PURIFICATION AND SEROLOGY OF APPLE CHLOROTIC LEAF SPOT VIRUS ISOLATED FROM PRUNUS GLANDULOSA

By

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Department of Plant Science
The University of British Columbia
Vancouver, Canada
Date April 17, 1990
Abstract

Two isolates (A and B) of apple chlorotic leafspot virus (ACLSV) associated with line pattern symptoms in Prunus glandulosa were isolated in Chenopodium quinoa and purified by a simple method using bentonite clarification, polyethylene glycol precipitation, and caesium sulphate isopycnic centrifugation. Symptoms on herbaceous hosts were as previously reported for ACLSV. No symptoms were observed on a range of ACLSV woody host indicators.

Isolates A and B had a coat protein MW of 26 and 24.5 kilodaltons respectively, ssRNA of MW $2.6 \times 10^6$ daltons, and dsRNA of MW $5.6 \times 10^6$, $4.9 \times 10^6$, and $4.5 \times 10^6$ daltons. The particle widths were 12 nm and lengths were 782 nm (A) and 732 nm (B). Buoyant density in caesium sulphate was 1.27 g/cc$^3$. In thin sections of C. quinoa, flexuous rods were seen in the cytoplasm and nucleus of young sieve-tube members. Polyclonal antisera prepared against the A and B isolates had high background reactions and required cross-adsorption with host sap. Three monoclonal antibodies (MAB) against isolate A detected Prunus strains while a fourth detected both Malus and Prunus strains in C. quinoa with low background reactions. The broad spectrum MAB could not be used as a trapping antibody but could be directly conjugated with alkaline phosphatase or used in an indirect triple-antibody sandwich ELISA.

Tobacco mosaic virus (TMV) recovered from P. glandulosa was identical in serological and physical properties to the TMV-U1 type strain.
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Chapter 1 - General Introduction

Virus diseases of tree fruits were recognized as early as the eighteenth century. In France, sour cherry yellows was described in 1768 and peach yellows was discovered on a Philadelphia farm in 1791 (60). In this century much has been learned about the etiology, epiphytology, and physical characteristics of tree fruit viruses. Some diseases previously attributed to viruses were found to be caused by mycoplasmas, rickettsia or viroids. The viruses responsible for many diseases were isolated, purified, and their physical and serological properties established. In spite of these advances the etiological agents for many virus-like diseases of tree fruit remain unknown and the diseases are detected only by their graft-transmission to susceptible indicators.

In contrast to stone fruit viruses, many pome fruit latent viruses cause no symptoms in the common fruiting varieties. Examples are apple chlorotic leafspot (ACLSV), apple stem grooving and apple stem pitting viruses. These viruses may reduce growth and vigour depending on variety and rootstock (8). Although some evidence exists for natural spread of these viruses (65,71) they are spread mainly by graft transmission and their importance is due to their widespread distribution in clonally propagated stock.

ACLSV occurs in more tree fruit species than any other virus and causes a wide range of diseases ranging from symptomless
infections to severe stock/scion incompatibility and fruit symptoms. In apple it is considered latent in most fruiting cultivars, however, some crabapples are sensitive and are used as virus indicators (54, 59). In Japan the clonally propagated Maruba-kaido (*Malus prunifolia* Borkh. var *rinkii* Rehd.) rootstock is widely used. ACLSV infected cultivars on this rootstock show a general tree decline with xylem pitting and phloem necrosis in the rootstock (58). Two diseases causing fruit symptoms in apple, ringspot and russet ring have been associated with ACLSV, the former in combination with apple stem pitting (60, 75).

ACLSV causes pear ring pattern mosaic (14). Symptoms consist of light green rings and line patterns and, in sensitive cultivars, the fruit shows sunken greenish rings. Incompatibility between infected pear cultivars and quince rootstocks, particularly quince E, can occur (76).

The diseases caused by ACLSV in stone fruits are more severe than in pome fruits and are more prevalent in Europe than North America. Peach dark green sunken mottle virus produces sunken spots and wavy lines on peach leaves (12) and can cause scion/stock incompatibilities in some cultivars (53). Plum bark split virus causes sunken dark areas on the stems which eventually crack and split. The trees become stunted with a proliferation of suckers and are uneconomical (21). Some ACLSV infected plum cultivars show chlorotic rings and lines known as pseudopox symptoms (17). In apricots ACLSV causes incompatibility with some seedling rootstocks (55) and pseudopox, where the fruit becomes
deformed with a sunken dark necrosis (64). ACLSV is latent in most cherry cultivars (30) but some are sensitive and sunken necrotic spots develop on the fruits (17). There is one report of the virus infecting Prunus glandulosa Thunb. 'sinensis' and causing pinpoint chlorotic lesions and necrotic leaf tips (11). Other natural host plants of ACLSV are Amelanchier canadensis Med., Chaenomeles spp., Crataegus monogyna Jacq., C. oxyacantha L. emend Jacq. and Mespilus germanica L. (74).

Control of ACLSV is based on the use of healthy propagative material, both scion and rootstock. The virus is detected on herbaceous and woody host indicators and by serological techniques, however, one method may not be effective for screening the various biological and serological strains. ACLSV is relatively easy to eliminate by conventional hot air heat therapy (79) and can be eliminated by foliar application of the anti-viral drug ribavirin (36) or by incorporation of the drug in shoot culture media (37).

The purpose of this study was to identify the sap-transmissible virus isolated from dwarf flowering almond (P. glandulosa 'sinensis'), to develop a suitable serological detection technique, and to investigate the biological and serological properties of this virus. Preliminary evidence from host range studies and particle morphology suggested the virus was a closterovirus, probably apple chlorotic leafspot virus.
Chapter 2 - Biological Detection

Introduction

Apple chlorotic leafspot (ACLSV) can be detected on both herbaceous and woody indicator plants. The virus was first detected from apple with the indicators *Malus platycarpa* Rehd. (54) and the Russian rootstock clone R12740-7A (58). In *Prunus* the recommended indicator is GF 305 seedlings (*Prunus persica* (L.) Batsch) but others such as *Prunus tomentosa* Thunb. and *Prunus domestica* L. 'P 707' have been used. Pear ring pattern mosaic is detected on quince C7/1 (*Cydonia oblonga* Miller), or *Pyrus communis* L. 'Hardy' and 'Nouveau Poiteau'. Many authors have pointed out the difficulties in detecting the various ACLSV strains with one indicator. Marenaud et al. (56) inoculated 9 ACLSV strains from *Malus, Prunus* and *Pyrus* to 11 woody indicators and found, with one exception, that the *Prunus* strains were not detected on R12740-7A. Only *M. platycarpa* detected all strains while *P. tomentosa* and quince C7/1 detected all but one strain. Slightly different results were obtained by Paunovic (63) where *M. platycarpa* detected only 4 of 21 isolates whereas peach GF 305 detected all isolates. Dunez et al. (21) reported that the reactions of the isolates from *Malus* and *Prunus* were generally different but a few exceptions were found in each genus with some apple isolates showing symptoms on *Prunus* indicators and vice versa. Detection on woody indicators is further complicated by the uneven distribution of the virus in the host. Generally, buds
near the proximal end of the shoot are more likely to be infected while the tip buds are often healthy (26).

ACLSV is sap transmissible to herbaceous plants (13). Chenopodium quinoa Willd. is the most reliable indicator plant but other species such as Chenopodium amaranticolor Coste & Reyn. and Chenopodium murale L. are also infected. Some ACLSV isolates described as "super-latent" by Gilmer and Mink (31) are detected by C. quinoa but not the standard apple indicators. The time of year and tissue type influence the reliability of ACLSV herbaceous testing as results are variable later in the growing season. Blossoms and young leaves are the best inoculum source (67,51). In C. quinoa, infectivity is decreased by an inhibitory substance, probably a ribonuclease, which is inactivated by bentonite or the concentration reduced by dilution of the infected sap (68). Just as results are variable on woody indicators with ACLSV isolates from different species, the same is true on herbaceous hosts. Chairez and Lister (9) and Paunovic (63) both found differences in symptom type and severity on C. quinoa with isolates from Malus and Prunus.

Materials and Methods
Virus Isolates.

Two sources of dwarf flowering almond (Prunus glandulosa Thunb. 'sinensis’) were obtained from the Agriculture Canada, Plant Quarantine Station, Sidney, British Columbia. Accession Q1114-01 originated from Ontario, Canada and accession Q1292-01
was from Washington State, U.S.A. These are referred to as isolate A and isolate B respectively. Both sources were maintained as potted plants in a glasshouse at 20-25 °C during experimentation. Other isolates of ACLSV used for characterization of the monoclonal antibodies were propagated in *C. quinoa* and included the following: American Type Culture Collection (ATCC) PV 60 from *Malus* (51); P 205 isolated from *Malus* in Japan (81); S1 isolated from *Prunus avium* L. by A. J. Hansen, Summerland, B.C.; UBC isolated from *Malus sylvestris* Mill. 'Spartan' at Vancouver, B.C.; and Q1000-04 isolated from *M. sylvestris* 'Katja' which originated in Sweden.

Transmission

After initial local lesion tests on *Chenopodium quinoa* to determine the optimum buffer for rub inoculation, infected tissue was routinely ground in 0.01 M Tris, pH 8.5, with 0.01 M MgSO₄ (TM buffer) and rubbed on test plants previously dusted with carborundum. For isolations from woody host leaves, flowers and roots, 1.0% nicotine was added to the buffer and the plants were rinsed with water after being inoculated. Both A and B isolates were passed twice through single local lesions to reduce the chance of a mixed infection. Host range studies were done using infected *C. quinoa* sap and purified virus. All test plants were back inoculated to *C. quinoa* and assayed by ELISA to detect symptomless infections. The following herbaceous plants were tested with the A and B isolates: *Beta vulgaris* L. 'Detroit Dark

The effects of the pH of the grinding buffer on transmission was investigated using TM buffer at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 and inoculating C. quinoa immediately and 24 hr later. To test the effects of dilution on symptom expression in C. quinoa, isolate A infected tissue was ground 1:3 (w/v) in TM buffer and serially diluted in five two-fold steps to 1:96. The six samples were inoculated to C. quinoa.

To determine symptoms on woody host indicators, buds from each isolate were T-budded onto field indicators (two per test). Pome fruit indicators had both the indicator and inoculum buds simultaneously applied to the rootstock while Prunus indicator buds were applied the previous summer. Also, some potted rootstocks were double budded with the indicator and the virus infected source in August, moved to 4 C in October and brought into a 20 C glasshouse in January for symptom expression. The A and B isolates were budded to the following woody indicators: Prunus avium 'Bing', 'Sam' (field); P. serrulata Lindley
'Kwanzan' (field); *P. persica* 'Halford' (field); *P. persica* 'Siberian C' seedlings (greenhouse); *P. armeniaca* L. 'Tilton' (field); *Pyronia veitchii* (Trab.) Guillaum (greenhouse); *Malus platycarpa* (greenhouse); *M. sylvestris* 'Spy 227', 'R12740-7A' (greenhouse).

**Results**

The host ranges for the A and B isolates were narrow, infecting only three of twenty species tested (Table 1). No cases were found of symptomless infections. The A and B isolates produced local lesions on *C. quinoa* after 2 weeks and systemic symptoms after 2-4 weeks (Fig. 1 B). If plants were inoculated at a young stage (four leaves), systemic symptoms appeared sooner than when older plants were inoculated. In *C. amaranticolor* the B isolate produced only systemic symptoms and no local lesions whereas the A isolate showed only local lesions. Back inoculation of symptomless *C. amaranticolor* leaves to *C. quinoa* gave negative results. The A isolate was unique in this respect as the five other isolates also became systemic in *C. amaranticolor*. Transmissions from *P. glandulosa* to *C. quinoa* could be made with leaves, flowers or roots.

Both *P. glandulosa* mother plants showed a chlorotic line pattern and vein break symptom which increased in intensity as the season progressed (Fig. 1 A). Since TMV was recovered from only one of these sources, it is assumed that the symptoms were not associated with TMV but could be attributed to ACLSV. Without
Table 1. The herbaceous host range of isolates A and B.

<table>
<thead>
<tr>
<th>Species</th>
<th>Symptoms produced by isolate^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td><strong>Beta vulgaris</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Chenopodium amaranticolor</strong></td>
<td>nec ll</td>
</tr>
<tr>
<td></td>
<td>dist, lp</td>
</tr>
<tr>
<td><strong>C. murale</strong></td>
<td>chl ll</td>
</tr>
<tr>
<td></td>
<td>sys les</td>
</tr>
<tr>
<td><strong>C. quinoa</strong></td>
<td>nec ll</td>
</tr>
<tr>
<td></td>
<td>sys les</td>
</tr>
<tr>
<td></td>
<td>mo, lp</td>
</tr>
<tr>
<td><strong>Cucumis sativa</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Datura stramonium</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Gomphrena globosa</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Lactuca sativa</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Nicotiana benthamniana</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. clevelandii</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. glutinosa</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. occidentalis</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. rustica</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. tabacum 'Harrownova'</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. tabacum 'Samsun'</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>N. tabacum 'Xanthi'</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Petunia 'Coral Satin'</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Phaseolus vulgaris 'Black Turtle'</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Vigna unguiculata</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Zinnia elegans</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

^aKey: ll = local lesions; sys = systemic; les = lesions; lp = line pattern; dist = distortion; nec = necrotic; mo = mottle; chl = chlorotic; 0 = no infection
Fig. 1. Symptoms associated with apple chlorotic leafspot virus (isolate B) in *Prunus glandulosa* (A) and *Chenopodium quinoa* (B).
confirmation by Koch's postulates these symptoms must be described as ACLSV associated.

Various grinding buffer additives affected the number of local lesions produced during rub transmission tests (Table 2). The greatest number of lesions occurred with additive C (0.01M MgCl$_2$). Without magnesium there was a complete loss of infectivity after 24 hr. Over the range of pH 6.0 to 8.5 there was no difference in the severity or amount of systemic symptoms produced in C. quinoa after rub inoculation using TM buffer. Likewise, dilution of infected tissue ground in TM buffer from 1:3 to 1:96 had no effect on systemic symptoms in C. quinoa.

After inoculation with isolates A and B, no symptoms were found in any of the greenhouse indicators and after 1 year no symptoms were expressed in the field indicators. ACLSV clones from apple and pear used as positive checks produced typical symptoms on P. veitchii, M. platycarpa, Spy 227, and R12740-7A but were not used on the Prunus indicators. Positive checks for other viruses normally detected by the Prunus indicators induced typical symptoms in the appropriate hosts.
Table 2. The effects of buffer additives on *P. glandulosa* isolate B local lesion number in *Chenopodium quinoa*.

<table>
<thead>
<tr>
<th>Additive&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Inoculated immediately</th>
<th>Inoculated after 24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>*&lt;sup&gt;d&lt;/sup&gt;</td>
<td>*&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>76</td>
<td>28</td>
</tr>
<tr>
<td>D</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>57</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tissue was ground 1:3 (w/v) with the appropriate additive, stirred 1 hr at 4°C, and clarified with the addition of bentonite followed by a low speed centrifugation.

<sup>b</sup>Average number of lesions per leaf (12 leaves)

<sup>c</sup>All buffers made with 0.01M Tris pH 8.5

- A = no additives
- B = 0.01M MgCl₂, 0.15% sodium thioglycollate, 0.1% pectinase, 3% Triton X-100.
- C = 0.01M MgCl₂.
- D = 0.2% 3-3′-diaminodipropylamine. (DD)
- E = 0.01M MgCl₂, 0.1% pectinase.

<sup>d</sup>Leaves damaged by buffer to extent that lesions could not be counted.
Discussion

The herbaceous host range of the ACLSV from *P. glandulosa* appears to be narrower than that reported from other woody plants. A number of hosts reported by other authors such as *Beta vulgaris, Datura stramonium, Phaseolus vulgaris, Petunia hybrida,* and *Nicotiana tabacum* (51, 67) showed no symptoms when inoculated with the A and B isolates. These differences do not appear to be unique to *Prunus* strains because the strains used for host range studies by Lister et al. (51) and by Saksena and Mink (67) both originated from apple yet differed in their host range and symptomology. The *P. glandulosa* isolate host ranges were identical using purified virus or infected *C. quinoa* as inoculum so the effects of an inhibitor substance from *C. quinoa* did not influence infectivity.

The A and B isolates produced slightly different symptoms on *C. amaranticolor* and *C. murale* (Table 1). When the virus was first isolated from the woody host and present in low concentration, only local lesions would appear until after a few passages through *C. quinoa*. This was not the case for the A isolate, as repeated inoculations of *C. amaranticolor* with purified virus or infected *C. quinoa* never produced any systemic symptoms and back inoculations of these symptomless leaves to *C. quinoa* were negative. This lack of systemic invasion is in contrast to the results reported in the literature and the results of the other ACLSV isolates used in this study, all of which produced systemic symptoms in *C. amaranticolor*. The age of
inoculated *C. quinoa* affected the type and severity of symptoms expressed. This is explained in terms of the long distance movement of viruses through the phloem tissue in association with metabolites. Samuel (70) showed that TMV spread from the inoculated leaf, first to the roots, then the young leaves and meristem, and finally the older leaves. Therefore the amount of time for the virus to move systemically in a young plant and the number of symptomless leaves between the inoculation point and the growing tip is less than in an older plant.

Factors which influence the choice of extraction buffer for purification are preservation of particle integrity and infectivity. Magnesium best preserved infectivity over a 24 hr period while pectinase, which was added to aid in the release of particles from the phloem tissue, had a negative effect on infectivity (Table 2). Divalent cations are required by viruses such as southern bean mosaic for stabilizing protein-protein interactions (39,40) and magnesium was also essential for stabilizing ACLSV during purification (52). The polyamine DD, which has been recommended as a stabilizing agent (20) was not as effective in stabilizing the virus as was magnesium.

The results obtained from woody indicators were unexpected because none of them showed symptoms. Besides the one report of a "super-latent" strain which did not show on woody indicators (31), all other ACLSV isolates have produced symptoms on at least one indicator cultivar. In this study two important ACLSV *Prunus* indicators were not available, namely GF 305 and *P. tomentosa,*
which may have detected the *P. glandulosa* isolates. *P. glandulosa* is usually propagated by cuttings rather than budding on understock, although it is compatible with peach and some plum rootstocks. This fact together with the small diameter of the shoots may explain the lack of transmission to the woody indicators or the *P. glandulosa* isolates may simply have a very narrow host range.
Chapter 3 - Virus Purification

Introduction

ACLSV has been purified by a number of workers (51, 69, 52, 20, 4, 49, 50). All of the methods have in common a clarification step using bentonite followed by polyethylene glycol (PEG) precipitation and/or high-speed centrifugation, then rate zonal density-gradient centrifugation. The effect of bentonite on viruses and plant constituents was determined by Dunn and Hitchborn (22). Ribosomes, 18 S protein, green pigments, and some viruses were absorbed by bentonite while other viruses were not absorbed. ACLSV is very labile and early purification attempts resulted in high losses due to particle instability and degradation. Lister and Hadidi (52) showed these losses can be counteracted by using magnesium in the extraction buffer to stabilize the particle, probably through increased protein-protein interaction and by inactivation of ribonuclease activity with bentonite. Chairez and Lister (9) compared strains of ACLSV from Malus and Prunus and found the yield from the Prunus strains was one-fifth that of the apple strains and was not significantly improved with magnesium in the extraction buffer. For the plum bark split strain, Dunez et al. (20) found that a high pH (9.0-9.5) extraction buffer with 0.2% 3,3'-diaminodipropylamine (DD) was needed to prevent particle aggregation and instability. This strain was less stable than ones from apple and gave poor yields when purified by the method of Lister and Hadidi. A modification was used by Bar-Joseph et al. (4) whereby virus purified by the
usual method was run on a CsSO₄ isopycnic gradient. In CsSO₄ gradients the virus is stable and does not degrade as it does in CsCl. Another variation was used by Legrand and Verhoyen (49). They helped preserve particle integrity with 0.25% formaldehyde and used a sucrose density gradient superimposed on a reverse PEG gradient to separate the virus. A purification method which did not use bentonite for clarification was described by Thomas (72) using diethyl ether and carbon tetrachloride which gave a high yield (1 mg/100 g tissue) and A₂₆₀/₂₈₀ ratio of 1.72.

A point stressed by many authors is the variable amount of bentonite needed for clarification. In the above mentioned references the quantity varies and some authors use a single dose while others prefer multiple doses with smaller volumes. The age and condition of the plant host affects the amount of bentonite required and excess bentonite eliminates infectivity (51).

An unusual property of ACLSV is the abnormally high A₂₆₀/₂₈₀ ratio (1.7 - 1.8) caused by a lack of aromatic amino acids which absorb in the 280 nm range (4). Factors which can lower the ratio are contaminating proteins and particle breakage.

Another property of ACLSV is the difficulty in separating the virus from host material. Lister et al. (51) found two zones of host origin in their density gradient tubes. Zone A, which resembled Fraction I protein, was present in healthy and infected samples whereas zone B, consisting of phytoferritin, was only found in infected samples.

Occasionally, the removal of all plant host constituents from
a purified virus preparation proves difficult with standard methods because the treatments damage the virus, the host material is tightly bound to the virus, or the contaminant has chemical and physical properties similar to the virus. A specific method for eliminating these contaminants is to precipitate them with an antiserum prepared against host antigens (32,28). The host-antibody complexes can then be removed by low-speed centrifugation.

Materials and Methods

The most effective and simplest ACLSV purification method was to homogenize systemically infected C. quinoa leaves in a Waring blender with three volumes of TM buffer plus 0.02M 2-mercaptoethanol (2-ME) and 15 ml/100 g tissue of a bentonite suspension prepared by a variation of the method described by Dunn and Hitchborn (22). Twenty grams of bentonite (Fisher lab grade) was homogenized in 400 ml of 0.01 M Tris pH 8.5 and centrifuged 1 min at 400 g. The supernatant was centrifuged 10 min at 6000 g and the pellet resuspended in 150 ml of the same buffer. This produced a suspension of 50-55 mg/ml bentonite. The homogenized sap was squeezed through cheesecloth, stirred for 10 min at 4 C, then centrifuged for 15 min at 9000 g. More bentonite (15 ml/100 g) was added slowly and the mixture stirred and centrifuged as above. The supernatant was mixed with 8.0% polyethylene glycol (PEG), stirred 1 hr at 4 C, then centrifuged at 14,500 g for 20 min. The pellets were resuspended in 6.0 ml
total of TM buffer containing 1.0% Triton X-100 and stirred for 1-2 hr at 4 C. Caesium sulphate (3.5 g) was added and the sample brought to 10 ml with TM buffer, centrifuged 16 hr at 171,000 g and the thin band of virus was drawn off with a needle and syringe. The sample was then centrifuged for 90 min at 138,000 g through a 1.5 ml 20% (w/v) sucrose cushion made in TM buffer and the pellet resuspended in a small volume of TM buffer for 16 hr on a shaker. Various stages of the purification and the final product were examined in the electron microscope and scanned in a spectrophotometer from 230-360 nm. Some samples were analyzed for virus concentration by a Bradford protein assay (Bio-Rad). Also, some samples were analyzed for purity by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in the following section and some samples were tested by double antibody sandwich ELISA (DAS-ELISA) for virus content. To test the relative amounts of virus left in the pellet and supernatant after centrifugation the pellets were resuspended in an equal volume of buffer and both samples assayed by ELISA.

A sucrose-PEG gradient similar to that used by Legrand and Verhoyen (49) was tried. A 5-30% sucrose gradient in TM buffer was superimposed on an inverse PEG gradient by preparing a 5% sucrose solution with 6% (w/v) PEG and a 30% sucrose solution. The gradients were prepared with a gradient maker which gradually mixes the dilute solution into the concentrated solution as the tube is filled. The tubes were layered with 200 µl of isolate B purified by the standard method and centrifuged 30 min at 13,000
The tubes were scanned at 254 nm with a gradient fractionator (Isco).

A sucrose-caesium step gradient of the type used for the purification of other closteroviruses (82) was also tried. Caesium sulphate solutions of 0.0M, 0.4M, 0.8M, and 1.2M were prepared with 30% sucrose (w/v) in TM buffer and 1.0 ml of each (1.2M on bottom) was layered in an SW 41 tube. Resuspended PEG pellets were diluted to 8 ml, layered over the step gradient and centrifuged for 90 min at 175,000 g. The tubes were then scanned at 254 nm on a fractionator with fluorinert FC-70 (Sigma) used to displace the gradient from the bottom of the tube. This was followed by centrifugation in the standard caesium sulphate isopycnic gradient described earlier.

The purification method described by Lister and Hadidi (52) was compared with the earlier described standard method. Lister and Hadidi’s technique was identical up to the resuspension of the PEG pellets. The sample was then centrifuged 5 min at 2,000 g, the supernatant was centrifuged 75 min at 138,000 g and the pellet resuspended in TM buffer. After a low speed centrifugation of 5 min at 2,000 g the supernatant was layered over a 10-40% sucrose density gradient and spun for 90 min at 175,000 g. The tubes were scanned at 254 nm, the peaks collected and centrifuged 75 min at 138,000 g. The pellet was resuspended in 250 μl of TM buffer.

Other variations of the standard ACLSV purification method were tried. The virus was fixed in 2% glutaraldehyde for 6 hr
prior to centrifugation in caesium sulphate, a second caesium sulphate gradient was done, the extraction homogenate was stirred for 10 min after the addition of the bentonite, a Pollähne roller press was compared to a blender for tissue homogenation, and infected tissue was frozen in liquid nitrogen prior to homogenation. Other factors investigated were the use of two clarifications with one half bentonite volumes each versus one clarification with full volume bentonite, the omission of MgSO₄ from the extraction buffer, the use of 20% versus 30% bentonite for clarification, a tissue to buffer ratio of 1 : 4.5 (w/v), the addition of 1% NaCl during PEG precipitation, the age of the infected tissue, and a comparison of Triton X-100 and urea for resuspension of the PEG pellets.

To test the effects of various factors on ACLSV integrity, purified virus was treated as follows and observed in the electron microscope. Samples were diluted 1:5 with either pH 8.5, 7.5, 6.5, or 5.5 TM buffer. Other samples were diluted 1:5 with either 0.1M, 0.05M, 0.01M, or 0.005M TM buffer. Virus was also diluted 1:5 in TM buffer with either 0.0%, 1.0%, 3.0%, or 5.0% Triton X-100. The effect of 20% ethanol, 8.0% butanol and 16.6% chloroform were tested separately on purified virus.

Host antigens were prepared by homogenizing C.quinoa tissue in two volumes 0.1M borate plus 0.1M EDTA pH 8, squeezing through cheesecloth and stirring for 1 hr with 2.0% Triton X-100. The sample was centrifuged 20 min at 14,500 g, the supernatant spun for 90 min at 111,000 g and the pellets resuspended in 4.0 ml
buffer. Four grams of caesium sulphate were added and the mixture brought to 10 ml, then centrifuged 16 hr at 171,000 g. An opalescent band was extracted with a needle and syringe and dialysed against the borate buffer. The host antigens were injected intramuscularly into a New Zealand White rabbit to produce antiserum. Four hundred μl of anti-healthy C. quinoa gamma globulins were mixed with 1/5 volume (80μl) Protein A/sepharose C1-4B (Sigma), centrifuged 5 min at 325 g, washed with phosphate buffered saline (PBS), incubated 5 min and centrifuged again. Four hundred μl of purified isolate A were mixed with the Protein-A/sepharose gamma-globulin pellet, incubated 30 min at room temperature (RT), centrifuged and the supernatant saved. The pellet was washed with PBS, incubated 5 min, centrifuged and the supernatant saved again while the pellet was resuspended in 400 μl PBS. All samples were then analyzed by electron microscopy, spectrophotometer adsorption scan, ELISA and SDS-PAGE electrophoresis.

Results

The described ACLSV method of purification would typically yield 0.1 to 0.25 mg virus per 100 g of tissue, calculated from the UV absorption scan using an extinction coefficient $E_{260}(1 \text{ mg/ml}) = 2.0$ (4). The $A_{260/280}$ ratio was 1.5 to 1.7 (corrected for light scattering). The virus concentration estimated from the absorption at 260 nm was very close (1.03 versus 0.9 mg/ml) to the concentration determined by a Bradford protein assay. The
high 260/280 ratio was a good estimate of purity and mirrored the results from the electron microscope and SDS-PAGE. Samples run on SDS-PAGE had a contaminating host band at MW 50 and 66 kilodaltons (kDa). The 50 kDa band corresponded to a heavy band of the same weight found in healthy C. quinoa clarified sap. The critical stage in the purification procedure was the bentonite clarification. If the supernatant was light green rather than amber, the final product was contaminated despite the use of a number of techniques that were intended to eliminate the host material. If Triton X-100 (1.0%) was added to the extraction buffer in an effort to break up host material and liberate more virions, clarification became difficult. More bentonite was required and because of the green pigments released, it was difficult to assess the degree of clarification. Similar results occurred when 8% butanol or 0.2% DD were tried in the extraction buffer. Clarification became difficult and although the final product had more virus, it was irreversibly contaminated to the point where purification was not possible. Attempts to separate host component by using a second caesium gradient, sucrose density gradients (10-40% and 20-50%), and a sucrose-PEG gradient failed. After the caesium sulphate gradient, the virus was typically contaminated with high molecular weight host material and many irregular shaped spheres of 10-12 nm diameter (Fig. 2). The most effective way to eliminate these spheres was a high speed centrifugation through a 20% sucrose cushion. The $A_{260/280}$ ratio measured before and after this
treatment was 0.86 and 1.2 respectively and the spheres were almost completely eliminated when viewed in the electron microscope.

A sucrose-caesium step gradient could be used to eliminate most of the contaminating spheres from a purified preparation but the virions were fragmented after this procedure. When the procedure was incorporated into the standard one before the isopycnic caesium gradient no band was formed.

When assayed by DAS-ELISA, the average loss of virus in the

Fig. 2 Purified isolate B ACLSV from a contaminated preparation showing spheres. Bar = 100 nm.
first low-speed clarification pellet averaged 28% (range = 11-38%) and the loss for the second clarification step averaged 14% (range = 5-24%). Earlier attempts at purification included a low-speed centrifugation step after the resuspension of the PEG pellets and at this stage the average loss of virus in the pellet averaged 54% (range = 37-64%) even if the speed and time were reduced to 5 min at 2000 g.

The method of Lister and Hadidi (52) could be used to purify ACLSV provided the tissue was clarified sufficiently. The yield and purity of virus from this method were equal to the method described in this paper when assayed by SDS-PAGE and electron microscopy.

The following additional variables were investigated for their effect on virus yield and contamination during purification. When the virus was fixed with 2% glutaraldehyde for 6 hr prior to centrifugation in caesium sulphate, no band was formed. After a second caesium sulphate gradient the viral band was much smaller than the first band and equally contaminated. Clarification was improved by stirring after the addition of bentonite for 10 min at 4 C. Host material was reduced with no loss of virus. In a comparison between a Pollähne roller press and a blender for tissue homogenization there was no difference in particle fragmentation and the roller press sample was slightly more contaminated. If tissue was powdered in liquid nitrogen in a coffee mill prior to blending, no virus band was formed. The use of two clarifications with one half bentonite volumes each versus
one clarification with full volume increased virus yield (0.35 to 0.41 mg/ml), and host contamination, as judged in the electron microscope, was slightly less. The omission of MgSO₄ from the extraction buffer resulted in partial particle degradation. When 20% bentonite (20 ml/100 g tissue) was used rather than the standard 30% the yield was increased (0.32 mg/ml to 1.05 mg/ml) but the sample was more contaminated. A tissue to buffer ratio of 1 : 4.5 (w/v) did not improve the yield or reduce contamination.

The addition of 1% NaCl during PEG precipitation increased contamination (A₂₆₀/₂₈₀ 1.2 versus 1.7) and also increased virus yield (0.91 versus 0.46 mg/ml). Twenty day old tissue was less contaminated, especially with spheres, than 35 day tissue. Urea (0.5M) was not better than Triton X-100 (1.0%) for decreasing particle aggregation during resuspension of the PEG pellets.

Various treatments were tried to break up the aggregations of host and virus in a contaminated purified preparation. The pH of the TM buffer had an effect on virus stability. When purified isolate A was diluted (1:5) with pH 7.5, 6.5, and 5.5 buffer, the rods were degraded when viewed in the electron microscope while the untreated virus remained intact. The ionic strength of the pH 8.5 TM buffer had no effect on viral stability over the range 0.1M to 0.005M. One percent and 3.0% Triton X-100 had no effect on viral stability or clumping of virus and host, however, when 5% was used the clumps of virus and host were broken up but the virus was partially degraded. Twenty percent ethanol had no effect on the virus whereas 8% butanol and 16.6% chloroform gave
results similar to 5.0% Triton X-100 where both the virus and host were degraded.

Even with the optimum ACLSV purification procedure small amounts of host material still remained. The antiserum against healthy C. quinoa reacted against five other species (N. glutinosa, N. benthamniana, C. amaranticolor, V. unguiculata, and C. sativa) in agar gel double diffusion tests indicating the antiserum detects one or more components common to a number of different plants. After purified isolate A virus was cross-adsorbed with anti-host antiserum, the two contaminating bands observed in the pre-adsorbed preparation in SDS-PAGE were still present and in the electron microscope the two samples appeared identical. The UV absorption scan of both preparations showed the virus concentration dropped from 0.365 mg/ml to 0.278 mg/ml after cross adsorption. The pellet from the cross adsorption step was resuspended and centrifuged again and the supernatant and pellet examined by ELISA and SDS-PAGE for evidence of non-specific and specific binding of virus. By ELISA the A405 for the cross adsorbed supernatant was 0.388, the pellet supernatant was 0.166 and the resuspended pellet was 0.06. In SDS-PAGE a small virus band was seen in the pellet supernatant and a smaller band in the resuspended pellet (Fig. 3). These data indicate that the contaminating host component could not be removed by cross-adsorption and that a small amount of virus was lost by specific and non-specific binding to the Protein A/gamma globulin sepharose bead.
Fig. 3. SDS-PAGE of cross-adsorption (C/A) of isolate A. Lanes A and H = low MW standards (Bio-Rad). B = pre-C/A virus. C = supernatant from C/A. D = supernatant from pellet centrifugation. E = pellet from pellet centrifugation. F = ACLSV monoclonal gamma-globulin. G = anti-host gamma-globulin. Note contaminating bands at 50 and 66 KDa in lanes C and D and virus band at 26 KDa in lanes D and E.

Discussion

The described ACLSV purification method is simple and quick, and gives a product of equal concentration and purity when
compared to the standard method of Lister and Hadidi (52). It is also easier to use because homogenates that are not sufficiently clarified will still yield virus. If the clarification was poor, attempts to purify ACLSV by the standard method failed because of virus loss in low-speed and density-gradient centrifugation through particle aggregation. The described method avoids low-speed centrifugation after the initial clarification. Losses during clarification from virions aggregating with host tissue are substantial. Many methods designed to liberate more virions by breaking up aggregates of virus and host were unsuccessful resulting in more contamination or breakdown of virus. An area worthy of further research is how to avoid virus loss during clarification and to reduce contamination, a common consequence of increasing virus yield.

An unexpected result was the failure of the polyamine DD to improve purification since Dunez et al. (20) found DD to be essential for purifying the bark-split strain of ACLSV from Prunus domestica by preventing aggregation of the virus with ribosomal subunits. It may not be possible to assume that all ACLSV Prunus strains behave the same during purification.

The plant constituents most commonly found contaminating purified virus preparations are Fraction I protein (ribulose diphosphate carboxylase), phytoferritin, and ribosomal subunits (29). All of these have a diameter of approximately 10-12 nm. The MW of Fraction I protein subunits is 55 and 12 KDa (43), while phytoferritin is composed of 28 and 26.5 KDa polypeptides (48).
The identity of the contaminating spheres in my preparations is unknown. Unlike phytoferritin, these spheres did not contain an electron-dense core when unstained preparations were viewed in the electron microscope, however, the concentration of spheres increased in older plants, a characteristic of phytoferritin associated with its regulation of excess iron released from chloroplast breakdown. The MW of Fraction I protein resembles that of the spheres, however, the concentration of this enzyme should not increase in older plants. The other possible identity for the contaminating bands may be one of the ribosomal proteins as reported by Dunez (20).

It was hoped that a method could be devised to separate host constituents and virus in partially purified preparations but a number of these methods failed. The effects of low pH TM buffer on purified virus was unusual. At low pH the need for magnesium ions to stabilize ACLSV structure is reduced as it is with spherical viruses dependant on divalent cations for stabilization (52). The fact that low pH TM buffer partially disrupted the particles is even more unusual considering infectivity was not affected when infected C. quinoa was ground in these buffers. A pH dependant interaction between the virus and the uranyl acetate stain may be responsible for the decreased particle stability. Concentrations of Triton X-100 in the 3-5% range have been used in purifying grapevine leafroll (84) but these levels are detrimental to ACLSV.

The failure of the cross-adsorption with anti-host antiserum
bound to sepharose beads was expected in view of the ACLSV morphology. It is easy to imagine how long flexuous rods would become entangled on the sepharose beads and be lost in the centrifugation step. When the pellet from this step was resuspended and centrifuged again, both the supernatant and pellet contained virus which indicates that at least some of the virus was non-specifically bound to the beads and was released during resuspension of the pellet. The virus remaining in the pellet was either specifically bound to the anti-host immunoglobulins or because of its physical nature was not released during resuspension. This loss of virus could be justified if cross-adsorption worked, but the contaminating bands (Fig. 3) were not eliminated. For an effective cross-adsorption technique to be developed for ACLSV, a more specific anti-host gamma-globulin is needed in conjunction with a method which would avoid the non-specific binding of the virus, possibly an immunoprecipitating gel filtration column.
Chapter 4 - Cytopathology and Physical Properties

Introduction

Closteroviruses show a great diversity in features such as cytopathology and vector relationships and have therefore been placed into three subgroups (5). Subgroup A has particles 700 - 800 nm, subgroup B 1200 - 1500 nm and subgroup C 1500 - 2000 nm. Pathological changes found in subgroups B and C include virus particles in large masses, usually in the phloem or phloem parenchyma and cytoplasmic membranous vesicles (25). Other general necrogenic features such as chloroplast and mitochondria disintegration and accumulation of osmiophilic globules and phytoferritin in the chloroplast are found in all subgroups. The best studied closterovirus is the type member, beet yellows virus (BYV), which forms large masses of particles often associated with membranous vesicles referred to as "BYV-type" vesicles (23). These vesicles are also associated with a number of other closteroviruses. In contrast to the majority of closteroviruses which are found only in the vascular elements, Ohki et al. (61) found ACLSV, apple stem grooving virus and citrus tatter leaf virus in the mesophyll and phloem parenchyma cells, but not the epidermis or sieve elements of infected C. quinoa. ACLSV particles were observed as round aggregations or as crystallized parallelograms in the cytoplasm and nucleus.

The physical properties of ACLSV have been reported previously (4,72,81,). Analysis of ACLSV and other closterovirus dsRNA replicative forms by PAGE gave patterns that were distinct for
each virus but, with the exception of ACLSV, similar enough to be
diagnostic for closteroviruses (19).

Materials and Methods

Leaf tissue from P. glandulosa and C. quinoa was harvested
from systemically infected greenhouse grown plants in June. Small
pieces of petiole, midrib and leaf blade were fixed overnight
(0/N) in 4% glutaraldehyde in pH 7, 0.1M cacodylate buffer,
washed twice with cacodylate buffer and left in 1% OsO₄ in pH 7,
0.1M cacodylate buffer for 1 hr. Samples were put through a
dehydration series of two washes each in 50%, 70%, 95% and
absolute ethanol (EtOH) and then washed three times in propylene
oxide before being infiltrated 0/N in a 50/50 mixture of
propylene oxide and Epon 812. Finally, the samples were
transferred to embedding molds filled with fresh Epon. The
embedded samples were sectioned on a Reichert Om U2 microtome
with glass knives to obtain sections with gold and silver
diffraction colours which were transferred to 100 mesh copper
grids. Sections were stained with 4% aqueous uranyl acetate (UA),
washed with distilled water (dH₂O), stained with Reynolds lead
citrate diluted 1:4 in 0.01N NaOH, washed again and blotted dry.

Four hundred mesh copper grids were floated on drops of virus
purified from C. quinoa (virus concentration 0.5 to 1.0 mg/ml) in
pH 8, 0.01M Tris buffer with 0.01M MgSO₄, rinsed on a drop of
dH₂O and stained with 8 drops of 2% aqueous UA or phosphotungstic
acid (PTA). All micrographs were taken on an Hitachi 600
electron microscope at 75 Kv accelerating voltage. The virus modal lengths were calculated by measuring non-fragmented particles on micrographs with a calibrated tracing wheel at final magnifications of 37,500 to 150,000. Modal lengths were determined by taking the mean of the most frequent histogram cell and the contiguous cells. Measurements of virus width and pitch were made from micrographs with a final magnification of 150,000 to 375,000 using a Leitz 8x magnifier. The microscope was calibrated at each magnification using a 2160 lines per cm diffraction grating.

Viral coat protein molecular weights were determined by discontinuous polyacrylamide gel electrophoresis with a 4% stacking gel and a 12% or 15% running gel in a mini-vertical slab gel apparatus (Bio-Rad) with the buffer system of Laemmli (47). Samples were boiled for 4 min in 0.06M Tris, pH 6.8 with 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% 2-ME and 0.00125% bromophenol blue before loading on the gel. Standards were Bio-Rad low molecular weight standards (92 to 14.4 KDa). The amperage was set at 10 mA/gel until the samples reached the running gel, then 25 mA/gel until the tracking dye reached the bottom of the gel. The gels were stained with Coomassie Brilliant Blue R-250 in 25% methanol and 70% acetic acid for 1 hr then destained O/N in 25% methanol, 7% acetic acid.

For western blot analysis gels were not stained but soaked in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 30 min and transferred to an Immobilon membrane
(Millipore) in a Bio-Rad trans-blot cell for 1.5 hr at 100 V at 4 C. The blots were blocked O/N in phosphate buffered saline (PBS) with 0.05% Tween-20 (PBS-Tw) and 1% non-fat milk powder (Blotto) (41), washed three times in PBS-Tw and soaked in polyclonal antiserum diluted 1:500 with clarified C. quinoa sap for 2 hr at room temperature (RT). The blots were washed three times with PBS-Tw then soaked 1.5 hr in \textsuperscript{125}I- labelled Protein-A prepared according to Bolton and Hunter (6). The blots were then washed four times in PBS-Tw, blotted dry and autoradiographed O/N at -70 C with intensifying screens.

For RNA extraction 500 \textmu l of purified isolate A was mixed with 100 \textmu l 0.05M Tris pH 8.9, 25 \textmu l 20% SDS, 200 \textmu l chloroform-isoamyl alcohol (24:1) and 200 \textmu l redistilled phenol saturated with dH\textsubscript{2}O. After a low speed centrifugation the aqueous phase was extracted with 300 \textmu l phenol and 300 \textmu l chloroform-isoamyl alcohol and then with 600 \textmu l chloroform-isoamyl alcohol. The RNA was precipitated with 0.1 volume sodium acetate pH 5.4 and 2.25 volumes absolute ethanol in a liquid nitrogen bath for 5 min. After centrifugation for 30 min, the RNA pellet was washed twice with 70% ethanol and resuspended in sterile deionized dH\textsubscript{2}O. The RNA was quantified spectrophotometrically using an extinction coefficient of 25 (mg/ml)\textsuperscript{-1}cm\textsuperscript{-1} at 260 nm. Electrophoresis of denatured RNA was done in a 1% agarose gel containing 5 mM methylmercuric hydroxide using a modification of the method of Bailey and Davidson (1). The gel was run for 1 hr at 100 V and stained with ethidium bromide (1 \mu g/ml) after the addition of
0.07% 2-mercaptoethanol. RNA standards consisted of an RNA ladder from 9.5 to 0.3 Kb (Bethesda Research Laboratories).

Double-stranded RNA (dsRNA) was purified by the method of Kurppa and Martin (46). Twenty gram samples of infected C. quinoa tissue were powdered in liquid nitrogen and extracted with 2 volumes of 2x STE (1x = 50 mM Tris, 100 mM NaCl and 1 mM EDTA pH 7.1), 0.5 volumes 10% SDS and 1.5 volumes STE saturated phenol. The aqueous phase was adjusted to 16% ethanol and poured over 2.5 g CF-11 cellulose (Whatman), washed with STE containing 16% ethanol (STE-EtOH) and the bound nucleic acids eluted with 15 ml STE. Samples were digested sequentially with T\textsubscript{1} RNAase and DNAase 1 (Sigma) adjusted to 20% EtOH and 1/30 volume 0.5M EDTA was added. The material was bound to APX cellulose (Serva) and washed on a column with STE-EtOH, then eluted with STE and precipitated with 0.1 volume 3M sodium acetate pH 5.2 and 2.5 volumes EtOH. Samples were stored at -20 C. Electrophoresis of the dsRNA was in 1% agarose gels with 1 µg/ml ethidium bromide made with TAE buffer (40 mM Tris, 40 mM acetate and 2 mM EDTA, pH 8.0) for 2-3 hr at 40 V. Standards were Pst I or Hind III cut lambda phage DNA.

To determine buoyant density a small amount of caesium sulphate was withdrawn from a sister tube at the same level as the virus band after isopycnic centrifugation and the refractive index read on a refractometer and converted to density using the formula of Vinograd and Hearst (78).
Results

The particles were disrupted in 2% PTA and only 2% UA was used. Purified virus particles were long, very flexuous rods with a striated cross-banding pattern. The axial canal was not visible and full length and fragmented particles were observed, frequently intertwined in aggregates with host material. Also, small (10-12 nm) irregular spheres were found (Fig. 2). Measurements of particle width for the B isolate made from five particles from five separate purifications averaged 11.5 nm and the helical pitch measured from three particles from three separate purifications averaged 3.25 nm.

The particle length distribution for the A isolate was plotted using a histogram cell width of 50 nm (Fig. 4). The modal length was 782 nm and the three contiguous cells around the mode contained 71% of the particles measured. The length distribution of the B isolate (Fig. 5) was more difficult to interpret. The length most frequently observed is 600 to 650 nm and with the two contiguous cells includes 39.5% of the particles, however, another peak occurs around the 750 to 800 nm cell which also contains 39.5% of the particles. The majority of particles fell in the 550 to 900 nm range, the average of this group being 732 nm.

Thin sections from both P. glandulosa isolates and infected C. quinoa were examined for evidence of particles in vivo and for cytoplasmic inclusions such as vesicles or cross-banded bodies which have been associated with other closteroviruses (25). In
Fig. 4. Modal length distribution of 46 particles of ACLSV isolate A measured from purified preparations. Histogram cell width = 50 nm. Modal length = 782 nm.
Fig. 5. Modal length distribution of 38 particles of ACLSV isolate B measured from purified preparations. Histogram cell width = 50 nm. Modal length = 732 nm.
the woody hosts, long flexuous strands were observed in sieve tube members and companion cells but not in the mesophyll. The strands were found loose within the cell or associated with sieve plates (Fig. 6 A). In C.quinoa thin flexuous rods were seen loosely arranged in young sieve-tube members in the cytoplasm and nucleus and also aligned in parallel bundles (Fig. 6 B). Some bundles appeared in cross-section as a tightly packed array of dots. No vesicles were found. It is not known if the flexuous rods were P-protein, which is found in the sieve-tube members of dicotyledonous plants, or virions.

Three virus specific dsRNA bands were extracted from ACLSV infected C.quinoa of MW $5.6 \times 10^6$, $4.88 \times 10^6$ and $4.45 \times 10^6$ Da (Fig. 7). The ssRNA extracted from purified isolate A had a major band at $2.66 \times 10^6$ and minor bands at $1.53 \times 10^6$ and $0.65 \times 10^6$ Da (Fig. 8). The buoyant density of ACLSV isolates A and B in caesium sulphate was $1.26 \text{g/cc}^3$ and $1.28 \text{g/cc}^3$ respectively. The coat protein MW of the ACLSV isolates was estimated at 24.5 KDa (B) and 26.0 KDa (A) (Table 3). The slight difference in MW between the two isolates was consistent over four different gels. There were also major host component contaminating bands at 49 and 66 KDa. That these bands were not of viral origin was confirmed by Western blot analysis where the membrane was soaked in antibody diluted in clarified healthy C. quinoa sap and probed with $^{125}$I-labelled Protein-A. The 24.5 and 26 KDa bands showed strong reactions while there was only a faint reaction at the 49 KDa band with the more contaminated B isolate (Fig. 9).
Fig. 6. Virus-like particles in apple chlorotic leafspot virus infected tissues. A. Particles associated with a sieve-plate in infected Prunus glandulosa. Bar = 1μm. B. Bundles of virus-like particles in a sieve-tube member of infected Chenopodium quinoa. Bar = 1μm. Arrows = bundles in cytoplasm and nucleus.
Fig. 7. Electrophoretic visualization of dsRNA extracted from apple chlorotic leafspot infected Chenopodium quinoa. Lane A = PstI cut lambda, Lane B = isolate A, Lane C = isolate B, Lane D = healthy C. quinoa, Lane E = HindIII cut lambda. Line marks position of three virus-specific bands at MW $5.6 \times 10^6$, $4.88 \times 10^6$ and $4.45 \times 10^6$. 
Fig. 8. Electrophoretic visualization of ssRNA extracted from apple chlorotic leafspot infected Chenopodium quinoa. Lanes A & D = RNA ladder from 9.5 to 0.3 Kb, Lanes B & C = isolate A.
Table 3. Physical characteristics of ACLSV from *P. glandulosa*

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<th></th>
<th>Coat Protein (kDa)</th>
<th>ssRNA ($\times 10^6$)</th>
<th>dsRNA ($\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLSV-A</td>
<td>26.0</td>
<td>2.66</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.53</td>
<td>4.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.65</td>
<td>4.45</td>
</tr>
<tr>
<td>ACLSV-B</td>
<td>24.5</td>
<td>-</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.45</td>
</tr>
</tbody>
</table>
Fig. 9. A. Electrophoretic visualization and Western Blot of apple chlorotic leafspot virus coat protein. Std = low molecular weight standards (92.5 to 14.4 KDa), 1114 = isolate A, C.q. sap = raw Chenopodium quinoa sap, Clar sap = clarified C. quinoa sap, 1292 = isolate B. Virus coat protein band = 24 to 26 KDa. B. Western blot of Fig. 9A gel probed with isolate B polyclonal antiserum diluted with clarified C. quinoa sap followed by $^{125}$I-labelled Protein A.
Discussion

Closteroviruses exhibit some unique cytopathological properties which can be diagnostic to the group level (35) and others which are used to divide them into subgroups (25). Banded bodies in the phloem are characteristic of closteroviruses while within the group, BYV-type vesicles are indicative of subgroup I (subgroup B according to Bar-Joseph and Murant, (5)). ACLSV is placed in subgroup II.

A common problem in interpreting the cytopathological effects of closteroviruses is distinguishing them from P-protein which may occur in several forms (granular, filamentous, fibrillar, tubular and crystalline) in the sieve-tube members of dicotyledonous plants and in some monocots. The function of P-protein is unknown but possible roles include the sealing of sieve-plate pores in injured sieve tubes or long distance transport of substances in the phloem (23). Occasionally, P-protein and virions can be distinguished in the same micrograph but in this work it is not known if the filamentous material observed in the micrographs is P-protein or virus particles. Immunogold staining would be a useful technique to make this distinction, however an antiserum without any background reactivity against host components would be needed. In contrast to the results of Ohki et al. (61) no evidence of ACLSV virus particles was found outside of the vascular tissue. This may be because different virus isolates were studied.

The particle modal lengths for the A and B isolates (782 and
732 nm respectively) are close to the previously reported values of 795 nm for an ACLSV isolate from Prunus domestica. The particle measurements were 30 nm longer (825 vs 795) when measured from leaf sap as compared to purified virus (72). Values for apple isolates of ACLSV are between 620 to 740 nm. The difference in modal lengths between the A and B isolates may be real or simply reflect the increased susceptibility to breakage by the B isolate during purification. By reporting the modal length of rod-shaped viruses, the effects of end to end aggregation can be eliminated and viruses with particles of two sizes (eg. tobacco rattle) can be detected. However, when easily damaged rods from purified preparations are used for modal length determination, the value reported may be the length to which the particles are most frequently broken and not the true length of the virus in vivo. Attempts to answer this question by trapping particles from clarified sap were not successful, probably because the polyclonal antiserum had a large component directed against host proteins or the virus concentration was too low.

The particle width of 11.5 nm is consistent with other estimates for ACLSV of 12 nm (4) as is the pitch estimate of 3.25 nm compared with a reported value of 3.8 nm (3).

The difference in coat protein MW between ACLSV isolates A and B is small and both estimates are within the range reported previously for ACLSV (81,9). The function of viral coat proteins goes beyond that of protecting the viral RNA. Dawson et al. (15) showed that TMV mutants with insertions or deletions in the coat
protein gene affected the type of symptoms expressed in Xanthi tobacco. Further research should be conducted on whether the difference in the coat protein of isolate A and B is responsible for the lack of systemic movement in C. amaranticolor by isolate A. The Western blot analysis confirmed that the 24.5 and 26 KDa proteins were of viral origin. After cross-adsorption of the detecting antibody with clarified C. quinoa sap, the faint reaction with the 49 KDa band indicates that the polyclonal antiserum contains a component directed against this host protein which could not be removed.

Double-stranded RNA analysis has been used as a diagnostic tool for detection and classification of closteroviruses. Dodds and Bar-Joseph (19) reported a dsRNA of MW $4.6 \times 10^6$ and three minor bands at approximately $1.5 \times 10^6$. Interestingly, this pattern was not similar to the three other closteroviruses and the authors questioned the inclusion of ACLSV in the closterovirus group. Yoshikawa and Takahashi (81) found three major (MW ($X 10^6$) 4.9, 4.3, 3.9) and two minor (4.5 and 3.1) bands from ACLSV infected C. quinoa. Both A and B isolates showed similar dsRNA patterns consisting of three bands (MW ($X 10^6$) 5.60, 4.88, 4.45) in the same relative position as the three bands reported by Yoshikawa and Takahashi (81) but at higher molecular weights. It is not known if this is due to the source of the isolates being Prunus rather than Malus or reflects the different techniques used. The MW of the slowest migrating band ($5.66 \times 10^6$) was slightly more than double that of the major
ssRNA band \((2.66 \times 10^6)\).

With the exception of the virions not being found in the mesophyll, the physical properties and cytopathology of the A and B isolates agree with those previously reported for ACLSV.
Chapter 5 - Serological Detection

Introduction

ACLSV has been detected by serological techniques such as ring interface (67), agar gel diffusion (9), ELISA (24,18,27,2,80,66), and immunosorbent electron microscopy (ISEM) (42,44). Saksena and Mink (67) could detect ACLSV from blossoms but not from leaf tissue by the ring precipitin test. The virus from blossoms required a partial purification before being used. Agar gel diffusion tests can be done with infected C. quinoa sap but not from Malus or Prunus leaf tissue (9). The omission of stabilizing agents such as polyamines or MgCl\textsubscript{2} degrades the virus and improves diffusion through the agar (52). Because of the difficulties in detecting ACLSV in woody hosts by gel diffusion and ring precipitin tests, the ELISA technique is preferred for routine detection. Flegg and Clark (24) used a modification of the basic technique where the sample was incubated simultaneously with the antibody-conjugate. They speculated that the virus in the virus-antibody complexes formed by this step were less susceptible to degradation. Detienne et al (18) found that the standard ELISA method could be used if MgCl\textsubscript{2}, 3,3'-diaminodipropylamine (DD) and nicotine were added to the extraction buffer and this method could detect ACLSV from all sources except apricot. A F(\text{ab}')\textsubscript{2} based ELISA was used by Barba and Clark (2) to detect ACLSV in all woody hosts except pear. Monoclonal antibody technology, first developed by Köhler and Milstein (45), has allowed for the routine use of serological
techniques without the problems associated with polyclonal antiserum such as limited supply, variable quality and non-specific reactions (57). Host components are often found in purified ACLSV preparations and polyclonal antiserum produced from these preparations must be cross-adsorbed with host antigens. To avoid this problem Poul and Dunez (66) produced monoclonal antibodies against the plum bark split strain of ACLSV and developed an ELISA test with ten-fold increased sensitivity over a test using polyclonal antiserum.

The serological variability of ACLSV was first reported by Chairez and Lister (9) when they found differences between strains from Malus and Prunus. In contrast Detienne et al. (18) found limited antigenic variation among ACLSV strains using polyclonal antiserum against a Prunus strain. Barba and Clark (2) showed that antiserum prepared against an apple isolate reacted well with homologous antigens but did not detect a Prunus isolate causing viruela of apricot. Conversely, an antiserum prepared against a plum isolate detected isolates from Prunus and apple. These reports of serological variation emphasize the need for adequate screening of the antiserum specificity before adopting a serological testing program. For example, a commercially available ACLSV antiserum failed to detect the virus isolated from apple trees in Finland (50).

Materials and Methods

Polyclonal antiserum against the A and B ACLSV isolates, and
healthy C. quinoa were produced in New Zealand White rabbits. The immunization schedule consisted of four weekly intramuscular injections of 0.1 to 0.5 mg antigen with Freund's incomplete adjuvant followed a week later by one interveinal injection of 0.2 to 0.3 mg without adjuvant. Ten and 15 days later the rabbits were bled. Gamma-globulins were purified by ammonium sulphate precipitation and passage through DEAE cellulose, then adjusted to 1 mg/ml using an $A_{280} = 1.4$. Conjugation of antibodies with alkaline phosphatase was according to Clark and Adams (10) using glutaraldehyde. The double antibody sandwich (DAS) ELISA was done according to Clark and Adams (10) with a few modifications. Linbro polystyrene plates (Flow Laboratories) were coated with antibody in coating buffer for 1 hr at 37 C followed by phosphate buffered saline (PBS) with 0.05% Tween-20 and 0.1% Blotto (PTB) for 1 hr at room temperature. Samples ground 1:10 (w/v) in PTB (2% PVP added for woody tissue) were added to the wells and left overnight at 4 C. The specific conjugates were diluted in healthy C. quinoa sap, either fresh or previously frozen, ground 1:10 (w/v) with PTB and filtered through two layers of Kimwipes, left 10 min and then added to the plates for 3 hr at 37 C. For comparison, the appropriate amount of stock conjugates were added to a volume of healthy C. quinoa extract (1:10 w/v) equal to 1/10 the volume of diluted conjugate required for the test, left 10 min at room temperature (RT) and then diluted with PTB and added to the plates (33). After the addition of substrate tablets, (p-nitrophenyl phosphate) (Sigma) the plates were read at 405 nm on
an automated plate reader (Titertek Flow Laboratories) after 1 to 2 hr and after standing overnight.

In attempts to lower background reactions, ACLSV polyclonal antiserum was cross adsorbed with host preparations. Healthy C. quinoa was purified to the PEG precipitation stage described in the virus purification chapter. One half a millilitre of this preparation was added to 1 ml of antiserum and 8.5 ml of dH₂O, left 16 hr at RT and centrifuged 20 min at 14,000 g followed by ammonium sulphate precipitation and purification of immunoglobulins on DEAE cellulose.

Agar gel double diffusion tests were made in 1.0% agar gels containing 0.5% sodium dodecyl sulphate (SDS), 0.85% NaCl and 0.02% sodium azide. Three hundred microlitres of 10% SDS were added to the TM grinding buffer for each gram of sample.

Monoclonal antibodies (MABs) against the A isolate were produced by injecting Balb/c mice subcutaneously with 35 μg of purified virus disrupted with 1% SDS and mixed equally with Freund’s incomplete adjuvant. After a 25 day rest the mice were injected intraperitoneally with 50 μg virus and sacrificed 4 days later to remove their spleen. Fox-NY myeloma cells were cultured in Dulbecco’s Modified Eagle’s media (DME) plus 10% fetal calf serum (FCS) (Hyclone, Logan, Utah) and fused with splenocytes according to the method of Kohler and Milstein (45) using 50% PEG. The cells were resuspended in 20% FCS DME with aminopterin, hypoxanthine, adenine, thymine and a thymocyte feeder layer. Antibody secreting hybridomas were identified by an indirect
triple antibody sandwich (TAS) ELISA. Half of the plates were coated with isolate A polyclonal antibodies at 2 μg/ml for 1.5 hr at 37 C followed by a blocking step with PTB. Healthy C. quinoa sap ground 1:10 in PTB was left on the plates for 16 hr. Culture fluid (90 μl) was then added for 1 hr at 37 C, followed by a rabbit anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research) for 1 hr at 37 C and then P-nitrophenyl phosphate tablets in substrate buffer. The other half of the plates were direct coated with purified isolate A virus (2.5 μg/ml) in coating buffer for 16 hr at RT, blocked with PTB, and followed by the procedures described above. Clones which reacted to direct coated virus and not host antigens were selected for further testing. Positive cell lines were cloned by limiting dilution using a thymocyte feeder layer, grown in cell culture and either injected intraperitoneally into mice for production of ascitic fluid (62) or grown in 1 L culture bottles. Antibodies were purified from ascites fluid by precipitation with 50% saturated ammonium sulphate followed by passage through a DEAE cellulose column. One litre batch cultures were purified by low speed centrifugation to remove hybridoma cells and followed by freeze drying or precipitation with ammonium sulphate. The subclass of each antibody was determined with a hybridoma immunoglobulin typing kit (Behring Diagnostics).

The triple antibody sandwich (TAS) ELISA was used to test the MABs against the ACLSV isolates. One MAB was directly conjugated to alkaline phosphatase and used in the DAS-ELISA with polyclonal
coating antibody. A comparison was made of the following grinding buffer additives in the TAS-ELISA system: 2.5% nicotine, 0.01M MgSO₄, 0.2% DD, nicotine plus MgSO₄ plus DD, and nicotine plus MgSO₄.

**Results**

The two polyclonal ACLSV antisera gave high background reactions without some form of cross-adsorption against healthy C. quinoa (Table 4). The specific antibody-enzyme conjugate from both treatments was also diluted in healthy C. quinoa sap or PTB. The highest infected/healthy ratio was achieved with unadsorbed antiserum where the conjugate was diluted in host sap. A separate test was done comparing dilution of conjugate in sap with the method of Gonsalves et al. (33) where the appropriate volume of stock conjugate was incubated with a small volume of host sap and then diluted with PTB. Reactions to healthy C. quinoa were 1.6 times higher and reactions to infected tissue were one half to one quarter lower with the latter method. The detection limit of homologous purified virus by DAS-ELISA was approximately 25 ng/ml. ACLSV was also detected in P. glandulosa leaves and flowers but was erratically distributed and the signal weak, typically in the range of two to five times that of healthy C. quinoa.

In agar gel diffusion tests with purified virus the A and B isolates formed continuous precipitin lines (Fig. 10) however this test could not be used with crude sap because of non-
Table 4. Comparison of infected/healthy absorbance ratio in double antibody sandwich ELISA for cross-adsorbed versus non cross-adsorbed apple chlorotic leafspot virus antiserum.

<table>
<thead>
<tr>
<th>Antiserum(^a)/conjugate diluent(^b)</th>
<th>Infected/Healthy Ratio(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Unadsorbed/PTB</td>
<td>1.29</td>
</tr>
<tr>
<td>A Unadsorbed/sap</td>
<td>14.50</td>
</tr>
<tr>
<td>A Adsorbed/PTB</td>
<td>1.55</td>
</tr>
<tr>
<td>A Adsorbed/sap</td>
<td>5.08</td>
</tr>
<tr>
<td>B Unadsorbed/PTB</td>
<td>1.70</td>
</tr>
<tr>
<td>B Unadsorbed/sap</td>
<td>7.52</td>
</tr>
<tr>
<td>B Adsorbed/PTB</td>
<td>2.25</td>
</tr>
<tr>
<td>B Adsorbed/sap</td>
<td>3.20</td>
</tr>
</tbody>
</table>

\(^a\)A and B refer to the two isolates from Prunus glandulosa

\(^b\)Conjugate diluted either in PBS, 0.05% Tween, 0.1% skim milk powder (PTB) or host sap. Infected and healthy Chenopodium quinoa ground at 1:10 tissue to buffer ratio.

\(^c\)Ratios were determined from the average of eight wells/treatment
Fig. 10. Agar gel double diffusion test with purified apple chlorotic leafspot virus isolates A and B. As = isolate B polyclonal antiserum. Wells B and E = isolate A. Wells C and F = isolate B. Well A = Healthy Chenopodium quinoa. Well D = buffer only.
specific host reactions.

The technique of diluting conjugate in sap worked well, however, fresh C. quinoa leaves may not be available so a comparison was made using frozen sap and sap stored at 4 C with 0.02% sodium azide. Results with fresh or stored sap were equivalent but infected/healthy ratios were reduced from 12 and 13.5 respectively to 2.5 with frozen sap. The ratio was also lower when the PTB buffer was supplemented with MgSO₄ or TM buffer was used (Table 5).

Initial fusions done after injecting mice with whole virus failed to produce any virus-specific hybridomas. When the virus was disrupted with 1% SDS before injection, six positive clones out of 480 screened were detected. Further testing revealed that two of the six clones could detect the virus when coated directly to the polystyrene plate but not in infected C. quinoa sap while the four other clones could detect the virus under both conditions. After recloning, these four hybridomas were retested against seven isolates of ACLSV (Table 6). Clones A2, C3 and D4 reacted with the Prunus isolates only whereas clone B2 detected all isolates but did not give as strong a reaction as the other three clones.

The subclass of immunoglobulins secreted by the hybridomas was IgG1 for clones A2, C3 and D4 and IgG2a for clone B2.

In a TAS-ELISA the B2 MAB was diluted in two-fold steps from 1/100 to 1/51200. When left overnight, the absorbance values for
Table 5. Comparison of three grinding buffers and three methods of diluting conjugate on the absorbance ratio of infected/healthy C. quinoa in double antibody sandwich ELISA.

<table>
<thead>
<tr>
<th>Sap Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Buffer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fresh</th>
<th>Frozen</th>
<th>Stored</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>8.5</td>
<td>4.7</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>10.0</td>
<td>3.4</td>
<td>8.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Sap Type - (ground 1:10 in PTB)

Fresh = Healthy C. quinoa sap

Frozen = Healthy C. quinoa sap stored at -20 C

Stored = Healthy C. quinoa sap stored at 4 C

<sup>b</sup>Buffer

A = PBS, 0.05% Tween-20, 0.1% Blotto (skim milk powder) (PTB)

B = PTB, 0.01M MgSO<sub>4</sub>

C = 0.05M Tris, 0.01M MgSO<sub>4</sub>, 0.1% Blotto, 0.05% Tween-20

<sup>c</sup>Ratios were determined from the average of three wells/treatment
Table 6. Reactions of four monoclonal antibodies (MAB) to seven isolates of apple chlorotic leafspot virus in Chenopodium quinoa by triple antibody sandwich ELISA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>A2</th>
<th>B2</th>
<th>C3</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (Prunus)</td>
<td>0.640&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.190</td>
<td>0.490</td>
<td>0.450</td>
</tr>
<tr>
<td>A (Prunus)</td>
<td>0.670</td>
<td>0.250</td>
<td>0.470</td>
<td>0.710</td>
</tr>
<tr>
<td>ATCC (Malus)</td>
<td>0.028</td>
<td>0.210</td>
<td>0.017</td>
<td>0.071</td>
</tr>
<tr>
<td>P205 (Malus)</td>
<td>0.031</td>
<td>0.260</td>
<td>0.019</td>
<td>0.058</td>
</tr>
<tr>
<td>UBC (Malus)</td>
<td>0.034</td>
<td>0.210</td>
<td>0.020</td>
<td>0.047</td>
</tr>
<tr>
<td>S1 (Prunus)</td>
<td>0.420</td>
<td>0.300</td>
<td>0.280</td>
<td>0.420</td>
</tr>
<tr>
<td>1000-04 (Malus)</td>
<td>0.030</td>
<td>0.230</td>
<td>0.017</td>
<td>0.061</td>
</tr>
<tr>
<td>Healthy C. quinoa</td>
<td>0.010</td>
<td>0.006</td>
<td>0.005</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average of three wells
infected C. quinoa at the 1/51200 dilution were between 0.50 and 0.94 and the healthy values were between 0.003 and 0.024.

A buffer containing PTB plus 2% PVP, 2.5% nicotine and 0.01M MgSO₄ was the best for detecting the homologous antigen from woody and herbaceous hosts (Table 7).

Although the B2 MAB could detect all seven isolates in TAS-ELISA it could not be used as a trapping antibody in DAS-ELISA. However, B2 could be conjugated directly to alkaline phosphatase and used with a polyclonal trapping antibody. In such tests the absorbance values were higher than when the same MAB was used in TAS-ELISA with no increase in healthy background reactions.
Table 7. The effects of six different grinding buffer additives on the detection of apple chlorotic leafspot virus isolate A in Chenopodium quinoa and Prunus glandulosa by triple antibody sandwich ELISA

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. quinoa</td>
<td>0.757&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.990</td>
<td>0.375</td>
<td>0.123</td>
<td>1.221</td>
<td>1.885</td>
</tr>
<tr>
<td>P. glandulosa</td>
<td>0.228&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.119</td>
<td>0.109</td>
<td>0.097</td>
<td>0.181</td>
<td>0.350</td>
</tr>
<tr>
<td>Healthy C. quinoa</td>
<td>0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.015</td>
<td>0.010</td>
<td>0.006</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Buffer only</td>
<td>0.012&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.003</td>
<td>0.001</td>
<td>0.008</td>
<td>0.003</td>
<td>0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup>The standard buffer was PBS with 0.05% Tween-20, 0.1% skim milk powder, 2% PVP. Various compounds were added as follows: 1 = no additives; 2 = Nicotine (2.5%); 3 = MgSO<sub>4</sub> (0.01M); 4 = diaminodipropylamine (DD) (0.02%); 5 = Nicotine, MgSO<sub>4</sub>, DD; 6 = Nicotine, MgSO<sub>4</sub>

<sup>b</sup>Average of three wells

<sup>c</sup>Single well only
Discussion

The ACLSV polyclonal antiserum had a high proportion of antibodies directed against host components and without cross-adsorption could not discriminate between infected and healthy tissue. The infected/healthy ratios listed in Table 4 show that diluting the antibody-enzyme conjugate in host sap is the most effective way of removing non-specific reactions. Freshly prepared host sap or sap stored at 4°C could be used to dilute the conjugate but not previously frozen sap. Freezing has been used as a clarification technique for precipitation of plant proteins during virus purification. Therefore, the reduced infected/healthy ratio seen when previously frozen sap was used as the diluent (Table 5) probably resulted from the precipitation of host proteins needed for cross-adsorption. The more commonly used technique of cross-adsorbing the raw antiserum before purification of gamma-globulins resulted in a lower ratio, probably due to non-specific removal of antibodies against the virus. Other authors (2,18) have reported high background reactions with ACLSV antiserum and the need for cross-adsorption. The A and B isolates are serologically identical although they differ slightly in symptoms caused on herbaceous hosts (see Chapter 2). In agar gel diffusion tests, a continuous precipitin line was formed between the two isolates (Fig. 10) and they reacted identically in the ELISA test.

The initial fusions for MAB production were done using whole virus and resulted in clones specific for host components only.
Because of the virus morphology, the particles frequently become entangled with themselves and host material to form large aggregates which are difficult to separate. When injected into the mouse, such aggregates would illicit a high response to host antigens. When virus disrupted with SDS was used for immunization, six positive clones out of 480 screened were detected and fewer clones against host antigens were produced. Whether this success was due to injection of disrupted virus is not known. If only a small percentage of virus-specific hybridomas are produced (6/480 = 1.25%) these may have been missed during the screening process after injection of whole virus as not all hybridomas are stable and survive.

Most MABs are conformation-specific and recognize separate regions of amino acid sequence brought together by the folding of the polypeptide chains (77). The four MABs capable of detecting the virus in sap were separated into two categories, those that detect only strains from Prunus (A2, C3, D4) and those that detect all strains used in this study (B2). The clones specific for the Prunus strains gave a much stronger reaction than the broad spectrum clone and probably are directed against a conformational epitope on the surface of the virion while the broad spectrum clone may be specific for an internal epitope common to all strains. This is supported by the fact that the B2 clone cannot be used as a trapping antibody in ELISA. To verify this, the effects of adding substances such as SDS to the grinding buffer which would denature the virus and expose
internal epitopes should be investigated. Of the five grinding buffer additives investigated the best results were obtained with the standard buffer (PTB with 2% PVP) supplemented with 2.5% nicotine and 0.01M MgSO$_4$ (Table 7).

This research indicates that a monoclonal antibody (clone B2) may be useful as a broad spectrum detection tool for the many different biological strains of ACLSV in fruit trees, however, further characterization of the MABs must still be done. For instance, the B2 clone should be checked for heterospecific reactions against other closteroviruses. All clones should be tested against more ACLSV isolates in woody hosts, especially those that are difficult to detect virus from, such as pear and apricot. Different types of woody host tissues such as leaves, blossoms, cambium, and roots could be investigated. Also, the broad spectrum and Prunus-specific clones could be compared in competitive inhibition assays and possibly used to study antigenic variation in ACLSV strains.
Chapter 6 - Isolation of Tobacco Mosaic Virus

Introduction

Tobacco mosaic virus (TMV) is occasionally found as a latent infection in fruit trees, however, no symptoms have ever been observed on woody indicators (60). Infected trees show no symptoms and in sweet cherry the virus concentration is so low that attempts to isolate TMV from known infected plants often failed (30). In Hungary, Burgyan et al. (7) isolated TMV from plum which was serologically identical to TMV-U1 but differed slightly in symptomology on herbaceous hosts. The plum isolate occasionally produced a systemic latent infection in C. quinoa and produced a stem necrosis in Nicotiana glutinosa L.

Materials and Methods

The buffer used for transfer of the TMV isolates was 0.05M sodium phosphate, pH 7.5, containing 2% polyvinylpyrrolidone (PVP). For isolations from woody host leaves, flowers and roots, 1.0% nicotine was added to the buffers and the plants were rinsed with water after being inoculated. The following herbaceous plants were tested with the TMV isolates: Chenopodium amaranticolor Coste & Reyn., C. quinoa, Cucumis sativa L. 'Straight 8', Datura stramonium L., Nicotiana benthamiana Domin., Nicotiana clevelandii Gray, N. glutinosa, Nicotiana tabacum L. 'Harrowova', 'Xanthi', 'Samsun', Nicotiana rustica L., Vigna unguiculata (L.) Walp., and Zinnia elegans Jacq.

TMV-1292 was purified from N. benthamiana. The tissue was
homogenized in two volumes of 0.05M sodium phosphate pH 7.5 and 
clarified with 8.0% butanol. The supernatant was precipitated 
with 8.0% polyethylene glycol (PEG) and 1.0% NaCl, resuspended in 
0.1 volume phosphate buffer and given one low speed 
centrifugation of 20 min at 14,000 g. The sample was centrifuged 
at 111,000 g for 90 min and resuspended in 4.0 ml of buffer 
followed by isopycnic centrifugation in 40% caesium chloride for 
16 hr at 171,000 g. The viral band was withdrawn and dialysed 
against phosphate buffer.

Production of antiserum to TMV-1292 was as described in 
Chapter 5 for ACLSV.

Results

The original isolation of TMV-1292 was from the systemic 
leaves of isolate B ACLSV infected C.quinoa to one of four N. 
benthamniana. Further tests from P. glandulosa were done in which 
six of six N. benthamniana became infected but no local lesions 
appeared on N. tabacum 'Xanthi'. After one more successful 
isolation subsequent attempts failed to detect any TMV-1292. To 
test if the systemic host was actually more sensitive than the 
local lesion host, a series of 10-fold dilutions of purified TMV-
1292 from 1 mg/ml to 1 ng/ml were rub inoculated to the local 
lesion host N. glutinosa. The dilution endpoint was 10 ng/ml. 
Subsequently, four N. glutinosa and four N. benthamniana were 
inoculated with 10 ng/ml and 1 ng/ml purified virus. At the 
higher concentration one of four N. glutinosa developed lesions
while three of four N. benthamniana became infected. At the lower concentration, no N. glutinosa were infected and one of four N. benthamniana developed symptoms. The infected plants from both dilutions were back inoculated to N. glutinosa and N. benthamiana and all plants developed typical TMV symptoms. The herbaceous host range of TMV-1292 is consistent with other TMV isolates (Table 8).

The coat protein of the TMV-1292 was estimated at 17.9 KDa and migrated identically to TMV-U1 on the same gel. When dsRNA was extracted from TMV-1292 infected N. benthamniana the MW of the major band was $4.3 \times 10^6$ daltons with minor bands at $2.4 \times 10^6$ and $0.8 \times 10^6$ daltons. The ssRNA was estimated at $2.07 \times 10^6$ daltons and migrated at the same rate as TMV-U1 RNA on the same gel.

The TMV-1292 antiserum detected the homologous and U1 isolates in Nicotiana species by ELISA and purified virus by ELISA and agar gel diffusion. Repeated attempts to detect TMV in P. glandulosa failed as did attempts to re-isolate the virus from the woody host after the first two successful isolations.
Table 8. The herbaceous host range of tobacco mosaic virus (TMV) isolated from Prunus glandulosa.

<table>
<thead>
<tr>
<th>Species</th>
<th>Symptoms*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenopodium amaranticolor</td>
<td>nec 11</td>
</tr>
<tr>
<td>C. quinoa</td>
<td>nec 11</td>
</tr>
<tr>
<td>Cucumis sativa</td>
<td>11, sys dist</td>
</tr>
<tr>
<td>Datura stramonium</td>
<td>11</td>
</tr>
<tr>
<td>Nicotiana benthamiana</td>
<td>stunt</td>
</tr>
<tr>
<td>N. clevelandii</td>
<td>sys les</td>
</tr>
<tr>
<td>N. glutinosa</td>
<td>11</td>
</tr>
<tr>
<td>N. rustica</td>
<td>sys les</td>
</tr>
<tr>
<td>N. tabacum 'Harrownova'</td>
<td>sys les</td>
</tr>
<tr>
<td>N. tabacum 'Samsun'</td>
<td>sys les</td>
</tr>
<tr>
<td>N. tabacum 'Xanthi'</td>
<td>11</td>
</tr>
<tr>
<td>Vigna unguiculata</td>
<td>0</td>
</tr>
<tr>
<td>Zinnia elegans</td>
<td>11</td>
</tr>
</tbody>
</table>

*Key: 11 = local lesions; sys = systemic; les = lesions; dist = distortion; nec = necrotic; 0 = no infection
Discussion

The host range for TMV-1292 differed from the type strain range (38) only in the reaction on C. sativa (local lesions and systemic distortion). The unique symptoms in N. glutinosa reported by Burgyan et al. (7) for a plum isolate of TMV were not observed. TMV is known to give rise to a wide range of mutant types so this is not unusual (83). When isolating viruses from woody hosts, the virus may be missed because of low titre or may only produce a few lesions. In such cases it is necessary to build up the titre by a passage through the herbaceous host before attempting to determine the host range. This fact was demonstrated with the isolation of TMV from P. glandulosa when the usual local lesion indicators were not infected. The possibility of TMV contamination can be ruled out because extreme precautions were taken and none of the control plants showed any evidence of infection. The best explanation is that the TMV occurs in a low concentration in P. glandulosa and/or is irregularly distributed as in cherry (30).

The physical and serological characteristics of TMV-1292 were close to those previously reported (83,16) for a tobacco isolate of TMV.
Chapter 7 - General Discussion

In general the biological, physical and serological properties of the two ACLSV isolates studied were the same as those previously reported and confirm the diagnosis of the isolates as ACLSV. Some differences were noted in symptomology on herbaceous and woody indicators. Isolate A differed from the other ACLSV isolates by not moving systemically in C. amaranticolor and both A and B isolates failed to infect some species reported as susceptible by other authors. Further, the lack of symptoms on the woody indicators was unexpected. This variability has been reported previously (56,21) and can be expected for a virus that causes such a wide spectrum of diseases in it’s numerous hosts (17).

Closteroviruses are among the most difficult viruses to purify. Yields are typically low, as the viruses are phloem limited, and the particles frequently damaged. The particles also aggregate and are adsorbed to host membranes (3). The purification method described in this paper is simple and not as dependent on the initial clarification step as are other ACLSV purification methods. The ELISA values for the purification pellets and supernatant show that a considerable amount of virus is lost during low speed centrifugation. If suitable conditions of ionic strength or molarity could be found under which the particle adsorption to host tissue was decreased, the virus yield could be substantially increased. The second area of ACLSV purification needing improvement is the elimination of the
contaminating spherical structures responsible for the serological reactions to host tissue observed with the polyclonal antisera. The spheres could not be purified by the ACLSV method from healthy C. quinoa and may be present in large numbers only in virus-infected or stressed plants. If an antiserum specific for the spheres were produced, they may be eliminated by cross-adsorption.

This study agrees with previous reports that the typical beet yellows type of vesicles found associated with subgroup I closteroviruses are not present in ACLSV infected tissue (25). The particle length, width and pitch are also similar to those previously reported (4,72). Unfortunately, the long flexuous rods seen in the vascular elements could not be distinguished from P-protein. As part of the further characterization of the monoclonal antibodies, their ability to bind to intact virions and react with protein-A colloidal gold marker should be investigated. This technique would allow for a definitive identification of the flexuous rods in the sieve tube elements and facilitate further studies on ACLSV cytopathology, especially in light of the recent report of ACLSV particles in the mesophyll of C. quinoa (61).

As more research is done on the closteroviruses, it is clear that the group is not homogeneous, but made up of a number of subgroups which are related only on the basis of gross morphology. ACLSV differs from the type member beet yellows in particle length, cytopathology (lack of vesicles, not phloem
limited), no aphid vector, sap transmission and dsRNA pattern (25,19,81,61) and will probably be separated from the closteroviruses in the future as more information is gathered on the genome organization of group members.

The serological results with the polyclonal antisera emphasize the problems encountered with antisera produced from contaminated virus preparations. In this case the contaminating protein was difficult to eliminate and the antiserum needed cross-adsorption by diluting the enzyme-conjugate in host sap. Results for ACLSV detection in P. glandulosa by this method were variable, probably due to irregular virus distribution (26) or low virus titre. A certification program based solely on serological techniques using polyclonal antiserum would clearly be inadequate unless extensive testing of the most suitable type of host tissue, the optimum time of year for sampling and the reactions against the various strains were done. Grüntzig and Fuchs (34) found that unless the ELISA test was done using petals in April to May, a biological test on C. quinoa should be used to ensure a 90% reliability rate.

Monoclonal antibodies offer increased detection sensitivity over polyclonal antibodies and give low background levels. With the appropriate screening methods, clones can be selected with varying degrees of serological specificity (57). Poul and Dunez (66) produced two monoclonal lines which recognized 17 ACLSV isolates from Prunus and Malus. Their ELISA test used one MAB for coating and the second was labelled with alkaline phosphatase.
These types of experiments must still be done with the isolate A monoclonals. The ultimate goal is to devise an ELISA technique for routine detection of ACLSV from woody plants and evaluate the optimum tissue type and time of year for sampling. The B2 MAB is the only one with potential for this type of application unless the tests were limited to Prunus species. Other areas of research such as virus elimination, vector relationships and surveys could now be done using the MABs.

All of the isolates of TMV recovered from stone fruits resemble the tomato or Dahlemense isolates (30). The TMV isolated from P. glandulosa does not differ in serological or physical characteristics from the Ul type strain. As in other fruit trees, TMV probably occurs in P. glandulosa as a latent infection in low concentrations.
References Cited


