV or related diseases, in December 1998. The effectiveness of an IC-RT-PCR test for ASPV from bark samples of these trees (protocol and primers 1 & 3, Schwartz and Jelkmann, 1997) was assessed. ASPV was detected in only 23 of these trees (21 apples, two pears) by PCR using these primers (ASPV1 and 3) and there were questionable product bands in the two replicates of another sample. A new primer (ASPV5) was therefore designed to a well-conserved region upstream of primer 3, using the sequence information of Schwartz and Jelkmann (1997), and IC-RT-PCR was repeated in February 1999. These tests included samples from an extra seven pear trees. ASPV was confirmed by ISEM in these trees, in all the apples and in one of the two pears tested previously. Using primers ASPV1 and ASPV5, clear product bands were visible with the sample that gave a doubtful result with primers ASPV1 and ASPV3 in December. A further six samples that could not be amplified by those primers were successfully amplified by the new primer pair. However, samples from two apples and seven pear trees could not be amplified by either primer pair, including the stony pit-infected pear tree 7/40. Tests carried out in May (using leaf material) showed little difference in the ability of the 2 primer pairs to amplify these ASPV isolates (Table 6)

A number of isolates that were not or poorly detected by the primers ASPV1 and ASPV3 have been sequenced. Although there are sequence differences in these ASPV isolates, the region to which primer ASPV3 was designed is no more varied than ASPV1 and does not really explain why the ASPV5 and ASPV1 combination is sometimes more efficient at amplifying ASPV.

**Table 6.** Number of samples in which apple stem pitting virus was detected in extracts from apple and pear bark by PCR in December 1998 and February 1999 and in leaf extracts in May 1999.

| Isolates detected by: | December | February | May |
|---|---|---|---|
| Primers 1 + 3 | 23 | 20 | 29 |
| Primers 1 + 5 | - | 27 | 27 |
| 1 + 3 and not 1 + 5 | - | 0 | 3 (pear) |
| 1 + 5 and not 1 + 3 | - | 7 (apple) | 1 (apple) |
| ISEM | 28 | 35 | - |
| No. of isolates tested | 29 | 36 | 36 |

Failure of PCR to detect ASPV in all these trees was probably not due to a lack of sensitivity. Tests on two trees taken at random, 1 apple and 1 pear, gave positive results with extract dilutions of $10^{-4}$ and PCR is more sensitive than ISEM. Despite the good performance of the IC-RT-PCR test there is obviously still a requirement for more genome information to be able to detect all the isolates of ASPV that we have in woody plants. We attempted to obtain such information from isolate 7/40 as it was not possible to detect ASPV by PCR using any of the primers either in samples from the pear tree 7/40, or in samples from this isolate that had been transferred to *N. occidentalis*. This isolate is of particular interest as tree 7/40 has symptoms of pear stony pit for which ASPV is thought to be the cause.

Obtaining nucleotide sequence data for ASPV isolate 7/40

ASPV isolate 7/40 shows typical symptoms in the herbaceous host *Nicotiana occidentalis* and there was no indication of contamination by another virus because inoculation to other herbaceous plants (*Chenopodium quinoa, C.amaranticolor, Nicotiana clevlandi,* and *Cucumis sativus*) failed to produce symptoms. Virus particles of 7/40 were similar in appearance to other ASPV isolates in ISEM and particles were heavily decorated with ASPV antiserum (Yanase). The same antiserum was used in western blot analysis. A faint cross reaction was observed to a protein

from 7/40-infected *N. occidentalis* that migrated similarly to the standard ASPV isolate VYA, indicating that the coat protein of 7/40 is of the correct size for ASPV.

*cDNA library experiments*

Attempts were made to obtain cDNA clones of ASPV from viral RNA in two ways. In one, virus was trapped on plates coated with ASPV (Yanase) antiserum, to concentrate the virus. In the other, virus was concentrated centrifugally and reverse transcription primed with oligodT. Concentration of the cDNA was attempted by PCR with random primers and oligodT. None of the resulting clones in pUC18 proved to be virus related.

*PCR experiments*

In further PCR experiments, various sources of template were used. cDNA was made from immunocaptured ASPV isolates 7/40 and VYA and also from total RNA extracted from VYA and 7/40-infected *N.occidentalis* leaves (RNeasy plant kit, Qiagen). An oligodT-anchor primer was used in the reverse transcription procedure and PCR carried out using a variety of primers (Table 7). In each case a product of the expected size was amplified with VYA cDNA but nothing was seen in the 7/40 samples. There was the possibility that 7/40 did not have a polyA tail, but amplification using primers ASPV5 & 1 and ASPVF1& ASPVR5 from cDNA primed with random primers also failed to produce products for 7/40.

**Table 7.** Primer combinations used for attempts to amplify ASPV isolates 7/40 and VYA

| Forward primer | Reverse primer | Expected product | Region |
|---|---|---|---|
| ASPV5 | ASPV1 | 0.38 kb | coat protein |
| ASPV3 | ASPV1 | 0.26 kb | coat protein |
| ASPV5 | Anchor | 0.43 kb | coat protein |
| ASPV3 | Anchor | 0.31 kb | coat protein |
| ASPVFB | ASPV1 | 1.33 kb | coat protein |
| ASPVFB | Anchor | 1.38 kb | coat protein |
| ASPVFB | SPR | 1.24 kb | coat protein |
| ASPVF1 | ASPVR5 | 0.29 kb | RNAdependent RNA polymerase |

*Cloning from dsRNA*

An alternative route to provide viral RNA template for cDNA synthesis is to use purified double stranded RNA (dsRNA) replication intermediates isolated from infected *N.occidentalis* (Jelkmann *et al.*, 1992). Double stranded RNA is extremely stable and has proved to be the best target for sensitive, reproducible PCR detection of ASGV (Kummert *et al.*, 1998).

DsRNA was isolated from healthy and 7/40 infected *N.occidentalis* plants (Fig. 1). No dsRNA was isolated from healthy material, so *N.occidentalis* does not contain non-viral dsRNAs. This meant that the dsRNA observed from 7/40 infected plants is of viral origin. Very little ASPV dsRNA was isolated from infected leaf material and the optimum time for harvesting for maximum dsRNA presence is still unknown. Dodds (1993) observed that the host and season have effects on dsRNA quantity and quality and that there is a poor correlation between relative dsRNA levels and relative virus concentration.

The small quantity of dsRNA (200ng) isolated from 7/40 infected herbaceous host plants is not sufficient for standard cloning techniques which have been reported (Asamizu *et al.*, 1985; Antoniw *et al.*, 1986). However, sequence data has been obtained from several nanograms of dsRNA for two viruses that infect cherry (Jelkmann 1995; Keim-Konrad and Jelkmann 1996) but this technique involves the use of a very toxic compound, methyl mercuric hydroxide. Heat denaturation and reverse transcription of dsRNA is reported to be relatively inefficient (Jelkmann *et al*., 1989) but was successful in our hands for construction of a PCR amplifiable cDNA library immobilised on oligo$(dT)_{25}$ beads (Dynal). PCR amplification was necessary due to the small amount of first strand cDNA produced so several forward primers were tried in combination with oligodT-anchor. A product approximately 440bp was obtained with the primer combination ASPV5- adapter (lane 3, fig. 2). This PCR product was cloned and sequence analysis confirmed the viral origin (fig. 3). The sequence of 7/40 does not fully explain why this isolate was not detected by the IC/RT-PCR test, although it must be noted that there are several mismatches within the 7/40 sequence for the diagnostic primers.

It is possible that the lack of detection of 7/40 in previous PCR experiments was due to improper annealing of the reverse primer ASPV1. It is possible to amplify 7/40 using ASPV5 and oligo(dT)-adapter primers from dsRNA so PCR amplification experiments were carried out on total RNA extracted from healthy, and both VYA and 7/40 ASPV isolates in *N.occidentalis*. As for the diagnostic IC/RT-PCR test the cDNA was primed using an oligo(dT)-anchor primer but amplified using the primer combination ASPV5 and adapter. A product of the expected size was amplified from VYA infected plant material but nothing was amplified from the healthy or 7/40 infected plants (data not shown). This is surprising because we have used this primer combination for successful amplification from 7/40 dsRNA.

Discussion and proposed future work

The immunocapture PCR procedure using primers ASPV1 and ASPV5 was successful in amplifying many but not all isolates of ASPV. Sequence was obtained of an isolate (7/40) from pear that was consistently not detected in either the immunocapture PCR or in PCR from total RNA extracts from pear and from *N. occidentalis*. The sequence indicated that differences at the ASPV1 site might partially account for the failure to amplify this isolate, however, further sequence is needed for a detailed comparison of 7/40 with other isolates of ASPV.

The novel technique that was used to obtain the cDNA library from very small amounts of dsRNA is promising, particularly as it used heat rather than the extremely toxic methyl mercuric hydroxide to denature the dsRNA.

Antiserum is essential to the immunocapture PCR protocol and is unavailable commercially. It should be possible to utilise the protein expressed in objective 3 in *E. coli* for this purpose. This will probably not be satisfactory for ELISA but the results with ISEM emphasised the importance of considering serology as a test for this virus and indicated that it may be more successful than a nucleic acid based method because of the variability of the genome.

A small amount of development of the results obtained here should further improve the PCR assay for ASPV and should also facilitate investigation of the relationship between pear stony pit and ASPV.

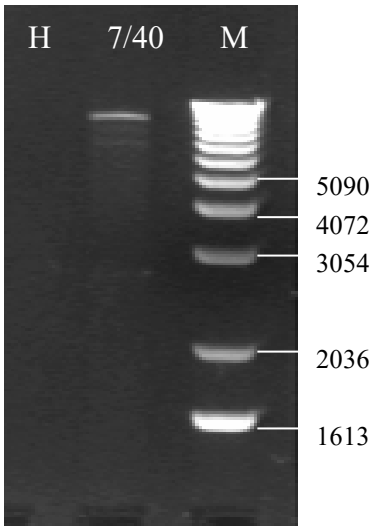


**Figure 1**. dsRNA isolated from healthy (H) and 7/40 infected *N.occidentalis* (7/40) loaded onto a 0.8% agarose gel along with 1Kb (M) markers (Gibco BRL).

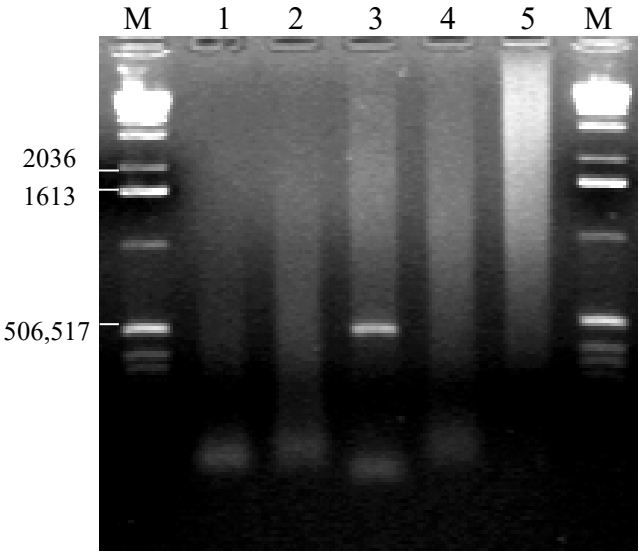


**Figure 2.** PCR amplification products generated from the immoblised 7/40 cDNA library using the oligo(dT)-adapter and various ASPV forward primers in lanes:

(1) ASPV 7956

(2) ASPV F1

(3) ASPV 5

(4) ASPV 3

(5) random primers

**Figure 3.** The sequence of the ASPV (isolate 7/40) PCR product with the positions and sequence of the diagnostic primers ASPV5, ASPV3 and ASPV1 (in lower case).

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ASPV5
atgtctggaa cctcatgctg c
ATGTCTGGAA CCTCATGCTG CAGACTCAGA GCCCCCCAGC GAACTGGGTT

GGTAAAGAAT TCAAATTTGA AACTAGGTAT GCAGCTTTCG ACTTCTTCTT

               ASPV3  ctcttgaacc agctgatggc
CGGAGTTGAA AGCTCTGCAG CACTAGAACC TGCTGATGGG CTCGTGAGGC

TTCCCACCCA GGCTGAGAGA GTTGCAAACG CCACAAGCAA AGAGATTCAG

ATGTATCGAA TCCGCTCTAT GGAAGGCACA CAAGCTGTCA ACTTCGGGGA

AGTCACAGGT GGAAAAGTGG GGCCAAAGCC GGTTTTATCC ATCAGGAAGT

GATTAATTTT GTCTGTTCAC ATCCTCCCTA TCTTAATATT TATGCTTTTG

AATAAAGTTG ATTCCGACCT AATTGGAACG GCTAAGTGTG TTTTAATTTT
                aacct aaccggggcg gctat  ASPV1

CATGCTTTAG CTTATTTTTG TTTTAACTAG ATTTTCAAAA
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