DETECTION, PURIFICATION AND CHARACTERIZATION OF AND HOST RESPONSE TO WHEAT SPINDLE STREAK MOSAIC VIRUS

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ABSTRACT

DETECTION, PURIFICATION AND CHARACTERIZATION OF
AND HOST RESPONSE TO
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Wheat spindle streak mosaic (WSSM) is a prominent virus disease of winter wheat in most of the wheat growing regions in the United States and Canada. Until now, accurate diagnosis of WSSMM was difficult, as symptoms mimic those of other wheat virus diseases, and wheat spindle streak mosaic virus (WSSMV) particles occur sparsely in infected tissues. A modification of the serological technique immunosorbent electron microscopy (ISEM) was developed as a rapid, specific and sensitive technique for the detection of WSSMV in infected tissues. ISEM was greater than two hundred times more sensitive than conventional electron microscopy for detecting WSSMV in leaf extracts.

At present, most of the commercially-grown winter wheat cultivars are susceptible to WSSMV, and recommended cultural practices have not controlled the disease. To identify resistant germplasm, commercial cultivars and experimental lines of soft white and soft red winter

wheats were rated for their reaction to WSSMV based on symptom expression and virus titer in infected leaves as determined by ISEM. Several cultivars and lines showed resistance to WSSMV; this resistance will be incorporated into adapted lines showing high yield potential to improve cultivar performance. Cultivars and lines resistant to the virus were susceptible to the fungal vector, indicating that resistance is due to resitance to the virus rather than the vector.

ISEM was also used to monitor the presence of virus in each step during the development of a purification protocol for WSSMV. A procedure which yielded sufficient intact virus for biochemical characterization consisted of clarification of tissue extracts in chloroform, concentration of particles by polyethylene glycol and further purification by sucrose-cesium sulfate density gradient centrifugation. Purified particles had a modal length of 800 nm, a buoyant density of 1.20-1.21 g/cm^3 and an $A_{260/280}$ absorption ratio of 1.12-1.25. These data, in addition to the inclusion protein molecular weight of 69,000 and molecular weights of 36,000, 32,000 and 29,000 for coat proteins, provide evidence for inclusion of WSSMV in the potyvirus group.

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INTRODUCTION

Wheat spindle streak mosaic (WSSM) commonly occurs in most of the wheat growing regions in the United States and Canada. Diagnosis is based on foliar symptoms, the presence of cytoplasmic inclusions in leaf ultrathin sections, and long, sparsely-occurring wheat spindle streak mosaic virus (WSSMV) particles in leaf-dip preparations, all of which may be diagnostic for other wheat virus diseases. Because it is important for the wheat grower as well as the researcher to be able to accurately identify WSSM, a sensitive and specific assay for the rapid detection of WSSMV in infected wheat was sought. Chapter I is devoted to the development of this assay.

At present, most of the commercially-grown and newly-released winter wheat cultivars are susceptible to WSSMV. Because recommended cultural practices have not effectively controlled the disease, it is important to identify germplasm with resistance to WSSMV so that cultivars with WSSMV resistance can be developed. Chapter II is devoted to studies of host reactions to WSSM and the identification of cultivars and lines with resistance to WSSMV.

Although much is known about WSSM symptomatology, epidemiology and cytopathology, little is known about the disease agent and its properties. Only one purification protocol (Usugi and Saito, 1979) for WSSMV has been reported. Particles in purified preparations were highly

fragmented and therefore unsuitable for biochemical characterization. It was the purpose of the third phase of this research to develop a purification scheme which would yield sufficient, intact particles for characterization purposes, and to identify and characterize the genome, capsid protein and inclusion protein of WSSMV. Chapter III is devoted to the development of a suitable purification protocol and the characterization of WSSMV-associated proteins. Experiments on the identification of genomic nucleic acid and double-stranded RNA species from WSSMV are reported in the Appendix.

Chapter I has been published in a condensed form (Haufler and Fulbright, 1983). Chapter II has been accepted for publication (Haufler and Fulbright, 1986), and Chapter III is being prepared for publication.

LITERATURE REVIEW

Disease description and geographical distribution

One of the most prominent virus diseases of soft red and white winter wheat (*Triticum aestivum* L.) in most of the wheat growing regions in the United States and Canada is wheat spindle streak mosaic (WSSM). The disease was first recognized on winter wheat in southern Ontario, Canada in 1957 by Slykhuis (20), and during the next several years annual surveys were conducted throughout Ontario to determine the distribution of the disease. Approximately 60% of the fields examined during these years contained infected plants, and 36% of the plants in these fields showed WSSM symptoms (7, 11, 22, 27). Estimates of reductions in grain yield ranged from 3-59% (7, 11, 22).

Slykhuis (20, 21) first described the disease as "Ontario soil-borne wheat mosaic", characterized by bronzing and necrosis of lower leaves and a light green to yellow mosaic with spots and short streaks on younger leaves. Dashes and streaks were parallel to the leaf axis and many were spindle-shaped. The chlorotic markings were first apparent near the tip of the leaf blade; they then increased and diffused throughout the blade, eventually coalescing into large chlorotic areas which later became necrotic. Plants with symptoms were less vigorous than normal plants when observed in early May, but appeared near-normal when observed again in mid-June. Symptoms were readily found on most commercially-grown wheat cultivars, but no symptoms were found on winter barley (*Hordeum vulgare* L.) or rye (*Secale*

cereale L.) (21, 22).

"Wheat variegation," as it was called in Michigan, was first observed in the Thumb area of that state in 1961 (33). By 1968, the disease was widespread throughout the wheat-growing regions of the lower peninsula. Its rapid expansion throughout Ontario, Michigan and other northeastern states prompted the initiation of a conference on wheat variegation at Michigan State University in early 1969. Researchers from 11 states and Canada presented reports on the occurrence of the disease in their area. Data on disease distribution, transmission, cytopathology and varietal reactions were exchanged and discussed.

Mosaic diseases of wheat caused by soil-borne viruses had previously been identified in the U.S. (14), Japan (31) and Italy (5). WSSM differed from the diseases caused by either soilborne wheat mosaic virus (SBWMV) or wheat streak mosaic virus (WSMV) in that it affected neither rye nor barley (21,22) and did not develop at temperatures above 15 C (22). Less than a year after the wheat variegation conference, the etiological agent of WSSM was identified as a sparsely-occurring, thread-like virus particle much different from the shorter, thicker rods of SBWMV; the virus was designated wheat spindle streak mosaic virus (WSSMV) (22,27).

Once WSSM had been identified as a new disease caused by a previously unknown virus, reports of its occurrence throughout the U.S. and Europe became more common. The disease was observed in several areas of Kentucky (35), where most of the wheat varieties and breeding lines were rated moderately to highly susceptible to WSSMV. Jackson et al. (9) reported having periodically observed WSSM throughout Indiana

since the early 1960's. Leaf-dip preparations from susceptible cultivars revealed flexuous virus particles up to 5000 nm in length. WSSM significantly reduced plant growth and grain yield in Pennsylvania red wheat in 1977-1978 (15). The disease was also reported in India (1), France (19), New York and Maryland during the mid-1970's. In 1982, Brakke and coworkers (4) reported the first observation of WSSM in Nebraska. Cultivars resistant to SBWMV, commonly found in Nebraska, were susceptible to WSSMV. A mixed infection of SBWMV and WSSMV was found in Kansas (13) in a wheat cultivar considered resistant to SBWMV. The authors suggested that the presence of WSSMV may somehow have altered resistance to SBWMV in this cultivar. The first published report of WSSM in the southeastern U.S. came from Georgia (3), where significant yield losses due to the disease occurred in 1984. Additional evidence of the occurrence of WSSM in the U.S. has come from Tennessee (B.B. Reddick, personal communication), Virginia (S.A. Tolin, personal communication), Oklahoma (J.L. Sherwood, personal communication) and Ohio (O.E. Bradfute, personal communication). In Europe and Asia, West Germany reported the occurrence of WSSM in 1983 (17). Antiserum made to WSSMV isolated from Michigan wheat reacted serologically with particles in West German plants (H. Kleinhempel, personal communication). Researchers in both Italy (C. Rubies, personal communication) and South Africa (M.B. Von Wechmar, personal communication) are currently using Michigan WSSMV antiserum to determine the identity of a flexuous virus with characteristics similar to WSSMV.

It is likely that WSSM was present in some of these areas prior to its actual identification, as symptoms may have been masked by or

confused with, those of soilborne wheat mosaic (SBWM), wheat streak mosaic (WSM) and Agropyron mosaic (26), Septoria leaf blotch, and damage caused by low spring temperatures (9) and wet soil conditions.

Host range

Until recently WSSM was known to affect only winter wheat and durum wheat (Triticum durum Desf.). Slykhuis (21) originally observed that only wheat became infected when winter barley, rye and wheat were grown in flats of soil from fields in which diseased wheat had been found. These results were later confirmed in field plot studies (22). Many gramineaceous and dicotyledonous species were tested for WSSM symptom development following mechanical inoculation or growth in infectious soil (10, 17, 22). No local lesion hosts were identified among the dicotyledonous plants tested. However, rye was recently identified as a host for WSSMV (3, 17). Proeseler and Stanarius (17) in 1983 reported the presence of a flexuous rod occurring in low concentrations in leaf dips from wheat and rye. Virus particle morphology, infected cell ultrastructure and temperature requirements for disease development all were characteristic of WSSM. Michigan WSSMV antiserum reacted serologically with these particles from rye (H. Kleinhempel, personal communication), thereby confirming the identity of the virus. Symptoms of WSSM were also found on rye in Georgia (3).

Virus transmission

Although WSSM symptoms were considered attributable to a soil-borne agent when the disease was first observed in Canada, it was not until 18 years later that a potential virus vector was identified. Researchers at the Wheat Variegation Conference discussed the

possibilities of fungal, nematode, aphid and mite vectors; L.R. Nault from Ohio claimed to have achieved a low level of transmission with the eriophyid mite Aceria tulipae. A year later, Slykhuis (22) reported several conditions favoring transmission and development of WSSM. He observed that infectivity was found in several soil types and that it persisted for many years even when soils were dry. Infectivity was associated with an agent that readily passed through fine (44 um) screens, and was eliminated by heating for 30 minutes at 52 C or by treating soil with various chemicals, including fungicides. High levels of urea, ammonium nitrate, manure and sucrose greatly reduced or eliminated disease development. Infectivity was not transmitted through seed.

A few years later, Barr and Slykhuis (2) examined roots of wheat plants grown in soil samples previously tested for WSSM from collections made in Canada and the U.S. Although several species of zoosporic fungi such as Olpidium brassicae, Rhizophydium graminis and Pythium spp. were found associated with roots of plants grown in known infectious soil, Polymyxa graminis and Lagena radicicola were found the most frequently. Because P. graminis was most abundant in infectious soil samples and was absent in non-infectious samples, and because of its reported association with SBWMV (18), the authors suggested that P. graminis was the vector of WSSMV.

In 1978 Slykhuis and Barr (29) provided additional evidence for P. graminis as a vector of WSSMV. Transmission of WSSMV by unifungal cultures of P. graminis, O. brassicae, R. graminis, L. radicicola and Pythium spp. was studied by inoculating wheat seedlings with the fungi

in sterile soil and mechanically inoculating leaves with virus-infected sap. Plants showing symptoms were removed and planted with test seedlings which were later removed and placed at 10 C. Only those test plants which had been placed in soil containing P. graminis eventually developed symptoms. The authors were unsuccessful, however, in achieving transmission to seedling roots via water in which roots of naturally diseased plants had soaked, as had been demonstrated for SBWMV (18). In addition, no test seedlings developed WSSM symptoms from growing in association with pieces of roots from diseased plants bearing specific zoosporic fungi, including P. graminis. Nolt et al. (16) successfully transmitted WSSM by the root-washing and root-piece transmission methods, but transmission rates were low and poorly reproducible, and roots of infected plants contained either O. brassicae or L. radicicola in addition to P. graminis. Although much of the evidence supports the conclusion that P. graminis is the vector of WSSMV, the association between virus and possible vector will remain only correlative until natural transmission with pure cultures of the vector is unequivocally demonstrated.

Factors affecting disease development

The conditions necessary for WSSM infection and symptom development have been studied extensively (22, 23, 25). Temperature is the most important factor affecting disease development. Wheat sown in infested soil in the autumn becomes infected after emergence when soil temperature is between 8 and 18 C, with infection occcurring optimally at 15 C (23, 25). This is within the range of temperatures (15-22 C) favoring development of P. graminis (29). Symptoms become evident and

develop rapidly when growth resumes in the spring at temperatures between 5 and 15 C, with symptoms developing optimally at 10 C (23, 25).

Spring infections can occur but are rare. Symptoms have not been found on spring-infected wheat probably because the period is too short for infection and incubation to be completed before warmer temperatures inhibit symptom development (25).

Soil transmission under controlled conditions can be achieved in several ways. WSSM symptoms developed on wheat grown in infested soil and placed in a growth chamber at 5-13 C (10 C optimum) with 10,000-15,000 lux of light 12 hours/day (22). Symptoms usually became apparent after 60 days in the growth chamber. Wiese and Hooper (32) reported that a period at lowered temperatures, either for 2 months in a 1 C growth chamber or 1-2 months in an outdoor cold frame (with soil temperature 3±5 C), markedly increased both symptom severity and the percentage of test plants affected. This vernalization effect probably mimics the sequence of temperatures to which field wheat is exposed.

Slykhuis (26) identified factors stimulating the development of WSSM in plants growing in infested soil at 15 C for 4 weeks. Transplanting and other manipulations causing slight injury to the root system, such as shaking or washing soil from roots, and transferring plants to soil containing quartz sand or amendments such as ammonium nitrate, stimulated the development of symptoms at 10 C. Overwintering plants in nature are subjected to freezing and thawing of soil which damages roots, similar to the effects of transplanting and root washing, thereby possibly promoting fungal zoospore infection of roots. Physical damage caused by freezing and thawing may be the reason why outdoor cold

frame treatments were more effective in promoting disease development than growing plants in a 1 C growth chamber (32).

Factors governing the mechanical transmissibility of WSSMV have also been investigated (10, 22, 24, 28). Initial attempts at manual transmission involved grinding infected leaves in distilled water to which celite or 600-mesh carborundum was added, and rubbing the mixture on leaves of two-leaf stage plants (22). Some plants developed symptoms after 4-8 weeks at 8-12 C, but results were erratic. Slykhuis and Polak (28) later reported that sap from infected plants used as inoculum retained infectivity longer at pH 7.0 and above than at lower pH levels. They compared three methods of mechanical inoculation and found that the leaf tissue rub method, which used freshly-abraded infected leaves as an inoculum source, produced the highest percentage of infected plants. The artist's airbrush method was not as satisfactory as the leaf tissue rub method, but was superior to the conventional leaf rub method in promoting infection. For the latter two inoculation methods, inoculum prepared by grinding diseased leaves in 0.1 M phosphate buffer, pH 7.0, 0.5 M borate buffer, pH 9.0, or in 0.1 M sodium sulfite at a high concentration (1 g leaves:3-4 ml diluent) was the most effective for virus transmission (24). The virus was more readily transmitted from older, severely chlorotic leaves than from younger, lightly mottled leaves, and plants in the two- to five-leaf stages were most susceptible. Jackson et al. (10) found that the artist's airbrush method gave a nearly three-fold greater proportion of infected plants than did the leaf tissue rub method, contrary to the results obtained by Slykhuis and Polak (24).

Disease effects

Of primary importance to the plant pathologist when a new disease is being investigated are data pertaining to the effects of the disease on the host crop(s). Although such data for WSSM are of somewhat limited value due to experimental differences, they will be briefly reviewed here. WSSM-affected plants are slightly stunted and have reduced seed weight per head and reduced 1000-kernel weight; these parameters are reduced by less than 10%, however (34). The major effect of WSSM is reduced tillering, and, therefore, reduced seed yield. Wiese et al. (34) reported that numbers of tillers were reduced by as much as 37% in susceptible cultivars.

Estimates of grain losses due to WSSM vary widely, ranging from 3-64% (3, 7, 11, 15, 22, 34). Yield losses are usually only light to moderate and are dependent upon the duration of cool weather in the spring and on the susceptibility of the cultivar planted. The earliest estimates of yield loss from WSSM were low: 3-5%, or approximately 2.2 bushels per acre (7, 11). Slykhuis (22) reported grain losses of 7-59% in small experimental plots. In one farmer's field, however, disease symptoms were rated moderately severe in early May, yet a record wheat yield was reported after harvest in July (22). By combining yield losses per plant with the incidence of WSSM per cultivar, Wiese et al. (34) calculated grain losses of 3-18% among the susceptible cultivars commonly grown in Michigan. Nguyen and Pfeifer (15) reported significant plant height reduction and grain losses from 7-64% among susceptible cultivars in Pennsylvania, while Bays et al. (3) found a 35%

yield reduction in one susceptible cultivar in Georgia.

Control

Suggested cultural practices and soil treatments for controlling WSSM have had only limited success. Because early planting increased disease incidence in susceptible cultivars (9, 15), late-autumn planting was suggested as a cultural means of controlling WSSM. This practice reduced disease incidence but resulted in greater yield losses due to increased winter kill of late-planted wheat (15, 22, 25). As previously mentioned, vector control by chemicals such as urea, manure and ammonium nitrate, and fungicides such as methyl bromide were partially effective in suppressing disease development (15, 22) but are impractical for use over large acreages.

The most efficient and effective method of controlling WSSM is the use of resistant cultivars. Moderate resistance to WSSMV has been reported for cultivars Monon (10), Yorkstar, Yamhill and Tecumseh (34), and Hart, Ruler and Blueboy (15), but most of these cultivars have been replaced with higher-yielding cultivars, which are susceptible to WSSMV. Resistance was also found in selected Purdue breeding lines (10).

Cytopathology

Ultrathin sections of leaf tissues from WSSMV-infected plants exhibit characteristic cytopathic abnormalities not present in tissues from healthy plants. Hooper and Wiese (8, 32) first examined ultrathin sections of WSSMV-infected leaves and found cytoplasmic cylindrical (pinwheel) inclusions and rod-like particles within cells from green and chlorotic areas of diseased leaves. Both pinwheel inclusions and virus-like particles were observed most frequently in mesophyll cells, but

also occurred in epidermal and vascular parenchyma cells. The inclusions were characteristic of those observed in plants infected with long flexuous viruses (6). Virus-like particles occurred as aggregated bundles in the cytoplasm and as single particles evenly spaced and aligned along pinwheel arms. Particles were 20-22 nm in diameter and of an undetermined length. In addition to pinwheel inclusions, complex membranous bodies and crystalline material associated with aggregates of virus-like rods were found in all leaf cell types except mature sieve elements and differentiated xylem cells. Inclusions were also observed in cortical, endodermal and vascular parenchyma cells in roots of affected plants. The authors proposed a developmental sequence of inclusion formation which first involved the formation of membranous bodies from tubes or sacs of unknown origin. The bodies became ordered into membranous sheets which interconnected at regular points to form pinwheels. Evaginations of the membrane between each pinwheel arm possibly provided a surface for inclusion or viral synthesis. Virus-like rods appeared in conjunction with the development of pinwheel inclusions, and their length frequently exceeded 2500 nm. All inclusion types were readily apparent by the time symptoms were evident. In severely chlorotic and necrotic tissues, the cellular constituents and inclusions degenerated, filling cells with debris. Langenberg and Schroeder (12) confirmed Hooper and Wiese's proposed inclusion developmental sequence and found that the membranous bodies consisted of convoluted plates and tubules originating from the endoplasmic reticulum.

Virus properties

While WSSM epidemiology, symptomatology and tissue cytopathology are well-documented, less is known about the disease agent and its properties. Slykhuis and Polak (28) observed long, slightly flexuous virus particles 12.8 nm in diameter and 190-1975 nm in length in leaf-dip preparations from WSSM-affected plants. Peaks in particle length frequency occurred at 275 nm and 625 nm. Particles ranging from 200-1,500 nm in length (30) and those with a diameter of 14 nm and exceeding 2,000 nm in length (9) in leaf dips have also been reported. In ultrathin leaf sections, particles measured 18-20 nm in diameter and frequently exceeded 3,000 nm in length (8, 12).

The virus appears to be unstable in undiluted sap, as infectivity was lost within 1 hour at 10 C (28). Extracts diluted in water lost infectivity within 1 hour at 20 C, while extracts similarly prepared in 0.5 M sodium borate (pH 9.0), 0.1 M potassium phosphate (pH 7.0) or 0.1 M sodium sulfite were still infectious after 4 days at 10 C. Reported thermal inactivation points were 45-50 C for 10 minutes (28, 30), and a dilution endpoint of 1:1,000-5,000 was recorded (30).

Initial attempts to purify WSSMV were unsuccessful (28). A partially-purified preparation was obtained by grinding WSSM-infected leaves in 0.5 M sodium borate, pH 9.0, clarifying with butanol and chloroform, centrifuging the emulsion, then subjecting the top aqueous phase to differential centrifugation and resuspending the pellet in phosphate buffer. The resulting preparations contained particles 90-1,000 nm in length, with peaks in frequency at 300 nm and 540 nm. The partially-purified preparations were not infectious, possibly because

longer, more intact particles were necessary for infectivity. Attempts to develop an antiserum from these preparations were unsuccessful, as it was discovered that WSSMV particles flocculated spontaneously at pH 7.0 in microprecipitin tests. Hooper and Wiese (unpublished) also observed particles under 1,000 nm in partially-purified preparations, and efforts to further purify the virus also were unsuccessful.

In 1979 Usugi and Saito (36) successfully purified WSSMV as follows: infected leaves were ground in 0.1 M citrate or phosphate buffer, pH 7.0, and the resulting extract was filtered through cheesecloth and then clarified with 20% (v/v) carbon tetrachloride or chloroform. The clarified preparation was subjected to three cycles of differential centrifugation followed by either sucrose or cesium chloride density gradient centrifugation. The purified preparation was infectious and contained particles 300-600 nm in length. This suggested that infectivity was associated with particles whose length was much less than 1,000 nm, contradicting Slykhuis and Polak's idea (28) that only particles longer than 1,000 nm are infectious. The buoyant density of WSSMV in cesium chloride was 1.28 g/cm^3. No additional data on particle properties or composition were reported.

The Japanese workers also were the first to prepare an antiserum to WSSMV (36). Investigating the relationship between wheat yellow mosaic virus (WYMV) in Japan and WSSMV from Canada using antisera specific for both viruses, they found that WYMV and WSSMV were closely related but not identical. In addition, a challenge infection by WSSMV was completely suppressed in plants latently infected with WYMV, demonstrating cross-protection between these two viruses. Based on

these results as well as other similarities between the viruses, such as similar inactivation and dilution endpoints, particle morphology and length distribution, and buoyant density, the authors concluded that WSSMV was a North American strain of WYMV.

LITERATURE CITED

1. Anlawat, Y.S., Majumdar, A., and Chenulu, V.V. 1976. First record of wheat spindle streak mosaic in India. Plant Dis. Reptr. 60:782-783.

2. Barr, D.J.S., and Slykhuis, J.T. 1976. Further observations on zoosporic fungi associated with wheat spindle streak mosaic virus. Can. Plant Dis. Surv. 56:77-81.

3. Bays, D.C., Cunfer, B.M., and Demski, J.W. 1985. Occurrence of wheat spindle streak mosaic virus on winter wheat in Georgia. Plant Dis. 69:1094-1096.

4. Brakke, M.K., Langenberg, W.G., and Samson, R.G. 1982. Wheat spindle streak mosaic virus in Nebraska. Plant Dis. 66:958-959.

5. Canova, A. 1964. Richerche sulle malattie da virus delle Graminaceae. I. Wheat soil-borne mosaic. Phytopathol. Mediterranea 3:86-94.

6. Edwardson, J.R., Purcifull, D.E., and Christie, R.G. 1968. Structure of cytoplasmic inclusions in plants infected with rod-shaped viruses. Virology 34:250-263.

7. Gates, L.F. 1969. Incidence and effects of wheat spindle streak mosaic in Essex and Kent counties, Ontario, 1967-68. Can. Plant Dis. Surv. 51:24-31.

8. Hooper, G.R., and Wiese, M.V. 1972. Cytoplasmic inclusions in wheat affected by wheat spindle streak mosaic. Virology 47:664-672.

9. Jackson, A.O., Bracker, C.E., Huber, D.M., Scott, D.H., and Shaner, G. 1975. The occurrence and transmission of a disease in Indiana with properties of wheat spindle streak mosaic virus. Plant Dis. Reptr. 59:790-794.

10. Jackson, A.O., Bracker, C.E., and Shaner, G. 1976. Mechanical transmission, varietal reactions and electron microscopy of a disease in Indiana with properties of wheat spindle streak mosaic. Plant Dis. Reptr. 60:202-206.

11. James, W.C. 1971. Importance of foliage diseases of winter wheat in Ontario in 1969 and 1970. Can. Plant Dis. Surv. 51:24-31.

12. Langenberg, W.G., and Schroeder, H.F. 1973. Endoplasmic reticulum-derived pinwheels in wheat infected with wheat spindle streak mosaic virus. Virology 55:218-223.

13. Lommel, S.A., and Willis, W.G. 1984. The role of wheat spindle

streak and wheat soilborne mosaic viruses in an epiphytotic of resistant wheat in Kansas. (Abstr.) Phytopathology 74:844.

14. McKinney, H.H. 1931. Differentiation of viruses causing green and yellow mosaics of wheat. Science 73:650-651.

15. Nguyen, H.T., and Pfeifer, R.P. 1980. Effects of wheat spindle streak mosaic virus on winter wheat. Plant Dis. 64:181-184.

16. Nolt, B.L., Romaine, C.P., Smith, S.H., and Cole, H., Jr. 1981. Further evidence for the association of Polymyxa graminis with the transmission of wheat spindle streak mosaic virus. Phytopathology 71:1269-1272.

17. Proessler, G., and Stanarius, A. 1983. Nachweis des Weizenspindel-strichelmosaik-Virus (wheat spindle streak mosaic virus) in der DDR. Arch. Phytopathol. u. Pflanzenschutz, Berlin 19:345-349.

18. Rao, A.S., and Brakke, M.K. 1969. Relation of soil-borne wheat mosaic virus and its fungal vector, Polymyxa graminis. Phytopathology 59:581-587.

19. Signoret, P.A., Alliot, B., and Poinso, B. 1977. Presence en France du wheat spindle streak mosaic virus. Ann Phytopathol. 9:377-379.

20. Slykhuis, J.T. 1960. Evidence of a soil-borne mosaic of wheat in Ontario, Canada. Can. Plant Dis. Surv. 40:43.

21. Slykuis, J.T. 1961. The causes and distribution of mosaic diseases of wheat in Canada in 1961. Can. Plant Dis. Surv. 41:330-343.

22. Slykhuis, J.T. 1970. Factors determining the development of wheat spindle streak mosaic caused by a soil-borne virus in Ontario. Phytopathology 60:319-331.

23. Slykhuis, J.T. 1974. Differentiation of transmission and incubation temperatures for wheat spindle streak mosaic virus. Phytopathology 64:554-557.

24. Slykhuis, J.T. 1975. Factors critical to mechanical transmissibility of wheat spindle streak mosaic virus. Phytopathology 65: 582-584.

25. Slykhuis, J.T. 1975. Seasonal transmission of wheat spindle streak mosaic virus. Phytopathology 65:1133-1136.

26. Slykhuis, J.T. 1976. Stimulating effects of transplanting on the development of wheat spindle streak mosaic virus in wheat plants

infected from soil. Phytopathology 66:130-131.

27. Slykhuis, J.T., and Polak, Z. 1969. Verification of wheat spindle streak mosaic virus as a cause of mosaic of wheat in Ontario. Can. Plant Dis. Surv. 49:108-111.

28. Slykhuis, J.T., and Polak, Z. 1971. Factors affecting manual transmission, purification, and particle lengths of wheat spindle streak mosaic virus. Phytopathology 61:569-574.

29. Slykhuis, J.T., and Barr, D.J.S. 1978. Confirmation of Polymyxa graminis as a vector of wheat spindle streak mosaic virus. Phytopathology 68:639-643.

30. Usugi, T., and Saito, Y. 1979. Relationship between wheat yellow mosaic virus and wheat spindle streak mosaic virus. Ann. Phytopathol. Soc. Jpn 45:397-400.

31. Wada, E., and Fukano, H. 1937. On the difference and discrimination of wheat mosaics in Japan. Jpn. Imp. Agr. Exp. Sta. 3:93-128.

32. Wiese, M.V., and Hooper, G.R. 1971. Soil transmission and electron microscopy of wheat spindle streak mosaic. Phytopathology 61:331-332.

33. Wiese, M.V., Saari, E.E., Clayton, J., and Ellingboe, A.H. 1970. Occurrence of wheat streak mosaic and a new variegation disorder, wheat spindle streak mosaic, in Michigan wheat. Plant Dis. Reptr. 54:635-637.

34. Wiese, M.V., Ravenscroft, A.V., and Everson, E.H. 1974. Incidence of wheat spindle streak mosaic among ten wheat cultivars and its effects on yield. Plant Dis. Reptr. 58:522-525.

35. Williams, A.S., Pirone, T.P., Slykhuis, J.T., and Tutt, C.R. 1975. Wheat spindle streak mosaic virus in Kentucky. Plant Dis. Reptr. 59:888-889.

CHAPTER I

DETECTION OF WHEAT SPINDLE STREAK MOSAIC VIRUS BY IMMUNOSORBENT ELECTRON MICROSCOPY

CHAPTER I

Detection of Wheat Spindle Streak Mosaic Virus by Immunosorbent Electron Microscopy

Abstract

Wheat spindle streak mosaic virus (WSSMV) was readily detected by immunosorbent electron microscopy (ISEM) in roots, crowns and leaves of wheat plants suspected to be infected with this virus. ISEM was at least two hundred times more sensitive than conventional electron microscopy for detecting WSSMV in crude leaf extracts from field and cold-frame plants. ISEM also enhanced detection of particles in various tissues prior to appearance of foliar symptoms. WSSMV particles were 16 nm in diameter and ranged from 370 to 3,800 nm in length, with a modal length of 1,775 nm. ISEM provides a routine rapid assay for detecting WSSMV-infected plants.

Introduction

Wheat spindle streak mosaic (WSSM) commonly occurs on winter wheat during cool spring seasons in many of the wheat-growing areas in the United States and Canada (2, 4, 10, 11, 15, 18, 25, 27). Diagnosis of WSSM is usually based on foliar symptoms (18), the presence of the suspected vector Polymyxa graminis in infected roots (14), the presence of cytoplasmic (pinwheel) inclusions in leaf ultrathin sections (26) and long, sparsely-occurring wheat spindle streak mosaic virus (WSSMV) particles in leaf-dip preparations (20). WSSM symptoms can easily be confused with those of soilborne wheat mosaic (SBWM), which is also favored by low temperatures (24), as well as symptoms of wheat streak mosaic (WSM), Agropyron mosaic (AM) and barley yellow dwarf (BYD). P. graminis is also the reported vector of soilborne wheat mosaic virus (SBWMV) (3), and fungal propagules of P. graminis can readily be found in roots of virus-free wheat plants (14). Preparation of leaf ultrathin sections is difficult and time-consuming, and pinwheel inclusions are also diagnostic for wheat streak mosaic virus (WSMV) (24). Electron microscopic examinations of crude sap can also be misleading, as particles of WSMV and soil-borne viruses such as oat mosaic virus (OMV) are morphologically similar to WSSMV. WSSMV concentration in infected tissues appears to be low, and particles are fragile and break easily, making accurate identification using conventional transmission electron microscope methods difficult. Because it is important for the grower as well as the researcher to be able to distinguish WSSM from other wheat diseases, a sensitive and specific assay for the rapid detection of

WSSMV in infected wheat was sought.

Several methods for the detection and identification of plant viruses using serological techniques have been developed (13). Of the methods combining serology with electron microscopy, the procedure described by Ball and Brakke (1) included electron microscopic examination of virus-antiserum mixtures that had been dried onto grids. Salts and cellular components which make virus particle visualization difficult, in addition to low numbers of particles attached onto grids, limit the usefulness of this approach. In 1973 Derrick (6) introduced a method for quantitative assay of plant viruses attached to electron microscope grids coated with virus-specific antiserum. In serologically specific electron microscopy (SSEM), or immunosorbent electron microscopy (ISEM) as it is now called (17), virus particles are specifically trapped and concentrated on the surface of grids to which a film of specific antiserum has been absorbed. Two- to one thousand-fold increases in numbers of particles observed on grids when ISEM was used have been reported (6, 8, 12). Salts and other components in the virus extract are removed by washing, allowing rapid visualization and quantification of virus particles with the electron microscope.

Another very useful serological technique for virus detection is enzyme-linked immunosorbent assay (ELISA) (5). In this procedure, virus in the test sample is selectively trapped and immobilized by specific antibody adsorbed onto plastic microtiter plates instead of microscope grids as in ISEM. Enzyme-labeled specific antibody is added to the plate and, after excess enzyme-labeled antibody is removed, the

remaining enzyme is assayed by adding a suitable substrate. Substrates which yield colored reaction products allow rapid detection of virus in samples as well as a quantitative measurement of virus concentration.

Both ISEM and ELISA are widely accepted and useful immunosorbent techniques for detection of plant viruses. Because the sensitivity of ISEM is comparable to or greater than that of ELISA (7, 13), and because much smaller volumes of virus extract and antiserum are required, ISEM was chosen for the detection of WSSMV in wheat grown in the field and cold frame during the 1980-1981 and 1981-1982 growing seasons.

Materials and Methods

Field studies. Triticum aestivum L. cv. Ionia plants showing WSSM symptoms were collected from fields in Allegan County, MI in May, 1982. Field plants were transplanted into 20 cm-diameter plastic pots containing greenhouse soil, 3-4 plants per pot, and were placed in a growth chamber at 10 C with 10,000 lux of light for 10 hr/day (19), where they were maintained until heading.

Cold-frame studies. Cold-frame studies were initiated to provide controlled environmental conditions for promotion of uniform infection and to determine if there were any differences between field- and cold frame-infected wheat. In November, 1980 and 1981, Ionia winter wheat seed was planted in wooden flats containing infested soil collected from wheat fields in Saranac and East Lansing, MI in which WSSM-affected plants had previously been identified. Uninfected control plants were grown in flats containing sterilized greenhouse soil. After germination, plants were kept in the greenhouse 5-8 days at 20 $\pm$ 3 C. Flats were then transferred to a cinderblock cold frame with a metal hardware cloth covering where they remained for 30-60 days (26). After the 1-2 mo vernalization period, flats were transferred to a 10 C growth chamber and observed for symptom development.

Additional soil transmission studies. Attempts were made to achieve soil transmission of WSSMV through various planting and temperature treatments (21). For all treatments, Ionia wheat seed was planted in flats of infested soil. Flats remained in the greenhouse 5-8 days after germination. One set of flats (four flats per set) was placed

in a 1 C growth chamber for 30 days, then transferred to a 10 C growth chamber. A second set was placed in a 15 C growth chamber and treated similarly. One flat of wheat planted in greenhouse soil was included in each treatment as a control. A set of five flats, four containing infested soil and one containing sterilized soil, was placed directly in a 10 C growth chamber 8 days after seeds germinated. A fourth set of flats was placed in a 15 C growth chamber for 30 days, and then individual plants were subjected to the various treatments described below, transplanted into 13 cm plastic pots, and placed in a 10 C growth chamber. Treatments consisted of removing plants from one flat and transplanting directly into pots of infested soil, rinsing roots of plants from a second flat with distilled water prior to transplanting, rinsing roots of plants from a third flat and transplanting into infested soil amended with 5 g ammonium nitrate per kg soil, and rinsing roots of plants from a fourth flat and transplanting into pots containing quartz sand. Plants from one flat planted in sterilized soil and placed at 15 C for 30 days were also subjected to the treatments indicated.

Mechanical transmission studies. Ionia and Genesee wheat seed were planted in 13 cm plastic pots, 12 seeds per pot, containing greenhouse soil. At the three- to four-leaf stage, pots were transferred from the greenhouse to a 10 C growth chamber. Two methods of mechanical transmission were tested: the conventional leaf rub method (10) and the leaf tissue rub method of Slykhuis (20). For the leaf rub method, WSSMV-infected Genesee wheat collected from the field and frozen

for storage was thawed, ground in four volumes of 0.06 M sodium phosphate buffer (ISEM buffer), pH 7.0, and rubbed onto leaves dusted with 600-mesh carborundum. For controls, plants were inoculated with similarly prepared extracts from uninfected wheat and with buffer alone. For the leaf tissue rub method, frozen WSSMV-infected Genesee leaves were thawed overnight at 4 C, abraded using fine, wet emery paper dusted with 600-mesh carborundum and rubbed onto leaves of test plants. Control plants were inoculated with uninfected leaves as described above. All plants were inoculated 1-2 wk after transfer to the 10 C growth chamber.

Plants in all transmission studies were fertilized every 4 wk with Peter's solution except plants in pots of quartz sand which were fertilized weekly.

Sampling. Wheat roots, crowns and leaves were assayed for virus every 2 wk after field and cold-frame plants were transferred to the 10 C growth chamber for a 12 wk period. Tissues were sampled from randomly chosen plants per flat; only lower leaves bearing distinct symptoms were harvested to maintain a uniform sampling procedure. Only leaf tissue from rub-inoculated plants was assayed for virus.

Extracts from infected tissues were obtained by first cutting 0.5-1.0 g of tissue into small pieces and then grinding in a mortar and pestle with liquid nitrogen. Three to five ml of ISEM buffer was added to the ground tissue to obtain an aqueous suspension, resulting in a final dilution of about 1:5 tissue:buffer. Tissue extracts from uninfected plants were prepared similarly.

ISEM. WSSMV antiserum with a titer of 1/320 in complement fixation was obtained from T.Usugi. A further modification of the Derrick technique (6) as modified by Milne and Luisoni (12) was used for ISEM. Carbon-coated Parlodion-filmed 300-mesh grids were floated on 30 ul drops of a 1:500 dilution of antiserum in ISEM buffer to coat grids with specific antiserum. Drops of antiserum were placed on parafilm-wrapped microscope slides which were incubated in a petri dish containing moistened filter paper. Grids were incubated at room temperature (23 C) and 37 C for 1-3 hr, then rinsed twice for 10 min in ISEM buffer. Grids were then briefly drained and floated on 30 ul drops of plant extract. To determine the minimum length of time necessary for maximum trapping of virus particles, grids were incubated for 1,3, 5 and 7 hr, and overnight, at 4 C and 23 C. After incubation, grids were briefly drained and then negatively stained with 2% ammonium molybdate, pH 7.0. To enhance visualization of virus particles, most grids were coated again (decorated) with antiserum (12). For decoration, grids incubated overnight were drained and then floated on 30 ul drops of a 1:500 dilution of antiserum for 1-3 hr at 4 C and 23 C. Grids were then drained and negatively stained with ammonium molybdate.

For grids not pretreated with antiserum, 30 ul drops of plant extract prepared as described previously were placed on carbon-coated Parlodion-filmed grids for 1 min. Grids were drained and negatively stained with ammonium molybdate.

All grids were examined at 20,000X in a Philips 201 transmission electron microscope (TEM) operated at 60 kV. An estimate of particle

numbers was made by counting particles on 20 randomly chosen 300-mesh grid squares (GS) on each of six grids in duplicate (240 GS) from six different extracts of each tissue assayed.

Examination of roots for P. graminis. Roots from plants in soil transmission studies were stained and examined for the presence of P. graminis according to the method of Phillips and Hayman (16). Roots were excised from plants, washed in distilled water and placed in vials containing 10 ml 10% KOH. Vials were heated to 90 C for 1 hr. After washing in distilled water, roots were acidified by soaking for 1 hr in 0.1 M HCl and then stained by heating for 5 min in 0.05% trypan blue in lactophenol. Excess stain was rinsed off with lactophenol, and roots were examined with a light microscope at 600X.

Tissue fixation. Segments of leaves bearing symptoms from field and cold-frame plants were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.0, rinsed in 0.1 M phosphate buffer, and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer. Fixed tissues were dehydrated in a graded ethanol series, transferred to acetone, and embedded in Spurr's resin (22). Ultrathin sections were positively stained with 5% uranyl acetate in ethanol followed by 0.4% lead citrate in water. Grids were examined with the Philips 201 TEM.

Results

Field studies. Plants collected from the field showed distinct, characteristic symptoms that continued to develop on new leaf tissue as the plants matured in the 10 C growth chamber (Fig. 1). When leaf tissue was removed from plants for assay, a 2-3 cm piece was left attached to the culm. Until plants neared maturity, new leaf tissue bearing bright, distinct symptoms was produced from intercalary meristems, causing cut leaves to elongate to nearly their original length. In this way, new leaf tissue produced from growth of tillers and intercalary meristems provided a continuing source of virus-infected tissue for several months.

Resting spores of P. graminis were found in all roots sampled. Spores were particularly abundant and clustered in small lateral roots (Fig. 2).

Characteristic pinwheel inclusions, some with attached laminated arms, and aggregates of virions were commonly found in leaf bundle sheath and mesophyll cells in ultrathin sections of infected cells (Fig. 3). Some cells also contained abundant thread-like rods and ordered membranous tubes in addition to pinwheel inclusions (Fig. 4). Chloroplasts and mitochondria were somewhat distorted in cells from mildly chlorotic tissues, whereas cytoplasmic constituents were greatly disrupted in severely chlorotic and necrotic tissues.

For ISEM, optimum conditions for coating grids with antiserum were 1 hr at 37 C or 2-3 hr at 23 C. A 7 hr incubation period at either 4 C or 23 C was necessary to achieve maximum trapping of virus particles.

Figure 1. WSSMV-infected wheat plants collected from the field and transferred to the 10 C growth chamber.

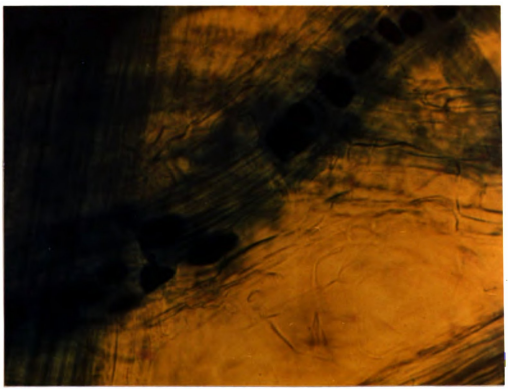


Figure 2. Resting spores of P. graminis in roots of WSSMV-infected wheat plants

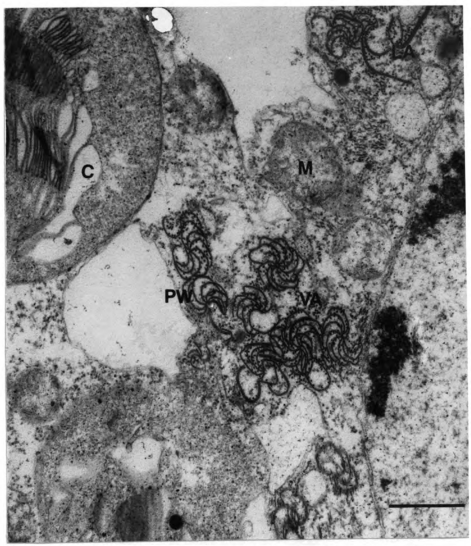


Figure 3. WSSMV-infected wheat leaf cell showing pinwheel inclusions and virus aggregates. C, chloroplast; M, mitochondrion; PW, pinwheel inclusions; VA, virus aggregates; LA, laminated arms. Scale bar = 5 μm.

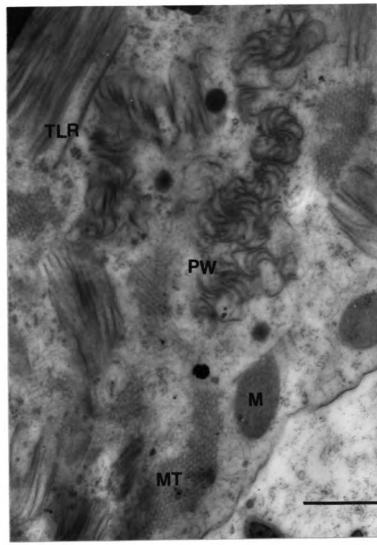


Figure 4. WSSMV-infected wheat leaf cell showing thread-like particles and membranous tubes. M, mitochondrion; PW, pinwheel inclusions; TLP, thread-like particles; MT, membranous tubes. Scale bar = 5 μm.

For decoration, a 1 hr incubation period at either 4 C or 23 C was sufficient.

Examination of extracts of roots and crowns by ISEM revealed few to no virus particles in these tissues. Two to three particles per GS were found in these tissues during initial sampling periods, whereas no particles were found when tissues were sampled 4 wk after plants were transferred to the growth chamber, resulting in an average of less than one particle per GS in these tissues. Particles were not detected in extracts from either tissue on grids not treated with antiserum.

An average of one particle per GS was found in extracts from infected leaves when grids not treated with antiserum were examined, whereas many particles were found on serum-coated grids in both undecorated and decorated preparations (Fig. 5). Using ISEM, an average of 220 particles per GS was found in leaf extracts, resulting in more than a 200-fold increase in the numbers of particles detected when ISEM was used. Because particles decorated with antiserum were seen more easily and could therefore be detected more rapidly, all subsequent ISEM preparations were decorated.

Measurements were made of both decorated and undecorated particles. Lengths of particles from both treatments ranged from 600 to 3,800 nm, with a modal length of 1,750 (Fig. 6). Undecorated particles had an average diameter of about 16 nm, whereas the diameter of decorated particles was somewhat larger and difficult to measure.

Cold-frame studies. Because symptoms on wheat planted in 1980 were barely discernible and were therefore attributed to stress rather

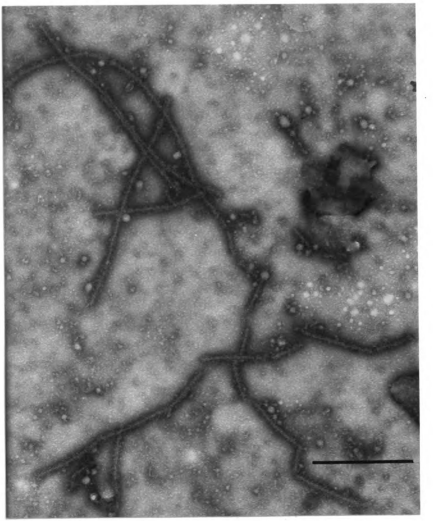
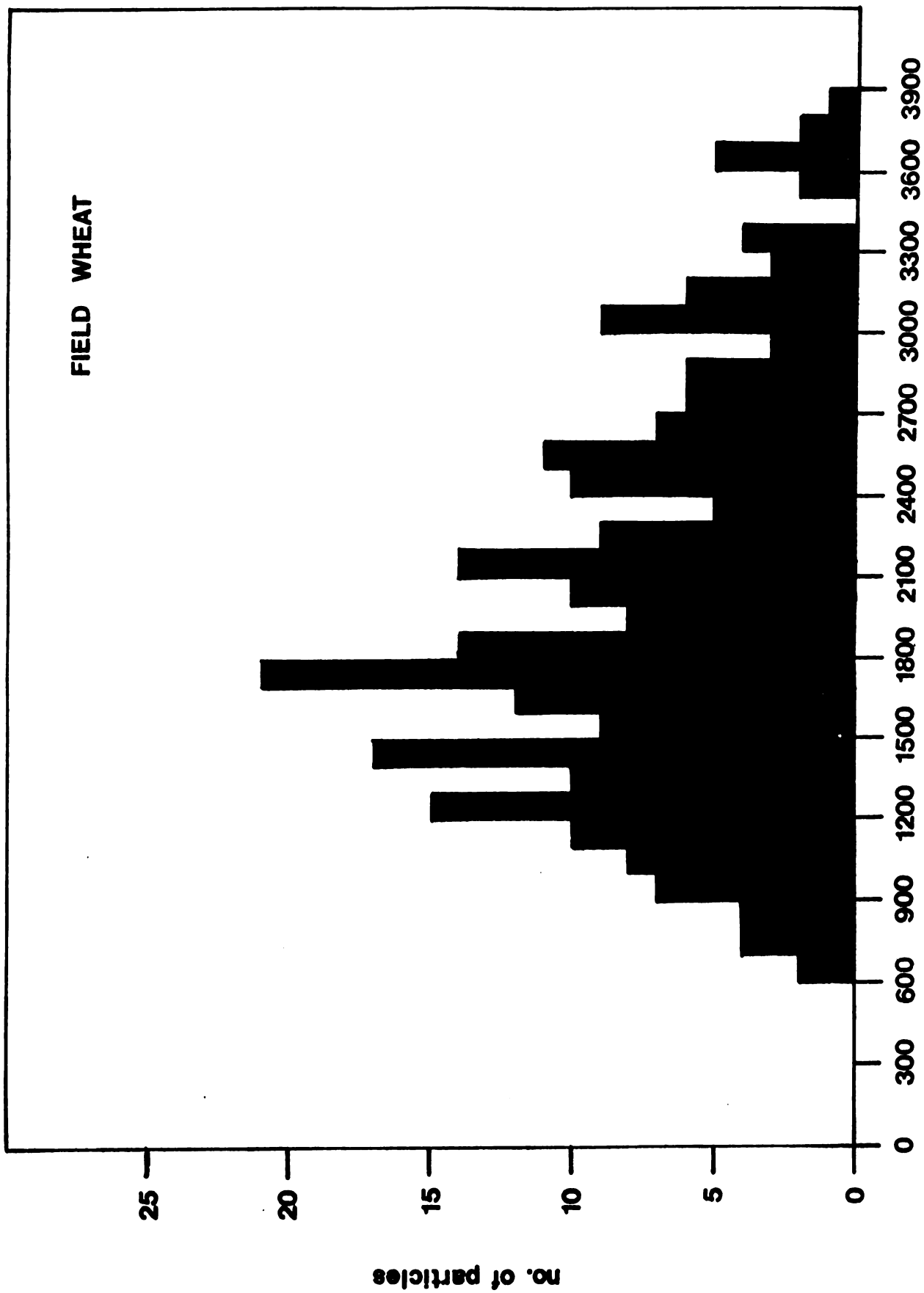


Figure 5. WSSMV particles coated with antiserum (decorated). Scale bar = 0.5 μm.

Figure 6. Distribution of lengths of WSSMV particles found in wheat leaf tissue from plants transferred from the field to the 10 C growth chamber. Modal particle length = 1,750 nm.


FIELD WHEAT
no. of particles
0
5
10
15
20
25
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
3600
3900

than virus infection, and because P. graminis was not found in randomly sampled roots, these plants were not assayed for virus and were discarded. Therefore, data reported are from wheat planted in 1981 and sampled in 1982.

Symptoms appeared on vernalized wheat 4-6 wk after transfer to the 10 C growth chamber. Symptoms consisted of a diffuse chlorotic mottle, with a few chlorotic streaks on the foliage. In general, symptoms on plants grown in the growth chamber were less intense than those found on infected wheat grown in the field. In addition, cold-frame plants were slower to tiller and elongate and remained immature longer than plants collected from the field. Until plants began to head, new leaf tissue replacing the distal portion removed for assay was produced, providing a source of infected tissue for several months.

P. graminis resting spores were found in roots from infected plants, and cytoplasmic inclusions and virions were found in cells of infected leaves, as reported for field wheat. Neither resting spores nor inclusions were found in tissues from uninfected plants.

Using ISEM, virus particles were found in extracts of roots and crowns from infected plants for a period of 8 wk starting on the day flats were placed in the growth chamber. An average of three particles per GS was found in root extracts whereas an average of less than one particle per GS was found in crown extracts during the 12 wk sampling period. No particles were detected in infected preparations on untreated grids or in preparations from uninfected plants.

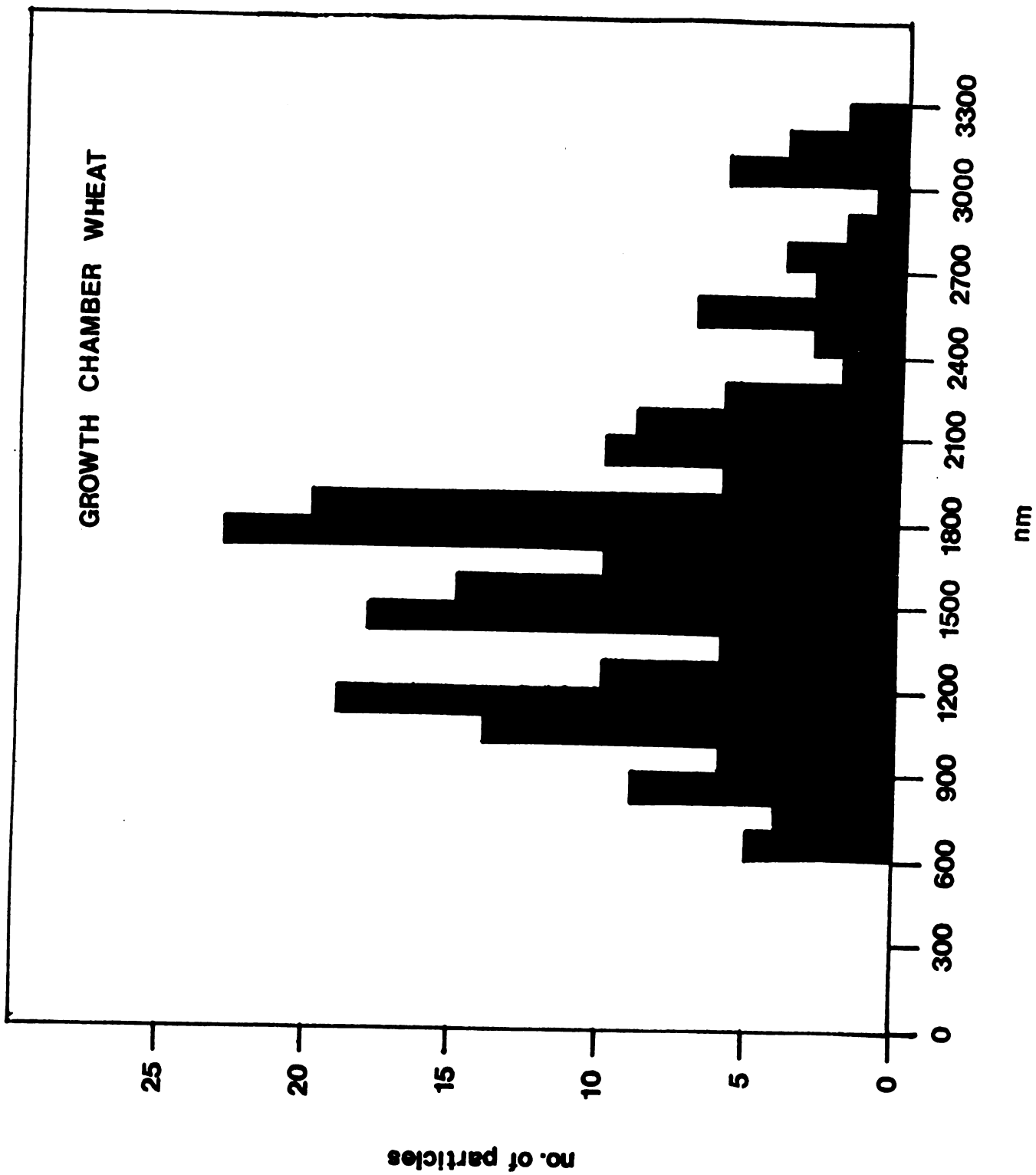
An average of 10 particles per GS was found on serum-coated grids

of extracts from infected leaves when sampled before symptoms developed. After symptoms became apparent, the virus content of infected leaves rose sharply, with an average of 205 particles per GS found in leaf extracts as determined by ISEM. No virus was found in extracts on uncoated grids when leaves were sampled prior to symptom development, whereas an average of one particle per GS was found on uncoated grids of extracts from leaves with symptoms. Particle lengths ranged from 600-3,300 nm, with a modal length of 1,800 nm (Fig. 7). No particles were found in leaf extracts from uninfected plants. Based on the Student's t test, there was no significant difference at $p < 0.01$ between numbers of particles on ISEM grids of leaf extracts from cold-frame and field plants.

Other soil transmission studies. None of the plants in the additional soil transmission studies developed characteristic WSSM symptoms, although a few transplanted plants showed a slight chlorosis which was attributed to stress. P. graminis resting spores were not found in roots from randomly chosen plants from any of the treatments. Because of this, these plants were not assayed for virus and were discarded.

Mechanical transmission studies. A mild chlorosis uncharacteristic of WSSM developed on some leaves of plants inoculated via the leaf tissue rub method. Examination of grids using ISEM revealed no virus particles in extracts from leaves rubbed with infected tissue. Control plants rubbed with uninfected tissue showed similar symptoms, probably as a result of injury due to vigorous rubbing. No

Figure 7. Distribution of lengths of WSSMV particles found in wheat leaf tissue from plants transferred from the cold frame to the 10 C growth chamber. Modal particle length = 1,800 nm.


GROWTH CHAMBER WHEAT
no. of particles
0
5
10
15
20
25
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
nm

virus was found in leaf extracts from these plants.

A mild chlorosis developed on plants in three out of nine pots inoculated via the conventional leaf rub method, and an average of five particles per GS was found in leaf extracts using ISEM. Particles ranged in length from 370-2,500 nm. No symptoms were found on plants rubbed with buffer alone, and WSSMV particles were absent in these plants.

Discussion

Until now, accurate diagnosis of WSSM could not easily be made because of similarities in symptomatology with other wheat virus diseases and inconclusive results in leaf-dip and tissue preparations. ISEM with decoration has proven to be a sensitive and specific technique for rapid detection of WSSMV in infected tissues. In preparations using grids pretreated with antiserum, over two hundred times more particles were detected in leaf extracts from infected field and cold-frame wheat than in preparations on grids not pretreated. This is comparable to other reported increases in sensitivity when ISEM was used instead of conventional electron microscopy for detecting certain other plant viruses (6, 8, 12).

Addition of decoration to the ISEM procedure provided enhanced visualization of virus particles with a high degree of specificity. Although long, rod-shaped viruses are easier to detect than spherical viruses, even in preparations from crude extracts the halo of antibody molecules surrounding decorated particles greatly enhanced visualization of particles and also provided conclusive identification of the particles as WSSMV.

A few differences between cold-frame wheat and field wheat in relation to WSSM were apparent. Although numbers of virus particles in roots, crowns and leaves from field plants were not significantly different from numbers of particles in the same tissues from cold-frame plants, there were differences in other reactions between the two sets of plants. As previously mentioned, symptoms on cold-frame plants were

less intense than those on field wheat. Because cold-frame plants were also slower to mature, leaves remained smaller and more narrow for a longer period than leaves from field wheat, which actually facilitated easier tissue grinding and virus extraction. Because cold-frame plants were transferred to the growth chamber at an earlier growth stage than field plants, differences between the two may be attributable to growth chamber conditions which may not have been optimal for plant growth and symptom development rather than differences in disease reactions.

Age of host tissue may have had an effect on virus location within the plant. Examination of roots of young, symptomless plants apparently is important for the early detection of WSSMV-infected plants. Some researchers routinely screen for WSSM by examining roots of young plants for WSSMV (S.A. Tolin, personal communication). Virus is also present at low levels in leaves of symptomless plants. Virus concentration greatly increases in leaf tissues as symptoms develop, whereas few to no particles can be found in roots or crowns at this stage, suggesting movement of virus from lower to upper portions of the plant. Because field wheat was at the three- to four-leaf stage when placed in the growth chamber, a few virus particles were found in roots and crowns only during the first three sampling dates, whereas particles were found in these tissues during the first nine sampling dates from cold-frame wheat which had been transferred in January when little viable foliage was evident.

Plants in cold-frame studies were vernalized for 30 days in 1980-1981 and 60 days in 1981-1982. Although Wiese and Hooper (26) reported

100% infection when plants were outdoors for 30 or 60 days, a longer period of vernalization may enhance disease development, since symptoms were more apparent on wheat planted in 1981 than in 1980. However, although P.graminis was absent in the few roots sampled from wheat planted in 1980, plants escaping zoospore infection within a flat are not uncommon. In addition, these 1980 plants bearing indistinct but nevertheless chlorotic markings were not assayed for virus and thus may have been infected with WSSMV afterall. Similarly, although conditions for disease development may not have been optimal, some plants from the additional soil transmission studies may have also been infected.

Test plants inoculated via the leaf tissue rub method failed to become infected possibly because they were too old when inoculated. In addition, virus concentration in the inoculum tissue may have been very low since the tissue had been frozen and then thawed over a long period of time. Similarly, although inoculations via the leaf rub method were somewhat successful (33% infection), test plants may have been too old and virus concentration in the inoculum too low for efficient transmission, as virus concentration in test plants was low (5/GS).

A diameter of 16 nm for WSSMV reported in this work is within the 12-20 nm range reported by other researchers (4, 10, 23, 26). Variations in length measurements are greater, however, with reported measurements ranging from 100-5,000 nm in leaf-dip preparations (4, 9, 14, 23, 26, 27). In this work a range of 600-3,800 nm was found, with a modal length of 1,775 nm, for particles extracted from infected leaves by grinding in liquid nitrogen. Because WSSMV particles are long and

thin, they are fragile and subject to breakage during preparation of leaf dips and extraction from tissue. Although some short particles were obtained in our extracts, grinding in liquid nitrogen was a more satisfactory procedure than grinding in buffer for quick release of relatively intact virus particles from infected tissues.

Results of this study indicate that ISEM with decoration is an accurate and sensitive technique for the diagnosis of WSSM in asymptomatic as well as symptomatic plants.

Literature Cited

1. Ball, E.M., and Brakke, M.K. 1968. Leaf-dip serology for electron microscopic identification of plant viruses. Virology 36: 152-155.

2. Bays, D.C., Cunfer, B.M., and Demski, J.W. 1985. Occurrence of wheat spindle streak mosaic virus on winter wheat in Georgia. Plant Dis. 69:1094-1096.

3. Brakke, M.K., Estes, A.P., and Schuster, M.L. 1965. Transmission of soil-borne wheat mosaic virus. Phytopathology 55:79-86.

4. Brakke, M.K., Langenberg, W.G., and Samson, R.G. 1982. Wheat spindle streak mosaic virus in Nebraska. Plant Dis. 66: 958-959.

5. Clark, M.F., and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483.

6. Derrick, K.S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56: 652-653.

7. Gillett, J.M., Morimoto, K.M., Ramsdell, D.C., Chaney, W.G., Baker, K.K., and Esselman, W.J. 1982. A comparison between the relative abilities of ELISA, RIA and ISEM to detect blueberry shoestring virus in its aphid vector. Acta Hortic. 129: 25-29.

8. Harrison, B.D., and Roberts, I.M. 1979. Detection of potato leaf roll and potato mop-top viruses by immunosorbent electron microscopy. Ann. Appl. Biol. 93: 289-297.

9. Jackson, A.O., Bracker, C.E., Huber, D.M., Scott, D.H., and Shaner, G. 1975. The occurrence and transmission of a disease in Indiana with properties of wheat spindle streak mosaic virus. Plant Dis. Reptr. 59: 790-794.

10. Jackson, A.O., Bracker, C.E., and Shaner, G. 1976. Mechanical transmission, varietal reactions and electron microscopy of a disease in Indiana with properties of wheat spindle streak mosaic. Plant Dis. Reptr. 60: 202-206.

11. Lommel, S.A., and Willis, W.G. 1984. The role of wheat spindle streak and wheat soilborne mosaic viruses in an epiphytotic of resistant wheat in Kansas. Phytopathology (Abstr.) 74: 844.

12. Milne, R.G., and Luisoni, E. 1977. Rapid immune electron microscopy of virus preparations. Pages 265-281, IN: K.

Maramorosch and H. Koprowski, eds. Methods in Virology. Vol. 4. Academic Press, New York. 396 pp.

13. Milne, R.G., and Lesemann, D.-E. 1984. Immunosorbent electron microscopy in plant virus studies. Pages 85-101, IN: K. Maramorosch and H. Koprowski, eds. Methods in Virology. Vol. 8. Academic Press, New York. 396 pp.

14. Nolt, B.L., Romaine, C.P., Smith, S.H., and Cole, H., Jr. 1981. Further evidence for the association of Polymyxa graminis with the transmission of wheat spindle streak mosaic virus. Phytopathology 71: 1269-1272.

15. Nguyen, H.T., and Pfeifer, R.P. 1980. Effects of wheat spindle streak mosaic virus on winter wheat. Plant Dis. 64: 181-184.

16. Phillips, J.M., and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55: 158-162.

17. Roberts, I.M., Milne, R.G., and van Regenmortel, M.H.V. 1982. Suggested terminology for virus/antibody interactions observed by electron microscopy. Intervirology 18: 147-149.

18. Slykhuis, J.T. 1960. Evidence of a soil-borne mosaic of wheat in Ontario, Canada. Plant Dis. Surv. 40: 43.

19. Slykhuis, J.T. 1974. Differentiation of transmission and incubation temperatures for wheat spindle streak mosaic virus. Phytopathology 64: 554-557.

20. Slykhuis, J.T. 1975. Factors critical to mechanical transmissibility of wheat spindle streak mosaic virus. Phytopathology 65: 582-584.

21. Slykhuis, J.T. 1976. Stimulating effects of transplanting on the development of wheat spindle streak mosaic virus in wheat plants infected from soil. Phytopathology 66: 130-131.

22. Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.

23. Usugi, T., and Saito, Y. 1979. Relationship between wheat yellow mosaic virus and wheat spindle streak mosaic virus. Ann. Phytopathol. Soc. Jpn. 45: 397-400.

24. Wiese, M.V. 1977. Compendium of Wheat Diseases. The American Phytopathological Society, St. Paul. 106 pp.

25. Wiese, M.V., Saari, E.E., Clayton, J., and Ellingboe, A.H. 1970. Occurrence of wheat streak mosaic and a new variegation disorder, wheat spindle streak mosaic, in Michigan wheat. Plant Dis. Reptr. 54: 635-637.

26. Wiese, M.V., and Hooper, G.R. 1971. Soil transmission and electron microscopy of wheat spindle streak mosaic. Phytopathology 61: 331-332.

27. Williams, A.S., Pirone, T.P., Slykhuis, J.T., and Tutt, C.R. 1975. Wheat spindle streak mosaic virus in Kentucky. Plant Dis. Reptr. 59: 888-889.

CHAPTER II

IDENTIFICATION OF WINTER WHEAT CULTIVARS AND EXPERIMENTAL LINES RESISTANT TO WHEAT SPINDLE STREAK MOSAIC VIRUS

CHAPTER II

Identification of winter wheat cultivars and experimental lines resistant to wheat spindle streak mosaic virus

Abstract

Five commercial cultivars and eight experimental lines of soft white and soft red winter wheats, selected from hundreds of cultivars and lines screened in field plots, were rated for their reactions to WSSMV under field and growth chamber conditions. Ratings were based on symptom expression and virus particle counts using immunosorbent electron microscopy. Two of the cultivars and all of the experimental lines exhibited some resistance to WSSMV. Virus was not detected in leaves from three of the experimental lines. Resting spores of the fungal vector Polymyxa graminis were found in roots from susceptible and resistant cultivars, indicating that resistance to WSSMV may be due to resistance to virus infection or multiplication.

Introduction

Wheat spindle streak mosaic (WSSM) has been prevalent in Michigan winter wheat fields the past several growing seasons. In the past, preliminary diagnosis of WSSM was based on characteristic foliar symptoms during cool spring weather. Recently, immunosorbent electron microscopy (ISEM) with decoration (4) has been used to confirm the identity of the causal agent, wheat spindle streak mosaic virus (WSSMV).

Considerable variation in WSSM symptom expression has been observed in many wheat cultivars and experimental lines when plots in several Michigan nurseries were rated for resistance to WSSMV over the last 10 years. During any one season, symptoms among the different cultivars and lines have ranged from few to no visible chlorotic streaks on lower foliage to a severe mosaic up to the flag leaf.

Similarly, several other researchers (5, 6, 10, 14, 15) have observed a wide range in WSSM symptom severity and other reactions among different wheat lines and cultivars. Williams et al. (15) rated 24 cultivars and numbered entries for WSSM symptom expression and found that nearly 70% showed prominent symptoms, indicating moderate to severe infections in these cultivars and entries. Three of the cultivars and entries showed no symptoms. Wiese et al. (14) found that out of 10 cultivars examined, only one showed no effects of the disease while the other nine showed varying degrees of symptom expression and negative effects on yield. Nguyen and Pfeifer (10) reported three levels of tolerance to WSSMV among five cultivars rated on the basis of symptom severity and effects on yield. Jackson et al. (5) reported a wide

variation in symptom expression among nine red wheat cultivars grown in Indiana. The authors observed a mean of 16 virus particles per 300-mesh grid square (GS) in leaf-dip preparations from 10 breeding lines considered susceptible to WSSMV, whereas less than one particle per GS was found in preparations from 10 resistant lines.

Although data pertaining to yield losses due to WSSM are of limited value because of experimental differences, crop loss estimates range from 2-64% (3, 10, 14, 15). While a few of the older winter wheat cultivars showed some resistance to WSSMV (6, 10, 14, 15), at present most of the commercially-grown and newly-released cultivars are susceptible (6, 10, 13, 14). Because recommended cultural practices such as crop rotation and late planting have not been effective in controlling the disease, it has become necessary to identify germplasm and develop cultivars resistant to WSSMV. Thus, the purpose of this study was to identify winter wheat cultivars and experimental lines resistant to WSSMV on the basis of ratings for symptom expression and virus titer in infected leaves. In addition, the relationship between disease rating and the presence of the fungal vector Polymyxa graminis was investigated.

Materials and Methods

Field studies. Field plots of winter wheat (Triticum aestivum L.) were planted in mid-October, 1980-1983, and rated for symptoms in May of the following year. Symptoms were rated on a 0-2 scale with a rating of 0 = symptomless plants, 1 = plants bearing either indistinct symptoms or symptoms of questionable origin and 2 = plants with distinct, characteristic symptoms. Plants were located in advanced yield trial breeding nurseries containing five-row plots of 30 entries which were replicated three times. Plants chosen for virus titer studies performed during the 1982-83 and 1983-84 growing seasons were collected from plots, transferred to flats and placed in a 10 C growth chamber prior to sampling for virus. Lower leaves from randomly-chosen plants from each cultivar in the growth chamber were harvested for virus sampling. Fresh and frozen leaves were ground in liquid nitrogen, and buffer was added in a 1 ml:5 g tissue:buffer ratio to extract virus as described previously (4). For virus particle counts, ISEM was performed also as described previously (4). Six virus particle counts were made each year (1983 and 1984) for each cultivar or line. Each count consisted of the average number of particles found on 10 randomly-chosen GS per 300-mesh grid.

Cold-frame studies. Cold-frame studies were initiated to provide stable environmental conditions for promotion of uniform infection and to compare disease reactions with those from field-grown plants. Five commercial cultivars and eight experimental lines of winter wheat were selected for further study on the basis of field symptoms during the

1980-1984 growing seasons and virus titer studies during the 1982-83 and 1983-84 growing seasons. These cultivars included Ionia, Augusta and Tecumseh, soft white wheats developed at Michigan State University, Genesee, a soft white cultivar from New York and S-76, a soft red cultivar from Pioneer (Pioneer Hi-Bred International, Inc., Johnston, IA). Six of the eight experimental lines represented soft white wheats, while the remaining two (accession numbers B7321 and B9028) were soft red wheats. All of the experimental lines except I2724 were developed through the Michigan State University breeding program and have pedigrees of diverse genetic origin (Table 1). Seed of these cultivars and lines were planted in flats of infested soil and germinated in the greenhouse. The flats were then placed in an outdoor cold frame for 2 mo and transferred to a 10 C growth chamber as described previously (4). Twelve rows of seed were planted per flat, two rows per cultivar or line, each of which was replicated three times among different flats.

WSSM disease ratings were based on symptom expression and virus particle counts in infected leaves as determined by ISEM. Symptoms were rated as for field plants on a 0-2 scale, except that a rating of 1 = plants bearing indistinct or mild symptoms. Symptom ratings were made 6 wk after flats were placed in the growth chamber. Leaf tissue was prepared and virus particle counts were performed as described for field material. Plants were checked for virus biweekly beginning the day flats were transferred from the cold frame to the growth chamber until heading (10 annual counts).

Examination of roots for P. graminis. Roots from all cultivars

and lines vernalized in the cold frame were examined for the presence of P.graminis. Roots were cleared and stained as described in Chapter I.

Results

Although symptoms on cold-frame plants were not as intense as those on field plants, field and cold-frame symptom ratings were not significantly different for either the experimental lines or commercial varieties (Tables 1 and 2). Similarly, virus particle counts from cold-frame plants were somewhat lower than counts from field plants, but this difference also was not significant (Table 2). Virus counts reported from cold-frame plants were averages of 12 particle counts (120 GS) per cultivar or line taken during the weeks of peak virus titer, approximately 2 mo after placement of flats in the growth chamber. Particle counts for both field and cold-frame plants ranged from 0-400 particles/GS. Because counts for each cultivar and line consistently fell within a narrow range, four groups of particle counts were arbitrarily chosen to represent each range. These groups were: 0 particles/GS, 1-20 particles/GS, 21-70 particles/GS and >70 particles/GS. Based on the two criteria of symptom severity and virus particle count groups, cultivars and lines were assigned WSSM susceptibility ratings (Tables 1 and 2). Thus, plants in the 0/GS group were usually considered resistant to WSSMV, plants in the 1-20/GS group were usually rated moderately resistant, those in the 21-70/GS group were usually rated moderately susceptible and those with >70 particles/GS were considered susceptible to WSSMV.

Three of the commercial cultivars selected, Ionia, Genesee and Augusta, were rated susceptible to WSSMV. Ionia leaves showed very intense, distinct symptoms and frequently yielded virus particle counts

Table 1. Wheat spindle streak mosaic virus particle counts of eight wheat lines showing no symptoms in field or cold-frame studies.

Accession Number	Origin	Particle count[a] Cold Frame	Disease rating[b]
B2231	USA	2	MR
B4135	USA, Japan	8	MR
B4145	USA, New Zealand	15	MR
B6018	USA, Japan	0	R
B7321	Russia	4	MR
B7322	Russia	2	MR
B9028	Yugoslavia, Mexico	0	R
I2724	USA	0	R

[a]Numbers are ISEM averages of 120 GS for each individual cold-frame wheat line.

[b]MR = moderately resistant, R = resistant. Disease rating is based on symptom severity rating and virus particle counts per entry.

Table 2. Winter wheat commercial cultivar reactions to WSSM.

Variety	Symptom expression[a]		Particle count[b]		Disease rating[c]
	Field	Cold Frame	Field	Cold Frame	
Ionia	2.0	1.8	350	325	S
Augusta	1.8	1.8	180	150	S
Genesee	1.8	1.5	160	130	S
Tecumseh	1.2	0.8	40	30	MS
S-76	0.8	0.5	15	6	MR

[a]Numbers represent averages of six symptom ratings each for field and cold-frame wheat cultivars. Differences between field and cold-frame ratings were not significant by the Student's t test ($p < 0.05$).

[b]Numbers are the ISEM averages per GS obtained by examining 120 GS for individual field and cold-frame wheat cultivars. Differences between field and cold-frame ratings were not significant by the Student's t test ($p < 0.05$).

[c]S = susceptible, MS = moderately susceptible, MR = moderately resistant, R = resistant. Overall disease rating is based on symptom severity rating and virus particle counts per entry.

as high as 400/GS (Table 2). Augusta and Genesee were also considered susceptible to WSSMV, although particle counts from leaves of these cultivars were somewhat lower (70-200/GS). Less severe symptoms were found on leaves from Tecumseh than were found on leaves from susceptible cultivars, and particle counts were much lower (15-50/GS). Tecumseh was therefore considered to be only moderately susceptible to WSSMV. Symptoms on S-76 leaves were even milder and less distinct than those on Tecumseh leaves, and particle counts were low (0-25/GS). Thus, S-76 was rated moderately resistant to WSSMV.

The eight experimental lines selected from field studies (Table 1) failed to develop distinct WSSM symptoms in either cold-frame or field tests, although a light mottle was occasionally found on leaves from field plants of accession number B4145. Virus particles were not found in leaves from three of the experimental lines (B6018, B9028 and I2724); these lines were rated resistant to WSSMV. Fewer than 5 particles/GS were found in leaves from three of the other lines (B2231, B7321 and B7322), while particle counts from the remaining two lines (B4135 and B4145) were slightly higher (5-20/GS). These five lines were therefore rated moderately resistant to WSSMV.

Resting spores and, occasionally, zoosporangia of P. graminis were found in roots from all cold-frame plants, regardless of their disease susceptibility rating. Colonizations varied from light to heavy, with no obvious pattern regarding host disease susceptibility. In addition, moderate colonizations of resting spores and zoosporangia (in approximately equal numbers) of the parasitic fungus Olpidium brassicae,

which has been suggested as a possible vector of WSSMV, were found in approximately 15% of the samples examined.

Discussion

Resistance to WSSMV was present in two out of the five commercial soft white and red winter wheat cultivars and in all eight breeding lines examined. Disease susceptibility ratings were based not only symptom expression but also on pathogen titer which affords a better definition of genetically resistant cultivars and lines. Earlier work by Jackson et al. (5) investigated the association of WSSMV particles with disease symptoms, but a systematic survey of and disease susceptibility ratings for various cultivars and lines showing varied reactions to WSSM were not reported. In this work, a direct correlation between WSSM symptom severity and virus titer was evident, as cultivars and lines with moderate to severe symptoms had high virus particle counts, whereas few to no particles were found in cultivars and lines with less intense or no symptoms.

Because neither symptoms nor virus particles were found associated with lines B6018, B9028 and I2724 (Table 1), some may consider these lines immune rather than resistant to WSSMV. Although no virus was detected in these plants via soil transmission, it is possible that these lines may be susceptible to WSSMV via manual inoculation or in protoplast culture (12). Jackson et al. (6) reported the successful transmission of WSSMV using an artist's air brush to a few cultivars that were symptomless in the field. Thus, because such stringency has not been met in this study, these lines were considered resistant but not necessarily immune to WSSMV.

Until recently, no information on the inheritance of reaction to

WSSMV had been reported. Based on the identification of cultivars and lines representing a range of phenotypic responses to WSSMV in this study, Van Koevering (13) chose three cultivars and four experimental lines as parents in a diallel mating design to study the mode of inheritance of resistance to WSSMV. Virus particle counts as determined by ISEM served as the basis for analysis of parental and F_1 progeny reactions to WSSM. Resistance to WSSMV was found to be highly heritable and controlled by completely dominant genes (with some additive effect). Similarly, results from the analysis of a five-parent winter wheat diallel cross indicated that resistance to soilborne wheat mosaic virus (SBWMV) was monogenic dominant over susceptibility (2).

Because P. graminis resting spores were found not only in roots from cultivars and lines susceptible to WSSMV but also in roots from resistant lines indicates that disease resistance may be due to resistance to virus infection or multiplication rather than to the fungal vector. While this is the only known report of a possible mechanism of resistance to WSSM, several researchers have investigated the mechanism of resistance to soilborne wheat mosaic (SBWM), with conflicting results. Palmer and Brakke (11) concluded that resistance to SBWM is due to resistance to the fungal vector P. graminis because both field-resistant and field-susceptible cultivars were susceptible to virus infection when manually inoculated. Kucharek et al. (7) indicated that resistance is to the virus because P. graminis resting spores were found in roots of field-resistant cultivars. Campbell et al. (1) found that both field-resistant and field-

susceptible cultivars showed symptoms with manual inoculation and harbored P. graminis resting spores. They suggested that resistance to SBWM expressed in the field results from resistance to or nonpreference for P. graminis. T. T. Hebert (unpublished) also found that wheat cultivars resistant to SBWM were as susceptible to the vector as susceptible cultivars. More recently, Larson et al. (9) reported that wheat cultivars susceptible and resistant to SBWM were susceptible to P. graminis. The authors further stated that root-system biomass was reduced by 71% in SBWMV-infected plants, indicating that the mechanism of field resistance to SBWMV lies within the roots of resistant plants. Langenberg (8) also found that field-resistant cultivars were hosts of P. graminis and suggested that field resistance may be the result of hypersensitivity to virus infection in the roots rather than resistance to P. graminis. As previously mentioned, when studying mechanical transmission of WSSMV, Jackson et al. (6) found that while some field-resistant breeding lines were resistant to manual inoculation, others developed mild symptoms and contained virus particles after manual inoculation. However, these field-resistant cultivars and lines susceptible to manual inoculation were rated field resistant on the basis of symptom expression alone and may actually have been only moderately resistant to WSSMV according to the results from this study. Results from all of the studies reviewed here suggest that for both WSSM and SBWM there may be resistance to both the fungus (or, more likely, fungal transmission) and resistance to the virus through an inhibition of infection or multiplication.

Because germplasm with resistance to WSSMV has been identified, based on both symptom expression and virus titer, it is hoped that this more accurate assessment of resistance will facilitate improvement of cultivar performance by allowing the wheat breeder to incorporate WSSMV resistance into adapted lines of high potential yield. A better assessment of yield loss due to WSSM could then be made by using isogenic lines representing genotypes susceptible, moderately susceptible, moderately resistant and resistant to WSSMV.

Literature Cited

1. Campbell, L. G., Heyne, E. G., Gronau, D. M. and Niblett, C. 1975. Effect of soilborne wheat mosaic virus on wheat yield. Plant Dis. Reptr. 59: 472-476.

2. Dubey, S. N., Brown, C. M., and Hooker, A. L. 1970. Inheritance of field reaction to soil-borne wheat mosaic virus. Crop Sci. 10: 93-95.

3. Gates, L. F. 1969. Incidence and effects of wheat spindle streak mosaic in Essex and Kent counties, Ontario, 1967-68. Can. Plant Dis. Surv. 51: 24-31.

4. Haufler, K. Z., and Fulbright, D. W. 1983. Detection of wheat spindle streak mosaic virus by serologically specific electron microscopy. Plant Dis. 67: 988-990.

5. Jackson, A. O., Bracker, C. E., Huber, D. M., Scott, D. H., and Shaner, G. 1975. The occurrence and transmission of a disease in Indiana with properties of wheat spindle streak mosaic virus. Plant Dis. Reptr. 59: 790-794.

6. Jackson, A. O., Bracker, C. E., and Shaner, G. 1976. Mechanical transmission, varietal reactions and electron microscopy of a disease in Indiana with properties of wheat spindle streak mosaic. Plant Dis. Reptr. 60: 202-206.

7. Kucharek, T. A., Walker, J. H., Barnet, R. D. 1974. Effect of cultivar resistance and soil fumigation on soilborne wheat mosaic virus in Florida. Plant Dis. Reptr. 58: 878-881.

8. Langenberg, W. G. 1984. No resistance found to Polymyxa graminis, vector of soilborne wheat mosaic virus, in sorghum, corn and wheat cultivars. Ann. Wheat Newsl. 30: 145-146. Univ. Communic., Colorado State Univ.

9. Larson, H., Brakke, M. K., and Langenberg, W. G. 1984. Soilborne wheat mosaic virus (SBWMV) and wheat streak mosaic virus (WSMV). Ann. Wheat Newsl. 30: 144-145. Univ. Commun., Colorado State Univ.

10. Nguyen, H. T., and Pfeiffer, R. P. 1980. Effects of wheat spindle streak mosaic virus on winter wheat. Plant Dis. 64: 181-184.

11. Palmer, L. T., and Brakke, M. K. 1975. Yield reduction in winter wheat infected with soilborne wheat mosaic virus. Plant Dis. Reptr. 59: 469-471.

12. Tavantzis, S. M. 1984. The use of terms for responses of plants to viruses: a reply to recent proposals. Phytopathology 74: 379-380.

13. Van Koevering, M. 1985. The inheritance of resistance of wheat spindle streak mosaic virus (WSSMV) in winter wheat. M.S. Thesis, Michigan State University. 51 pp.

14. Wiese, M. V., Ravenscroft, A. V., and Everson, E. H. 1974. Incidence of wheat spindle streak mosaic virus among ten wheat cultivars and its effect on yield. Plant Dis. Reptr. 58: 522-525.

15. Williams, A. S., Pirone, T. P.. Slykhuis, J. T., and Tutt, C. R. 1975. Wheat spindle streak mosaic virus in Kentucky. Plant Dis. Reptr. 59: 888-889.

CHAPTER III

PURIFICATION AND PARTIAL CHARACTERIZATION OF WHEAT SPINDLE STREAK MOSAIC VIRUS

Chapter III

Purification and Partial Characterization of Wheat Spindle Streak Mosaic Virus

Abstract

A purification procedure which yielded sufficient (up to 55 µg/g), relatively intact virus for biochemical characterization was developed for wheat spindle streak mosaic virus (WSSMV). Virus was purified by sucrose-cesium sulfate density gradient centrifugation after clarification of tissue extracts in chloroform and concentration of virus particles by polyethylene glycol (PEG). Sodium sulfite and sodium diethyldithiocarbamate were added to the extraction buffer to maintain particle stability, and use of urea and Triton X-100 in the resuspension buffer facilitated recovery of particles from PEG-precipitated virus pellets. Purified particles had a modal length of about 800 nm and a buoyant density of 1.20-1.21 g/cm^3 in cesium sulfate. Estimated molecular weights for viral capsid proteins were 36,000, 32,000, and 29,000, and an estimated molecular weight for WSSMV cylindrical inclusion protein was 69,000. These data suggest that WSSMV is a probable member of the potyvirus group.

Introduction

Wheat spindle streak mosaic (WSSM) commonly occurs in winter wheat growing areas of the United States, Canada and several other countries (see Literature Review). The disease epidemiology, symptomatology and cytopathology have been well-documented (see Literature Review). However, little information is available on the causal agent, wheat spindle streak mosaic virus (WSSMV). Virus particles occur sparsely in infected tissues and are therefore found only with some difficulty in leaf-dip preparations (16). Reported particle measurements vary widely, ranging from 200-5,000 nm in length and 12.8-22 nm in diameter (see Literature Review and Chapter I).

Slykhuis and Polak (16) and Hooper and Wiese (unpublished data) were unsuccessful in purifying WSSMV. In 1979 Usugi and Saito (15) successfully purified infectious virus using three cycles of differential centrifugation followed by cesium chloride density gradient centrifugation. Purified particles ranged in length from 100-1,300 nm, with the majority ranging from 200-600 nm.

Although virus purified according to the Japanese method was infectious, it is not known whether infectivity was associated solely with the shorter (< 600 nm) particles or whether the longer, more intact particles contributed to or were necessary for infectivity. Although particles in these preparations were highly fragmented, this material was suitable for antiserum production. However, for biochemical characterization purposes, preparations of intact virus are

necessary. Therefore, it was the purpose of this study to investigate other purification protocols which would yield large quantities of intact virus suitable for biochemical characterization. Purified WSSMV was characterized for inclusion and capsid protein molecular weights, particle buoyant density and genomic nucleic acid type.

Materials and Methods

Virus source. WSSMV was extracted from leaves of susceptible winter wheat cultivars (Ionia, Genesee and Augusta) obtained from natural infections in the field or soil transmission studies in the cold frame. Most of the field and cold-frame material was placed in a 10 C growth chamber for virus propagation and maintenance (see Chapter I). Alternately, leaf tissue was harvested, placed in plastic bags and stored at -20 C or -70 C until used for virus isolation.

Virus extraction, clarification and concentration. Adaptations and modifications of several published purification methods for other filamentous virus particles were investigated. Various extraction buffers containing several different additives such as urea and 2-mercaptoethanol (3) were incorporated into some of these purification schedules to find those components necessary for extracting the highest concentration of intact virus from infected leaves. Different procedures for releasing virus from tissues such as grinding leaves in buffer, liquid nitrogen or homogenizing in a Waring blender were also tested. Organic solvent treatments and low-speed centrifugation for sap clarification were investigated, as were polyethylene glycol (PEG) precipitation and high-speed centrifugation for virus concentration.

For further virus purification, centrifugation in various density gradients was tested. All purification steps were done at 4 C or on ice. The presence of virus during steps of each purification protocol was monitored by immunosorbent electron microscopy (ISEM) using antiserum provided by T. Usugi or antiserum prepared by the author.

Procedure 1:

Initial attempts to purify WSSMV were made following the protocol of Usugi and Saito (17). Infected leaves were ground in three to six volumes 0.1 M sodium phosphate buffer, pH 7.0, with a mortar and pestle. The extract was filtered by expressing through four layers of moistened cheesecloth. The pulp was reextracted with buffer, and one-fifth volume of carbon tetrachloride was added to the filtrate. The suspension was stirred for 15 min at 4 C, then the emulsion was broken by centrifuging in a Sorvall SS-34 rotor at 4,000 rpm for 15 min. The top aqueous phase was centrifuged at 9,000 rpm for 15 min., and the supernatant was centrifuged in a Beckman Type 40 rotor at 34,000 rpm for 1 hr. The pellet was resuspended overnight in buffer (one-tenth of the original sap volume) then subjected to two more cycles of differential centrifugation.

Procedure 2:

In a similar attempt, the method of Slykhuis and Polak was used (16). Leaves were ground in one to two volumes of 0.5 M sodium borate buffer, pH 9.0, and the extract was filtered through moistened cheesecloth. After reextracting of the pulp, one-half volume of n-butanol:chloroform (1:1, v/v) was added to the filtrate and the mixture

was stirred for 15 min. The emulsion was broken by centrifuging at 5,000 rpm for 15 min in a Sorvall SS-34 rotor, and the top aqueous phase was centrifuged as before. The supernatant was subjected to two cycles of differential centrifugation, and the final pellet was resuspended in phosphate buffered saline, pH 7.0.

Procedure 3:

A legume carlavirus purification method of Veerisetty and Brakke (18) was also investigated for purifying WSSMV. After grinding leaves in two to three volumes of 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer, pH 9.0, containing 0.1% sodium diethyldithiocarbamate (NaDIECA) and 0.5% 2-mercaptoethanol (2-ME), the extract was filtered through cheesecloth and centrifuged for 10 min at 8,000 rpm in a Sorvall SS-34 rotor. The supernatant was clarified by the addition of one-twentieth volume of 0.2 M sodium-phosphate and one-one hundredth volume of 1.0 M calcium chloride with stirring, followed by low-speed centrifugation. Six percent (w/v) PEG MW 8,000 was added to the clarified supernatant and the mixture was stirred for 2 hr. The precipitated virus was collected by low-speed centrifugation and resuspended for 2 hr in buffer (one-tenth of the original sap volume) containing 1% (v/v) Triton X-100. After a second low-speed centrifugation, the virus suspension was layered on a 10 ml pad of 20% (w/v) sucrose in buffer containing 1% Triton X-100 and centrifuged at 34,000 rpm for 3 hr in a Beckman Type 40 rotor. Pellets were resuspended in buffer containing 1% Triton X-100 and subjected to a low-speed centrifugation, and the supernatants were layered on 10 ml pads of

30% (w/v) sucrose in buffer containing 1% Triton X-100 and centrifuged as before. Pellets were resuspended in buffer and centrifuged at 8,000 rpm for 10 min. prior to further purification.

Procedures 4 and 5:

Purification protocols for citrus tristeza virus were adapted for WSSMV purification (2, 6). Tissue was ground in a mortar and pestle in the presence of liquid nitrogen, and two volumes of 0.1 M tris (tris hydroxymethyl aminomethane)-HCl buffer, pH 8.4, were added to the powder. After filtering through cheesecloth and reextracting the pulp, the filtrate was centrifuged at 6,000 rpm for 10 min in a Sorvall SS-34 rotor. Polyethylene glycol MW 8,000 and NaCl were added to the supernatant to make final concentrations of 4 and 0.8% (w/v), respectively. After stirring for 1 hr, the suspension was centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in two volumes of 0.04 M potassium phosphate buffer, pH 8.0, for 1 hr, then centrifuged at low speed, and virus was precipitated from the supernatant with PEG and NaCl as before. After 1 hr, the virus was collected by centrifugation and resuspended for 1 hr in 0.015 M potassium phosphate buffer (one-fifth of the original sap volume), pH 8.0. The suspension was clarified by low-speed centrifugation. Alternatively (Procedure 5), infected leaves were ground in liquid nitrogen, five volumes of 0.01 M tris-HCl buffer, pH 7.8, were added to the powder, and the extract was filtered as described previously. The filtrate was centrifuged for 10 min at 5,000 rpm in a Sorvall SS-34 rotor, then the supernatant was centrifuged for 5 min at 8,000 rpm. The supernatant was filtered as before, and

one-fifth volume of 30% (w/v) PEG MW 8,000 dissolved in 0.6 N NaCl plus one-fiftieth volume of 20% (w/v) NaCl was added to the filtrate. The mixture was stirred briefly and then stored for 1 hr. After the virus precipitate was collected by centrifugation, the pellet was resuspended in 0.04 M sodium phosphate buffer (one-fourth of the original sap volume), pH 8.2, for 1 hr and then further concentrated by low-speed centrifugation.

Procedures 6 and 7:

Attempts to purify WSSMV according to potyvirus purification methods were also made. As described by Gonsalves and Ishii (7), leaves were ground in liquid nitrogen and two volumes of 0.5 M potassium phosphate buffer, pH 7.5, containing 0.01 M EDTA and 0.1% (w/v) sodium sulfite (Na_2SO_3) were added to the powder. After filtration through moistened cheesecloth, one-half volume each of chloroform and carbon tetrachloride were added to the filtrate. After stirring, the emulsion was centrifuged at 10,000 rpm for 15 min in a Sorvall SS-34 rotor. Eight percent (w/v) PEG MW 8,000 was added to the supernatant and the mixture was stirred for 2 hr. Precipitated virus was recovered by centrifugation and resuspended by stirring for 1 hr in 0.1 M potassium phosphate buffer (one-fifth of the original sap volume), pH 7.0, containing 0.01 M EDTA. After low-speed centrifugation, virus was precipitated from the supernatant by adding 5% (w/v) PEG MW 8,000 and bringing the supernatant to 0.3 M with NaCl. Virus was collected by centrifugation and resuspended in a small volume of buffer. According to Yang et al. (19)(Procedure 7), five volumes of 0.5 M potassium

phosphate buffer, pH 7.2, containing 1.0 M urea, 0.01 M NaDIECA and 0.5% (v/v) 2-ME were added to infected leaves ground in liquid nitrogen. After filtration through cheesecloth, one-fifth volume of chloroform was added to the filtrate and stirred for 15 min to overnight. The emulsion was broken by centrifuging at 9,000 rpm for 15 min. in a Sorvall SS-34 rotor, and the top aqueous phase was decanted through a glass wool pad. Virus was precipitated by the addition of 4% (w/v) PEG MW 8,000 and made to 0.25 M with NaCl. The mixture was stirred for 1 hr, and virus was collected by centrifugation. The pellet was resuspended for several hr to overnight in buffer (one-tenth of the original sap volume) containing 1.0 M urea and 1-4% (v/v) Triton X-100. After low-speed centrifugation, the supernatant was subjected to a second PEG precipitation, and virus pellets were resuspended for several hr in a small volume of buffer.

Density gradient centrifugation. Depending upon the extraction, clarification and concentration methods used, the partially purified preparations were subjected to various additional steps for further purification. Resuspended high-speed pellets from Procedures 1 and 2 were layered on top of 30% (w/v) CsCl and centrifuged for 18 hr in a Beckman SW 50.1 rotor at 36,000 rpm. Partially purified preparations from Procedure 3 were layered on 15-35% (w/v) sucrose gradients and centrifuged for 3 hr at 22,000 rpm in a Beckman SW 25.1 rotor.

The majority of the partially purified preparations, however, were subjected to cesium sulfate (CS_2SO_4) density gradient centrifugation. Supernatants (about 4.0 ml) from low-speed centrifugations of

resuspended PEG pellets were mixed with CS_2SO_4 to produce a 30% (w/v) solution which was layered on top of a 53% (w/v)CS_2SO_4 cushion (about 0.7 ml). Tubes were centrifuged in a Beckman SW 50.1 rotor at 30,000 rpm for 18 hr. Sucrose-cesium sulfate cushion step gradients were also used for further purification. Virus suspensions (about 1.0 ml) were layered on top of 1.6 ml fractions (steps) of 0, 15, 22.5 and 30% (w/v) CS_2SO_4 dissolved in 0.04 M sucrose. Tubes were centrifuged for 2 hr in a Beckman SW 41 rotor at 31,000 rpm. Other step gradients were prepared by layering virus extracts (about 1.0 ml) on top of 0.6 ml fractions of 0.0, 0.5, 1.0 and 1.5 M CS_2SO_4 in 30% (w/v) sucrose or 0.6 ml fractions of 0.0, 0.4, 0.8 and 1.2 M CS_2SO_4 in 30% (w/v) sucrose. Tubes were centrifuged for 2 hr in a Beckman SW 50.1 rotor at 38,000 rpm.

For buoyant density determinations, virus suspensions (about 4.0 ml) were mixed with sufficient CS_2SO_4 to yield a 22% (w/w) solution with a density of 1.21 g/cm^3. Tubes were centrifuged for 18 hr at 39,000 rpm in a Beckman SW 50.1 rotor. Bouyant density measurements were made using a Bausch and Lomb refractometer.

Gradient fractions were removed from tubes either by piercing the bottom with a needle and collecting the fractions by hand or by monitoring at 254 nm with an ISCO (Instrumentation Specialties Co., Lincoln, NE) UA-5 fractionator and hand-collecting fractions. All fractions were examined for virus using ISEM. Virus-containing fractions were pooled, diluted with buffer and centrifuged at 34,000 rpm in a Beckman Type 55.2 Ti rotor for 2 hr. Alternatively, pooled virus fractions were subjected to a second cycle of CS_2SO_4 density gradient

centrifugation, and virus in fractions was concentrated by high speed centrifugation as before.

As controls for WSSMV-infected tissues in protein coat and nucleic acid isolations, healthy wheat antigens were prepared by processing uninfected wheat leaves according to a modification of the potato virus Y purification protocol (19).

Cytoplasmic inclusion isolation and electrophoresis. A modification of the method described by Dougherty and Hiebert (5) for purification of tobacco etch and pepper mottle virus cylindrical inclusions was used for isolating WSSMV inclusions. Leaves from uninfected control plants and WSSMV-infected plants were ground in liquid nitrogen with a mortar and pestle and then ground in two volumes of 0.5 M potassium phosphate buffer (PB) pH 7.3, containing 0.25% Na_2SO_3. One-fourth volume each of chloroform and carbon tetrachloride was added to the extract. The mixture was homogenized with a Janke and Kunkel Ultra-turrax Tissumizer^R (Tekmar Co., Cincinnati, OH) at high speed for 1 min and then centrifuged at 2,500 rpm for 5 min in a Sorvall GSA rotor. The pellet was reextracted with 0.5 ml of 0.5 M PB per gram of original tissue, homogenized and recentrifuged as before. The two supernatants were combined and centrifuged at 9,000 rpm for 15 min in a Sorvall SS-34 rotor. The pellets were resuspended with the Tissumizer in 0.5 ml of 50 mM PB containing 0.1% (v/v) 2-ME and 5% (v/v)Triton X-100 per gram of original tissue. After stirring for 1 hr, the mixture was centrifuged in a Sorvall SS-34 rotor at 13,000 rpm for 15 min. The pellet was resuspended in 50 μl of 20 mM PB containing 0.1% (v/v) 2-ME

per gram of original tissue. The resuspended material was homogenized for 30 sec and then layered on a sucrose step gradient made up of 50% (7 ml), 60% (7 ml) and 80% (10 ml)(w/v) sucrose in 20 mM buffer. The gradient was centrifuged for 1 hr at 21,000 rpm in a Beckman SW 25.1 rotor. Inclusion proteins were located on top of the 80% sucrose zone; about 2 ml of material at the interface between the 80% and 60% sucrose zones was collected dropwise from the bottom of the centrifuge tube. The inclusions were diluted five times with 20 mM PB to remove sucrose and then centrifuged at 13,000 rpm for 15 min. The pellet was resuspended in a small volume of 20 mM PB.

Samples were prepared for electrophoresis by dissociation in one volume of 0.01 M sodium phosphate buffer, pH 7.0, containing 1% (v/v) sodium docecyl sulfate (SDS), 1% (v/v) 2-ME, 8M urea, 10% (v/v) glycerol and 0.015% (w/v) bromphenol blue (disruption buffer). Samples were heated at 100 C for 3-5 min prior to electrophoresis. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 14 cm x 16 cm vertical slab gels 1 mm thick composed of a 12% resolving and 5% stacking gel and the buffer system of Laemmli (12). Electrophoresis was for 4-5 hr at room temperature at 40 mA. Gels were silver stained according to the method of Morrissey (13).

The molecular weight values estimated for WSSMV structural and non-structural proteins were based on the line of best fit of the mobilities of marker proteins plotted against the log of their molecular weights. The following BRL (Bethesda Research Laboratories, Gaithersburg, MD) marker proteins were used for extrapolation of

molecular weight estimates: β-lactoglobulin (18,400 MW), α -chymotrypsinogen (25,700 MW), ovalbumin (43,000 MW) and bovine serum albumin (68,000 MW). Bio-Rad (Bio-Rad Laboratories, Richmond, CA) marker proteins were also used: soybean trypsin inhibitor (21,500 MW), carbonic anhydrase (31,000 MW), ovalbumin (45,000 MW) and bovine serum albumin (66,000 MW). Potato virus Y coat protein was also used as an additional marker.

Single- and double-stranded (ds)RNA extraction and electrophoresis. Several RNA extraction methods were investigated in attempts to isolate viral genomic nucleic from purified WSSMV, and several attempts were made to extract dsRNA from WSSMV-infected roots and leaves. Results of these studies are presented in the Appendix.

Total plant protein isolation and electrophoresis. During early stages of WSSMV infection when there was insufficient infected leaf tissue for virus purification and subsequent capsid protein isolations, a comparison of proteins present in infected and uninfected leaves was made according to a modification of the method described by Jackson et al. (10) for isolating protein from healthy and barley stripe mosaic virus-infected barley. Leaves were ground in liquid nitrogen and then ground in four volumes of 50 mM tris-HCl buffer, pH 7.6, containing 60 mM KCl and 6 mM 2-ME (SSB). After squeezing through four layers of cheesecloth, the extract was centrifuged at 4,000 rpm in a Sorvall SS-34 rotor. The supernatant fraction was diluted with one-third volume of 40 mM tris-HCl, pH 8.0, containing 4 mM EDTA, 40% (v/v) glycerol, 8% (w/v) SDS, 20% (v/v) 2-ME and 0.01% (w/v) bromphenol blue (4X TESS) and boiled

for 5 min to solubilize proteins. The SSB pellet was resuspended in one volume of SSB and centrifuged as before. The resulting pellet was resuspended in one-fourth volume of 1X TESS and boiled for 5 min. Both the 4X TESS (original supernatant) and the 1X TESS (original pellet) preparations were centrifuged to remove remaining insoluble material. Supernatants were electrophoresed in 10% SDS-polyacrylamide gels and silver stained as for WSSMV cylindrical inclusion proteins.

Capsid protein isolation and electrophoresis. To isolation coat protein pellets from healthy and purified virus preparations were resuspended in a small volume of disruption buffer and heated at 100 C for 3-5 min prior to electrophoresis. Samples were electrophoresed and silver stained as described previously for inclusion proteins.

Serology. For the initial injection, purified virus (after one cycle of gradient centrifiguation) resuspended in sterile distilled water or in 0.01 M potassium phosphate buffer, pH 7.0, was emulsified 1:1 with Freund's complete adjuvant and injected into the hip muscle of a white New Zealand rabbit. Virus was emulsified 1:1 with Freund's incomplete adjuvant for subsequent injections given 2, 4, 8 and 12 wk after the initial injection. About 1 mg of virus was injected over the 12 wk period. Blood was collected at about 10-day intervals starting 4 wk after the initial injection. Serum was stored frozen or lyophilized.

For immunosorbent electron microscopy (ISEM), a 1:300-1:500 dilution of antiserum was used for coating grids and for decoration. ISEM was performed as described previously in Chapter I.

Electron microscopy. Virus preparations were examined

occasionally without using ISEM. Carbon-coated Parlodion- or Formvar-filmed grids were floated on 30 µl drops of virus preparations for 1-60 min, drained and negatively stained with 2% ammonium molybdate, pH 7.0. Grids were examined at 20,000X in a Philips 201 transmission electron microscope.

Results

Virus purification. Three methods for releasing virus from infected leaves were investigated: grinding in liquid nitrogen, grinding in buffer and homogenizing in a Waring blender. Although all three methods yielded comparable amounts of virus from the same amount of tissue, cutting leaves into small pieces followed by grinding in liquid nitrogen was the most satisfactory method for the quick release of relatively intact virus particles from moderate amounts (10-150 g) of infected tissues. Grinding in buffer alone was slow and tedious, particularly when grinding mature leaves with much sclerenchyma. Only when small amounts (< 20g) of material were processed was grinding in buffer used. Similarly, homogenizing wheat leaves in a blender was tedious primarily because of the binding effect of the sclerenchyma on the blades of the blender. When large (> 150 g) amounts of tissue were processed, however, a blender was used sometimes.

In initial trials, three purification protocols utilizing low speed centrifugation for sap clarification were used for purifying WSSMV (Procedures 1-3). Purification steps were monitored using conventional electron microscopy, as WSSMV antiserum was not yet available. Relative to the particle concentration in purified preparations, considerable loss of virus occurred when crude extracts or partially clarified supernatants were subjected to low speed centrifugation (Table 3). In addition, particles in purified preparations prior to final high speed pelleting were highly fragmented, with the majority less than 800 nm in length (Fig. 8). An average of approximately one particle per grid

Table 3. Loss of virus due to low speed centrifugation[a] during WSSMV purification as determined by conventional electron microscopy.

Purification protocol[b]	Virus particles count in low speed pellets[c]	Virus particle count in purified preparations[d]
Slykhuis and Polak, 1971	3	5
Usugi and Saito, 1979	2	5
Veerisetty and Brakke, 1978	2	6

[a]Centrifugation at 4,000-9,000 rpm in a Sorvall SS-34 rotor.

[b]Thirty grams of tissue were extracted in each protocol.

[c]Values are average numbers of virus particles counted per 300-mesh grid square on 20 grid squares. Presence of virus in low speed pellets indicates loss during purification.

[d]Values are average numbers of virus particles per grid square obtained by examining 20 grid squares and represent virus present in preparations prior to final high speed pelleting.

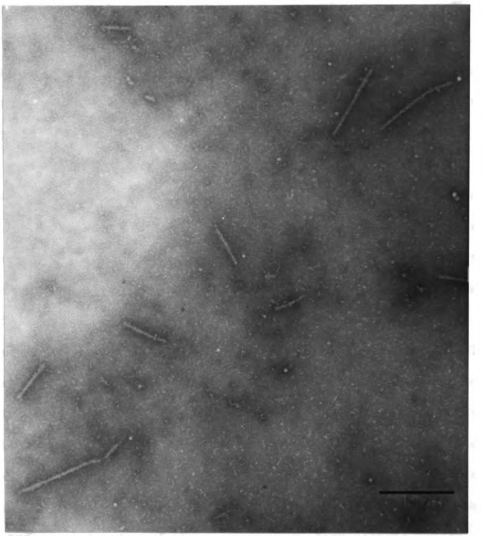


Figure 8. WSSMV particles purified according to Procedure 2. Scale bar = 0.5 µm.

square was found in low speed pellets during subsequent cycles of differential centrifugation. Thus, because irrecoverable losses of virus occurred when preparations were centrifuged to remove host material, and because remaining virus particles were highly fragmented due to repeated pelleting, low speed centrifugation and differential centrifugation for sap clarification and concentration, respectively, were not studied further.

Modifications of protocols for purifying citrus tristeza virus and several potyviruses (Procedures 4-7) were suitable for purifying WSSMV in general. These protocols incorporated PEG precipitation for virus concentration, which was more satisfactory for maintaining particle integrity and recovering virus from clarified supernatants than differential centrifugation. Some loss of virus due to PEG precipitation was evident, but losses were slight (4-15 particles/GS) when compared with virus concentrations in purified preparations (> 400/GS) as determined by ISEM (Table 4). Some purification attempts utilized the addition of 1-3% Triton X-100 to the filtrate prior to PEG precipitation to solubilize membranes and facilitate virus release. Other attempts incorporated clarification with organic solvents followed by PEG precipitation of the aqueous phase from centrifugation of the emulsion. In some instances PEG was added directly to the filtrate following a brief low speed centrifugation. In most cases virus loss during these steps was minimal (Table 4), and PEG precipitation was adopted for concentrating WSSMV particles. However, unless PEG-precipitated virus pellets were resuspended by stirring slowly for

Table 4. Loss of virus due to clarification and concentration procedures during WSSMV purification as determined by ISEM.

Procedure	Particle count[a]	% loss[b]
Clarification by low speed centrifugation	40-200	15-40%
Chloroform or carbon tetrachloride clarification	1-8	0.3-1%
PEG precipitation (with 10 min resuspension)	20-150	7-19%
PEG precipitation (with overnight resuspension)	4-15	1-3%

[a]Values represent the range of virus particles counted per 300-mesh grid square (GS) on 30 GS.

[b]Calculations based on approximately 400-1,000 particles counted per 300-mesh GS (obtained by extracting 300 g of leaves) on 120 GS of purified virus preparations prior to high speed centrifugation.

several hours to overnight, recovery of virus from pellets was reduced (Table 4), possibly because of PEG-induced aggregation.

Various extraction buffers were tested to find the one(s) most suitable for extracting the largest number of virus particles from infected leaves (Table 5). Fifty mM citrate buffer, pH 5.4 and 0.1 M potassium phosphate buffer, pH 7.0 were comparable in extracting the greatest number of virus particles and producing small, white PEG-precipitation pellets. PEG pellets from tissue extracted with 0.1 M citrate-sodium phosphate buffer, pH 6.4 were also small and clean, but virus yields were reduced. Extraction in 20 mM HEPES buffer, pH 7.0, 0.1 M tris-HCl, pH 7.8 and 0.1 M borate, pH 8.6 resulted in greatly reduced virus yields and large PEG pellets contaminated with green host material. Citrate and potassium phosphate buffers were therefore considered best for virus extraction.

Several additives to extraction and resuspension buffers were also tested for their ability to improve virus yields or reduce aggregation. The addition of 0.5-1.0 M urea to extraction and resuspension buffers slightly improved yields from PEG-precipitated WSSMV but had little effect on aggregation, as side-to-side (Fig. 9) and end-to-end (Fig. 10) aggregation was present in purified preparations. A reducing agent, either 0.5% 2-ME or 0.1% Na_2SO_3, and NaDIECA in the extraction buffer were important for maintaining particle integrity, as purified suspensions prepared without these additives contained little virus. The addition of 2-4% Triton X-100 to the virus extract filtrate facilitated release of virus from host material, and addition of Triton

Table 5. Efficiency of extraction buffers for WSSMV purification[a]

Buffer	PEG pellet diameter (mm)	Virus particle count[b]
0.05 citrate, pH 5.4	2	94
0.1 M citrate-sodium phosphate,pH 6.4	3	47
0.1 M potassium phosphate, pH 7.0	4	92
0.02 M HEPES, pH 7.0	17	30
0.1 M tris-HCl, pH 7.8	17	10
0.1 M borate, pH 8.6	20	3

[a]Five grams of tissue per buffer were extracted.

[b]Numbers are ISEM averages per 300-mesh grid square from counting 20 grid squares. Values are numbers of particles present in purified preparations following density gradient centrifugation.

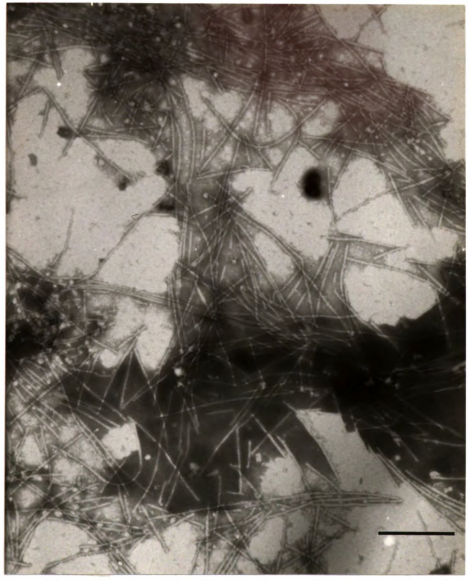


Figure 9. Side-to-side aggregation in WSSMV particles purified according to Procedure 7. Scale bar = 0.5 µm.

Figure 10. End-to-end aggregation in WSSMV particles purified according to Procedure 7. Scale bar = 0.5 µm.

X-100 to the resuspension buffer facilitated resuspension of PEG-precipitated virus.

For routine purification, centrifugation in sucrose-cesium sulfate cushion step gradients was found the most suitable for further purifying WSSMV to near-homogeneity. Sucrose gradients were not suitable because virus particles were found distributed throughout much of the gradient. Although Usugi and Saito (17) utilized CsCl gradients for WSSMV purification, results from this study indicate that not only did the CsCl frequently react with the copper grids to obscure virus particle visualization but also particles purified in CsCl occasionally appeared more flexuous than normal (Fig. 11). Because of this and because Gonsalves et al. (6) found that citrus tristeza virus particles were unstable in CsCl unless fixed with formaldehyde, Cs_2SO_4 was chosen for density gradient centrifugation.

Depending upon the extraction buffer used as well as clarification and concentration methods, prominent bands of green material were visible at the top of and about two-thirds of the way down in gradient tubes following Cs_2SO_4 density gradient centrifugation. Extraction in citrate and phosphate buffers and concentration with PEG eliminated most of the green host material. In subsequent step gradient centrifugations, however, a discrete opalescent to solid white band, occasionally containing white flocculent material, was consistently found about two-thirds of the way down in gradient tubes, corresponding to an area just above the top of the 22.5% Cs_2SO_4 step. Similarly treated healthy preparations also showed this band to a lesser extent.

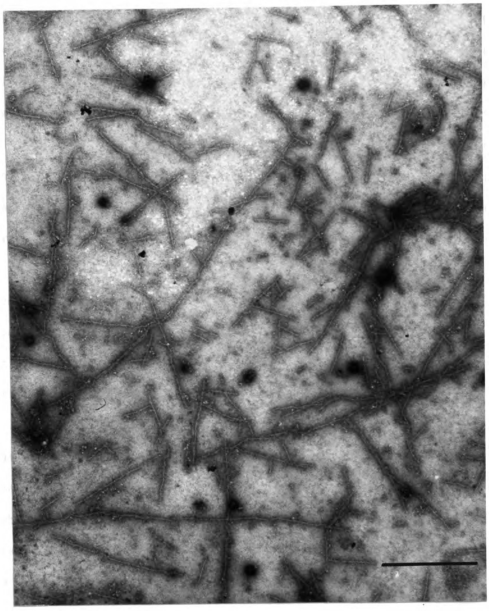


Figure 11. WSSMV particles partially-purifed according to Procedure 7 and further purified by CSCl density gradient centrifugation. Scale bar = 0.5 μm.

Using ISEM, virus was found to occur in a zone within and just below the opalescent band; no distinct virus band per se was evident (Fig. 12). A second cycle of gradient centrifugation eliminated much of the opalescent band, but considerable loss of virus also occurred. In attempts to separate the white material from virus within the gradient, various concentrations of Cs_2SO_4 dissolved in different concentrations of sucrose and centrifuged in larger tubes (Beckman SW41 rotor instead of SW50.1 rotor) were investigated. Although some small improvement with some of the treatments was evident, the majority of the virus remained in close association with the contaminating material, as the buoyant density of this proteinaceous material was apparently similar to that of WSSMV. Therefore, removal of this contaminating material prior to density gradient centrifugation was necessary to attain clean virus preparations. Overnight clarification with chloroform or clarification with a 20% mixture of chloroform and n-butanol (8:1, v/v) for several hr eliminated most of the contaminating host proteins.

ISCO gradient fractionator scans of Cs_2SO_4 gradients containing virus extracted in citrate or phosphate buffer and clarified and concentrated other than as indicated above revealed larged amounts of UV-absorbing material near the top of the tube and in the area of the opalescent band (Fig. 13A). During attempts to isolate the viral genome from virus purified in this manner, broadly stained areas of high molecular weight material identified as DNA by nuclease treatments were observed in 1% agarose gels (see Appendix). Dougherty and Hiebert (4) found that using phosphate buffer for purifying tobacco etch and pepper

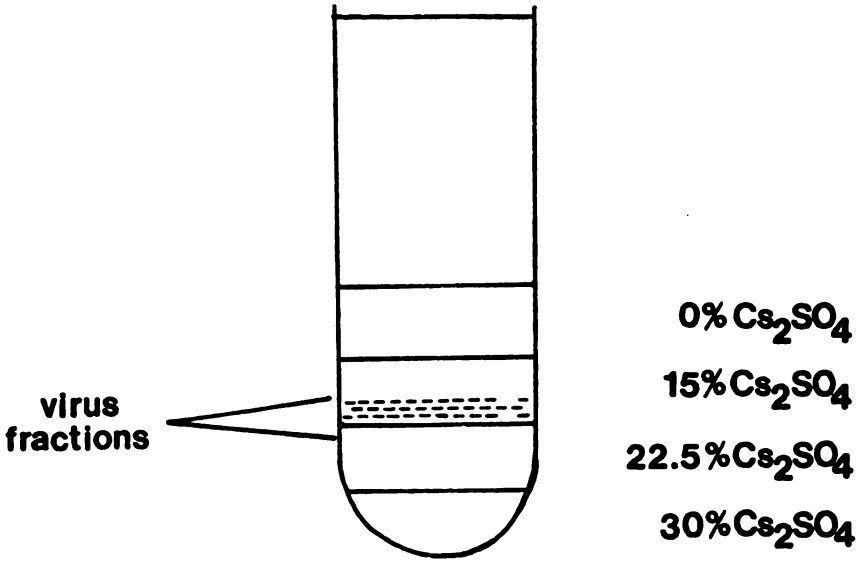


Figure 12. Schematic of gradient tube following Cs_2SO_4 density gradient centrifugation.

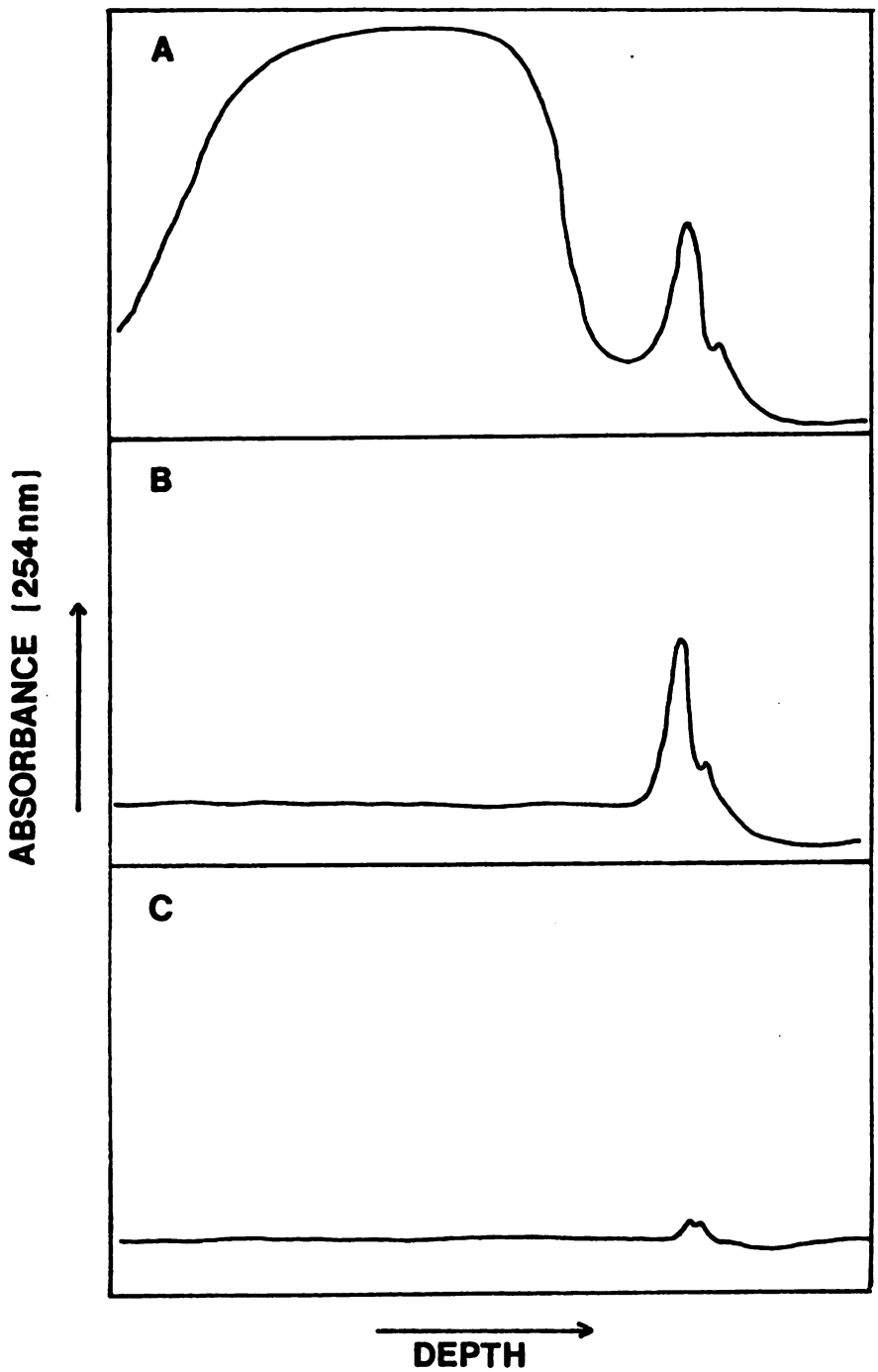


Figure 13. ISCO scans of gradient tubes following Cs_2SO_4 density gradient centrifugation: (A) Virus extracted with phosphate buffer and clarified with chloroform for 10 min. (B) Virus extracted with HEPES buffer and clarified with chloroform for 10 min. (C) Virus extracted with HEPES buffer and clarified with chloroform overnight.

mottle viruses yielded virus preparations contaminated with host DNA, while virus purified with HEPES buffer yielded DNA-free preparations. Similar results were obtained when WSSMV was purified using HEPES buffer instead of phosphate buffer (Fig. 13B), although virus yields were reduced. When overnight clarification in chloroform was utilized, ISCO scans revealed that much of the host proteinaceous material had been eliminated (Fig. 13C).

The method finally adopted for purifying WSSMV (and healthy antigens) was a modification of the procedure described by Yang et al. (19) for purifying potato virus Y. All steps were done at about 4 C. Leaves were ground in liquid nitrogen and then ground in buffer for maximum release of virus particles. About 75-100 g of tissue were processed each time in duplicate or triplicate, and the resuspended PEG pellets from each grinding were pooled. Buffers suitable for extraction were 0.1 M potassium phosphate buffer, pH 7.0, 0.05 M citrate buffer, pH 5.4 and 0.02 M HEPES buffer, pH 7.0. One molar urea, 0.01 M NaDIECA and 0.1% (w/v) Na_2SO_3 were included in the extraction buffer. After squeezing the extract through four layers of moistened cheesecloth, one volume of chloroform was added to the filtrate, and the mixture was stirred for 6 hr and then allowed to settle overnight. The emulsion was broken by centrifuging at 9,000 rpm for 15 min in a Sorvall SS-34 rotor, and 5% (w/v) PEG MW 8,000 was added to the supernatant which was made 0.25 M with NaCl. The mixture was stirred for 1 hr, and precipitated virus was pelleted by centrifugation for 20 min at 10,000 rpm. The pellet was resuspended in buffer (one-tenth of the original sap volume)

containing 1.0 M urea and 4% (v/v) Triton X-100 by stirring several hr to overnight. The suspension was centrifuged at low speed and the supernatant was subjected to a second PEG precipitation. The virus suspension (about 1.0 ml) was layered on top of a sucrose-cesium sulfate cushion step gradient consisting of 0.6 ml steps containing 0.0, 0.4, 0.8 and 1.2 M Cs_2SO_4 dissolved in 30% (w/v) sucrose. Tubes were centrifuged for 2 hr at 38,000 rpm in a Beckman SW 50.1 rotor. Virus-containing fractions were diluted and centrifuged at 34,000 rpm for 2 hr in a Beckman Type 55.2 Ti rotor. To conserve virus, preparations occasionally were not subjected to a second PEG precipitation and usually were not subjected to a second cycle of density gradient centrifugation. Virus suspensions (prior to pelleting) were stored at 4 C for up to 5 days without apparent degradation, whereas virus suspensions stored at -20 C or as resuspended pellets at 4 C showed considerable aggregation.

The absorption spectrum of purified WSSMV with a maximum at 260 nm and minimum at 246 nm was typical of rod-shaped viruses (Fig. 14). The light scattering at 320 nm possibly indicated aggregation of the virus preparations, which was confirmed by electron microscopy (Fig. 9 and 10). Virus purified in phosphate buffer often had high $A_{260/280}$ absorption ratios (1.30-1.60) due to contaminated host DNA in the preparations (see Appendix). For virus purified in HEPES buffer, the $A_{260/280}$ ratio varied from 1.12-1.25 (not corrected for light scattering). This indicates a nucleic acid content of about 4-6%, which is typical of filamentous viruses (14).

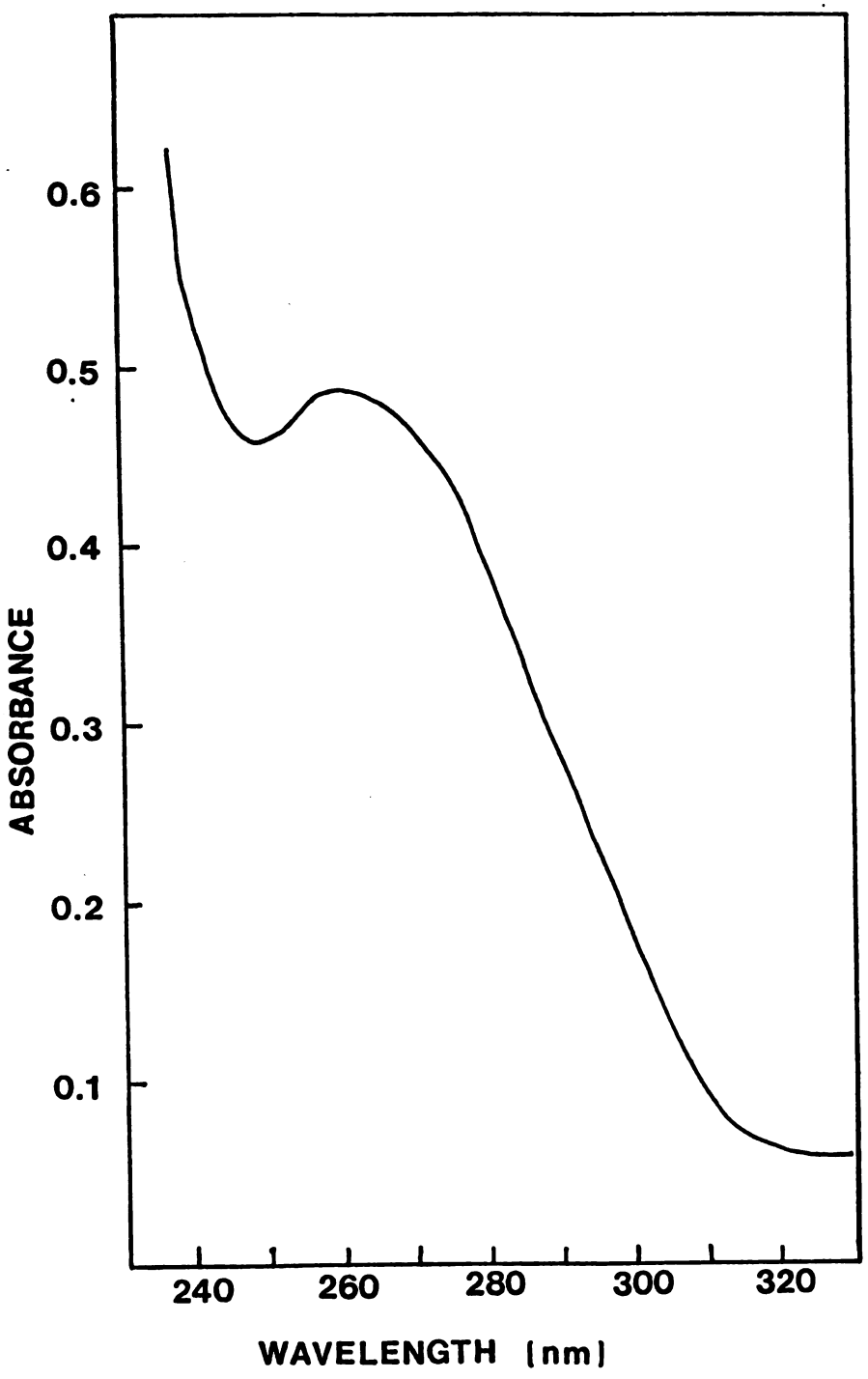


Figure 14. Absorption spectrum of WSSMV purified according to the final adopted protocol.

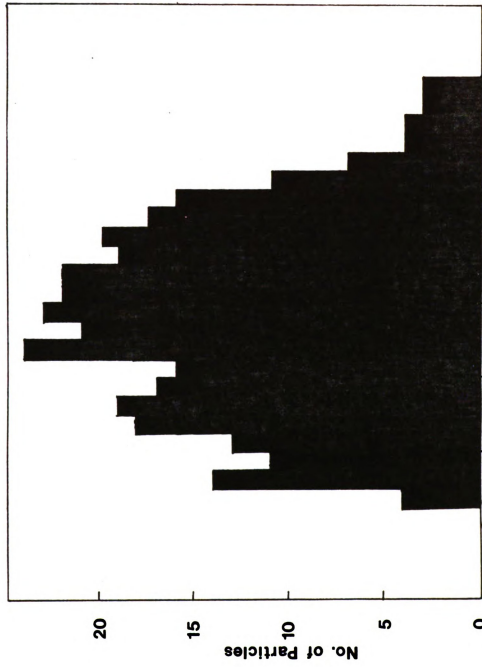
Initially, an extinction coefficient $E^{0.1\%}_{260\ nm}$ of 2.0 was assumed for purified WSSMV preparations based on work with citrus tristeza virus (6). Yields of purified virus calculated using this extinction coefficient ranged from 0.4-7.0 mg/100 g infected leaves (4-70 μg/g). However, because it may be more reasonable to assume an extinction coefficient of 2.4, which is similar to that used for potyviruses such as tobacco etch virus, yields were recalculated using this extinction coefficient. Yields of purified virus ranged from 0.3-5.5 mg/100 g when the potyvirus extinction coefficient was used.

Particles in purified preparations ranged in length from 260-1,400 nm, with a modal length of about 800 nm (Fig. 15). Most of the particles were monodisperse although some aggregation was evident as indicated previously (Fig. 16). Preparations of virus purified using earlier, similar protocols were infectious when manually rubbed onto leaves of susceptible wheat cultivars. Transmission levels were low, however, as plants in only five out of 14 pots (36%) became infected. Results from infectivity studies with preparations purified according to the final adopted protocol are not yet available.

A buoyant density of 1.20-1.21 g/cm^3 was recorded for WSSMV in CS_2SO_4.

Estimation of WSSMW cylindrical inclusion protein molecular weight. The purified inclusions were dissociated in disruption buffer and analyzed by SDS-PAGE. Electrophoresis of dissociated inclusion protein preparations resulted in the resolution of several bands in the WSSMV-infected samples after silver staining, whereas no bands were

Figure 15. Distribution of lengths of WSSMV particles from purified preparations. Modal particle length = 792 nm


No. of Particles
0
5
10
15
20
200
400
600
800
1000
1200
1400
nm

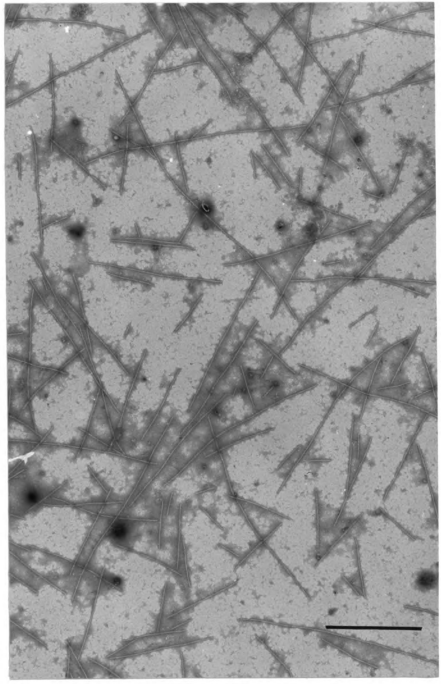


Figure 16. Purified WSSMV particles. Scale bar = 0.5 μm.

present in samples prepared from uninfected material (Fig. 17). A prominent band at about 69,000 MW present only in samples from infected tissues may represent WSSMV inclusion protein.

Estimation of WSSMV-infected plant protein and capsid protein molecular weights. Proteins from uninfected wheat were compared by electrophoresis in 10% SDS-polyacrylamide gels with those from wheat during early stages of WSSMV infection. Comparison of the protein profiles of uninfected and infected wheat revealed the presence of a WSSMV-specific protein of about 36,000 MW in extracts from supernatant (soluble protein) preparations from infected tissues (Fig. 18). No differences between proteins from uninfected and infected samples were detected in low speed pellet fractions.

Capsid protein was isolated from pellets of purified virus by resuspension in disruption buffer and electrophoresis in 12% SDS-polyacrylamide resolving gels. When citrate buffer was used for virus extraction, two distinct bands at about 32,000 MW and 29,000 MW were present in preparations from infected tissues but not in those from healthy tissue (Fig. 19). When phosphate buffer was used for extraction, three WSSMV-specific bands at about 36,000 MW, 32,000 MW and 29,000 MW were present in extracts from infected leaves (Fig. 20).

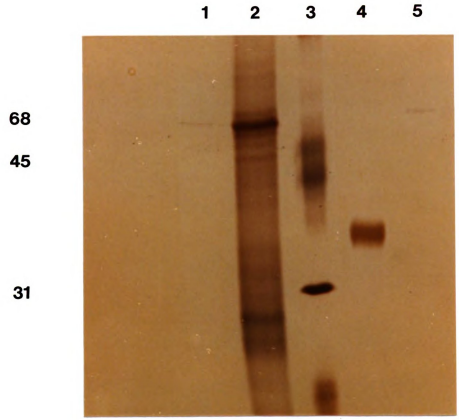


Figure 17. SDS-polyacrylamide gel showing proteins associated with purified WSSMV cylindrical inclusion preparations. Lane 1 = preparations from uninfected plants. Lane 2 = purified inclusion body proteins from infected plants. Lane 3 = protein standards; values at left are molecular weights (x 10^3) of standards. Lane 4 = potato virus Y coat protein, and lane 5 = bovine serum albumin.

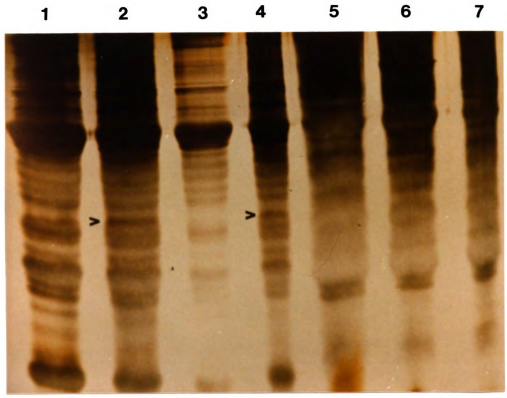


Figure 18. SDS-polyacrylamide gel showing plant proteins associated with uninfected and WSSMV-infected wheat. Lanes 1-4 = supernatant preparations and Lanes 5-7 are pellet preparations. Lanes 2, 4, 5 and 7 show protein profiles from infected wheat, and lanes 1, 3, and 6 show protein profiles from uninfected wheat. Arrow indicates band of MW of about 36,000.

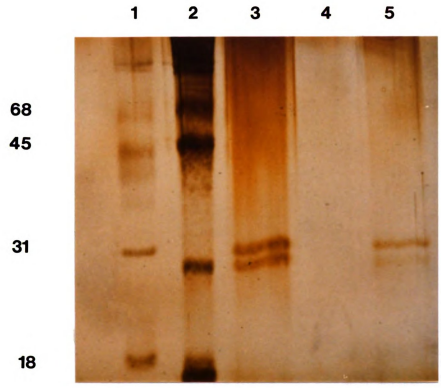


Figure 19. SDS-polyacrylamide gel showing coat proteins associated with purified WSSMV particles when extracted with citrate buffer. Lane 1 = Bio-Rad protein standards and Lane 2 = BRL standards. Values at left are molecular weights (x 10^3) of standards. Lanes 3 and 5 are coat proteins from infected wheat, and lane 4 shows coat protein preparations from uninfected plants.

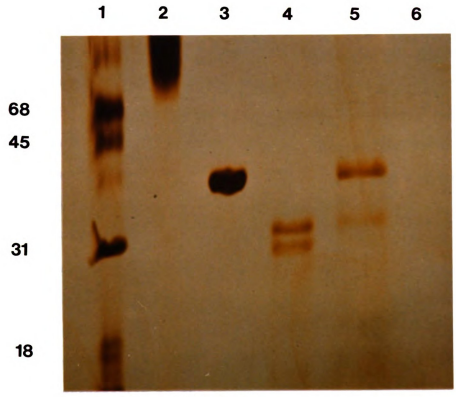


Figure 20. SDS-polyacrylamide gel showing coat proteins associated with purified WSSMV particles when extracted with phosphate buffer. Lane 1 = Big-Rad protein markers; values at left represent molecular weights (x 10^3) of standards. Lane 2 = bovine serum albumin standard and lane 3 = potato virus Y coat protein. Lane 4 = proteins from WSSMV extracted with citrate buffer. Lane 5 = proteins from WSSMV extracted with phosphate buffer, and lane 6 = preparations from uninfected plants.

Discussion

Only one group (17) has reported a purification protocol for WSSMV since Slykhuis and Polak (16) associated flexuous filamentous virus particles with wheat showing wheat spindle streak mosaic symptoms in 1971. This purification protocol involved CsCl buoyant density gradient centrifugation after clarification of tissue extracts in carbon tetrachloride and concentration of virus particles by several cycles of differential centrifugation. Particles in purified preparations were highly fragmented and therefore unsuitable for biochemical characterization. The purification scheme reported in this work consists of clarification of extracts in chloroform, concentration of virus by PEG precipitation and further purification in sucrose-cesium sulfate density gradients. This protocol minimizes repeated WSSMV pelleting; as a result, particles in purified preparations are relatively intact, a desirable feature for biochemical analysis. In most cases, virus yields using this protocol were sufficient for characterization studies. Furthermore, the new protocol provides a rapid concentration of the virus preparation and a rapid banding of virus particles in gradient fractions.

Preparations of particles purified by earlier procedures in this work as well as those purified by the protocol finally adopted were infectious. Thus, although purification protocols in this work yielded longer, more intact particles than the previously reported protocol (17), short particles were still present in these preparations. Therefore, the actual length of WSSMV particles, or the minimum particle

length necessary for infection, remains unknown.

ISEM was a valuable tool for monitoring the presence of virus during the development of a suitable WSSMV purification protocol. Both antisera prepared by the author and provided by T. Usugi were suitable for use in ISEM. Because considerable loss of virus was discovered when extracts were subjected to low speed centrifugation to remove host debris, and when PEG pellets were resuspended only briefly (Table 4) these steps were modified to reduce irrecoverable virus loss.

The reported buoyant density of 1.20-1.21 g/cm^3 in CS_2SO_4 compares well with the density of 1.28 g/cm^3 in CsCl reported previously (17). Although WSSMV particles morphologically resemble those of members of the closterovirus group, the buoyant density of WSSMV in Cs_2SO_4 is lower than that for closteroviruses (1.24-1.27 g/cm^3) (1) and more closely agrees with the range of values reported for carlaviruses (11) and potyviruses (9).

Other data reported in this work suggest that WSSMV may be a member of the potyvirus group. A molecular weight of 69,000 for cytoplasmic inclusion proteins (the formation of which is characteristic for potyviruses) is within the range (67,000-70,000) of cylindrical inclusion molecular weights reported for several potyviruses (8). The molecular weights (36,000, 32,000 and 29,000) of the coat proteins and their heterogeneity in size are also consistent with those characteristics of potyviruses (8). Also, other properties reported in this work such as absorption ratios of 1.12-1.25, in addition to previously reported properties such as a thermal inactivation point of

50 C for 10 min and a dilution endpoint of 1-5 X 10^3 (17) provide preliminary evidence for inclusion of this virus in the potyvirus group. Particles of members of the closterovirus group contain a single protein species of 23,000-25,000 molecular weight, and closterovirus absorption ratios are unusually high (1.3-1.8)(1). Carlaviruses are 600-700 nm in length and contain a single protein species of 31,000-34,000 molecular weight (11). Because properties and characteristics reported for WSSMV differ from those of closteroviruses and carlaviruses but are very similar to those of potyviruses, WSSMV is a probable member of the potyvirus group. To determine the relationship of WSSMV to other definitive potyviruses, serological techniques such as ELISA, ISEM or SDS-immunodiffusion will be included in future studies.

Literature Cited

1. Bar-Joseph, M., and Murant, A.F. 1982. Closterovirus group. No. 260 In: Descriptions of Plant Viruses, Commonw. Mycol. Inst. Assoc. Applied Biol., Kew, Surrey, England. Unpaged.

2. Bar-Joseph, M., Gumpf, D.J., Dodds, J.A., Rosner, A., and Ginzberg, I. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75: 195-198.

3. Damirdagh, I.S., and Shepherd, R.J. 1970. Purification of the tobacco etch and other viruses of the potato Y group. Phytopathology 60: 132-142.

4. Dougherty, W.G., and Hiebert, E. 1980. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Reaction conditions and identification of capsid protein as one of the products of *in vitro* translation of tobacco etch and pepper mottle viral RNAs. Virology 101: 466-474.

5. Dougherty, W.G., and Hiebert, E. 1980. Translation of potyvirus RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation and cylindrical inclusion protein as a product of the potyvirus genome. Virology 104: 174-182.

6. Gonsalves, D., Purcifull, D.E., and Garnsey, S.M. 1978. Purification and serology of citrus tristeza virus. Phytopathology 68: 553-559.

7. Gonsalves, D., and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70: 1028-1032.

8. Hiebert, E., and McDonald, J.G. 1973. Characterization of some proteins associated with viruses in the potato Y group. Virology 56: 349-361.

9. Hollings, M., and Brunt, A. 1981. Potyvirus group No. 245 In: Descriptions of Plant Viruses, Commonw. Mycol. Inst. Assoc. Applied Biol., Kew, Surrey, England. Unpaged.

10. Jackson, A.O., Dawson, J.R.O., Covey, S.N., Hull, R., Davies, J.W., McFarland, J.E., and Gustafson, G.D. 1983. Sequence relations and coding properties of a subgenomic RNA isolated from barley stripe mosaic virus. Virology 127: 37-44.

11. Koenig, R. 1982. Carlavirus group. No. 259 In: Descriptions of Plant Viruses, Commonw. Mycol. Inst. Assoc. Applied Biol., Kew,

Surrey, England. Unpaged.

12. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

13. Morrissey, J.H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117: 307-310.

14. Paul, H.L. 1959. Die Bestimmung der Nucleinsauregehaltes pflanzlicher Viren mit Hilfe einer Spectrophotometrischen. Methode. Z. Naturforsch., Teil C, Biochem., Biophys., Biol., Virol. 14b: 427-432.

15. Shepherd, R.J., and Purcifull, D.E. 1971. Tobacco etch virus. No. 55 In: Descriptions of Plant Viruses, Commonw. Mycol. Inst. Assoc. Applied Biol., Kew, Surrey, England. Unpaged.

16. Slykhuis, J.T., and Polak, Z. 1971. Factors affecting manual transmission, purification, and particle lengths of wheat spindle streak mosaic virus. Phytopathology 61: 569-574.

17. Usugi, T., and Saito, Y. 1979. Relationship between wheat yellow mosaic virus and wheat spindle streak mosaic virus. Ann. Phytopathol. Soc. Jpn. 45: 397-400.

18. Veerisetty, V., and Brakke, M.K. 1978. Purification of some legume carlaviruses. Phytopathology 68: 58-64.

19. Yang, L., Reddick, B., and Slack, S.A. 1983. Results of experiments on the purification of potato virus Y. (Abstr.) Phytopathology 73: 794.

APPENDIX

WHEAT SPINDLE STREAK MOSAIC VIRUS GENOMIC NUCLEIC ACID AND DOUBLE-STRANDED RNA ISOLATION ATTEMPTS

APPENDIX

Wheat spindle streak mosaic virus genomic nucleic acid and double-stranded RNA isolation attempts

Abstract

Attempts were made to isolate genomic nucleic acid from purified wheat spindle streak mosaic virus and double-stranded (ds)RNA from WSSMV-infected tissues. Only DNA and a low molecular weight single-stranded RNA, both possibly of host origin, were obtained in attempts to isolate genomic nucleic acid and dsRNA extracts. Because the relatively small amounts of tissues used were not highly infected, levels of both genomic nucleic acid and dsRNA possibly were too low to detect.

Introduction

Although the purification of wheat spindle streak mosaic virus (WSSMV) has been reported previously (12), no reports on the biochemical characterization of viral coat protein, inclusion protein or genomic nucleic acid have been published. Preliminary data on the characterization of WSSMV capsid and inclusion proteins are presented in Chapter III.

In this work, numerous attempts were made to isolate and identify viral genomic nucleic acid, presumably single-stranded (ss)RNA, from purified virus and replicative double-stranded (ds)RNA from WSSMV-infected tissues. The identification of the genome of WSSMV and its molecular weight will facilitate placement of this virus into a particular group, and patterns of dsRNA species that have been characterized for a particular virus or group of viruses can be used for routine and rapid virus disease diagnosis. Results from attempts to isolate ss- and dsRNA from WSSMV-infected plants are presented in this section.

Materials and methods

Extraction and electrophoresis of genomic nucleic acid. Virus purified according to the method finally adopted (except that clarification was for 30 minutes rather than overnight; see Chapter III) was resuspended in a small volume of 10 mM Tris (tris hydroxymethyl aminomethane) buffer, pH 8.0, containing 1 mM EDTA (TE buffer) and then subjected to various ssRNA isolation procedures. Extracts from healthy plants were prepared similarly and subjected to the same isolation procedures. All glassware was washed, rinsed several times with distilled water, coated with silicone (Sigmacote; Sigma Chemical Co., St. Louis, MO) and baked overnight at 225C. Buffers were made with water treated with diethyl pyrocarbonate (Sigma) and autoclaved to inactivate ribonucleases.

Initial extraction attempts were made following the standard phenol-sodium dodecyl sulfate (SDS) method (9). Phenol was distilled and then saturated with 1.0 M tris buffer, pH 8.0, containing 0.2% (v/v) 2-mercaptoethanol (2-ME) and 0.1% (v/v) 8-hydroxyquinoline prior to use. One volume of virus suspension, two volumes of phenol:chloroform (1:1, v/v) and 1% (w/v) SDS were mixed in sterile microfuge tubes or silicone-coated glass tubes and inverted gently or vortexed for 30 sec to 3 min. In some cases, one volume of 5% (w/v) SDS, 10 mg/ml bentonite and 5 mM EDTA was added to two volumes of virus suspension (3). An equal volume of water-saturated phenol was added, and the mixture was vortexed for 2-3 min. The emulsion was broken by centrifuging for 15 min at 10,000 rpm in a Sorvall SS-34 rotor, and the top aqueous phase was extracted as

before and recentrifuged. The two aqueous phases were combined and reextracted as before (sometimes this step was eliminated). After centrifugation, two and one-half volumes of absolute ethanol and one-tenth volume of 2.5 M sodium acetate, pH 5.2, were added to the final aqueous phase. The mixture was stored at -20 C or -70 C for 6 hr to overnight. After centrifugation for 30 min at 10,000 rpm, the pellet was dried under vacuum and resuspended in a small volume of TE buffer.

To avoid exposure of nucleic acids to shear forces and reduce nucleic acid loss during phase separation, isolation in a single-phase system was investigated (5). One volume of virus suspension was mixed with one volume of absolute ethanol; this solution was mixed with three volumes of phenol:ethanol (3:1, v/v) and stored on ice for 1 hr. After low speed centrifugation, the pellet containing nucleic acid was resuspended in 0.06 M sodium phosphate buffer, pH 7.0. The nucleic acid was precipitated twice with ethanol to remove phenol, and the final solution resuspended in buffer was clarified by centrifugation at 10,000 rpm for 20 min in a Sorvall SS-34 rotor to remove remaining traces of denatured protein.

Two extraction procedures involving disruption buffers instead of the phenol-SDS system were also employed (9). In the first procedure, disruption buffer [2.4% (w/v) SDS, 1% (v/v) 2-ME, 20% (w/v) sucrose, 0.08 M tris, 0.04 M sodium acetate, 0.02 M EDTA, pH 7.0] was added to an equal volume of virus suspension. The mixture was heated at 60 C for 5 min prior to electrophoresis. For the second procedure, one volume of disruption buffer consisting of 0.08 M tris, 0.4 M NaCl, 4 mM EDTA, 4%

(w/v) SDS and 2 mg/ml bentonite at pH 9.0 was added to three volumes of virus suspension. The mixture was allowed to incubate overnight at room temperature prior to electrophoresis.

A modification of the wheat streak mosaic virus RNA extraction procedure of Brakke and Van Pelt (2) was also investigated. One volume of virus suspension was added to a mixture of 0.2 M ammonium carbonate, 2 mM EDTA and 2% (w/v) SDS, pH 9.0. The mixture was layered on a 7.5-30% (w/v) sucrose linear gradient dissolved in 0.5 M tris-HCl buffer, pH 9.0. Gradients were centrifuged at 22,000 rpm for 20 h in a Beckman SW 25.1 rotor. After centrifugation, gradient columns were analyzed for UV absorbance with an ISCO (Instrument Specialties Co., Lincoln, NE) UA-5 monitor. The RNA peak was collected and precipitated with two and one-half volumes of absolute ethanol and one-tenth volume of 2.5 M sodium acetate. After overnight storage at -20 C, tubes were centrifuged at 10,000 rpm for 30 min in a Sorvall SS-34 rotor. The pellet was dried under vacuum and resuspended in TE buffer.

Nucleic acid suspensions mixed with one-third volume of tracking dye [5% (w/v) SDS, 25% (v/v) glycerol, 0.025% (w/v) bromphenol blue] were loaded on a 2.0 mm thick horizontal 0.7-1% (w/v) agarose gel in a Minisub gel apparatus (Bio-Rad Laboratories, Richmond, CA) and electrophoresced in E buffer (40 mM tris, 2 mM EDTA, 5 mM sodium acetate, pH 7.8) at 60 mA for about 2 hr. Gels were stained with ethidium bromide (1 μg/ml) for 15 min then destained in water for 5 min. Nucleic acids in the gel were visualized on a transilluminator (UV Products), and photographs were taken through a Wratten No. 4 filter

using Polaroid type 55 positive/negative film.

For the determination of nucleic acid type and strandedness, gels were treated with bovine pancreatic ribonuclease type 1A (10 μg/ml; Sigma) in 0.3 M NaCl for 2 hr (ssRNA will be degraded but not dsRNA) or 10 μg/ml ribonuclease in water for 2 hr (both ss- and dsRNA will be degraded) or deoxyribonuclease-1 (10 μg/ml; Sigma) and 10 mM magnesium chloride for 2 hr. Ribonuclease was made free of deoxyribonuclease by dissolving ribonuclease in 10 mM Tris-HCl, pH 7.5 and 15 mM NaCl and heating to 100 C for 15 min (7). Deoxyribonuclease was made free of ribonuclease by dissolving 1 mg/ml deoxyribonuclease in 20 mM tris, pH 7.5 and 10 mM $CaCl_2$ and heating to 37 C for 20 min. Proteinase K at a final concentration of 1 mg/ml was then added to the solution, and the incubation continued for 15 min (11). After enzyme treatments, gels were restained with ethidium bromide, destained and visualized with the transilluminator.

Restriction endonuclease digestion. Restriction endonucleases were purchased from BRL (Bethesda Research Laboratories, Gaithersburg, MD) and used as prescribed by the manufacturer. After incubation with enzymes for 20-40 hr, nucleic acids were ethanol precipitated as described previously.

Hyperchromicity studies. To determine the strandedness of the DNA isolated from WSSMV preparations, "melting curve" experiments were performed. Virus-associated DNA and calf thymus (ds)DNA (1 mg/ml) were diluted 1:20 in 0.01X SSC (1.5 mM sodium chloride, 15 mM sodium citrate)(10), and their A_{260} absorption readings were measured before

and after heating at 100 C for 30 min in a Gilford Model 240 spectrophotometer. The ratio corresponding to the increase in light absorption was then calculated. For ssDNA the ratio should remain close to 1.0, whereas for dsDNA an increase in optical density of about 50% should occur when preparations are heated, as the randomly-oriented separated strands absorb more UV light (9).

Extraction and electrophoresis of dsRNA. Attempts were made to extract dsRNA from roots and leaves of WSSMV-infected wheat by using a modification of the method of Morris and Dodds (8). Infected tissues were ground in liquid nitrogen and two to four volumes of STE buffer (0.1 M NaCl, 50 mM tris-HCl, 1 mM EDTA, pH 6.8), two to four volumes of STE-saturated phenol and one-third volume of 10% (w/w) SDS was added to the powder. The mixture was shaken on ice for 30 min and then centrifuged at 8,000 rpm for 15 min in a Sorvall SS-34 rotor. The aqueous phase was removed and adjusted to 15% (v/v) ethanol. The extract was passed through a 10 ml (2.5 g dry wt) column of chromatographic cellulose powder (Whatman CF-11 cellulose) equilibrated with 85% STE:15% ethanol and poured in a 20 ml disposable syringe. The charged column was washed with 80 ml STE:15% ethanol (in 20 ml aliquots) to elute ssRNA and DNA. The retained dsRNA was eluted with 15 ml STE buffer without ethanol. The dsRNA fraction was usually subjected to a second cycle of CF-11 chromatography. The dsRNA was then precipitated by the addition of two volumes of absolute ethanol and one-tenth volume of 2.5 M sodium acetate, pH 5.2. After overnight storage at -20C, the tubes were centrifuged, drained and the pellets resuspended in 1 ml STE.

The dsRNA was concentrated by ethanol precipitation and stored at -20 C prior to electrophoresis. Sample pellets were resuspended in E buffer containing 20% glycerol and were electrophoresed as for genomic nucleic acids. Gels were stained with ethidium bromide and subjected to nuclease treatments as described previously.

Results

Analysis of genomic nucleic acids. Results from extractions using the SDS-phenol, single phase and disruption buffer methods were usually similar (Fig. 21). A broadly stained area of fluorescence, corresponding to molecular weight standards of about 1.0×10^7 (marker shown in Fig. 22) was present in preparations from both healthy and infected plants. This area of fluorescence was usually more intensely stained in samples from infected preparations. In addition, a wide band of molecular weight of less than 0.75×10^6 was present only in preparations from infected tissues. The large molecular weight material was shown to be DNA by nuclease treatments, and the low molecular weight band was shown to be ssRNA. The low molecular weight RNA band was frequently absent when the single-phase extraction method was used and occasionally absent when the SDS-phenol and disruption buffer methods were used.

When the ammonium carbonate extraction method was used, no peaks of UV absorbance were detected. Because the RNA-rich fraction was usually found near the bottom of the tube (2), the bottom 8 ml were collected and treated as described previously. No nucelic acids were present in gels from these preparations.

To determine the strandedness (and possibly origin) of the DNA, three sets of experiments were performed. First, nucleic acids isolated by the SDS-phenol or disruption buffer methods were incubated with several different restriction endonucleases in an attempt to digest the DNA into a series of discrete fragments. If the DNA were host-derived

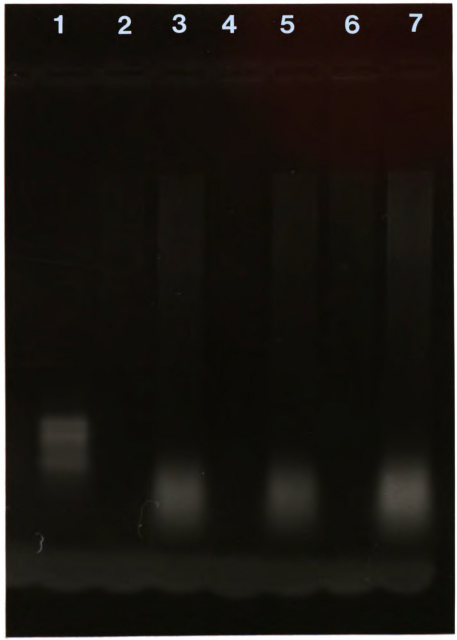


Figure 21. Agarose gel showing nucleic acids from WSSMV-infected and uninfected wheat extracted using the SDS-phenol method. Lane 1 = brome mosaic virus (BMV) ssRNA; values at left are molecular weights (x 10^6) of BMV RNA. Lanes 2, 4 and 6 are nucleic acids isolated from uninfected wheat. Lanes 3, 5 and 7 are nucleic acids isolated from infected wheat.

(dsDNA), it would be digested under the proper conditions, whereas if the DNA were single-stranded (viral DNA could be ssDNA or dsDNA), no digestion would occur, as ssDNA is not susceptible to restriction endonucleases. Although preparations of control DNA susceptible to specific restriction endonucleases were digested, WSSMV-associated DNA was not digested by any of the enzymes tested (Fig. 22).

In the second set of experiments, changes in UV absorbances (A_{260}) after heating were measured for DNA recovered from WSSMV purified preparations and calf thymus DNA (Table 6). The calf thymus (ds) DNA showed about a 39% increase in absorbance after heating whereas the increase in absorbance after heating WSSMV DNA was only about 12%, indicating that the virus-associated DNA may be single-stranded.

In the third set of experiments, to determine if the DNA were somehow adsorbed onto or in close association with WSSMV particles during purification, purified virus was treated with deoxyribonuclease for 2 hours as previously described prior to high speed pelleting of the virus suspension. In preparations pretreated with deoxyribonuclease, the high molecular weight smear of DNA was partially degraded and displaced as a lower molecular weight smear in the gel (Fig. 23). Longer periods of incubation with deoxyribonuclease eliminated most but not all of the DNA, indicating incomplete digestion of host DNA possibly because of contaminants in the nucleic acid sample.

It was later discovered that when phosphate buffer was used for the purification of certain potyviruses, virus preparations invariably were contaminated with host DNA (W. G. Dougherty, personal

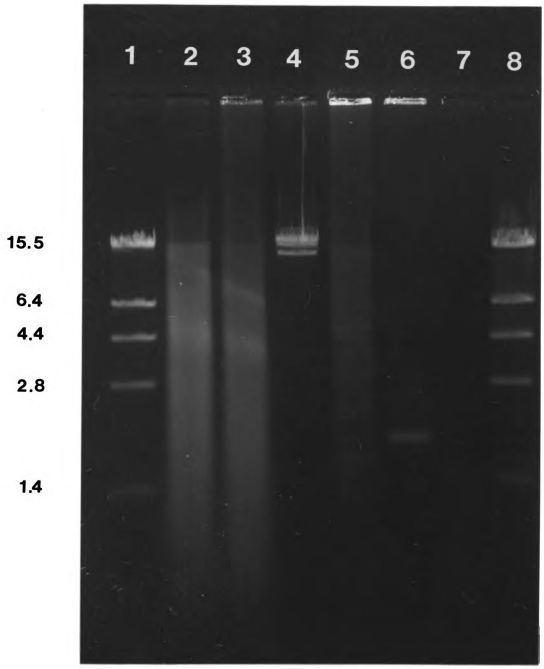


Figure 22. Agarose gel showing restriction endonuclease digests of WSSMV-associated DNA. Lanes 1 and 8 show size standards of bacteriophage lambda DNA digest by Hind III; values at left are fragment molecular weights (x 10^6). Lane 2 = WSSMV-associated DNA treated with Pst 1 and lane 3 = the DNA treated with Sau 3A. Lanes 4 and 5 = bacteriophage PEal(h) DNA digested with Pst 1 and Sau 3A, respectively. Lanes 6 and 7 = plasmid pUC8 digested with Pst 1 and Sau 3A, respectively.

Table 6. A_{260} absorption readings for WSSMV DNA and calf thymus DNA before and after heat treatment[a].

	A_{260} nm[a]			A_{260} after heating
	before heating	after heating	% change	A_{260} before heating
WSSMV DNA	0.144	0.160	12	1.11
calf thymus DNA	0.528	0.731	39	1.39

[a]Heat treatment = 100 C for 30 min.

[b]Values are averages from six readings.

Figure 23. Agarose gel showing treatment of purified WSSMV with deoxyribonuclease prior to high speed pelleting. Lane 1 shows partial digestion of WSSMV-associated DNA when intact virus was treated with deoxyribonuclease for 2 hr. Lane 2 shows WSSMV-associated DNA from untreated virus preparations.

communication, 6). Bar-Joseph et al. (1) also found that RNA preparations isolated from citrus tristeza virus were contaminated with host DNA. That WSSMV purified in phosphate buffer had high (1.30-1.60) $A_{260/280}$ ratios also indicated nucleic acid contamination. When WSSMV was purified using HEPES buffer, no DNA bands were found in purified preparations, and $A_{260/280}$ readings were much lower (see Chapter III). Therefore, because much of the evidence indicated that the DNA was of cellular rather than viral origin, no additional experiments to further characterize the DNA were performed.

Analysis of dsRNA extracts. Results from attempts to extract dsRNA from infected roots and leaves were similar. Low molecular weight bands and broadly stained areas were present in extracts from both types of tissue (Fig. 24). At least two bands were present in preparations from infected tissue that were absent in similarly prepared healthy extracts. Based on nuclease treatments, a large portion of the broadly stained region and the two upper bands were ssRNA, whereas the remaining fluorescence was due to DNA. The ssRNA bands had a molecular weight of about 1.0×10^6 based on appropriate markers. No dsRNA was isolated during any of the attempts.

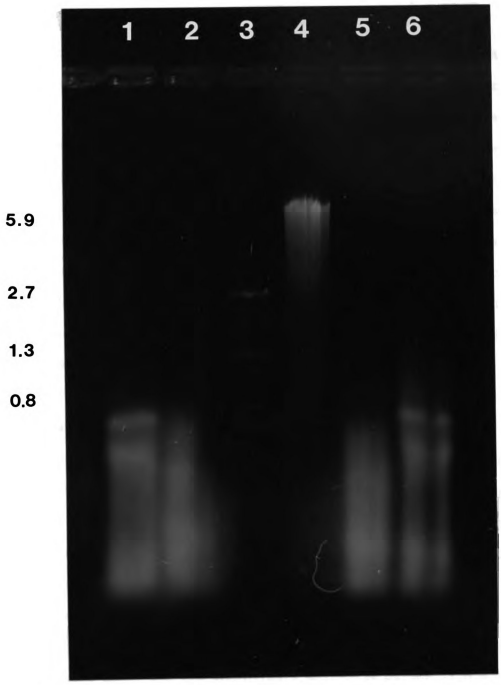


Figure 24. Agarose gel showing nucleic acids from dsRNA extractions of infected and uninfected leaf tissue. Lanes 1 and 6 = nucleic acids from infected preparations, and lanes 2 and 5 show nucleic acids from uninfected preparations. Lane 3 = reovirus type 3 dsRNA markers, and lane 4 = dsRNA from Endothia parasitica (Coli 5). Values at left are molecular weights (x 10^6) of markers.

Discussion

Attempts to isolate intact genomic nucleic acid and dsRNA were unsuccessful. Several explanations for this are possible. For genomic nucleic acid isolations, additional measures to protect preparations from nucleases may be necessary. Additives such as bentonite were used only infrequently and may need to be incorporated routinely into the extraction procedure. Except during initial trials, due to the unavailability of fresh tissue, most isolation attempts were made from virus purified from one- to two-year-old frozen tissue, the virus yield from which was usually much less than 1 μg/g. Thus, the concentration of genomic nucleic acid may have been too low to detect. In addition, purified virus may have had little to no infectivity, since only a low level of manual transmission was attained with purified preparations (see Chapter III).

Based on results from the restriction endonuclease and "melting curve" experiments, the virus-associated DNA appeared to be single-stranded. However, the most likely explanation for the inability of the DNA to be digested or "melted" is that the preparation contained dsDNA contaminated with protein. Such contamination would render the dsDNA less sensitive to endonuclease activity and heat treatment. In addition, the DNA was absent in preparations when other extraction buffers were used, and its approximate molecular weight (1.0×10^7) roughly corresponds to that reported for mitochondrial DNA (15,000 base pairs) (4).

While the DNA was most likely host-derived, the origin of the

ssRNA in extracts from both ss- and dsRNA isolation procedures is less certain. It most likely does not represent full-length genomic nucleic acid, as the molecular weight of the ssRNA (about $0.7\text{-}1.0 \times 10^6$) is too low for potyvirus genomic nucleic acid ($3.0\text{-}3.5 \times 10^6$), closterovirus genomic nucleic acid ($2.5\text{-}6.5 \times 10^6$) or the molecular weight of the genome of any filamentous virus known. The ssRNA may represent viral genomic RNA that has been partially degraded due to nuclease activity. Another possibility is that the RNA represents ribosomal RNA, possibly 18S RNA which has a molecular weight of about 0.7×10^6 (4), although this RNA was not found in extracts from healthy plants. In any case, the nature of the genome of WSSMV remains unknown. Additional attempts to isolate the genome are planned and will include utilizing more stringent extraction conditions and extracting from larger quantities of tissue with higher concentrations of virus.

Why only ssRNA and DNA but not dsRNA were isolated from infected tissues also is unknown. Because dsRNA isolation attempts utilized 10 g of tissue or less, and because it has been suggested that 20 g of tissue or more are needed for isolating dsRNA from potyviruses (Ramon Jordan, personal communication), future dsRNA isolation attempts will also utilize greater amounts of highly-infected tissue.

Literature Cited

1. Bar-Joseph, M., Gumpf, D.J., Dodds, J.A., Rosner, A., and Ginzberg, I. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75: 195-198.

2. Brakke, M.K., and Van Pelt, N. 1970. Properties of infectious ribonucleic acid from wheat streak mosaic virus. Virology 42: 699-706.

3. Bruening, G.E. 1972. Virus degradation and nucleic acid isolation. Pages 444-465, IN: C.I. Kado and H.O. Agrawal, eds. Principles and Techniques in Plant Virology. Van Nostrand Reinhold Company, New York. 688 pp.

4. DeRobertis, E.D.P., and DeRobertis, E.M.F. 1980. Cell and Molecular Biology. Saunders College/Holt, Rinehart and Winston, Philadelphia. 673 pp.

5. Diener, T.O., and Schneider, I.R. 1968. Virus degradation and nucleic acid release in single-phase phenol systems. Arch. Biochem. Biophys. 124: 401-412.

6. Dougherty, W.G., and Hiebert, E. 1980. Translation of potyvirus RNA in a rabbit reticulocyte lysate: reaction conditions and identification of capsid protein as one of the products of *in vitro* translation of tobacco etch and pepper mottle viral RNAs. Virology 101: 466-474.

7. Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 545 pp.

8. Morris, T.J., and Dodds, J.A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69: 854-858.

9. Ralph, R.K., and Bergquist, P.L. 1967. Separation of viruses into components. Pages 464-545 IN: K. Maramorosch and H. Koprowski, eds. Methods in Virology, Vol. 2. Academic Press, New York. 682 pp.

10. Sela, I., Assouline, I., Tanne, E., Cohen, S., and Marco, S. 1980. Isolation and characterization of a rod-shaped, whitefly-transmissible, DNA-containing plant virus. Phytopathology 70: 226-228.

11. Tullis, R.H., and Rubin, H. 1980. Calcium protects DNase I from

proteinase K: A new method for the removal of contaminating RNase from DNase I. Analyt. Biochem. 107: 260-264.

12. Usugi, T., and Saito, Y. 1979. Relationship between wheat yellow mosaic virus and wheat spindle streak mosaic virus. Ann. Phytopathol. Soc. Jpn. 45: 397-400.