CHARACTERIZATION OF INDIVIDUAL RIBONUCLEIC ACIDS OF BARLEY STRIPE MOSAIC VIRUS STRAINS

by

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INTRODUCTION

Researchers attempt to understand the biological processes in many living things by examining the basic material of heredity, the nucleic acid. The problems involved in such studies are compounded by the difficulty of isolating and characterizing nucleic acids from a complex plant or animal system without loss of viability. Fortunately, simple living entities such as viruses, can easily be stripped to the nucleic acid level for study, both in vivo and in vitro. Nucleic acids from viruses may differ in structure and function from cellular nucleic acids but their general constituents are similar.

Plant pathologists are primarily interested in a virus due to its ability to cause a disease. Although disease severity may vary depending upon the virus, suscept and environment, there are instances of great economic losses as a result of virus diseases. In order to have a more efficient and practicable control measure, however, an elucidation of the infecting unit is essential.

Barley stripe mosaic virus (BSMV) has many advantages for the study of function of viral nucleic acid. It has several strains and every strain has at least two ribonucleic acid (RNA) components (41, 49). Type, ND18 and AM strains contain 2, 3, and 4 RNA components, respectively. Each RNA fraction varies in the proportion present, size, and probable function. Based on gel electrophoresis, component I is the slowest migrating component while IV is the fastest migrating component.

BSMV may be a multicomponent virus with a defective or satellite particle(s). It requires at least two of the largest RNA components for infectivity. The primary objective of this study is to characterize the

individual RNA components of BSMV to ascertain the fundamental differences within and between strains. Thus, the possible role of the various components in the total viral genome may be determined. This objective has been approached in several ways, one of which is through RNA-RNA hybridization of dsRNA and radioactively-labeled ssRNA. Information from the hybridization experiments and the isolation of RNA early in infection could facilitate the determination of the function and synthesis of the RNA pieces. Recombination of components and assay of recombinants, infectivity at close to dilution end-points, and the effects of temperature and aging on the RNA in vivo were investigated to further characterize the compositional features of BSMV RNA.

LITERATURE REVIEW

The virus now called BSMV was first described by McKinney in 1951 as barley false stripe disease (55). The virus is seed- and pollen-borne (73) and is also easily transmitted mechanically. The only natural hosts are wheat (Triticum aestivum L.) (27) and barley (Hordeum vulgare L.) but several species of Gramineae and some dicotyledenous species can be inoculated (3). Chenopodium amaranticolor Coste & Reyne., C. quinoa L. and C. album L. are local-lesion hosts. There is no known vector.

Several strains of the virus which cause symptoms ranging in severity from mild mosaic to lethal necrosis have been isolated (57). Decline of infectivity with subsequent extinction of culture lines has been reported with some weak strains (58) and Type strain (65). The reason for loss in infectivity has not been adequately explained. Some strains are incapable of seed transmission (37). Based on electron microscope studies, Carroll (20) concluded that NSP strain was not seed transmissible because of the exclusion of the virus from the embryo and hence from developing seeds. The reason for the exclusion was unknown.

Mixed infections with brome mosaic virus (BMV) and BSMV resulted in synergism (47, 56, 62) and BSMV RNA coated with BMV protein was recovered (68). Dodds and Hamilton (25) reported that BSMV stimulated the replication of tobacco mosaic virus (TMV) in doubly-infected barley plants.

Electron microscopy has shown the virus to be a rigid rod of about 125 x 20 nm (34). BSMV had a tendency to aggregate (10), which appeared to interfere with the infectivity assay. The aggregated virus was

dispersed by Igepon T-73 (a detergent) and stabilized by the addition of healthy barley extracts (11). The aggregation problem resulted in the use of Igepon and Triton x-100 in recent purification procedures in our laboratory.

McKinney and Greeley (57) published a comprehensive review and study of the biological characteristics of BSMV. The study included characterization of disease symptoms, interstrain competition, blocking effect of established strains against reinfection by related strains, histories and characteristics of strains, and grain yield and seed passage test of infected barley and wheat. Meanwhile, other workers studied the physical and chemical properties of BSMV in either a semi-purified or purified state (2, 11, 45, 63, 64, 69). In barley sap, the thermal inactivation point is about 70^{0} C for 10 min (45, 55), and a dilution end-point of about 10^{-3}. Murayama, et al. (63) reported a loss in infectivity after 7 days at room temperature but not until 12 days at 4^{0}C.

BSMV has a sedimentation coefficient of about 185s (3) and a molecular weight of about 26×10^{6} daltons (2). The virus is also highly immunogenic (38, 61). After degradation of BSMV by calcium chloride, BSMV protein gave a major antigen showing partial affinity to BSMV virions and minor antigens identical to those formed on the degradation of BSMV or BSMV protein with an anionic detergent (4, 36).

The multicomponent nature of BSMV was suggested by Palomar (65) who found that slowly sedimenting virions had a low specific infectivity. Pring (71) reported that ND18 produced two RNA species sedimenting at 21.3 and 19.5s upon density gradient centrifugation while Atabekov and Novikov (2) working with a Russian strain found 20 and 21s RNA corres-

ponding to molecular weights of 8.8×10^5 and 9.7×10^5. By electron microscopy, Harrison, et al. (39) showed a distribution of virions into 3 particle lengths, 111, 128 and 148 nm. Chiko (21) confirmed the results of Harrison and co-workers and was able to distinguish BSMV strains by particle size. The reports referred to above provided indirect evidence of multiple component RNA in BSMV strains. Jackson and Brakke (41) and Lane (49) confirmed the multicomponent nature by gel electrophoresis and showed that at least two and possibly three of the major components were necessary for infection. The characteristics of multiple particle viruses have been discussed in detail in reviews by Hull (40), Kassanis (44), Lane (50), Matthews and Ralph (54), and van Kammen (78).

BSMV is approximately 4% RNA, which is single-stranded (2). The RNA has a A_{260}/A_{280} ratio of 2.2 to 2.5 (2) and buoyant density of 1.630 g/cc in Cs_2SO_4 (70). Its molecular weight, calculated from the molecular weight of the virion and the known content of RNA, is about 1×10^6 daltons (2). The molecular weight of the RNA was estimated to be 8.85×10^5 daltons, based on its sedimentation after formaldehyde treatment (71); without formaldehyde treatment it sediments as if it were $7\text{-}8 \times 10^5$ daltons, using BMV RNA component III (8×10^5 daltons) as standard. The molecular weight of the RNA components estimated by gel electrophoresis without formaldehyde treatment and against RNA markers from BMV and TMV were 1.40, 1.17, 1.04 and 0.94×10^6 daltons for components I, II, III, and IV, respectively (41); Lane (49) reported values of 1.50, 1.35, 1.20, and 1.05×10^6 daltons, respectively. He used formaldehyde-modified RNA's and ribosomal RNA's from E. coli as standards in gel electrophoresis. The molar percentage of nucleotides

were about 30.9, 20.3, 19.4, and 29.4 for adenine, guanine, cytosine, and uracil, respectively (2).

Although the virus spreads systemically the two top leaves contain the highest concentration in the infected plant. Virions accumulate both in the cytoplasm and the nucleus (33). They have been found to induce vesiculated chloroplasts and plastids which suggests that the plastids might be involved in the multiplication of BSMV (19).

MATERIALS AND METHODS

Abbreviations

A	optical absorbance
AM	Argentina Mild
APS	ammonium persulfate
bis	N,N'-methylene bis-acrylamide
BMV	brome mosaic virus
BSMV	barley stripe mosaic virus
DEP	diethyl pyrocarbonate
DNase	deoxyribonuclease
dsRNA	double-stranded RNA (Syn.- RF or replicative form RNA)
EDTA	ethylenediamine tetraacetic acid
ND18	North Dakota 18
nm	nanometer (Syn.- millimicron)
OD	optical density
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
ssRNA	single-stranded RNA
stains-all	1-ethyl-2(3-ethylnaphtho(1,2d)-thiazolin-2-ylidine)-2-methyl-propenyl)naphtho(1,2d)thiazolium bromide
TMV	tobacco mosaic virus
TEMED	N,N,N',N'-tetramethylethylenediamine
tris	tris(hydroxymethyl) aminomethane

Buffers

1. Dissociation buffer, pH 7.2 (Lane, personal communication)
   - 1.0% SDS
   - 0.5% mercaptoethanol
   - 10.0% sucrose
   - 0.001M Na_2EDTA
   - 0.05M Na_2HPO_4
   - 0.01M NaH_2PO_4
2. Disruption buffer, pH 9.0 (15)
   - 0.1M ammonium carbonate, pH 9.0
   - 0.001M Na_2EDTA
   - 1.0% SDS
   - 100.0 µg/ml bentonite
3. KKE, pH 7.3
   - 0.01M NaH_2PO_4
   - 0.02M Na_2HPO_4
   - 0.001M Na_2EDTA
4. RNA inoculation buffer, pH 9.2 (13)
   - 0.05M glycine
   - 0.03M K_2HPO_4
5. SOKE, pH 9.0 (71)
   - 0.5M sodium orthoborate
   - 0.01M KCl
   - 0.001M Na_2EDTA
6. SSC, pH 7.0
   - 0.15M sodium chloride
   - 0.015M sodium citrate

7. STE, pH 6.85 (29)

   0.1M NaCl

   0.05M Tris

   0.001M Na_2EDTA

8. TPE, pH 7.4 (41)

   0.02M Tris

   0.02M NaH_2PO_4

   0.001M Na_2EDTA

Growth of barley

The varieties of barley (Hordeum vulgare L) used were Black Hulless, Larker and Moore. Black Hulless barley, a highly susceptible cultivar, was the preferred host for assay and virus multiplication. Seeds were planted in clay pots filled with autoclaved soil and grown either in a growth chamber (14-hr days, 1200 ft-c., 21 C) or in the greenhouse (25 ± 1 C). The plants were watered daily and fertilized weekly with a Hyponex solution. Insecticides were applied as needed.

Source of virus

The Type strain (ATCC PV# 43) (1) and AM strain were supplied by H. H. McKinney in 1956 and 1966, respectively; ND18 was obtained from R. G. Timian in 1968; and Norwich strain was provided by L. C. Lane in 1973. All isolates were originally sent to M. K. Brakke and have been routinely propagated in this laboratory. Infected leaves were stored at 3 C over anhydrous calcium chloride to serve as virus stocks.

Centrifugation

Low speed centrifugations were done in a refrigerated Servall RC2-B centrifuge. A SS-34 rotor was used with Corex or polypropylene tubes for small volumes and a GSA rotor with polypropylene bottles (250 ml capacity) for large volumes. High speed centrifugations were carried out in type 30, 40, or 50Ti rotors in a Spinco Model L ultracentrifuge. Density gradient centrifugations were performed on Beckman L2-50 and L2-65B ultracentrifuges, equipped with rotor stabilizers, in SW 50.1, SW 41, or SW 27 rotors. The type rotors utilized polycarbonate bottles while the SW rotors used cellulose nitrate and polyallomer tubes. Sucrose gradients were prepared by layering sucrose solutions at least one day before used or overnight at 5 C to allow diffusion to occur and form a smooth gradient.

Spectrophotometry

A Cary model 14 recording spectrophotometer (Applied Physics Corp., Monrovia, Ca.) was used for ultraviolet spectrophotometric determination of concentration and relative purity of samples. The virion or nucleic acid preparation was scanned linearly from 320 to 220 nm at a chart speed of 1, 2, or 4 in/min and a scanning speed of 0.5 ^{o}A/sec.

Preparation of sucrose

Commercial sucrose was treated with decolorizing charcoal to remove the ultraviolet-absorbing materials and most of the nucleases. About 700 g of sugar was mixed with 50 g of charcoal and water was added to give a liter of solution. The mixture was boiled gently for 5-10 min or autoclaved for 5-10 min at 15 psi and vacuum-filtered hot through a pad

of charcoal in a Buchner funnel. The percentage of sugar was measured in a refractometer (Bausch & Lomb Incorp., Rochester, N. Y.) and the solution was diluted to 600 mg/ml.

Purification of bentonite

Bentonite powder was purified according to the method of Fraenkel-Conrat, et al. (28), adjusted to 10 mg/ml, and stored in polypropylene bottles in the refrigerator.

Inoculation and purification of BSMV

Barley seedlings were inoculated with BSMV 7 to 10 days after planting (2-leaf stage). Inoculum was prepared by grinding one gram of fresh infected leaves in 10 ml of distilled water or 0.01M phosphate buffer, pH 7.0 and adding 1% Celite (Johns-Manville diatomaceous earth) as an abrasive. Plants were inoculated by leaf rubbing. Leaves were harvested 10 to 14 days after inoculation, and the purification of BSMV was as previously described (65).

Isolation of BSMV ssRNA

BSMV ssRNA was isolated from purified virus by phenol extraction (35). Phenol crystals (80 g) were liquified by adding 20 ml of distilled water, with 0.1 g of 8-hydroxyquinoline. Extractions were carried out in Corex centrifuge tubes. Five ml of disruption buffer (15) were added to 5 ml of virus solution and mixed vigorously with 10 ml of 80% phenol. The mixture was centrifuged at 5,000 rpm for 10 min to separate the emulsions. The aqueous phase (top layer) was pipetted out carefully and placed in another centrifuge tube. Two

volumes of ethanol-acetate (97% ethanol, 0.15M NaAc) were mixed with one volume of the RNA solution and stored at -20 C for at least 2 hr to precipitate the RNA. After centrifugation for 30 min at 10,000 rpm, the pellet was suspended in TPE buffer and the RNA solution was ethanol precipitated again. The resulting pellet was taken up in TPE buffer, and the RNA concentration of this solution was determined spectrophotometrically assuming an absorbancy index of 25 OD/mg at 260 nm. The RNA preparations were kept in small test tubes or vials and stored in solution at -20 C until use.

Gel electrophoresis of BSMV RNA (7, 22, 26, 67)

Gel tubes 12 cm in length were cut from 6 mm (inside diameter) Plexiglas tubing with a sabre saw and washed with a detergent solution and completely rinsed with distilled water before each usage.

The following ingredients were used to make 6 gels containing 2.5% acrylamide and 0.5% agarose:

4.0 ml of acrylamide stock solution (15% acrylamide, 0.75% bis)
2.5 ml of 10x TPE buffer
8.3 ml of distilled water
10.0 ml of 1.3% agarose
0.02 ml of TEMED
0.10 ml of 10% APS

Agarose (200 mg) was placed in a50-ml Erlenmeyer flask containing 15 ml of distilled water, and dissolved by heating in a boiling water bath for 10-15 min. The agarose solution was then kept liquid in an oven at 50 C. Meanwhile, gel tubes were covered with two layers of parafilm at one end plus a strip of dialysis tubing over parafilm. The

dialysis tubing was held in place by a collar of rubber tubing. The tubes then were placed in the oven. Acrylamide, buffer, and distilled water were mixed in a 50-ml beaker and allowed to equilibrate at 50 C prior to the addition of 10 ml of agarose solution. TEMED and APS were added to the warm mixture before filling the gel tubes. The solution was allowed to gel in tubes for 15-30 min at 50 C and then brought to room temperature to complete the gelation. The gel tubes were sealed at the top with parafilm and stored at 5 C until needed.

Prior to electrophoresis, both ends of the gels were cut with a razor blade to get the desired length of 9 cm. The lower ends of the gel tubes were covered with a nylon mesh to prevent the gels from slipping out of the tube during electrophoresis. The gels were prerun for 1-2 hr at 5 mA per gel to remove the TEMED and persulfate. Unless stated otherwise, the buffer used in the upper and lower chambers was 1x TPE containing 0.1% diethylpyrocarbonate (DEP). Samples were layered on top of the gels with a micropipet or a hand drawn glass tubing. For a routine run, a sample contained about 5 µl of 0.1% bromphenol blue dye and 2-3 pellets of sucrose (or one drop of a 600mg/ml sucrose solution) in a volume of 0.10 ml or less.

After electrophoresis, the gels were individually marked at the bottom with India ink. Ultraviolet-absorbing materials were removed by soaking individual gels for approximately an hour either in a beaker of distilled water containing 2-3 drops of bentonite or in a solution of bentonite in 0.2M NaCl. The pH of the buffers of the upper and lower chambers were routinely checked after each run.

RNA bands were detected by optically scanning gels at 260 nm in a Gilford 2410 linear transport system or by staining. The gels were

placed overnight in stains-all (5.0 ml of 0.1% stains-all, 45 ml of formamide, 50 ml of distilled water) and destained in 1% acetic acid for 3-4 hr at room temperature under an indirect light. Stained gels were scanned at 570 nm in the Gilford.

Formaldehyde modification of BSMV RNA

Five µl of 37% neutralized formaldehyde were added to 0.05 ml solution containing 2-3 µg of BSMV RNA, 2 µg of TMV RNA and 2.5 µg of the two fastest sedimenting components of BMV RNA (components III and IV). The solution was heated for 10 min at 65 C and immediately placed on the surface of the gels. TMV RNA was prepared by heating TMV for 5 min at 60 C in dissociation buffer. The two smallest BMV RNA's were prepared by sucrose density gradient centrifugation of RNA extracted from BMV by phenol treatment.

The procedure for gel electrophoresis was as previously described in "Gel electrophoresis of BSMV RNA" but with these modifications:

1. The ingredients used to make 6 gels were as follows:

   3.6 ml of acrylamide stock solution
   2.5 ml of 10x KKE buffer
   8.7 ml of distilled water
   10.0 ml of 1.3% agarose
   0.02 ml of TEMED
   0.10 ml of 10% APS

2. The running buffer used was KKE with 0.1% DEP
3. The electrophoresis run was at 7.5 mA (70 volts) per gel for 5 hrs or twice the amount of time it would take for the marker dye to migrate through the gels.

The relative mobility of each BSMV RNA component was estimated using TMV RNA and BMV RNA as markers. The molecular weight of TMV and BMV were estimated using Escherichia coli RNA as standard.

Relative proportion of RNA components

The relative amount of each component was determined by using a planimeter to measure the area under the curve of the profiles of ssRNA after gel electrophoresis. Various RNA sources were used in the estimation of the percentage of the RNA components since the proportion of RNA components varied from one inoculation transfer to the next, especially with 3- and 4-RNA containing strains of BSMV.

Preparative fractionation of the ssRNA components

The gels used for fractionation contained 2.9% acrylamide and 0.5% agarose (79). The following ingredients were needed for 6 gels:

4.6 ml of acrylamide stock solution
2.5 ml of 10x TPE buffer
7.7 ml of distilled water
10.0 ml of 1.3% agarose
0.02 ml of TEMED
0.10 ml of APS

The procedure for gel electrophoresis was the same as previously described in "Gel electrophoresis of BSMV RNA" except that the electrophoresis was run overnight (16 hr) at 3 mA per gel at 5 C. Fifty µg of RNA were layered on each gel for fractionation. After electrophoresis, the gels were soaked in 0.1M NaCl + 2-3 drops of bentonite, scanned at 260 nm, and the ssRNA regions marked and excised.

Two procedures were used to extract the ssRNA's from the gel. In the earliest experiments, the RNA was recovered by grinding the frozen gel section in a cold mortar and pestle. The ground gel was then mixed in a small volume of TPE buffer and bentonite. After low speed centrifugation to remove the residual polyacrylamide, the RNA was ethanol precipitated from the supernatant and centrifuged. The pelleted RNA was dissolved in TPE buffer and centrifuged on sucrose density gradients (1.4, 1.4, 1.4, and 0.7 ml of 300, 225, 150, and 75 mg/ml sucrose in TPE buffer, respectively) at 45,000 rpm for 3½ hrs in a SW 50.1 rotor. The RNA zones were collected by fractionation and ethanol precipitated.

In another method of extracting RNA, the excised RNA-containing gel was placed 2 cm from the bottom of a gel tube (75) and was held in place by a collar of cellulose nitrate tubing. The RNA was eluted into a 2 cm space containing TPE buffer, which was contained by a piece of dialysis tubing wrapped around the bottom of the tube. After electrophoresis for 2-3 hr at 5 mA per tube (the time factor being dependent upon the size of the RNA being eluted), the eluent was collected and pooled, and the RNA was concentrated by ethanol precipitation.

Dilution end-point cultures

ssRNA extracted from cultures of barley plants infected by inoculation near the dilution end-point was analyzed to determine if any of the components had been lost in dilution. The loss of one or more components, especially from a 4- or 3-component RNA strain, would indicate the significance of the RNA fractions for infection and possibly suggest a duplication or substitution of function, i.e., coat protein synthesis, by certain components. Infected leaves (AM, ND18, and Norwich

strains) were ground in 0.01M phosphate buffer, pH 7.0 using a mortar and pestle. The crude sap was diluted in ten-fold steps to 10^{-3}, and each dilution was inoculated to barley plants. Usually 10 to 20% of the plants inoculated with the highest dilution became infected. When infection occurred in fewer than 10% of the plants inoculated with a dilution, infected plants were selected from those inoculated with the next most dilute inoculum. Ten days after inoculation, a single leaf from each infected plant was used as a source of inoculum for 20 to 30 healthy plants to increase the virus for purification. From 6 to 12 single leaves were used in every experiment. Infected leaves were harvested for purification 10-14 days after inoculation. A solution containing 150 µg of purified virions was mixed with an equal volume of 2x dissociation buffer and heated at 60 C for 5 min. The samples were placed on gels immediately after heating and addition of marker dye. Following electrophoresis, the number of components remaining in each sample after dilution end-point transfer was determined spectrophotometrically by scanning at 260 nm in the Gilford model 2410 or by staining. Each experiment was repeated 4 to 6 times.

Recombination of components and infectivity assays

It would be interesting to find if components III and IV of AM strain could be added to a 2-RNA containing Type strain to produce a 4-RNA Type strain. Along the same idea, failure to recombine components III and IV with a 2-RNA AM strain would indicate specificity of components within the same strain and possibly also show that components III and IV are defective particles that are unnecessary in the viral genome of BSMV. The method used for recombination consisted of adding AM RNA components I and II (eluted together from gels) to AM RNA components III and IV