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The Ohio State University, 1987
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PROTEINS AND SEROLOGICAL RELATIONSHIPS OF MAIZE MOSAIC VIRUS ISOLATES
AND REPLICATION OF THE VIRUS IN MAIZE (ZEAMAYS L.) PROTOPLASTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Rodolfo Gustavo Gomez Luengo, B.S., M.S.

* * * * *

The Ohio State University

1987

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PROTEINS AND SEROLOGICAL RELATIONSHIPS OF MAIZE MOSAIC VIRUS ISOLATES
AND REPLICATION OF THE VIRUS IN MAIZE (*Zea mays* L.) PROTOPLASTS

BY

Rodolfo Gustavo Gomez Luengo, Ph.D.

The Ohio State University, 1987

Professor Donald T. Gordon, Advisor

Virions of maize mosaic virus (MMV) isolates from Costa Rica (MMV-CR), Florida (MMV-FL), Hawaii (MMV-HI) and Iran (MMV-IR) were purified from greenhouse grown, inoculated maize (*Zea mays* L.). The use of analytical filter-aid celite was critical in the purification procedure for obtaining adequate amounts of purified MMV suitable for analytical work.

Analysis of virion proteins electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) showed identical banding patterns for MMV-CR, MMV-FL and MMV-HI. In contrast, MMV-IR had the same number of proteins but of lower molecular weight, except for the G protein. The G protein for all isolates was glycosylated. In addition, putative L, N, Ns, M₁ and M₂ proteins were detected. The G protein band of MMV-FL and MMV-HI was always associated with the envelope fraction after treatment of partially purified virions with non-ionic detergents, and the L, N, Ns, M₁ and M₂ proteins, with the
nucleocapsid fraction. The combination of these proteins and their association with the two virion fractions is unique among the plant rhabdoviruses.

Western blots of virion proteins immunostained with antiserum prepared to MMV-HI showed that MMV-CR, MMV-FL and MMV-HI were related, while MMV-IR appeared to have no serological relationship to these isolates. Monoclonal antibodies prepared to the G and N proteins of MMV-HI reacted identically with proteins of MMV-FL and MMV-HI. Based on these serological reactions, MMV-CR, MMV-FL, and MMV-HI should be classified as isolates of the same MMV strain, while MMV-IR is either a very distantly related strain or a different maize-infecting rhabdovirus.

Peptide maps of the G, N, M₁ and M₂ proteins of MMV-FL and MMV-HI showed only a few differences. Peptides were obtained by partial proteolysis using chymotrypsin, papain or staphylococcus V8 protease followed by electrophoresis in SDS-PAGE gels. A combined Coomassie Brilliant Blue G-250/silver stain proved highly sensitive for detecting peptide bands. These and the results from the serological and protein studies indicated that MMV-FL and MMV-HI (and most likely MMV-CR) have not diverged significantly in evolutionary development despite their occurrence in widely separated locations. The evolutionary relationship of MMV-IR to these isolates is uncertain.
Maize mesophyll protoplasts were infected with MMV-HI by fusion with polyethylene glycol (PEG). A time course study of virus concentration as measured by the Protein A-enzyme linked immunosorbent assay (PAS-ELISA) was conducted over the period of 0 to 48 hr post inoculation. Fluorescent, light and electron microscopy were used also to assess viral replication. Results indicated that at 0 hr there was some residual virus from the inoculum probably attached to the plasma membranes. By 12 hr, the concentration of virus had decreased, but by 24 hr it had increased to a level similar to that at 0 hr. At 48 hr, the virus concentration had doubled over that at 24 hr.
INTRODUCTION

RHABDOVIRUSES

Enveloped plant and animal viruses with bacilliform or bullet-shaped particles are grouped under the rhabdoviruses (Rhabdoviridae) (Bishop & Smith, 1977; Brown et al., 1979; Knudson, 1973; Matthews, 1982; Peters, 1981). The Rhabdoviridae were established around vesicular stomatitis virus (VSV) and rabies virus. The family name is derived from the Greek word rhabdos, meaning rod, in recognition of the rod-shaped particles of its members (Franck et al., 1985; Matthews, 1982).

The particle nucleocapsid is enclosed in a unit membrane envelope from which protein spikes protrude (Matthews, 1982; Peters, 1981). Enveloped particles of plant rhabdoviruses range from 50 to 95 nm in diameter and from 200 to 500 nm in length and sediment at about 1000 S (Francki et al., 1985). These viruses have been reported from most parts of the world including tropical, subtropical and temperate regions (Peters, 1981). They are transmitted by aphids, leafhoppers, planthoppers, lacebugs, and mites (Francki et al., 1981). The viruses are propagative in their vectors and most are transmitted to a restricted range of plant species (Francki et al., 1985). There are 38 plant rhabdoviruses sufficiently studied to
be classified as members of the group, and an additional 30 considered as tentative members (Peters, 1981).

PROTEINS OF Rhabdoviruses.

Proteins of rhabdovirus virions are designated, as proposed by Wagner et al. (1972), L (large), G (glycoprotein), N (nucleocapsid protein), Ns (non-structural), and M (matrix) or M1 and M2 (Peters, 1981). The roles of each protein have been investigated (Wagner, 1975), mainly for animal rhabdoviruses. The L and Ns proteins have transcriptase activity as determined for VSV (Bishop & Roy, 1972; De & Banarjee, 1984; Emerson & Wagner, 1972; Emerson & Yu, 1975; Moyer & Summers, 1974). The G protein is important in virus attachment to the plasma membrane during early stages of infection (Bishop & Smith, 1977; Wagner, 1975). This function has been suggested also for the G protein of potato yellow dwarf virus (PYDV), serotype SYDV (Gaedigk et al., 1986). The N protein is a structural protein complexed with virion RNA (Bishop & Smith, 1977; Francki & Randles, 1979; Knudson, 1973), and the M protein appears in close association with lipids and the G protein of the envelope (Bishop & Roy, 1972; Bishop & Smith, 1977; Knudson, 1973; McSharry, 1979; Wagner et al., 1972). More detailed information about each protein follows.
L PROTEIN.

A large (L) protein, with a molecular weight ranging from 190,000 to 241,000 daltons (Wagner et al., 1972) is present in small amounts in the VSV virion (Harmison et al., 1984) and is associated with the nucleocapsid. An L protein is also associated with the nucleocapsid of the rabies virus and other animal rhabdoviruses (Heyward et al., 1979).

Preparations of plant rhabdoviruses usually contain two or three high molecular weight polypeptides present in small amounts (Jackson et al., 1987). However, an L protein associated with the virion nucleocapsid has actually been demonstrated only for lettuce necrotic yellows virus (LNYV) (Dale & Peters, 1981).

G PROTEIN.

The glycosylated (G) protein is the spike protein which protrudes from the envelope of the virion (Wagner, 1975). A G protein has been identified in all plant rhabdoviruses so tested (Jackson et al., 1987). The molecular weight for this protein has varied from 70,000 to 90,000 daltons, and the protein has consistently stained positively for carbohydrate. The G protein is thought to be part of the viral envelope because it is readily released from the virion by mild non-ionic detergent treatment that

N PROTEIN.

The nucleocapsid (N) protein is tenaciously associated with the viral RNA and is an integral component of the transcriptase complex in VSV (Hunt et al., 1979). The molecular weight of this protein ranged from 50,000 to 62,000 daltons (Matthews, 1982). The protein appears to have an important regulatory role in replication of the viral genome as well as a role in determining particle morphology (Jackson et al., 1987). It has been detected from all plant rhabdoviruses so far studied (Dale & Peters, 1981; Falk & Tsai, 1983; Francki & Randles, 1975; Jackson, 1978).

Ns PROTEIN.

The Ns protein is a minor phosphorylated protein found in VSV virions (Jackson et al., 1987). Its molecular weight ranges from 40,000 to 50,000 daltons (Matthews, 1982). The original term Ns stood for non-structural protein (Wagner et al., 1972). In VSV, the Ns protein appears to promote binding of the L protein to the nucleocapsid (Williams & Emerson, 1984), and according to De and Banarjee (1985) it may also interact with the RNA-N protein complex to promote RNA unwinding during transcription.
A phosphorylated protein with the properties of the Ns protein has not been unequivocally identified from any plant rhabdovirus (Jackson et al., 1987). Because of similarities in migration patterns in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, several plant rhabdoviruses are thought to have an analogous protein to the Ns protein of VSV. Among these are LNYV and sonchus yellow net virus (SYNV) (Dale & Peters, 1981), maize mosaic virus (MMV) (Falk & Tsai, 1983), and wheat rosette stunt virus (WRSV) (Tsuhsun & Qiao-Xi, 1984). No chemical data are available on any of these putative Ns proteins of plant rhabdoviruses and thus it is not known if they are phosphorylated (Jackson et al., 1987).

M PROTEIN.

The M proteins, known as matrix proteins, apparently belong to two different classes of proteins. One class includes the membrane (M) protein of VSV (McSharry, 1979) and ranges in molecular weight from 20,000 to 30,000 daltons (Matthews, 1982). The second class, typified by rabies virus, has two M proteins; $M_1$ with molecular weight of 35,000 to 40,000 daltons and $M_2$ with a molecular weight of 22,000 to 25,000 daltons (Matthews, 1982). The M protein of VSV is hydrophobic and is intimately associated with the lipids of the viral envelope (McSharry, 1979) and, along with G protein, is important in maintaining viral structure (Jackson et al., 1987). The actual location and function of the two membrane proteins of rabies virus is
not clear. Evidence suggests that the rabies virus M₂ protein and
the M protein of VSV have a similar location in the virion and an
analogous function (Jackson et al., 1987). However, the M₁ protein
of rabies virus appears to be similar to the VSV Ns protein (Cox et
al., 1981).

We have no chemical data available about M proteins of any plant
rhabdovirus that could permit us to distinguish proteins
corresponding to the Ns and M proteins of VSV (Jackson et al., 1987).

STRUCTURE OF RHABDOVIRUSES.

Rhabdoviruses have the largest and most complex particle of all
known plant viruses, containing about 70% protein, 25% lipids, 4%
polysaccharides and 1% RNA (Matthews, 1982). Although the particle
often appears bullet-shaped in negatively stained preparations, it is
now generally accepted that nearly all rhabdovirus particles in an
unaltered state are bacilliform (Jackson et al., 1987).

Rhabdovirus particles are composed of several structural layers
and models have been proposed that show many similar features but
which differ in detail (Knudson, 1973). The single-stranded RNA is
intimately associated with the N protein. The L and Ns proteins are
loosely associated with the ribonucleoprotein and the matrix protein
or proteins of the nucleocapsid (Brown et al., 1979; Peters, 1981;
Wagner, 1975).
Three distinct layers of varying electron density are observed in cross sections of the virions. These layers are thought to be composed of surface projections (G protein), a unit membrane and a helical nucleocapsid surrounding a central canal (Jackson et al., 1987). The structure of the virion is visualized as follows. A nucleoprotein helix (nucleocapsid) forms a hollow cylinder that constitutes the internal component of the virion (Martelli & Russo, 1977). The exterior of the virion is composed of the lipoprotein envelope. The layer between the latter and the nucleocapsid is suggested to be formed by the matrix protein(s) (Knudson, 1973).

MORPHOGENESIS.

Cell membranes are undoubtedly involved in rhabdovirus morphogenesis as they are the site of particle assembly (Martelli & Russo, 1977). The sites of morphogenesis of plant and animal rhabdoviruses appears to differ. Assembly of plant rhabdoviruses is associated with the nuclear envelope or endoplasmic reticulum of plant cells, whereas the animal viruses assemble on the plasma and intracytoplasmic membranes of animal cells (Francki, 1973).

According to Jackson et al. (1987), plant rhabdoviruses can probably be divided into at least three groups depending on the site of nucleocapsid formation and assembly of virions. The first group includes viruses that mature in association with the inner nuclear membrane and accumulate in the perinuclear space, e. g., SYNV, PYDV
and eggplant mottled dwarf virus (EMDV). Members of the second group appear to mature in association with the endoplasmic reticulum and accumulate in vesicles within the cytoplasm, e. g., LNYV and maize sterile streak virus (MSSV). Members of the third group, represented by barley yellow striate mosaic virus (BYSMV) and northern cereal mosaic virus (NCMV) mature in association with a membrane-bound granular structure called viroplasm. After budding from membranes associated with the viroplasm, virions accumulate in vacuole-like structures within the cytoplasm.

CLASSIFICATION OF RHABDOVIRUSES.

At present, the Rhabdoviridae is divided into two genera, Vesiculovirus and Lyssavirus, with VSV and rabies virus as the respective type members. Members infecting plants have not been classified into genera (Francki, 1985; Matthews, 1982) but two subgroups have been proposed. Plant rhabdoviruses of subgroup I or A (LNYV, type member) have properties comparable to Vesiculoviruses, i. e., they assemble and accumulate in the cytoplasm, contain a single matrix or M protein, and possess readily detectable \textit{in vitro} transcriptase activity. In contrast, viruses in subgroup II or B (PYDV, type member) share properties with Lyssaviruses, i. e., they accumulate in the perinuclear space, possess $M_1$ and $M_2$ proteins, and have low \textit{in vitro} transcriptase activity (Matthews, 1982; Peters, 1981). These groupings were based on the suggestion of Dale and
Peters (1981) that plant rhabdovirus division could be based on cytopathology, transcriptase activity \textit{in vitro}, and association of proteins within the virion. Although this proposal provides a useful framework, more comprehensive comparisons of plant rhabdoviruses are essential before such taxonomic divisions can be accepted (Jackson et al., 1987).

**SEROLOGY OF Rhabdoviruses.**

Generally, plant rhabdoviruses are poor immunogens and the titer of antibodies raised in rabbits (polyclonal antibodies) is usually low (Jackson et al., 1987). In addition, many antiserum preparations also react with host antigens due to impurities in the viral preparations used for immunization. Nevertheless, serological procedures using polyclonal antibodies have been successful in studying virus relationships, detection, distribution and epidemiology.

The ability of monoclonal antibodies (raised in mice) to distinguish minor antigenic differences has made them useful probes with which to differentiate virus strains (Hsu et al., 1984). Monoclonal antibodies against proteins of a few animal rhabdoviruses have been reported, but, as yet, none against plant rhabdovirus proteins have been reported.
Among the animal rhabdovirus proteins, monoclonal antibodies have been prepared against the G, N, and M proteins of VSV (Bishnu et al., 1982; Pal et al., 1985; Volk et al., 1982; Ye et al., 1985) and the G, N and Ns proteins of rabies virus (Lafon & Wiktor, 1985; Lafon et al., 1983). Monoclonal antibodies have also been used to diagnose rabies virus infection and differentiate rabies virus from related viruses (Wiktor et al., 1980).

TRANSMISSION OF PLANT RHABDOVIRUSES.

Some plant rhabdoviruses have been transmitted experimentally by mechanical inoculation. This is almost certainly of no importance for their spread under field conditions since they all appear to be very unstable in vitro (Francki, 1973). There appears to be no reports of seed transmission (Francki et al., 1985). Insect vectors appear responsible for field-spread of the viruses which have been studied in detail, and the viruses all appear to be propagative in their vectors (Francki et al., 1981). The virus-vector relationship is highly specific with few, and often only a single insect species, being involved in spreading a particular virus (Francki et al., 1981; Francki et al., 1985).

Jackson et al. (1987) indicated that nine rhabdoviruses are known to have an aphid vector, 16 are transmitted by leafhoppers and planthoppers, one by a lacebug, one by a mite, and 14 others have no known vector.
Plant rhabdoviruses can multiply in both plant and insect cells (Jackson et al., 1987). The rhabdoviruses that cause serious diseases of vertebrates also multiply in their insect vectors (Brown & Crick, 1979). None of the viruses appear to affect adversely the insects which suggests a long evolutionary association between them (Jackson et al., 1987). Sylvester and Richardson (1970) pointed out that insects occupy a central position as hosts of plant and animal viruses, which suggests that rhabdoviruses may have originated as insect viruses.

MAIZE MOSAIC VIRUS.

Maize mosaic virus is a rhabdovirus (Francki, 1973; Francki & Randles, 1979; Herold, 1972; Martelli & Russo, 1977; Peters, 1981) transmitted by the planthopper, *Peregrinus maidis* (Ashmead) (Bradfute & Tsai, 1983; Herold, 1972) and that infects only members of the Gramineae. The virus occurs in many tropical and subtropical countries (Gordon & Ritter, 1987). It is not transmissible by inoculation of sap (Herold, 1972). Maize mosaic was first described in Hawaii in 1921 (Kunkle, 1921), but the characterization of the virus was not begun until 1960 with the study of a Venezuelan isolate (Herold et al., 1960). Since then, other isolates have been partially characterized including ones from Hawaii (MMV-HI) (Ammar & Nault, 1985; McDaniel et al., 1985), Florida (MMV-FL) (Bradfute & Tsai, 1983; Falk & Tsai, 1983), Venezuela (Herold & Munz, 1967;
Lastra, 1977), Mauritius (Autrey, 1980, 1983), Brazil (Kitajima & Costa, 1982), Guadeloupe and French Guyana (Migliori & Lastra, 1981), and more recently Iran (MMV-IR) (Izadpanah et al. 1983). These viruses have been designated as MMV isolates because of similarities in symptomatology, host range, particle morphology and less frequently serological relatedness.

Maize mosaic virus infected plants initially develop stripes between the leaf veins and the leaves may later turn yellow and become necrotic (Herold, 1972). If infection occurs early, the plants may be stunted with shortened internodes and deformed cobs (Jackson et al., 1987). Maize mosaic virus multiplies in both plant (McDaniel et al., 1985) and insect vector, P. maidis, (Ammar & Nault, 1985) cells. However, the assembly and accumulation of MMV-HI in P. maidis are somewhat different from these features in maize. Although in many insect tissues MMV-HI buds on the inner nuclear membrane and accumulates in the perinuclear space as it does in maize, in insect salivary glands and nerve ganglia it assembles more frequently on the plasma membrane and endoplasmic reticulum than on the nuclear membrane (Ammar & Nault, 1985).

PLANT PROTOPLASTS IN VIRAL STUDIES

Leaf mesophyll cells are the most common source of protoplasts for virus studies (Motoyoshi, 1985). In addition to mesophyll cells,
protoplasts isolated from cells in suspension cultures have been recently used (Jarvis & Murakishi, 1980; Kikkawa et al., 1982; Lesney & Murakishi, 1981).

Plant protoplasts provide an experimental system which allows synchronous infection for the study of the replication of viruses and viroids. Most of the protoplasts used in these studies are from dicotyledous plants. Muhlbach (1982) and Sander and Mertes (1984) indicate only four monocotyledous species from which protoplasts have been isolated and infected with plant viruses.

Members of many different plant virus groups have been shown to infect protoplasts. Among the rhabdoviruses, only three have been shown to infect protoplasts; these are PYDV (Riester & Adam, 1981), SYNV (van Beek et al., 1985a, b) and Festuca leaf streak virus (FLSV) (van Beek et al., 1985c). For all three rhabdoviruses the protoplasts infected were not from plants that were natural hosts. Also, for these plant rhabdoviruses protoplasts from no monocotyledous plants have been used, even though FLSV has a monocot as its natural host (Lundsgaard & Alberchtsen, 1976, 1979).

INOCULATION OF PROTOPLASTS WITH VIRUSES.

There are two principal requirements for successful inoculation; a concentrated virus suspension of high specific infectivity and a freshly prepared suspension of actively metabolising protoplasts.
(Wood, 1985). To carry out inoculation, the virus or viral nucleic acid and the cell have to be brought into intimate contact. There are basically four procedures for achieving this. First, the procedure used in the vast majority of successful protoplast infections has been employed polycations such as poly-L-ornithine that was originally introduced by Aoki and Takebe (1969). A second technique uses polyethylene glycol (PEG) which promotes cell fusion (Dawson et al., 1978; Maule et al., 1980). A variation of this method uses liposomes to carry the viral nucleic acid into the protoplast with the aid of PEG or polyvinyl alcohol (Fraley, 1982; Fukunaga et al., 1981). A third technique to introduce virus or nucleic acids into animal cells and fluorescent dyes into protoplasts is direct microinjection (Steinbiss & Stabel, 1983). The most recently developed procedure for introduction of foreign nucleic acids, particularly viral nucleic acids, is electroporation (Fromm et al., 1985; Nishiguchi et al., 1986; Okada et al., 1986).

POLYCATION (POLY-L-ORNITHINE) METHOD.

Most viruses as well as the protoplasts have a net negative charge at the pH at which inoculation is done (Wood, 1985). When virus is mixed with poly-L-ornithine, it is believed that a positive charge is imparted to the virus which forms aggregates that interact with the negatively charged protoplast membrane. The virus aggregates then penetrate the membrane by endocytosis possibly aided by poly-L-ornithine.
POLYETHYLENE GLYCOL METHOD.

Essentially, virus and protoplasts are mixed with a concentrated (20-40%) PEG (1500-6000 MW) solution, and the mixture quickly diluted with mannitol solution containing calcium ions (Wood, 1985). It is well known that PEG promotes membrane fusion (Kao & Michayulk, 1974), but its role in virus infection of protoplasts is not clear (Muhlbach, 1982). Casells and Cocker (1980) suggest that the virus particles are incorporated into localized areas of membrane by a fusion with the latter or that virus uptake occurs at destabilized areas of the plasmalemma between adjacent protoplasts.

LIPOSOME TECHNIQUE.

This technique involves the encapsulation of viral nucleic acid in aqueous solution inside the liposome, interaction of the liposome with the protoplast membrane in the presence of fusing agent (PEG or polyvinyl alcohol), and subsequent delivery of the nucleic acid into the protoplast cytoplasm where infection can be initiated (Wood, 1985).

MICROINJECTION TECHNIQUE.

Cells are attached to coverslips with polylysine, micropipettes with a diameter of about 0.3 μm are prepared and a microinjection system is assembled, consisting of microscope, micromanipulator and
injection apparatus. The glass needle tip is driven by the manipulator to bring it into contact with the cell surface where it pierces the membrane. The sample is injected by controlling the pressure of nitrogen in the needle.

ELECTROPORATION OR ELECTROFUSION.

This technique has been used to introduce tobacco mosaic virus RNA and cucumber mosaic virus RNA into tobacco protoplasts. The protoplast suspension is mixed with the viral nucleic acid solution. The mixture is then subjected to electroporation, which is an electrical pulse of high field strength (10 kv/cm). The pulse is repeated several times in short exposures (90 psec) with intervals between of 0.1 sec. The process is conducted in a chamber with a short distance between electrodes (200 μm).

METHODS FOR ASSAYING VIRUSES.

The progress of infection during incubation of inoculated protoplasts can be assessed in various ways depending on the requirements of the experiment (Wood, 1985). Probably the most common way is to monitor the proportion of infected protoplasts by testing for the accumulation of viral antigen in the protoplast using anti-viral serum and a fluorescein-labelled secondary antibody (a description of this technique is given in Chapter IV). A common practice with sap transmissible viruses is to monitor the
accumulation of infective virus by inoculation of protoplast preparations to a suitable local lesion host. Virus yields can also be assayed by sucrose density gradient centrifugation (Motoyoshi, 1985). Crude extracts are layered on top of sucrose density gradient columns, centrifuged, fractioned, and the virus quantities calculated by measuring the absorbance of the virus fraction at 260 nm (Motoyoshi et al., 1973). More recent techniques are direct observation of particles in the protoplast by electron microscopy (technique described in Chapter IV), the enzyme-linked immunosorbent assay (ELISA) for detecting accumulation of viral antigen (description in Chapter IV), and the dot-hybridization to detect accumulation of viral nucleic acid. In the latter, appropriately prepared samples are spotted onto nitrocellulose filters and viral nucleic acid detected by hybridization to a labelled, complementary DNA probe.

RESEARCH RATIONALE.

A number of MMV isolates have been reported and partially characterized but with little attention to comparing characteristics within the same laboratory, except for some preliminary serological and particle dimension comparisons. Further, there is lack of agreement among characteristics of these different isolates. For example, there are discrepancies on the number of M proteins, the presence of L and Ns proteins, and the classification based on both sites of assembly and accumulation and number of M proteins.
Exacerbating the problem is the limited information on the location of viral proteins within the viral particle, the presence of glycosylated proteins, and serological relationships among these various isolates. Also, there are no studies in which the MMV proteins have been compared by peptide mapping which is a very sensitive method for detecting differences among otherwise closely related viruses. Finally, since the sites of rhabdoviruses assembly and accumulation are of such importance for the classification of these viruses, study of MMV infection in a time course study involving synchronous infection of protoplasts would be expected to provide more precision in defining these sites. This approach seemed particularly germane since our previous studies of infected leaf cells showed an unexpected variety of sites that made classification of our MMV isolate by the criteria within the present guidelines impossible. Thus a study was conducted to compare the characteristics of several MMV isolates from widely separate geographical areas.

Results of this study are reported in the following four chapters. Chapter I deals with the analysis of proteins of MMV isolates and spatial distribution of viral proteins using detergents. Chapter II presents data on serological relations among the isolates using monoclonal and polyclonal antibodies. Chapter III presents results from the peptide mapping of the proteins of two isolates.
Finally, Chapter IV presents evidence for polyethylene glycol mediated infection of maize protoplasts by MMV.
CHAPTER I

Analysis of Protein Composition of Several Maize Mosaic Virus Isolates and Location of Proteins Within the Virion.

INTRODUCTION

One major criterion for the classification of viruses within the plant rhabdoviruses is protein composition of the virion (Matthews, 1982). Dale and Peters (1981) proposed the classification of plant rhabdoviruses into subgroups based on the presence of one or two M proteins. This approach proved to be useful and has since then been used as a helpful criterion in the characterization of plant rhabdoviruses.

At present, the information on protein composition of MMV is controversial. The protein composition of the MMV virion has been reported separately by different laboratories, and thus comparisons are difficult.

Maize mosaic virus has been classified in subgroup I or A based on the presence of a single virion M protein (Falk & Tsai, 1983; Lastra & Carballo, 1983). However, preliminary evidence for MMV-HI and MMV-FL indicated the presence of M₁ and M₂ proteins (L.L.
McDaniel and D. T. Gordon, unpublished), a feature of plant rhabdoviruses classified in subgroup II or B.

Corroborating evidence for the latter classification of MMV-FL has been obtained from electron microscopy studies of the sites of assembly of MMV in infected maize leaf cells (Bradfute & Tsai, 1983). Furthermore, the infected plant ultrastructural evidence presented by McDaniel et al. (1985) for MMV-HI suggests the possibility of another category of plant rhabdoviruses, represented by those that assemble at both the nuclear and cytoplasmic membranes. In this respect, AWSMV (Lee, 1970) and MMV-HI (McDaniel et al., 1985) appear to accumulate in both perinuclear space and in membrane-bound cytoplasmic structures. Similar findings have been reported for MMV-VZ (Martelli et al., 1975) and MMV-FL (Bradfute & Tsai, 1983), although the authors did not note it. At the time MMV-VZ was studied, the proposal for classification of plant rhabdoviruses into groups had not been made, whereas in the case of MMV-FL the authors did not make an attempt to fit the virus into either group based on their observations.

This chapter reports the results of studies on four isolates of MMV to determine the number of M proteins by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE). The chapter also compares the protein migration patterns of MMV-HI and MMV-FL following SDS-PAGE of virus treated with non-ionic detergents to
determine the association of the proteins with the soluble or the nucleocapsid fractions.
MATERIALS AND METHODS

VIRUS SOURCES.

The Hawaiian isolate of MMV and P. maidis were provided by R. Namba (Univ. of Hawaii, Honolulu). The MMV isolate from Costa Rica (MMV-CR) was supplied by R. Gamez (Univ. of Costa Rica) and L. R. Nault (The Ohio State Univ., OARDC, Wooster) and that from Florida (MMV-FL) was furnished by J. H. Tsai (Univ. of Florida, Fort Lauderdale). Finally, K. Izadpanah (Shiraz Univ., Shiraz, Iran) furnished the MMV from Iran (MMV-IR).

VIRUS AND VECTOR MAINTENANCE.

All MMV isolates were maintained in maize (Zea mays L.) (inbred Oh-28) by weekly inoculation with viruliferous (MMV exposed) planthoppers (P. maidis). Vector cultures were maintained as previously described (McDaniel et al., 1985).

To avoid contaminations, each MMV isolate was kept in an individual growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) during acquisition access (AAP) and inoculation access (IAP) periods. The insects were removed from plants within separate transfer hoods (Heady, 1985) after a 7-day IAP. Plants were sprayed with Resmethrin and later put in different rooms in the virus containment greenhouse.
The MMV-IR was injected abdominally into *P. maidis*, using glass needles, and allowed a 2 week incubation period before planthoppers were given an IAP on a new set of maize plants. Inoculated plants were treated identically to those for the other MMV strains.

**VIRION PURIFICATION.**

The following purification procedure (L. L. McDaniel, D. T. Gordon, R. G. Gomez, unpublished) was used. Infected maize leaves, 2-3 weeks post inoculation, were homogenized in four volumes of 0.1 M sodium citrate plus 0.25% thioglycollic acid (TGA), pH 7.5 (citrate buffer). The extract was pressed through a double cheesecloth layer, and 2 g Celite analytical filter-aid (Fisher Scientific, Springfield, NJ) were added per 25 g tissue. This slurry was passed through a 3-4 mm thick pad of Celite in a Buchner funnel with aspirator. The filtered extract was then layered onto a linear 100-400 mg/ml sucrose density gradient prepared in Beckman SW28 rotor tubes (Beckman Instruments, Inc., Palo Alto, CA) by layering 5, 7, 7 and 5 ml of 40, 30, 20 and 10% sucrose, respectively, in citrate buffer (13.5 ml/tube). The gradients were centrifuged at 25,000 rpm for 15 min at 4 C. The two light scattering virus bands located at the middle of the gradient were removed from the gradients by means of a hypodermic syringe inserted through the top of the gradient. The suspension containing these bands (11 ml) was layered onto a linear 250-550 mg/ml sucrose gradient, prepared in the SW28 rotor tubes by layering
5, 8, 8 and 5 ml of 25, 35, 45, and 55% sucrose in citrate buffer, respectively. Then, the gradients were centrifuged at 25,000 rpm at 4 C for 2.5 hr. Virus bands were removed as before, diluted three-fold with citrate buffer, and pelleted in SW28 rotor tubes centrifuged at 26,000 rpm for 30 min at 4 C. The pellets were resuspended in 0.1-0.3 ml of citrate buffer or 0.05 M Tris-HCl buffer, pH 7.4, depending on the experiment to be performed, per each 25 g of tissue. The amount of protein in purified viral preparations was determined by protein assay using Coomassie Brilliant Blue G-250 (Sedmak & Grossberg, 1977).

ANALYSIS OF VIRION PROTEINS.

Virion proteins were analyzed by SDS-PAGE (Laemmli, 1970) using 1.5 mm thick resolving gels of 10 or 15% acrylamide. Proteins from partially purified virus were reduced by adding an equal amount of 2X treatment buffer [0.125 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol] and sucrose dye solution (50% sucrose, 0.1% bromophenol blue). The samples were then heated to 100 C for 2 min, and 30-50 μl/well were placed on a 4% acrylamide stacking gel. Electrophoresis was done in a Hoeffer SE-400 unit (Hoeffer Scientific Instruments, San Francisco, CA) at 20 mA constant current until the sample reached the resolving gel; then the current was increased to 30 mA. Electrophoresis was done at room temperature and was stopped when the running front (sucrose dye) was about 0.5 cm
from the bottom of the gel. Maize mosaic virus-HI and MMV-FL were sometimes reduced and alkylated according to the procedure of Lane (1978). Protein bands were stained by placing gels in 20% methanol for 1 hr, then in Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Richmond, CA), prepared as described by Vesterberg et al. (1977), for 1 hr. Gels were destained in 7.5% acetic acid overnight.

Virion proteins were stained for carbohydrates as described by Falk and Tsai (1983). Schiff's reagent was prepared as described by Fairbanks et al. (1971).

ASSOCIATION OF PROTEINS WITH VIRION STRUCTURE.

Solubilization of viral proteins was conducted as described by Dale and Peters (1981). The only modifications were washing the precipitate once with ice cold 95% ethanol and letting the pellet dry for 30-60 min at room temperature. The detergents used were Nonidet P-40 and Triton X-100 (Sigma Chemical Co., St. Louis, MO) at 2% and 4% with either high (0.5 M) or low (0.1 M) NaCl concentrations. Analysis of protein patterns was done by SDS-PAGE using 10% or 15% gels stained with Coomassie Brilliant Blue G-250.
WESTERN BLOTTING.

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose membranes (Millipore Corporation, Bedford, MA) (Western blotting) was performed according to the procedure of Towbin et al. (1979). The only modification of the technique was that the electrode buffer (transfer buffer) contained 0.01% SDS (Towbin & Gordon, 1984) to facilitate transfer. Protein blotting was done using a Hoefer TE52 transfer unit operated at 40V for 4 hr. The unit was cooled with Hoefer TE47 heat exchanger. The nitrocellulose membranes were stained for total protein with Amido Black (0.1% Amido Black, 43% methanol, 10% acetic acid) for 3 to 5 min, then destained with several changes of 43% methanol, 10% acetic acid until the background was clear. Finally, membranes were washed with 55% methanol, 5% acetic acid, 2% glycerol for 5 min, blotted dry and stored.
RESULTS

VIRUS PARTIAL PURIFICATION.

This procedure using analytical filter-aid Celite provided adequate amounts of purified MMV (Plate IB) suitable for analytical work. The typical average yield of partially purified MMV was 0.6-0.8 mg/25 g tissue. Purity of preparations was evaluated by electron microscopic observation, electrophoresis on polyacrylamide gels (Plate IA), Western blotting and immunostaining (Chapter II) using MMV-HI antiserum or antiserum prepared against healthy tissue (Plate IIC). Patterns of healthy extract and partially purified virus were compared in gels and Western blots (Plate IIA, B), and presence of contaminating membranes was assessed by electron microscopy (Plate IIC). No contaminates were revealed by these methods of analysis.

Analytical filter-aid Celite gave reasonably clean and pure MMV, whereas with non-acid washed Celite the amount of impurities (green material) was high and they were difficult to eliminate by sucrose density gradients (results not shown). Partially purified MMV was further purified by repeating the semi-equilibrium centrifugation in the 250-550 mg/ml sucrose density gradients.
ANALYSIS OF VIRIAL PROTEINS.

Protein migration patterns in SDS-PAGE gels for MMV-CR, MMV-FL, and MMV-HI were identical, each showing six viral protein bands. Proteins of MMV-IR were different in relative banding position in SDS-PAGE gels, except for the protein G. The MMV-HI proteins migrated faster than the corresponding bands of the other isolates (Plate III). Bands were tentatively designated in increasing migration rate as L, G, N, Ns, M₁ and M₂. The molecular weights (Mr) of these proteins from MMV-HI had been determined previously and were found to correspond to the Mr's for these proteins from other rhabdoviruses. The banding positions of low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) were used as references for estimates of relative migration rate on all the gels. Of all the MMV protein bands, only the G protein tested positive for carbohydrates (Plate IV).

ASSOCIATION OF PROTEINS WITH VIRION STRUCTURE.

After treatment with Triton X-100 and Nonidet P-40, protein migration patterns of MMV-FL and MMV-HI were similar in all cases. The G protein was always recovered from the envelope or soluble fraction, while the L, N, Ns, M₁, and M₂ proteins were found in the nucleocapsid fraction (Plate V).
Sodium deoxycholate, SDS, and Tween 20 were also tested (results not shown). These detergents gave variable results. Deoxycholate at 0.1, 0.5, and 1% removed the G protein only partially; SDS at 0.01, 0.03, 0.1, and 0.3% solubilized all the proteins; Tween 20 at 1, 2, and 4% gave results similar to those obtained with Triton X-100 and Nonidet P-40, but the G band was hard to recover. Sonication and osmotic shock were also tested (results not shown). Sonication broke the particle into a soluble fraction and a nucleocapsid fraction similar to those obtained with Nonidet P-40 and Triton X-100; osmotic shock had no apparent effect on the particle (results not shown).
Plate I. Analyses of purified maize mosaic virus (MMV) for evidence of contaminants. A) Electrophoresed sodium dodecyl sulfate-polyacrylamide gel showing: 1. Low molecular weight standards (Phosphorylase B, 92.5 K daltons; Bovine Serum Albumin, 66.2 K daltons; Ovalbumin, 45 k daltons; Carbonic Anhydrase, 31 K daltons; Soybean Trypsin Inhibitor, 21.5 K daltons; and Lysozyme, 14.4 K daltons). 2. Healthy maize extract; 3. Purified Hawaiian isolate of MMV; and 4. Purified Florida isolate of MMV.
B) Sucrose density gradients: (left) gradient containing a clarified healthy extract and (right) a gradient containing MMV infected extract. Arrows point to the two bands that contained virus. C) Electron microscope micrograph showing rhabdovirus particles from purified MMV (magnification 15,000 x).
Plate I.
Plate II. Comparison of proteins of healthy maize extract and partially purified maize mosaic virus (MMV) stained by various techniques. Lane 1, purified Florida isolate of MMV (MMV-FL); Lane 2, purified Hawaiian isolate of MMV (MMV-HI); Lane 3, low molecular weight standard (see legend of Plate I for identity of protein standards and their respective molecular weights). A) Electrophoresed sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel stained with Coomassie Brilliant Blue G-250; B) Western blot of SDS-PAGE gel stained for total protein with Amido Black; and C) Immunostained Western blot of SDS-PAGE gel using polyclonal antibodies prepared against MMV-HI.
Plate II.
Plate III. Relative banding positions of proteins of maize mosaic virus (MMV) isolates and healthy extract electrophoresed in a sodium dodecyl sulfate 15% polyacrylamide gel. A) Low molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weights); B) Healthy maize extract; C) Costa Rican isolate (MMV-CR); D) Florida isolate (MMV-FL); E) Hawaiian isolate (MMV-HI); F) Iranian isolate (MMV-IR); and G) Low molecular weight standards. Letters identify the following virion proteins: g=glycosylated protein; n=N protein; ns=non-structural protein; and m₁ and m₂=the two matrix proteins.
Plate III.
Plate IV. Carbohydrate staining of G protein of maize mosaic virus (MMV) isolates. Lane 1, low molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weights); Lane 2, healthy maize extract; Lane 3, Costa Rican isolate (MMV-CR); Lane 4, Florida isolate (MMV-FL); Lane 5, Hawaiian isolate (MMV-HI); and Lane 6, Iranian isolate (MMV-IR). A) Electrophoresed sodium dodecyl sulfate polyacrylamide gel stained with Coomassie Brilliant Blue G-250 showing six protein bands; and B) Carbohydrate stained gel showing specific staining of the G protein of all the MMV isolates.
Plate V. Sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% acrylamide gels of dissociated virion proteins of maize mosaic virus (MMV) isolates after treatment with 2% Triton X-100 plus 0.5 M NaCl. A) Molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weights); B) Proteins from complete virion of Hawaiian isolate (MMV-HI); C) and D) Nucleocapsid associated proteins of MMV-HI and Florida isolated (MMV-FL) showing L, N, Ns, M1 and M2 proteins; E) and F) Envelope associated protein of MMV-HI and MMV-FL showing the G protein; in lanes A-D the asterisk denotes a protein that was infrequently seen in different virus preparations. In E and F two proteins of high molecular weight were observed. These were not observed in the nucleocapsid lanes. These are possibly aggregates, doublets and/or triplets of the G protein that resulted from detergent treatment.
Plate V.
DISCUSSION

The purification procedure resulted in relatively large yields of intact, partially purified virus particles. The use of analytical filter-aid Celite in the clarification was critical for eliminating chloroplastic materials and contaminating membranes. In our previous experiments Celite other than analytical filter-aid failed to eliminate contaminating materials. Celite filtration has also been used successfully as an aid in the purification of MMV-MR (Autrey, 1983), MMV-FL (Falk & Tsai, 1983), MMV-VZ (Lastra & Acosta, 1979), and SYNV (Jackson & Christie, 1977). The average thickness of the Celite pads used was within the range known to give good results. According to Jackson et al. (1987) the thickness of the pad is a critical variable; pads thicker than 7.5 mm seriously diminish virus yields, whereas pads less than 2.5 mm result in serious contamination with chloroplast fragments.

Results show that the virions of MMV-CR, -FL and -HI have proteins of the same size and relative amount, whereas corresponding MMV-IR proteins were of lower molecular weight, except for the G protein. These results suggest that MMV-IR is different from the other MMV isolates. Six viral proteins were identified for MMV-CR, -FL and -HI, and by comparison of relative banding positions in SDS-PAGE gels to those of MMV-HI proteins for which the Mr's were
calculated (L. L. McDaniel, R. G. Gomez, D. T. Gordon and N. H. Gordon, unpublished), they were tentatively designated as L (182,000), G (74,000), N (56,000), Ns (39,000), M₁ (32,000) and M₂ (31,000). In contrast, Falk and Tsai (1983) identified only three major proteins for MMV-FL, viz., G (75,000), N (54,000) and M (30,000); their G and N proteins are similar in Mr's to those of this study and their M protein appears to be equivalent to the M₂ of this study. On the other hand, for MMV-VZ Lastra and Acosta (1983) identified five proteins designated L (150,000), G (75,000), N (56,000), Ns (45,000) and M (33,000); their L and Ns proteins differ slightly in Mr from that estimated for the same proteins in this study, whereas the G and N proteins are similar to those of this study, and their M seems to be equivalent to the M₁ of this study.

The G protein in all the isolates tested was glycosylated. The G protein of MMV-FL and MMV-VZ were previously shown to be glycosylated (Falk & Tsai, 1983; Lastra & Acosta, 1983).

The staining methods used in this research (Coomassie Brilliant Blue G-250, silver stain, and Coomassie Brilliant Blue G-250/silver stain) revealed more bands than revealed by the fluorescence labelling used by Falk and Tsai (1983), indicating, that the former stains may be more sensitive methods. These authors mention the sporadic presence of protein bands that could have been L, Ns and M₂. The inconsistency in their staining results could be due to variability in their staining technique as well.
Maize mosaic virus appears to have two M proteins and an Ns protein. This combination has been suggested for only one other plant rhabdovirus, viz., SYNV. For the latter, Jackson (1978), reported a minor protein with an electrophoretic mobility near the N protein and suggested that it could be an Ns protein. While SYNV may have the combination of an Ns protein and two M proteins, it assembles on the inner nuclear membrane and accumulates in the perinuclear space, a distinctive feature of subgroup II or B of plant rhabdoviruses. In contrast, MMV assembles on both inner nuclear and cytoplasmic membranes and accumulates in both the perinuclear space and cytoplasm (Bradfute & Tsai, 1983; McDaniel et al., 1985). Thus, MMV appears to have a unique combination of M and NS proteins and sites of assembly and accumulation.

Results show the presence of a putative L protein for MMV. These results agree with those previously found in our laboratory by L. L. McDaniel (unpublished). This protein has been reported also for LNYV and SonV (sonchus virus) (Dale & Peters, 1981), SYNV (Jackson & Christie, 1977), SYVV (sowthistle yellow vesin virus) (Ziemiechi & Peters, 1976), WRSV (wheat rosette stunt virus) (Tsuhsun & Qiao-Xi, 1984), and AWSMV (Trefzger-Stevens & Lee, 1977) among the plant rhabdoviruses. After treatment with non-ionic detergent, the L protein was detected only in the nucleocapsid fraction of LNYV. Similar tests by Dale and Peters (1981) with SYVV, SYNV, and EMDV failed to reveal the presence of this protein in nucleocapsid
preparations of these viruses. Although this failure may have been a consequence of detergent treatment or experimental conditions (Jackson et al., 1987), our results may reflect yet another difference for MMV in comparison with other rhabdoviruses.

Another difference for the MMV proteins was in their association with the envelope and nucleocapsid of the virus after non-ionic detergent treatment. Only the G protein was associated with the envelope, whereas the L, N, Ns, M₁ and M₂ proteins were associated with the nucleocapsid. These results disagree with those of Falk and Tsai (1983) who reported that the G and M proteins of MMV-FL were associated with the viral envelope after Nonidet NP-40 treatment, and the N protein with the nucleocapsid. In the case of LNYV and SonV (Dale & Peters, 1981) the G and M proteins were associated with the envelope after incubation with 1% Nonidet NP-40, and the G and M₁ proteins with the envelopes of SYVV, SYNV, EMDV, (Dale & Peters, 1981), and PYDV (Gaedigk et al, 1986). The matrix proteins (M₁ and M₂) of the rabies virus, the type rhabdovirus for those having two matrix proteins, are both membrane-associated (Wagner et al., 1972). However, in a reevaluation of rabies virus proteins one of these proteins appeared associated with the nucleocapsid (Zaides et al., 1979). Our results suggest that the matrix proteins of MMV are more tightly bound to the nucleocapsid than the matrix proteins of other rhabdoviruses under the conditions tested.
Based on present and previously published evidence three characteristics of MMV place it outside the accepted classification for plant rhabdoviruses. First, it has the unique combination of two M proteins and a putative Ns protein, with SYNV possibly sharing this combination. Second, the G protein was associated with the envelope and the remaining L, N, Ns, M₁ and M₂ proteins with nucleocapsid fraction. Finally, its sites of assembly in plant cells were on both the nuclear envelope and endoplasmic reticulum and the sites of accumulation within the perinuclear space and cytoplasmic vesicles (McDaniel et al., 1985). These characteristics suggest that it belongs to an as yet undescribed subgroup of plant rhabdoviruses. Based on sites of assembly and accumulation as shown by AWSMV, Jackson et al. (1987) have suggested that several plant rhabdoviruses would be better classified in a subgroup other than the presently recognized subgroups. Clearly, the classification of MMV in this subgroup should be given careful consideration.

The CR, FL and HI isolates appear to be the same virus and should not be considered as distinct. In contrast, MMV-IR is apparently a different virus or a different strain of MMV as shown from these results and evidence from other investigations (Milne et al., 1986). The size of the MMV-IR particle (81 x 179 nm) in negatively stained preparations (Izadpanah et al., 1983) differs from that of MMV-HI (67 - 80 x 204 - 245 nm) (McDaniel et al., 1985). Further MMV-IR is transmitted in Iran by Ribautodelphax notabilis
Logv. (Izadpanah et al., 1983), a vector not reported to transmit MMV. When *P. maidis* was allowed to acquire MMV-IR by direct feeding on infected leaves, the rate of transmission was very low (Ca 0.5%) (R. G. Gomez and W. E. Styer, unpublished). However, abdominal injection of *P. maidis* with MMV-IR resulted in increased transmission rates (20%). It is not known whether *R. notabilis* will transmit the other MMV isolates of this study.
CHAPTER II

Serological Relations Among Four Isolates of Maize Mosaic Virus using Monoclonal and Polyclonal Antibodies.

INTRODUCTION

The principal criteria used for designating maize-infecting rhabdoviruses as isolates of MMV are symptomatology (Bradfute & Tsai, 1983), host range (Izadpanah et al., 1983), particle morphology (Kitajima & Costa, 1982), vector species (Trujillo et al., 1978), and in some cases serological relatedness (Falk & Tsai, 1983). The results from these tests have not been conclusive and the relationship of MMV isolates still remains obscure or at best unsettled, e.g., MMV-BR is serologically related, but other evidence suggests it is different (Kitajima & Costa, 1982).

The use of conventional polyclonal antibodies to study MMV isolates have not shown differences among them (Autrey, 1983; Bradfute & Tsai, 1983; Falk & Tsai, 1983; Kitajima & Costa, 1982).

This chapter presents a study of serological relationships among the proteins of MMV as assessed by Western blots immunostained using polyclonal and monoclonal antibodies prepared against MMV-HL.
MATERIALS AND METHODS

VIRION PURIFICATION.

The four isolates of MMV (CR, FL, HI, and IR) used in this test were purified using the procedure described in Chapter I.

ANALYSIS OF VIRAL PROTEINS.

Virion proteins were analyzed by SDS-PAGE (Laemmli, 1970) using 15% acrylamide gels, and the separated proteins were either stained with Coomassie Brilliant Blue G-250, as reference control, or further analyzed by Western blotting.

WESTERN BLOTTING.

Gels were further analyzed by Western blotting to nitrocellulose membranes (Towbin & Gordon, 1984; Towbin et al., 1979). Portions of the electroblotted membranes were stained with Amido Black for total protein and other portions were immunostained, as described below, using monoclonal or polyclonal antibodies.

PRODUCTION OF POLYCLONAL ANTIBODIES TO MMV-HI

Some of the polyclonal antiserum used in this research was previously prepared in our laboratory (L. L. McDaniel, unpublished).
An additional antiserum was prepared by the following technique using white New Zealand rabbits (4-4.5 kg). Preimmune serum was recovered from the blood taken from the marginal ear vein before starting the injections with MMV-HI. Three injections were made into the thigh muscles of rabbits at 7 day intervals using 2 ml of a 1:1 (v/v) mixture of Freund's complete adjuvant (Gibco Laboratories, Life Technologies, Inc., Chagrin Falls, OH) and 0.8 mg/ml (Chapter I) of partially purified MMV-HI for the first injection and 1 ml of a 1:1 (v/v) mixture of incomplete Freund's adjuvant (Gibco Laboratories) and 0.5 mg/ml of partially purified virus for the last two injections. Rabbits were bled three times from the marginal ear vein at 10-12 day intervals after the last injection. Blood, approximately 20 ml, was collected in centrifuge tubes that were incubated at room temperature for 1 hr and then overnight at 4°C. The tubes were then centrifuged at 2,500 rpm for 15 min in an IEC Centra-7 centrifuge (International Equipment Company, Needham Hts., MA) and the serum poured into clean tubes. Finally, the serum was diluted 1:1 with glycerol and stored in the freezer (-20°C).

PRODUCTION OF MONOCLONAL ANTIBODIES TO MMV-HI.

Monoclonal antibodies to MMV-HI were produced by standard techniques (Mishell & Shiigi, 1980; R. A. Simkins, unpublished; Appendix A). Partially purified MMV-HI (100 μg) in Freund's complete adjuvant was injected interperitoneally (i.p.) into Balb/c mice.
Then at 3 week intervals, two additional i.p. injections, 50 μg for the first time and 10 μg for the second time (both emulsified in Freund's incomplete adjuvant) were made. The serum of injected mice was assayed by indirect ELISA (enzyme-linked immunosorbent assay; as described below) to confirm the presence of MMV antibodies.

Spleen cells from immunized mice were fused by polyethylene glycol 4000 (Sigma Chemical Co.) with cells from the myeloma cell lines SP2.0-AG14. The medium from wells containing hybridomas was screened for binding to viral proteins by indirect ELISA. Hybridomas testing positive were expanded and cloned by limiting dilution. Hybridoma clones testing positive for viral protein were expanded. The monoclonal antibodies were isotyped as IgG's or IgM's by indirect ELISA using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim, Indianapolis, IN) and screened by Western blot analysis (Towbin & Gordon, 1980; Towbin et al., 1979) involving immunostaining (described below) to determine to which viral protein the antibody binds.

INDIRECT ELISA.

The indirect ELISA (von Wechmar et al., 1983) for testing the serum from injected mice, hybridoma supernatants, and purified monoclonal antibodies (Appendix B) involved the following steps. Wells were coated with partially purified MMV-HJ (1 μg/ml) 50 μl/well in 20 mM sodium carbonate buffer, pH 9.0, and incubated 2-4 hr at 37
C or overnight at 4 C. Unbound sites were blocked with 2% bovine serum albumin (BSA) (150 μl/well) in 20 mM sodium carbonate buffer, pH 9.0, and incubated 2-4 hr at 37 C. Mice sera, hybridoma supernatants or purified monoclonal antibodies were diluted (1:1000 to 1:3000) in TBS (25mM Tris-HCl, pH 8.0, 0.5 M NaCl) + 1% BSA (50 μl/ml), and incubated 2-4 hr at 37 C or overnight at 4 C. Secondary or detecting antibody was added, horseradish peroxidase conjugated goat anti-mouse IgG and IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN) (1:1000 to 1:3000) (50 μl/well), and incubated at room temperature for 2-3 hr. The substrate, ABTS [2,2'-azonobis (3-ethylbenzthiazoline sulfonic acid)] (Sigma Chemical Co.) (1 mM in 10 mM sodium citrate, pH 4.2), plus H₂O₂ (0.003%) were added (100 μl/well) and incubated at room temperature. Reaction was stopped when wells were dark enough (15-30 min) by addition of 5% SDS (50 μl/well). Absorbances were recorded at 495 nm with a Bio-Tek EIA Reader (Bio-Tek Instruments, Inc., Burlington, VT). There were three washes with TBS-T (TBS plus 0.05% Tween 20) between each of the above steps.

IMMUNOSTAINING OF NITROCELLULOSE MEMBRANES.

Electrophoresed gels of 10 or 15% acrylamide were blotted to nitrocellulose membranes and stained as described by Knecht and Dimond (1984) with two modifications: the Tris-HCl buffer system used contained 25 mM Tris-HCl, 500 mM NaCl (TBS), plus 0.05% Tween 20.
(Sigma Chemical Co.) (TBS-T), plus 5% horse serum (TBS-T + 5% HS) or 1% BSA (TBS-T + 1% BSA), pH 7.4; the 5-bromo-4chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.) was reduced to 0.1 mg/ml in the agarose solution.

Nitrocellulose membranes were sequentially incubated in the following solutions; TBS-T +5% HS or TBS-T to block unbound sites (30 min, 37°C), rinsed with TBS-T, incubated in primary antibody at an appropriate dilution in TBS-T + 1% HS or TBS-T + 1% BSA (1:1000 MMV-HI antiserum and IgG monoclonal antibodies, or 1:100 for IgM monoclonal antibodies) (90 min, 37°C), washed with TBS-T + 1% HS or TBS-T + 1% BSA (once, 10 min) and TBS-T (twice, 10 min/each), incubated in Protein A linked to alkaline phosphatase (Sigma Chemical Co.) (1:1500 v/v in TBS-T + 1% HS for 90 min at 37°C) or any of the following secondary antibodies (depending on the test being performed): a) goat anti-rabbit IgG linked with alkaline phosphatase (Miles-Yeda, Ltd., Israel) (1:2500 v/v in TBS-T; 90 min, 37°C) when rabbit polyclonal antibodies were used; b) affinity purified goat anti-mouse IgG and IgM linked to alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) (1:3000 v/v in TBS-T; 90 min, 37°C) when mouse monoclonal antibodies were being characterized; and c) affinity-purified goat anti-mouse IgM or goat anti-mouse IgG linked to alkaline phosphatase (Cooper Biomedical, Inc., Malvern, PA) (1:2000 v/v in TBS-T; 90 min, 37°C) when a specific mouse monoclonal antibody was used. After the detecting or secondary antibody
incubation, the membranes were washed again twice in TBS-T + 1% HS or TBS-T + 1% BSA (15 min/each), twice in TBS-T (15 min/each), and once in TBS (10 min). The membrane was blotted dry, then applied to the 1% agarose slab containing substrate (BCIP), and incubated 15 min at 30°C or until bands became visible; the incubation was continued for a couple more hours to overnight at room temperature in the dark.
RESULTS

PRODUCTION OF POLYCLONAL ANTIBODIES TO MMV-HI.

Both antiserum produced in rabbits reacted specifically with MMV proteins, and not to proteins from healthy plants or molecular weight standards (Plate VI B). In agar-gel double-diffusion the titer was only 1:32 as previously determined, L. L. McDaniel (unpublished). However, when used in Protein A sandwich ELISA (PAS-ELISA) (Edwards & Cooper, 1985) as described in Chapter IV, the useful titers were 1:1600 and 1:6000 for primary antibody and 1:100 to 1:200 for secondary antibody (Fig. 1).

PRODUCTION OF MONOCLONAL ANTIBODIES TO MMV-HI.

Fifteen monoclonal antibodies were produced; 12 IgM's and three IgG's. Ten IgM's were specific to N protein, and two were specific to G. One IgG was specific to G protein and two IgG's reacted broadly to N, Ns, and M₁.

IMMUNOSTAINING.

Using polyclonal antibodies produced against MMV-HI proteins, isolates MMV-CR, MMV-FL, and MMV-HI reacted indistinguishably from one another (Plate VI B). Isolate MMV-IR gave no reaction to the antiserum produced to MMV-HI (Plate VI). In tests using monoclonal
antibodies prepared against MMV-HI, the G proteins and N proteins of MMV-HI and MMV-FL reacted identically (Plate VII).
Fig. 1. Titration curves to determine the appropriate dilutions of two polyclonal antibodies used in different experiments. Antibodies were raised against isolate of maize mosaic virus from Hawaii (MMV-HI). Arrows indicate the dilution selected for use in subsequent tests. Selection was based on the point of inflection of the curve. Useful dilution 1:6000 (upper), 1:1600 (lower).
Fig. 1.
Plate VI. Immunostained Western blots of sodium dodecyl sulfate polyacrylamide gel electrophoretically separated proteins of maize mosaic virus (MMV) isolates. The bands were stained using polyclonal antibodies to healthy maize (A) and an isolate from Hawaii (B). Lane 1, Iranian isolate (MMV-IR); Lane 2, Hawaiian isolate (MMV-HI); Lane 3, Florida isolate (MMV-FL); Lane 4, Costa Rican isolate (MMV-CR); Lane 5, healthy maize extract; and Lane 6, low molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weights).

Letters identify virion proteins: L, N, Ns, M₁ and M₂; asterisk denotes a protein seen occasionally in gels.
Plate VII. Western blot analysis of virion proteins of maize mosaic virus (MMV) isolates from Florida (FL) and Hawaii (HI) with monoclonal antibodies. Protein preparations were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels transferred to nitrocellulose membranes and assayed with monoclonal antibodies. A) MMV-FL and B) MMV-HI immunostained with monoclonal antibody to N protein; C) MMV-FL and D) MMV-HI immunostained with monoclonal antibody to the G protein. Lanes E-H were stained for total protein with Amido Black: E) MMV-FL, F) MMV-HI, G) protein from healthy plants; and H) Molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weights).
DISCUSSION

The polyclonal antibodies raised in rabbits were specific to MMV isolates CR, FL and HI. The differences in titer were likely due to individual rabbit differences and time of bleeding after injection. The PAS-ELISA (Edwards & Cooper, 1985) was a more sensitive method to determine dilutions of the antiserum than the agar-double diffusion test.

The monoclonal antibodies produced to MMV-HI proteins were specific in all cases to viral proteins and failed to react with healthy maize proteins or the molecular weight standards used in the gels. Cross-reactivity of monoclonal antibodies, as in the case of two of our IgG's that reacted with the N, Ns, and M1 proteins, has been observed for monoclonal antibodies to the proteins of other rhabdoviruses. For example, Volk et al. (1982) reported that two IgG's to the G protein of VSV cross-reacted with the M protein. This cross reactivity could be due to similar epitopes on the two proteins.

Results with polyclonal antibodies indicated that MMV-CR, -FL, and -HI are similar if not identical, whereas MMV-IR was not serologically related to them. The results from immunostained Western blots agreed with those obtained by L. L. McDaniel, R. G.
Gomez and D. T. Gordon (unpublished) shown in Appendix C for MMV-CR, -FL and -HI; MMV-IR was not tested by these investigators.

The results with monoclonal antibodies to MMV-HI also showed that MMV-HI and MMV-FL were indistinguishable with respect to the reactions to the G or N proteins.

This work is the first in which immunostained Western blots have been used to study serological relationships among isolates of MMV and also the first in which more than one isolate was studied at a time. Previous experiments to establish the identity of MMV isolates (Bradfute & Tsai, 1983; Lastra, 1977) or the relationship among cereal rhabdoviruses (Greber, 1984; Milne et al., 1986) used double diffusion tests or the electron microscope decoration technique. The Western blots immunostained by monoclonal and polyclonal antibodies to MMV-HI were useful in establishing serological relationships among MMV isolates.

Evidence on MMV-IR from our studies suggest that MMV-IR should be considered a separate strain of MMV or a different maize-infecting rhabdovirus.
CHAPTER III

Peptide Mapping by Limited Proteolysis of Proteins of Two Isolates of Maize Mosaic Virus.

INTRODUCTION

Maize mosaic virus isolates have been reported from different parts of the world (Brewbaker, 1979; Gordon & Ritter, 1987). Some of the isolates have been partially characterized as MMV based on the morphology of the particle, the host, the vector or reactivity with MMV antiserum. All the isolates that have been identified as MMV share in common a similar symptomatology, a limited host range, obligate and persistent transmission by P. maidis, a typical rhabdovirus morphology in tissue sections and purified preparations, and serological reactivity with MMV-VZ antiserum. More detailed studies on the physicochemical properties of these isolates are limited.

Comparisons of MMV strains by conventional serological techniques have been useful in detecting antigenic similarity among them (Chapter II). However, this approach has been limited because it cannot detect all potential serological differences or similarities among the proteins of the isolates, i.e., it cannot
distinguish between different epitopes within the proteins. The serological tests used in Chapter II showed no differences between MMV-FL and MMV-HI. Peptide mapping following limited proteolysis has been used to identify conserved and distinct sequences in rhabdovirus proteins (Burge & Huang, 1979) and to measure the relative degree of relationship between four proteins of two strains of PYDV (Adam & Hsu, 1984). Thus, as an alternate approach for revealing differences between the proteins of these two isolates, partial proteolysis of their proteins followed by SDS-PAGE (Cleveland et al., 1977) was used to see if differences could be detected.
MATERIALS AND METHODS

VIRION PURIFICATION.

Purification of MMV-HI and MMV-FL was done as described in Chapter I.

ISOLATION OF PROTEINS.

The proteins of MMV-FL and MMV-HI were separated in SDS-PAGE gels containing 10% acrylamide, as described in Chapter I, without using the stacking gel; i.e., the protein mixture was layered onto the resolving gel directly after treatment with 2X buffer and boiling for 2 min. Sucrose-dye solution was added to the mixture after cooling.

The method of recovering proteins from gels was based upon the procedure of Haeger and Burgess (1980), i.e., the protein bands were visualized by soaking the gels in cold 0.25 M KCl for 5 min, and then in cold distilled water for 1 hr. The bands were then sliced from the gel and frozen until eluted.

For eluting protein, gel pieces were thawed, sliced into sections about 1 mm thick, and the sections placed in elution buffer [0.05 M Tris-HCl, pH 7.9, 0.1% SDS, 0.1 mM EDTA (disodium ethylenediamine tetraacetate, Fisher Scientific Co., Fair Lawn, N.J.), and 0.15 M NaCl]. After stirring slowly at room temperature
for several hours to overnight, liquid was drawn off and centrifuged at 10,000 rpm for 10 min in a Sorval SS 34 rotor to eliminate fine pieces of acrylamide. The protein was then precipitated by adding trichloroacetic acid (TCA) to a final concentration of 20%, and the mixture was incubated in the cold room for 2 hr and then centrifuged at 25,000 rpm for 30 min in a Beckman SW 41Ti rotor. The pellet was rinsed once with ice cold 95% ethanol, then dried at room temperature for 30-60 min, and resuspended in digestion buffer (0.125 M Tris-HCl, pH 6.8, 0.5% SDS, and 10% glycerol). Samples were kept frozen until used for peptide mapping.

PEPTIDE MAPPING.

Proteins were compared using the technique of Cleveland et al. (1977) as modified by Edwards and Gonsalves (1983). Protein samples to be compared following treatment with a particular enzyme were digested simultaneously and under identical conditions. Chymotrypsin, papain and staphylococcus V8 protease (product numbers C-4754, P-3125 and P-8400, respectively; Sigma Chemical Co.), were used at final concentrations of 150 µg/ml, 16.5 µg/ml and 150 µg/ml, respectively. Mixtures of viral protein and V8 protease were incubated at room temperature for 30 min. Mixtures of viral proteins with chymotrypsin or papain were not incubated, but rather layered onto gels immediately after mixing.
Protein digests were electrophoresed on discontinuous polyacrylamide minislab gels (4% stacking, 15% resolving) (Hoefer Mighty Small Vertical Slab Unit SE 250, Hoefer Scientific Instruments, San Francisco, CA), at 40 mA (20 mA/gel; constant current) during stacking, then once the front had reached the resolving gel the current was increased to 60 mA (30 mA/gel). Gels and buffers were prepared according to Laemmli (1970).

STAINING OF "PEPTIDE MAPPING GELS".

The gels were either silver-stained alone (Bio-Rad Bulletin 1089, 1982; Heukeshoven & Dernick, 1985; Marshall, 1984; Merril et al., 1984) or stained by a combined Coomassie Brilliant Blue G-250/silver stain (De Moreno et al., 1985). The modified technique used is described below. To begin, gels were soaked in 40% methanol, 10% acetic acid for 1 hr. They were stained with Coomassie Brilliant Blue G-250 for 30 min and destained in 5% acetic acid until background was clear. They were soaked in 50% ethanol, 10% acetic acid for 30 min; then in 25% ethanol, 10% acetic acid for 30 min; and finally in 10% ethanol, 5% acetic acid for 30 min. After soaking, gels were rinsed with deionized water, then soaked in oxidizer (0.0034 M potassium dichromate, 0.0032 N nitric acid) for 10 min, and washed with deionized water, using as many changes as needed to eliminate the yellow background caused by the oxidizer. Gels were then soaked for 30 min in 0.012 M silver nitrate, rinsed in deionized
water (1-2 min), and developed three times with 0.28 M sodium carbonate, plus 0.5 ml of formaldehyde/liter for 1, 3-5, and 2-3 min, respectively. A stock solution of sodium carbonate (200 ml) was mixed with 100 µl of formaldehyde just prior to use. The reaction was stopped by addition of 5% acetic acid for 5 min, and gels were rinsed and stored in deionized water.

If silver stained only, gels were soaked in the ethanol, acetic acid series described above for the Coomassie staining procedure. For a combined Coomassie Brilliant Blue G-250/silver stain, this soaking series were omitted.
RESULTS

PEPTIDE MAPPING.

Limited digestion of the proteins (G, N, M₁, and M₂) of the two isolates of MMV with chymotrypsin, papain, and V8 protease generated the band patterns showed in Plate VIII. Protein patterns of MMV-FL and -HI showed only a few differences. Digestions with chymotrypsin and papain were more complete, at least for G and N proteins, than were those with V8 protease.

With chymotrypsin, the G band of MMV-FL appeared to have two fragments not present in the Hawaiian isolate. With papain, the G band of MMV-FL showed one fragment not detected in MMV-HI. For the N band, MMV-FL had two doublets of lighter molecular weight and MMV-HI had one low molecular weight fragment not present in MMV-FL. In the case of V8 protease, the digestion was less extensive. Nevertheless, MMV-FL showed one extra band in both G and N protein patterns.
Plate VIII. Comparison of peptide fragments from the G, N, and M₁ and M₂ proteins of the Hawaiian (HI) and Florida (FL) isolates of maize mosaic virus (MMV) following partial proteolysis with chymotrypsin, papain or V-8 protease and electrophoretic separation on sodium dodecyl sulfate 15% polyacrylamide gels. A) Peptides from chymotrypsine treated viral proteins. Lanes 1 and 11: low molecular weight standards (see legend of Plate I for identity of protein standards and their respective molecular weight); Lane 2, proteins from virion of MMV-HI; Lane 3, proteins from virion of MMV-FL; Lane 4, peptides from G protein of MMV-HI; Lane 5, peptides from G protein of MMV-FL; Lane 6, peptides from N protein of MMV-HI; Lane 7, peptides from N protein of MMV-FL; Lane 8, peptide from M₁ and M₂ proteins of MMV-HI; Lane 9, peptides from M₁ and M₂ proteins of MMV-FL; Lane 10, chymotrypsine alone. B) Peptide from papain treated viral proteins. Lanes 1-11 contain the same proteins or peptides from viral proteins as in A. C) Peptides from V-8 protease treated viral proteins. Lanes 1, 4, 10, low molecular weight standards; Lane 2, proteins from virion from MMV-HI; Lane 3, proteins from virion of MMV-FL; Lane 5, peptides from G protein of MMV-HI; Lane 6, peptides of G protein of MMV-FL; Lane 7, peptides from N protein of MMV-HI; Lane 8, peptides from N protein of MMV-FL; Lane 9, V-8 protease alone. (Peptides from M₁ and M₂ proteins of MMV-HI and MMV-FL not shown).
DISCUSSION

The results clearly showed that the FL and HI isolates of MMV were nearly identical and classification as distinct strains would be inappropriate. Two isolates of MMV-VZ (Lastra, 1977) and three of MMV-MR (Autrey, 1983) have been separated into strains based on symptomatology; but serologically the strains were indistinguishable. So far the attempts to differentiate isolates of MMV by serological means [Chapter II; L. L. McDaniel, R. G. Gomez and D. T. Gordon, unpublished (see Appendix C)] have shown that they are all indistinguishable except for MMV-IR.

Maize mosaic virus isolates FL and HI were indistinguishable serologically but the peptide mapping technique of Cleveland et al. (1977) showed a few differences that may have significance. Similarly, a good correlation was found between serological typing and peptide mapping of tymovirus proteins (Koenig et al., 1981) and cucumber mosaic virus proteins (Edwards & Gonsalves, 1983). Peptide mapping is a sensitive and consistent means of supplementing symptomatological or serological classification (Edwards & Gonsalves, 1983) and has been of value in our work in providing evidence that indicated several differences between the FL and HI isolates.
That the MMV proteins (G, N, and M\textsubscript{1} and M\textsubscript{2}) differ little between MMV-FL and -HI indicates that the two isolates have experienced small changes during their evolutionary development although presumably geographically isolated from each other for a considerable time. Since similar results were obtained from serological comparisons (Chapter II), both sets of data support the idea that the two isolates have evolved from a common ancestor, but have not experienced significant changes leading to serotypes or different virus-vector specificities. In contrast, PYDV has different serotypes with different virus-vector specificities (Adam & Hsu, 1984) indicating greater evolutionary divergence of the virus.

Wilson (1985) points out that each replacement of an amino acid in a protein can be ascribed to a point mutation in a gene. Evidence shows that amino acid replacements accumulate at fairly steady rates over long periods of evolutionary time. Direct comparison of genes confirms the hypothesis that the steady evolution of proteins is rooted in the steady evolution of DNA. This suggests that probably isolates like MMV-FL, MMV-HI and MMV-CR, which are very similar have not experienced significant changes in their proteins, and thus still share common host, vector, symptomatology and physicochemical properties. On the other hand, MMV-IR could have evolved from the same common ancestor as these other isolates but because of its marked decrease in efficiency of transmission by P. maidis compared to that of the other isolates plus the other differences found in the
present study. These differences are that while having the same basic morphology the dimensions of virions are different (as discussed in Chapter I), and although the number of proteins is the same, their molecular weights are different except for the G protein (Chapter I; Plate III). On the other hand, similar characteristics are maize as a host, symptomatology in maize and similar cytopathological effects in infected cells (E-D. Ammar, unpublished). Still, we should not forget that convergent evolution of originally unrelated rhabdoviruses, as suggested by serology, could have lead to the appearance of these common characteristics.

Peptide mapping analysis could be expanded by using additional enzymes, perhaps revealing additional peptide differences not found with the enzymes used in this study. The silver stain and the combined Coomassie Brilliant Blue G-250/silver stain developed for these experiments increased the sensitivity for detecting peptide fragments compared to the regular Coomassie Brilliant Blue G-250 stain. Although the results of peptide mapping showed only a few differences between the MMV-HI and MMV-FL, other isolates that may show more differences are MMV-MR (Autrey, 1983) and MMV-VZ (Lastra, 1977) which have already been reported to show symptomatological differences and MMV-BR (Kitajima & Costa, 1982) which shows differences in the sites of assembly and accumulation within infected cells.
CHAPTER IV

Polyethylene Glycol-Mediated Infection of Maize Protoplasts with a Hawaiian Isolate of Maize Mosaic Virus.

INTRODUCTION

The potential usefulness of plant protoplasts for studies of virus infection was first demonstrated by Cocking (1960, 1965, 1966).

Protoplasts from several dicotylendous plants have been used to study multiplication of many types of plant viruses, but only two rhabdoviruses have been included, viz., SYNV and FLSV (festuca leaf streak virus) (van Beek, et al., 1985 a, b, c). Both rhabdoviruses were studied in cowpea [Vigna unguiculata (L.) Walp.] protoplasts, which is not a natural host of either virus.

There are several rhabdoviruses that infect maize, but no report of a study in which mesophyll protoplasts of maize have been infected with these viruses. There is only one report of maize protoplasts being infected by a virus, viz., brome mosaic virus which is not a rhabdovirus (Furasawa & Okuno, 1978).
Apart from information on the sites of assembly and accumulation as determined by electron microscopy, very little is known about the cellular and molecular events in the multiplication of plant rhabdoviruses. The main reason for this is that viral multiplication is difficult to study in systematically infected plants, because only a small fraction of cells are initially infected and later as more become infected they show different stages of infection.

Until recently, no experimental system permitted straightforward study of plant rhabdovirus multiplication. The ability to infect cowpea protoplasts with SYNV (van Beek et al., 1985 a, b; 1986) presents a possible way to overcome this impediment. The use of protoplasts permits nearly synchronous infection of a large fraction of cells that allows the study of the entire sequence of events in rhabdovirus multiplication and also conveniently allows treatment of infected cells with various inhibitors and radioactively labelled precursors.

The usefulness of having a maize protoplasts-MMV system is that it opens the possibility of: a) studying the ultrastructural features of infected protoplasts at various intervals from just after inoculation to the point where large number of virions have been assembled; b) determining the sites and sequence of synthesis, transport, modification, accumulation, and assembly of the virion proteins in situ and in cellular fractions; c) determining the sites of synthesis and sequence of appearance of putative messenger and
genomic RNA's by assay of cytoplasmic and nuclear fractions using cDNA probes complementary to viral RNA; and d) comparing multiplication of the different isolates of MMV as well as with other maize rhabdoviruses.

This chapter reports on a study of the infection of maize mesophyll protoplasts with MMV-HI in the presence of polyethylene glycol.
MATERIALS AND METHODS

VIRION PURIFICATION.

The Hawaiian isolate of MMV was purified as described in Chapter I. The only modification was that the virus was resuspended in a buffer of 0.4 mannitol plus 1 mM CaCl₂.

REAGENTS.

Pectinase (Macerozyme R-10) and cellulase R-10 were obtained from Yakult Biochemical Co., Ltd., Nishinomiya, Japan. Dextran T-20 was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was obtained from Calbiochem, La Jolla, CA. The rest of the reagents were purchased from either Sigma Chemical Co., or Fisher Scientific Company, Fair Lawn, NJ and were of analytical grade.

PROTOPLAST ISOLATION.

Maize protoplasts were prepared by a modification of the procedure of Rumpho and Edwards (1984) as follows. Ten to 12 day-old Aristogold Evergreen Bantam sweet corn plants were placed in the dark for several hours to reduce the amount of starch. The second true leaf (5 g) was chopped into 1 mm-wide pieces, the pieces were incubated in digestion medium (500 mM sorbitol, 1 mM MgCl₂, 1mM
MnCl₂, 0.05% bovine serum albumin (BSA), 2% cellulase R-10, 0.5% Macerozyme R-10, 5mM MES [2(N-morpholino)ethanesulfonic acid] buffer, pH 5.5, for 2-3 hr at room temperature with gentle agitation. The resulting preparation (Plate IX A) was passed through an 80 micron mesh to remove bundle-sheath strands and then centrifuged at 100xg for 5 min to collect the protoplasts (Plate IX B). Protoplasts were resuspended in Dextran medium [500 mM sucrose, 15% (w/v) Dextran T20, 1mM CaCl₂, 0.2% BSA, 5 mM HEPES buffer, pH 7.0], overlayed with sorbitol medium (500 mM sorbitol, 1mM CaCl₂, 0.2% BSA, 5 mM HEPES, pH 7.0), and centrifuged at 250xg for 7 min. Healthy, unbroken protoplasts were collected from the interface, washed with sorbitol medium to eliminate the Dextran, and centrifuged at 100xg for 5 min. Pelleted protoplasts were washed again with sorbitol medium and given a final wash with W5 medium (154 mM NaCl, 125 mM CaCl₂-2H₂O, 5 mM KCl, 5mM glucose) (Negrutiu et al., 1986) just prior to the start of the inoculation (Plate IX C,D). To avoid bacterial and fungal contamination, all solutions were filter-sterilized through 0.22 μm filters, the maize leaf surfaces sterilized by immersion in 20% Clorox (sodium hypochlorite) for 1 min followed by 3 washes in sterile water, and all work was done in a sterile transfer hood (Plate IX).
INOCULATION OF PROTOPLASTS.

Maize protoplasts were infected by a modified combination of the procedures of Negrutiu et al. (1986), van Beek et al. (1985), and Wood (1985). The resulting procedure follows. To the washed protoplasts (1-2 x 10^6 per ml) was added 100-150 μl of partially purified MMV (400 μg/ml) suspended in 0.4 M mannitol plus 1 mM CaCl₂. Then, an equal amount of 40% polyethylene glycol (PEG) 4000, plus 1 mM Ca(NO₃)₂ and 0.4 M mannitol, pH 9.0, were added and the mixture allowed to stand 20 sec before dilution with 5 ml of the W5 medium, added while shaking gently. The mixture was incubated at room temperature for 5 min, followed by centrifugation at 30-50xg for 5 min. The pellet was washed once with the W5 medium and resuspended in culture medium (0.2 mM KH₂PO₄, 1 mM KNO₃, 1 mM MgSO₄, 10 mM CaCl₂, 1 μM KI, 10 mM CuSO₄, 0.7 M mannitol, 1% sucrose, pH 5.6) and incubated in tissue culture plates (Falcon 3046, Becton Dickinson & Co., Oxnard, CA) at 27 C in the dark (Plate IX E,F).

MEASURING MMV INFECTION IN PROTOPLASTS BY IMMUNOFLUORESCENCE MICROSCOPY.

The technique (Nishiguchi et al., 1986) involved the following: a drop of protoplast suspension was placed on a glass slide previously coated with a thin layer of Mayer's albumin (Fisher Scientific, Orangeburg, NY); the slides were dried with a hair dryer; the protoplasts were fixed by immersing the slide in 95% ethanol for
15 min at room temperature; the slide was washed in phosphate-buffered saline (PBS, 0.14 M NaCl, 0.02 M sodium phosphate, pH 7.2) for 10 min; and excess PBS was removed from around the edge of the protoplast drop with filter paper. Then, a drop of 1:10 rabbit anti-MMV immunoglobulins was added to the fixed protoplasts and incubated for 30 min; the slide was washed in PBS for 10 min; 20 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) was added in PBS and the mixture was incubated for 30 min. The protoplasts were then washed twice, 10 min each, with PBS (0.14 M NaCl, 0.1 M sodium phosphate) at pH 8.0; the slides were mounted with a mixture of PBS (pH 8.0) and glycerol (2:3); and treated protoplasts were examined for fluorescence with a Zeiss fluorescence light microscope. When monoclonal antibodies were used, the secondary antibody was goat anti-mouse FITC (Sigma Chemical Co.).

MEASURING MMV INFECTION IN PROTOPLASTS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

Maize mosaic virus replication was determined by Protein A sandwich ELISA (PAS-ELISA) as described by Edwards and Cooper (1985) and briefly presented in the following. Aliquots of protoplasts infected with three different amounts of virus (25 µl, 50 µl and 100 µl, with starting concentration of 400 µg/ml) plus a healthy control were taken at 0, 12, 24, and 48 hr after inoculation and kept frozen
until assayed. A dilution series of purified MMV-HI starting at a 6 µg/ml dilution was included as a standard curve of virus concentration. The collected protoplasts and purified MMV-HI were diluted in three-fold steps to give seven dilutions. Along with the dilution series of each time interval and standard MMV preparation tested in a microtiter plate three negative controls were included, involving omission of Protein A, antigen, and detecting antibody from a set of two wells per omission.

Polyclonal antibodies to MMV-HI (Chapter II) where used in the PAS-ELISA involving the following steps between which there were three washes with TBS-T (25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05% Tween 20). To each well 50 µl of Protein A 3 µg/ml, in 0.05 M sodium carbonate, pH 9.6, was added and incubated for 2 hr at 37 C. Uncoated sites were blocked with 1% BSA in 0.05 M sodium carbonate, pH 9.6, (150 µl/well) and incubated at room temperature for 1 hr. Rabbit antiserum appropriately diluted in TBS-T was added (50 µl/well) and incubated for 2 hr at 37 C. Protoplasts were ruptured by thawing frozen samples and pipetting up and down a few times through a narrow orifice. A three-fold dilution series involving seven steps was prepared with TBS-T, 50 µl of each dilution were added per two wells, and the plates incubated overnight at 3-4 C or 2 hr at 37 C. Rabbit antiserum, again suitably diluted in TBS-T, was added (50 µl/well) and the mixture incubated for 2 hr at 37 C. Biotinylated Protein A (Amersham International, UK) diluted 1:1500 in
TBS-T was added (50 µl/well) and incubated for 2 hr at 37 C. Horseradish peroxidase-streptavidin (Amersham, UK) diluted 1:1000 in TBS-T was added (50 µl/well) and incubated at room temperature for 0.5 hr. The substrate, ABTS [2, 2'‐azinobis (3-ethylbenzthiazoline sulfonic acid)] (Sigma Chemical Co.) (1 mM in 10 mM sodium citrate, pH 4.2), plus H2O2 (0.003%) was added (100 µl/well) and incubated at room temperature until the positive control (purified MMV) showed an absorbance of 1.0 at 495 nm. To stop the reaction, 5% SDS was added (30 µl/well). Absorbances were recorded at 495 nm with a Bio-Tek EIA Reader (Bio-Tek Instruments, Inc., Burlington VT).

SODIUM DODECYL SULFATE‐POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTOPLASTS.

Frozen protoplasts from the MMV inoculation were thawed, 2X treatment buffer and sucrose dye were added, and the mixture boiled for 2 min. Treated samples were layered (40 µl/well) on a 4% acrylamide stacking gel and electrophoresed through a 7.5 to 17.5% acrylamide gradient gel made with a Hoefer XP077 Exponential Gradient Maker (Hoeffer Scientific Inc., San Fransisco, CA). Buffers and electrophoretic conditions were described in Chapter I. Gels were stained with the Coomassie Brilliant Blue G-250/silver stain described in Chapter III.
LIGHT AND ELECTRON MICROSCOPY OF INFECTED PROTOPLASTS.

Healthy and infected maize protoplast samples were collected by low-speed centrifugation (50xg/5 min), at varying times after inoculation. Protoplasts were prefixed by adding with gentle mixing an equal amount of 3% glutaraldehyde prepared in culture media to give a final concentration of 1-1.5% glutaraldehyde. The mixture was incubated at room temperature for 1-2 hr. Protoplasts were pelleted (50xg/5 min), the initial fixative replaced by a second amount of 3% glutaraldehyde in culture media and the mixture incubated in the cold room overnight at 4 C. Protoplasts were pelleted (50xg/5 min), the second fixative replaced with a third of 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, and the mixture incubated in cold room. The protoplasts were pelleted (50xg/5 min), embedded in 4% agar, and washed overnight in 0.1 M potassium phosphate buffer. The postfixation, embedding, sectioning and staining of protoplasts was done as described by McDaniel et al. (1985) for maize leaf pieces. Briefly, protoplast samples were postfixed in 1% OsO₄ in phosphate buffer for 3 hr, stained (en bloc) overnight in 1% uranyl acetate (UA), dehydrated in an ethanol-acetone series, and embedded in Spurr's medium (Spurr, 1969). Ultrathin sections, cut with a diamond or glass knife, were stained in 0.5% UA and 0.1% lead citrate and examined at 80 KV in a Philips 201 electron microscope (Philips Electronics Instruments, Inc., Mahwah, NJ). Some samples were also sectioned for light microscopy (2 µm; "semi-thin sections") using a
11800 Pyramitome ultramicrotome (LKB-Producter, Sweden). These sections were stained with 0.5% Toluidine Blue in 0.1% sodium carbonate for 30 sec on a hot plate (60 C), and examined in a Zeiss Photomicroscope II (Carl Zeiss, W. Germany) at 40x and 100x.
RESULTS

IMMUNOFLUORESCENCE MICROSCOPY.

Fluorescent labelling with polyclonal antibodies gave variable results due to background staining by antibodies produced to healthy material. Labelling with an IgG monoclonal antibody specific to G protein showed differences between infected and uninfected protoplast (Plate X), background staining was minimal and probably due to non-specific binding.

MULTIPLICATION OF MMV IN INFECTED PROTOPLASTS.

In the time-course study an appreciable amount of MMV was detected at 0 hr by PAS-ELISA. At 12 hr there was a decrease in viral concentration by at least two-fold. At 24 hr the concentration of virus increased to a level similar to that at 0 hr. At 48 hr, the increase in concentration doubled in some cases (viz., P3), that at 24 hr (Fig. 2).

ELECTROPHORETIC ANALYSIS OF PROTEINS FROM INFECTED PROTOPLASTS.

Electrophoretic protein patterns in gradient SDS-PAGE gels detected only the G protein from PEG-inoculated protoplasts. No viral bands were detected in lanes from either healthy (non inoculated) protoplasts or protoplasts inoculated with MMV without PEG. The rest of the MMV proteins (L, N, Ns, M₁ and M₂) were masked
by other protoplast proteins. In the case of the N protein it appeared embedded with three other proteins but the amount of staining at this position where it was expected indicated its probable presence (Plate XI).

LIGHT AND ELECTRON MICROSCOPY OF INFECTED PROTOPLASTS.

For the first inoculation trials examined by electron microscopy, protoplasts treated with PEG and PEG plus MMV showed extensive damage to the nucleus and chloroplast membranes. In contrast, protoplasts treated with virus only (no PEG) or untreated control protoplasts showed no appreciable damage (Plate XII).

In later fusions, the damage to nucleus and chloroplast membranes was reduced because of gentler handling during fusion. Protoplasts treated with PEG and PEG plus MMV showed extensive cytoplasmic vacuolation 24 hr after inoculation. Protoplasts that were inoculated with MMV plus PEG, besides vacuolation, showed membranes proliferating to the exterior of the cell to which viral particles were attached (Plate XIIIa). Viral particles were also seen attached and apparently budding from the outer membrane of the chloroplasts (Plate XIIIc). No viral particles were observed budding or attached to the nuclear membranes (Plate XIIIb).

At the light microscopy level, which in later experiments was used to determine the state of the protoplast prior to thin
sectioning, no damage to nuclei or chloroplasts was observed for any
treatment, but vacuolation was detected in PEG and PEG plus MMV
treated protoplasts (Plate XIV).
Plate IX. Condition of maize mesophyll protoplasts after different steps in the isolation, purification and inoculation procedures. A) Bundle sheath strands and free protoplasts during cell wall digestion (16x). B) Protoplasts before the Sorbitol-Dextran cushion step; arrows show broken protoplasts and free chloroplasts (16x). C) Protoplasts after Sorbitol-Dextran cushion; notice the absence of broken protoplasts. D) Purified protoplasts (40X). E) Protoplasts during fusion following exposure to polyethylene glycol in the inoculation procedure; arrows point to the sites where membrane fusion is taking place (16x). F) Fused protoplasts; arrows point to sites where membranes have fused (40x).
Plate X. Demonstration of infection of maize mosaic virus inoculated maize protoplasts and the absence of infection of non-inoculated protoplasts by immunofluorescent light microscopy. A) Inoculated maize protoplasts; notice the amount and distribution of fluorescence associated with protoplasts; arrows point to non-fluorescent protoplasts. B) Non-inoculated protoplasts; notice the dullness of the protoplasts; arrow points to a protoplasts showing fluorescence, probably due to non-specific binding of the antibody.
Fig. 2. Demonstration of virus replication in maize mosaic virus inoculated maize protoplasts by measuring relative virus concentration (ABS.) with the Protein A sandwich enzyme-linked immunosorbent assay at various times after inoculation. HT=healthy protoplasts; P1=protoplasts inoculated with 25 ul/virus; P2=protoplasts inoculated with 50 ul/virus; P3=protoplasts inoculated with 100 ul/virus; and MMV=maize mosaic virus at 0.6 ug/ml.
Fig. 2.
Plate XI. Demonstration of viral replication in maize mosaic virus (MMV) inoculated maize protoplasts by assaying for MMV G and N proteins using the sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis analysis of proteins from non-inoculated and inoculated protoplasts at 6 and 16 hr postinoculation. Lane 1, low molecular weight standards (see legend of Plate I for their identities and molecular weights); lane 2, MMV G, N, Ns, M1 and M2 proteins; lane 3, non-inoculated maize protoplasts; lane 4, MMV-inoculated maize protoplasts exposed to polyethylene glycol (PEG) (6 hr); lane 5, non-inoculated protoplasts exposed to PEG; lane 6, MMV-inoculated protoplasts but without exposure to PEG; lane 7, same conditions as in 4, but assayed at 16 hr; lane 8, same conditions as in 5, but assayed at 16 hr; lane 9, same conditions as in 6, but assayed at 16 hr. Solid arrow indicates the presence of G protein; open arrow indicates the position of N protein and its probable presence in inoculated protoplasts at 6 and 16 hr (lanes 4 and 7).
Plate XI.
Plate XII. Damage to maize protoplasts caused by polyethylene glycol (PEG) treatment. A) Non-PEG treated maize protoplast; notice the intactness of the nucleus (N), chloroplasts (C), plasma membrane (P) and the state of the cytoplasm (Cy). B) PEG-treated protoplasts, notice the absence of nucleus, the damage to the chloroplast, the vacuolation of the cytoplasm, and the stickiness of the plasma membrane to other protoplasts (fusion). Bar in A) represents 3 μm; in B) represents 2 μm. (Electron micrograph provided through the courtesy of Dr. E-D. Ammar)
Plate XIII. Location of rhabdovirus particles and alterations in infected protoplast ultrastructure in maize mosaic virus (MMV) inoculated maize protoplasts.

A) Maize protoplast infected with MMV; notice the amount of vacuolation (V), some of the damage to the chloroplasts (C), and the viral particles (Vr) attached to membranes associated with the chloroplasts and plasma membrane. B) Infected protoplast showing no virions associated with the nucleus (N). C) Higher magnification of A) showing viral particles in close association with chloroplast and plasma membranes. Note that for the latter virions are proliferating to the exterior of the protoplast.

Bar in A) represents 0.5 um; in B) represents 1 um; and in C) represents 0.5 um.

(Electron micrograph provided through the courtesy of Dr. E-D. Ammar)
Plate XIV. Demonstration of improved condition of maize mosaic virus (MMV) inoculated and non-inoculated maize protoplasts assessed by light microscopy after exposure to polyethylene glycol (PEG). A) Non-inoculated, non-exposed maize protoplasts; B) PEG-exposed protoplasts; notice reduced damage to nucleus (N); C) protoplast clumping due to exposure to PEG; D) protoplasts exposed to PEG but not to MMV-HI; notice reduced damage to protoplasts. V=vacuoles.

(Light micrograph courtesy of Dr. E-D. Ammar)
Plate XIV.
DISCUSSION

The results provided evidence that MMV-HI infects and replicates in protoplasts of maize. Results from fluorescent staining established that cells were infected after exposure to MMV in the presence of PEG. The highest amount of infected protoplasts were obtained 48 hr after inoculation. The percentage of infected protoplasts was not assessed by this method because individual protoplasts could not be recognized due to the amount of clumping caused by PEG.

The results from the time course study in which the relative MMV-HI antigen concentration was measured by the PAS-ELISA agreed with the observations of other researchers as summarized by Sander and Mertes (1984). The results of this study showed that at 0 hr there was some virus present which was probably residual inoculum. In the period from 0 to 12 hr after inoculation, viral concentration decreased, and from 12 to 24 hr, it increased so that at 24 hr it was similar to that observed at 0 hr. By 48 hr, the increase in concentration doubled over that at 24 hr. These findings suggest that virus nucleoprotein from the inoculum was present immediately after inoculation (0 hr) probably as virions bound to the protoplasts following inoculation as seen by electron microscopy. The decrease in viral titer at 12 hr post inoculation probably
represented an "eclipse period", and reflected the phase of uncoating during the initial stage of virus replication (Takebe, 1977). The presence of nucleocapsids of MMV in the infected protoplasts as well as abundant polyribosomes at 6 hr after inoculation (E. D. Ammar, R. G. Gomez and D. T. Gordon, unpublished) suggests this interpretation as does the observation from the PAS-ELISA data that the relative virus concentration had declined significantly between 0 and 12 hr post inoculation.

The "eclipse period" was followed by an increase of virus antigen for the two subsequent assay times (24 to 48 hr). Typically, this period of virus increase is followed by a plateau in concentration where there is no further increase, indicating that the cycle of virus replication is completed in the infected protoplasts. Since protoplasts were not assayed beyond 48 hr, this plateau in virus increase was not demonstrated. These findings suggest that MMV follows similar pattern to that observed for non-rhabdoviruses. Similar patterns have been observed for virus concentration in tobacco mosaic virus (TMV) infected tobacco (Takebe & Otsuki, 1969) and tomato (Motoyoshi & Oshima, 1975) protoplasts, tobacco rattle virus infected tobacco protoplasts (Kubo et al., 1975), and brome mosaic virus infected barley protoplasts (Okuno et al., 1977; Loesch-Fries & Hall, 1980).
In comparison to the two rhabdoviruses that have been studied in protoplasts (van Beek et al., 1985a, b, c; 1986), MMV behaves most like FLSV in that no structural changes were observed in the nucleus, but shares a characteristic with SYNV in that the first signs of infection occur at 12 hr after inoculation. Our evidence with ELISA suggests that the first changes in viral concentration occur from 12 to 24 hr and evidence from electron microscopy indicates that the first ultrastructural changes in MMV-infected protoplasts occur at 6 hr after infection (E. D. Ammar, R. G. Gomez and D. T. Gordon, unpublished). With SYNV at the ultrastructural level, the first changes were observed at 9 hr after inoculation. At that time, polysomes increased sharply in number indicating an acceleration of translational activity (van Beek et al., 1985b).

Maize mosaic virus appears to bud from chloroplast membranes, cytoplasmic membranes, and plasma membranes of infected protoplasts. With the plasma membranes, budding appears to be outward from the protoplast. In contrast, SYNV in cowpea protoplasts (van Beek et al., 1985b) buds from the nuclear membrane and accumulates in perinuclear space and endoplasmic reticulum. Festuca leak streak virus (van Beek et al., 1985c), also in cowpea protoplasts, seems to assemble on the outer membrane of chloroplast, and sometimes between the chloroplast and cytoplasmic membrane, but the virus never becomes abundant.
Results at the light and electron microscopic level suggested that PEG had harmful effects on maize protoplasts. van Beek et al. (1985a) also indicated the harmful effects of high concentrations of PEG in the inoculation process but showed no light or electron microscopic evidence. Brar et al. (1980) indicated that in experiments where sorghum and maize protoplasts were fused, most of the non-fused maize protoplasts did not survive PEG treatment. This suggests that maize protoplasts are very susceptible to PEG treatment and thus when working with these protoplasts one has to be particularly careful during fusion not to expose protoplasts to high concentrations of PEG or for over long periods of time if extensive damage is to be avoided.

The G protein was detected from extracts of maize protoplasts infected with MMV by SDS-PAGE between 6 and 16 hr after inoculation. The N protein appears to have been present too, but its resolution was not clear. Recently, van Beek et al. (1986) were able to detect G, N, M₁ and M₂ proteins of SYNV from infected protoplasts by immunoprecipitation between 8 and 12 hr after inoculation. Their methods of detection using radiolabelled compounds appear to be more sensitive than the ones used in this research.
REFERENCES


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HYBRIDOMA PRODUCTION: PREPARATION OF CELLS, FUSION AND CLONING

The following technique (R. A. Simkins, unpublished) was used.

MYELOMA CELLS.

Two days prior to fusion, SP2/0 cells were diluted to a density of $1 \times 10^5$ cells/ml; the next day the cells were diluted 1:1 in DMEM (Dulbecco's modified Eagles medium) containing 10% FBS (Fetal Bovine Serum). These cells were harvested from two or more 50 ml flasks to give 2-5 x $10^7$ cells. Cells were collected by centrifugation for 5 min at 200xg. The supernatant layer was aspirated and discarded, and the pelleted cells were resuspended in 50 ml of SPM (serum-free medium). Finally the suspended cells were pelleted by centrifugation just prior to the initial wash of the fusion procedure.

SPLEEN CELL PREPARATION.

Warm (37 C) medium (ca. 4 ml) was pipetted into wells of a 6-well culture dish. Immunized mice were killed by cervical dislocation and then wetted with 70% ethanol. Each mouse was placed on its right side and the skin below the ribcage to pelvis trimmed.
away using scissors and forceps. The exposed muscle layer was washed with 70% ethanol and a small incision was made with small iris scissors over the spleen, recognized as a dark mass under the muscle layer. The spleen then was pulled out and the connective tissue on underside trimmed away. The spleen was placed in SFM in a culture dish well and the remaining tissue trimmed away. The spleen was cut in two and teased gently with a spatula to release cells until the capsule was empty. The spleen cells were then pipetted up and down several times and transferred to a 15 ml sterile centrifuge tube. The large chunks were allowed to settle for 2 min and then the suspension was transferred to another centrifuge tube. The suspension was centrifuged for 5 min at 200xg and the supernatant fraction removed by aspiration. (The pellet was dark red). The pellet was resuspended in 3 ml of HLS (hypotonic lysing solution) buffer (25 mM HEPES, pH 7.5, 0.144 M NH₄Cl). The suspension was allowed to stand for 2 min, and then 5 ml of SFM were added. The diluted suspension was centrifuged for 5 min at 200xg and the supernatant fraction removed by aspiration. (The pellet was now light grey). The pellet was resuspended in 10 ml SFM.

FUSION.

Spleen cells and myeloma cells were washed separately twice in warm SFM and then mixed at a ratio of 2-5:1. The mixture was centrifuged at 200xg for 10 min in a 50 ml centrifuge tube. The
supernatant phase was removed and the cell pellet loosened by agitation. One ml of 50% (w/v) polyethylene glycol (PEG, M.W. 4000), plus 5% DMSO in DMEM, prewarmed to 37 C in a waterbath, was used for fusing 10^8 spleen cells with 10^7 myeloma cells. Then, 0.5-1.0 ml of the PEG solution was added dropwise over 30-60 sec with gentle agitation followed by dropwise addition of 1 ml of prewarmed SFM over a 1 min interval, again with gentle agitation. Treated cells were rested undisturbed for 1 min and an additional 1 ml was then added dropwise over 1 min. Ten ml of SFM were added dropwise over a 5 min period and the mixture incubated at 37 C for 10 min. The mixture was centrifuged for 5 min at 200xg and the supernatant medium was aspirated off. Twenty ml of HAT (0.1 mM hypoxanthine, 0.4 nM aminopterin, 16 nM thymidine in 80% SFM, 20% FBS) medium were very gently pipetted over the pellet and the latter flipped with a plugged pasteur pipette. The pellet was then incubated in a CO_2 incubator for 30-60 min at 37 C, pipetted up and down twice, and added to 80 ml of HAT medium. Four drops (ca. 0.2 ml) of the suspension were added to each well of as many 96-well tissue culture dishes as required to exhaust the suspension.

CLONING BY LIMITING DILUTION.

Spleen feeder cells were prepared exactly as were the spleen cells. The spleen feeder cells were diluted in 50-100 ml DMEM + 20% FBS or HT medium (hypoxanthine-thymidine). Hybridoma cells were
counted and diluted into spleen feeder cell medium to give three hybridoma cells/200 μl for each of 32 wells; one cell/200 μl for each of another 32 wells; and an average of 0.5 cells/200 μl for the remaining 32 wells. Four drops (ca. 200 μl) of the diluted cell suspension were added to each well. Plates were incubated at 37 C for 5 days and wells then examined for the presence of single colonies.

FREEZING CELLS.

Cells were grown to a density of 1-2 x 10^6 cells/ml and then harvested by centrifugation at 200xg for 5 min. The supernatant medium was aspirated from pellets and the latter resuspended in 2 ml of cold 90% FBS plus 10% DMSO. Then, 0.5 ml of the suspension was pipetted into a cryovial placed in a freezing box. The freezing box was placed in -20 C freezer overnight and then transferred to a -80 C freezer where it was kept for another 24 hr before being transferred and stored in liquid nitrogen.

THAWING CELLS.

Cells (0.5 μl) were thawed rapidly in a 37 C waterbath and then pipetted into warm (37 C) SFM (10 ml). The suspension was centrifuged at 200xg for 6 min. The supernatant medium was aspirated off and the cells resuspended in DMEM plus 20% PBS (5 ml). The suspended cells were added to a well of a 6-well culture dish.
APPENDIX B

PURIFICATION OF IMMUNOGLOBINS (IgG) FROM HYBRIDOMA SUPERNATANTS

The following technique was developed by R.A. Simkins (unpublished) based on the technique by Ey et al. (1978).

AMMONIUM SULFATE PRECIPITATION.

Antibodies were precipitated from cell-free medium by addition of ammonium sulfate to 40% saturation and stirred 2 hr to overnight at 4 C. The solution was spun at 10,000xg for 30 min, the supernatant was discarded, and the pellet was resuspended in 5 ml of TBS (25 mM Tris-HCl, 500 mM NaCl) by incubating at room temperature for 1 hr followed by vortexing. The solution was dialyzed against TBS (500 ml) with stirring in the cold room overnight. The contents of the dialysis tubing were removed and centrifuged at 10,000xg for 10 min. The pellet was discarded, and the supernatant was tested for monoclonal antibodies by indirect ELISA (Chapter IV). At this point, supernatant was stored for 2-3 weeks in the cold room (4 C) following the addition of sodium azide (NaN₃) to give 0.2%.

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PACKING OF CHROMATOGRAPHY COLUMN.

Sephadex-Protein A (Sigma Chemical Co.) was hydrated at room temperature for 1 hr in 20 ml of TBS-T (TBS plus 0.05% Tween 20) plus 0.2% NaN₃; the mixture was then placed in the cold room overnight to fully hydrate. The hydrated Sephadex-Protein A (1 ml) was poured into a 0.8 cm x 5 cm chromatography column (Isolab, Inc., Akron, OH) and packed by gravity.

PURIFICATION OF IMMUNOGLOBINS (IgG).

The column was washed three times with 3 ml of elution buffer (0.1 M glycine, pH 3.0) to free bound material, then equilibrated to pH 8 by washing six times with 3 ml of column buffer (20 mM Tris, 0.1 M NaCl, pH 8.0). The monoclonal antibody solution was poured into the column and column edges were rinsed twice with 500 µl of column buffer. The column was washed three times with 3 ml of column buffer to remove unbound proteins such as the IgM's and albumin. The elution of the IgG's was performed by first adding 1 ml of elution buffer and collecting the elutant, then by repeating this step once. Additional elution was accomplished by using five washes of 500 µl each, collecting each fraction in tubes containing 50 µl of 1 M Tris to neutralize the glycine. Protein concentrations of purified immunoglobins were determined by a protein assay using Coomassie Brilliant Blue G-250 (Sedmak & Grossberg, 1977). The IgG containing
fractions were combined and sodium azide (NaN₃) added to give 0.2%.

Aliquots were refrigerated (4 C) or frozen (-20 C).
APPENDIX C

SEROLOGICAL RELATIONS AMONG MAIZE MOSAIC VIRUS ISOLATES AND TWO OTHER CEREAL Rhabdoviruses


DOT IMMUNOBINDING.

Samples for dot immunobinding (Hawkes et al., 1982) were prepared by grinding leaf tissue (0.2 g) in two volumes of phosphate buffered saline (PBS) (0.01 M potassium phosphate, 0.15 M NaCl, pH 7.0, plus 0.02% sodium azide) in a mortar with pestle. Three-fold dilutions were prepared in test tubes with PBS and about 2 µl of each sample applied (spotted) on a 0.45 µm pore diameter nitrocellulose membrane. The nitrocellulose membrane was immunostained as described in Chapter II.
Table 1. List of sources of virus and antisera and viral acronyms.

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<th>Virus</th>
<th>Antiserum</th>
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<td>E. Luisoni, Instituto di Fitovirologia Applicata, Torino, Italy</td>
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<td>Cereal chlorotic mottle virus (CCMV)</td>
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<td>B. W. Falk, Univ. of Florida, Belle Gladea</td>
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<td>R. Namba, Univ. of Hawaii, Hawaii, Honolulu</td>
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<td>L. L. McDaniel(^b) and R. G. Gomez, Ohio State Univ., OARDC, Wooster</td>
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<td>Maize sterile stunt virus (MSSV)</td>
<td>R. S. Greber</td>
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<td>Wheat striate mosaic virus (WStrMV)</td>
<td>R. G. Timian, North Dakota State Univ., Fargo</td>
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a Presently, Univ. of California, Davis.
b Presently, Univ. of Florida, Ft. Lauderdale.
c Presently, Turrialba, Costa Rica.
Table 2. Serological relations among isolates of maize mosaic virus (MMV) from Florida (FL), Hawaii (HI), Costa Rica (CR), Mauritius (MR) and Venezuela (VZ), maize sterile stunt virus (MSSV) and cereal chlorotic mottle virus (CCMV).a

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</tbody>
</table>

aResults of dot immunobinding technique.

bMaximum dilution at which both nitrocellulose membrane and agar gel gave positive reactions (for description of technique see Materials and Methods, Chapter II, Immunostaining of nitrocellulose membranes).

cNo reaction detected.

dTissue from healthy maize plants grown in the greenhouse.