Morphological Comparisons of Echinochloa Ragged Stunt and Rice Ragged Stunt Viruses by Electron Microscopy

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ABSTRACT

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The morphological characters of Echinochloa ragged stunt virus (ERSV) and rice ragged stunt virus (RRSV) were compared by electron microscopy. Virions in negatively stained purified samples and dip preparations from leaves of *Echinochloa crus-galli* var. *oryzicola* infected with ERSV were 54–58 nm in diameter. Smaller B-spiked particles, 50 nm in diameter, were observed in purified preparations. Hexagonal particles measuring 72 nm in diameter, with A-spike projections, were present occasionally in crude dips prepared from glutaraldehyde-fixed leaves. In ultrathin sections of ERSV-infected plant tissues, particles 60–70 nm in diameter with densely stained cores occurred along the outer membranes of mesophyll chloroplasts and in viroplasms of phloem parenchyma cells. Particles approximately 55–75 nm in diameter were found in thin sections of cytoplasm of cells of the salivary gland, fat body, gut, brain, gastric caeca, and ommatidia cornea cells of the compound eye of viruliferous ERSV vectors, *Sogatella longifurcifera*. Purified RRSV consisted mostly of 55-nm-diameter subviral particles. Particles 62–66 nm in diameter were observed only in crude sap preparations of infected rice leaves fixed with glutaraldehyde. Particles of two different sizes, 40 nm and 55–70 nm in diameter, were scattered in the viroplasm in phloem cells of RRSV-infected rice plants. In thin sections of the viruliferous RRSV vector, *Nilaparvata lugens*, particles of 60–75 nm were found in the cytoplasm of salivary gland, fat body, seminal vescle, gut, and muscle cells.

Additional keywords: fijivirus, *Oryza sativa*, reoviridae, ultrastructure.

Echinochloa ragged stunt was first found in *Echinochloa crus-galli* var. *oryzicola* Ohwi in the rice paddy fields in central Taiwan in 1980 (5). The diseased plants exhibit severe dwarfing and a dark green color, with short, serrated leaves. Whitish vein enations appear on leaves, stems, and sheaths (5,6). *Triticum sativum* Lam. and *Setaria italica* Beauv. also are infected naturally. The planthopper *Sogotella longifurcifera* (Esai et Ishihara) transmitts Echinochloa ragged stunt virus (ERSV) in a persistent manner (4,5). With *S. longifurcifera* as vector, ERSV was transmitted to six of 19 Gramineous plant species tested (5). Based on symptomatology, host range, and vector relationship, Echinochloa ragged stunt is similar to plant diseases caused by reo-like viruses (3,18,22).

Symptoms of diseased *E. crus-galli* are indistinguishable from those produced by rice ragged stunt virus (RRSV), a member of the fijivirus subgroup, in rice plants (*Oryza sativa* L.). Both ERSV and RRSV are serologically related (4); however, they differ in many aspects. They are transmitted by different planthopper species (4,5,6,13) and contain different sizes of 10 double-stranded ribonucleic acid (dsRNA) genome segments (4,16,22). The objective of this study was to examine and compare the morphological characteristics of ERSV and RRSV by electron microscopy.

MATERIALS AND METHODS

Virus, vectors, and hosts. A field-collected isolate of ERSV was maintained in a screenhouse on *E. crus-galli* by serial transfers using the planthopper *S. longifurcifera*. The insects were originally collected from *Echinochloa* grass in rice paddy fields and raised on the same hosts in insectaries. Second or third instar *S. longifurcifera* nymphs were first fed on diseased *Echinochloa* plants for 2 wk and subsequently transferred to healthy *Echinochloa* seedlings. When the inoculated plants exhibited symptoms, the test insects were used to prepare ultrathin sections.

A culture of RRSV was obtained from Chiayi Agricultural Research Station, Chiayi, Taiwan. It was originally isolated from a rice plant collected from the experimental farm and subsequently maintained in rice plants using the vector *Nilaparvata lugens* Stl. Similar procedures used with *S. longifurcifera* were employed to prepare viruliferous *N. lugens* for ultrathin sections.

Virus purification. Both ERSV and RRSV were purified from infected plants by the same procedure. All work was carried out at 4 C. About 200 g of frozen leaves and stems from infected plants was homogenized in a Waring blender in 0.2 M potassium phosphate buffer, pH 7.5, (3 ml/g of tissue) containing 0.01 M disodium ethylenediaminetetraacetate and 0.01 $MgCl_2$. The solvent (1,1,2-trichloro-1-2,3-trifluoroethane) at 0.5 ml/g of tissue was added slowly as tissues were being ground. The homogenate was centrifuged at 4,500 *g* for 10 min, and 5% Triton X-100 was added to the supernatant and stirred in a beaker for 5 min. The homogenate was centrifuged at 5,900 *g* for 20 min. The supernant then was centrifuged at 36,900 *g* for 90 min. The pellet was resuspended in 0.02 M phosphate buffer, pH 7.0, and passed through a Sepharose CL-4B in a K9/60 column (Pharmacia, Inc., Piscataway, NJ). The effluent was centrifuged at 76,580 *g* for 60 min, and the pellet was resuspended in 0.02 M phosphate buffer. The virus preparation was further purified by isopycnic centrifugation. Cesium sulfate, final concentration 25% (w/v), was added to the virus suspension. Ten milliliters of the suspension was layered on each SW40Ti centrifuge tube containing 3 ml of 53% cesium sulfate (w/v). The tubes were centrifuged at 76,700 *g* for

20–22 hr. One visible band appeared at 4 cm from the bottom of the tube and was collected with a Pasteur pipet. The purified virus suspension was diluted with 0.02 M phosphate buffer and centrifuged at 76,700 *g* for 90 min to remove cesium salt. The pellets were resuspended in 0.01 M phosphate buffer and used for electron microscopy.

Crude sap preparation. Pieces of diseased leaf tissues, about 1 × 5 mm, were fixed by immerging in 2% glutaraldehyde in 0.1 M neutral phosphate buffer for 2–3 hr before crushing in a drop of 2% neutral phosphotungstic acid (PTA). The resulting suspension was placed on carbon-coated Formvar grids. The excess fluid was drawn off with filter paper, and the preparations were examined in a JEOL JEM-7 electron microscope (Japan Electron Optical Laboratories (JEOL), Ltd., Tokyo, Japan) (7).

Ultrathin section. Leaf tissues (1 × 5 mm) or insect organs were first fixed by immerging in 2% glutaraldehyde in 0.1 M neutral phosphate buffer for 2–3 hr and then in 1% osmium tetraoxide in distilled water for another 2–3 hr at 4 C. The tissues were dehydrated with acidified 2, 2-dimethoxypropane and embedded in Epon 812 (Shell Chemical Corp., San Francisco, CA) (7).

Epon blocks were cut with glass knives with a Leitz ultramicrotome (E. Leitz, GmbH Wetzlar, West Germany). Sections were placed on carbon-coated Formvar grids and stained in 2% uranyl acetate solution for 60 min and in lead tartarate for 15 min at room temperature for electron microscopy.

RESULTS

Negatively stained ERSV and RRSV. Purified preparations of ERSV stained with PTA contained numerous spherical particles approximately 54–58 nm in diameter (Fig. 1A). Smaller empty core particles (arrows) about 50 nm in diameter surrounded by a thin-layer protein also were observed in purified preparations (Fig. 1A). Projections (B-spikes) were observed on some degraded particles (Fig. 1B).

Particles approximately 54–58 nm in diameter similar to those in purified preparations also were observed in dip preparations from ERSV-infected leaves that were first fixed with glutaraldehyde

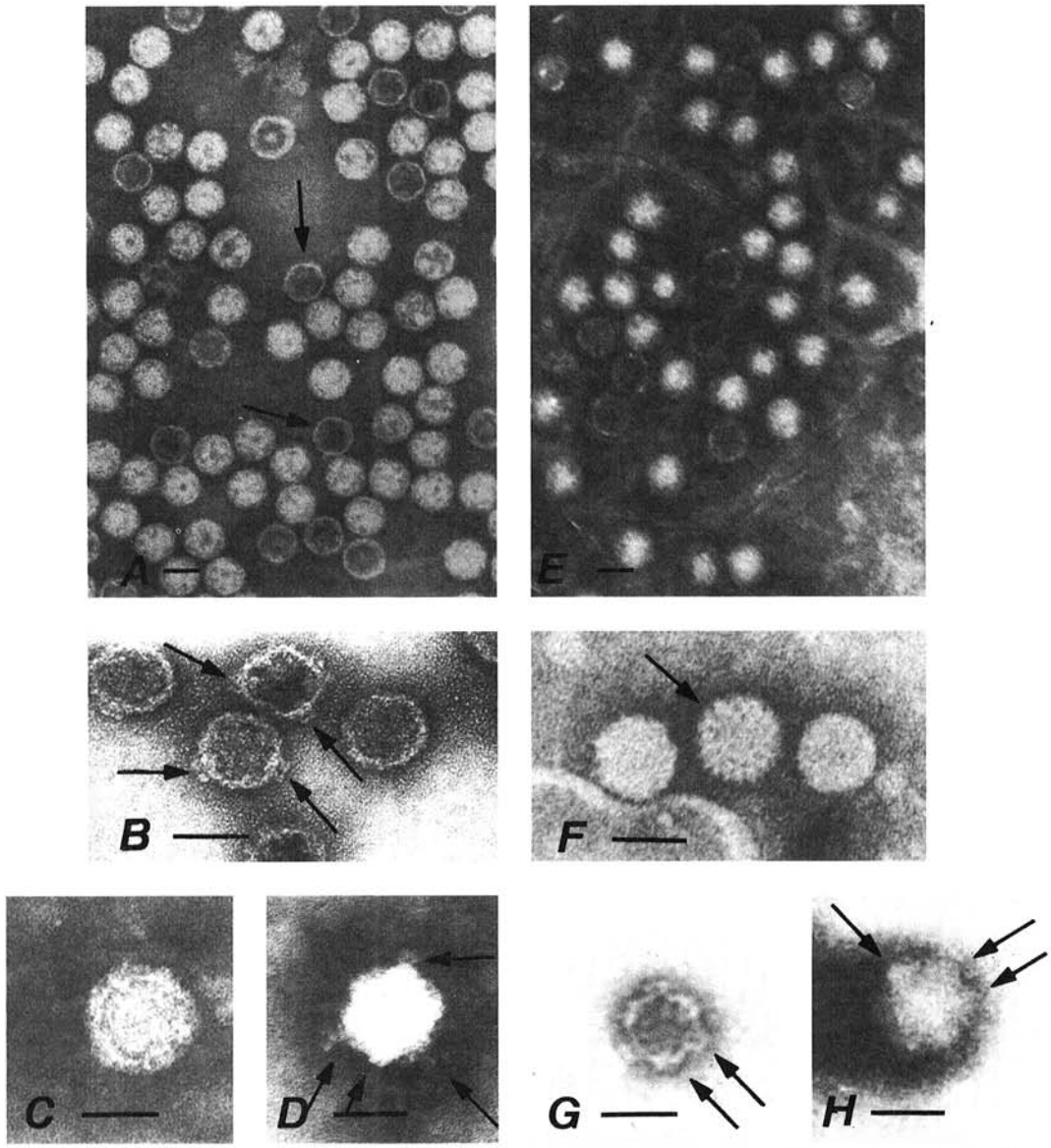

Fig. 1. Electron micrographs of Echinochloa ragged stunt virus (ERSV) and rice ragged stunt virus (RRSV), negatively stained with 2% phosphotungstic acid. Bars represent 50 nm. **A,** Purified preparation of ERSV. **B,** The B-spikes (arrows) of an ERSV particle from a purified preparation. **C,** A particle of ERSV with double shell, about 80 nm in diameter. Samples were fixed with 2% glutaraldehyde before negatively stained. **D,** A particle of ERSV with A-spikes (arrows) from a crude extract. Samples were fixed with 2% glutaraldehyde before negatively stained. **E,** Purified preparation of RRSV. **F,** A particle of RRSV (arrow) showing inner shell, about 60 nm in diameter from a purified preparation. **G,** A negatively stained RRSV subviral particle exhibiting B-spiked structure (arrows). **H,** A B-spiked subviral particle of RRSV in a crude preparation fixed in glutaraldehyde before negative staining. Arrows indicate B-spikes.

(data not shown). Larger intact particles having a double shell and a diameter of about 80 nm also were observed occasionally (Fig. 1C). These larger particles usually had A-spike projections on their surface (Fig. 1D). No particles were found in the preparations of healthy tissues.

Purified preparations of RRSV contained mostly subviral particles about 55 nm in diameter and some smooth cores about 50 nm in diameter when stained with 2% PTA (Fig. 1E). Particles of inner shell with a diameter about 60 nm also were observed in purified preparations (Fig. 1F). In negatively stained preparations, some particles exhibited B-spikes (Fig. 1G). B-spiked particles approximately 65–70 nm in diameter were observed in crude sap prepared from infected leaves with prior glutaraldehyde fixation followed by 2% PTA (Fig. 1H). They resembled the B-spiked subviral particles of RRSV reported by Milne (19) and Kawano et al (16).

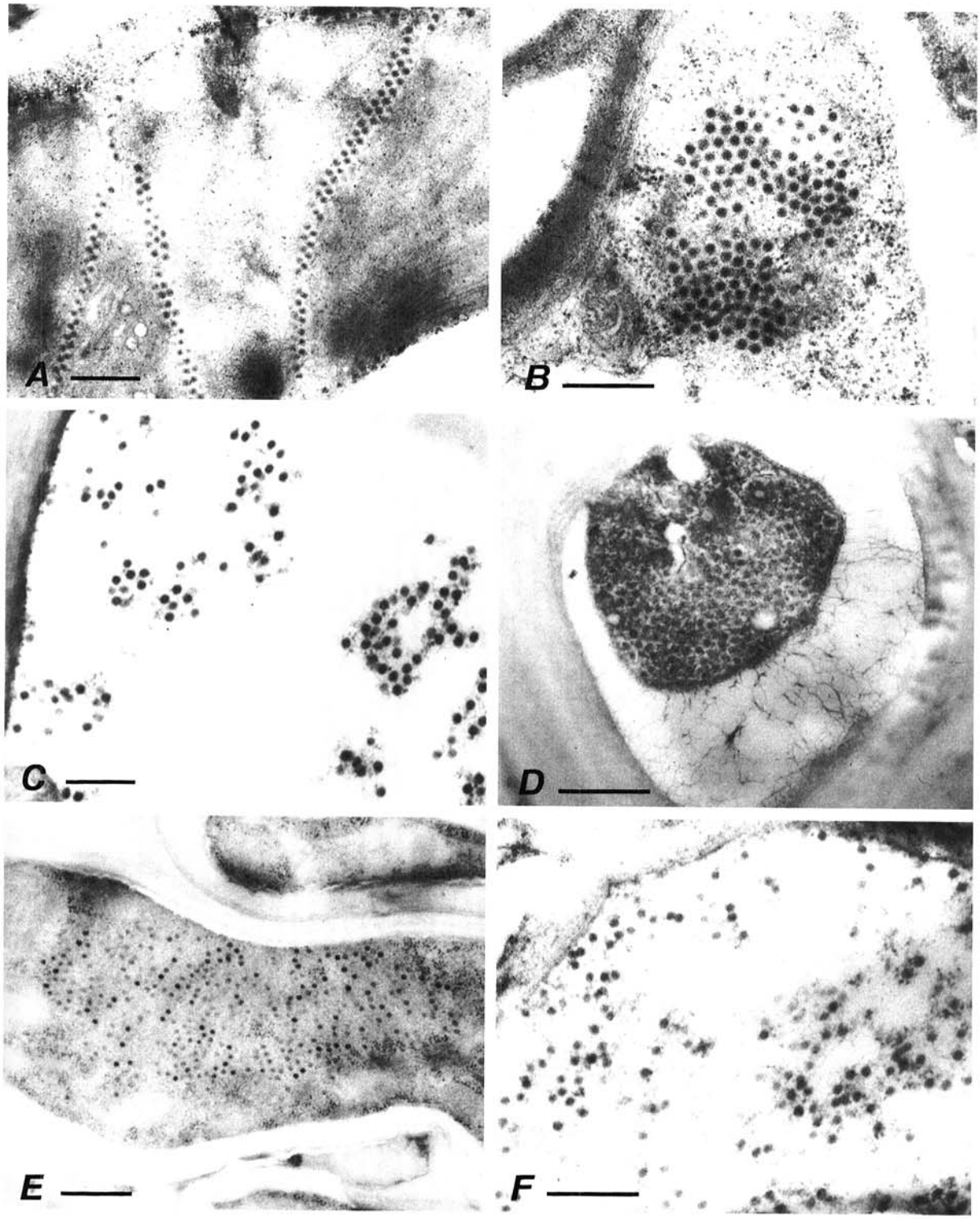

Fig. 2. Ultrathin sections of leaves infected with Echinochloa ragged stunt virus (ERSV) and rice ragged stunt virus (RRSV). Bars represent 500 nm. **A,** Virus particles of ERSV in arrays along the outside membrane of chloroplasts of mesophyll cells. **B,** Virus particles of ERSV aggregated in viroplasmlike structure. **C,** Virus particles of ERSV with threadlike structure scattered in a phloem cell. **D,** Large mass of virus particles of ERSV seem bounded by membranous materials inside a phloem cell that is undergoing plasmolysis. Some threadlike materials are connected between the membrane and the cell wall. **E,** Immature particles of RRSV about 40–45 nm in diameter scattered in a viroplasmlike inclusion of a phloem cell. **F,** Virus particles of RRSV and filaments in a phloem cell.

Ultrathin sections of virus-infected leaves. Ultrathin sections of ERSV-infected leaves of *E. crus-galli,* containing virus particles about 60–70 nm in diameter, occurred in a row in the outer membrane of chloroplasts of the mesophyll cells (Fig. 2A). The core of these 60- to 70-nm particles was densely stained and was clearly surrounded by a lightly stained thin layer. Similar particles also were scattered in viroplasms in the phloem cells (Fig. 2B). Particles about 60–66 nm in diameter were scattered in the vacuoles of the phloem cells, with threadlike structures about 20–250 nm long extending from the particles (Fig. 2C). Sometimes the phloem cells contained a large mass of particles surrounded by a membrane. In such cells, threadlike structures connecting the membrane and the cell wall occurred occasionally (Fig. 2D). Virus particles were not found in healthy leaf tissues of *E. crus-galli.*

In ultrathin sections of leaves from plants infected with RRSV, two types of particles, 40–45 nm and 55–70 nm in diameter,

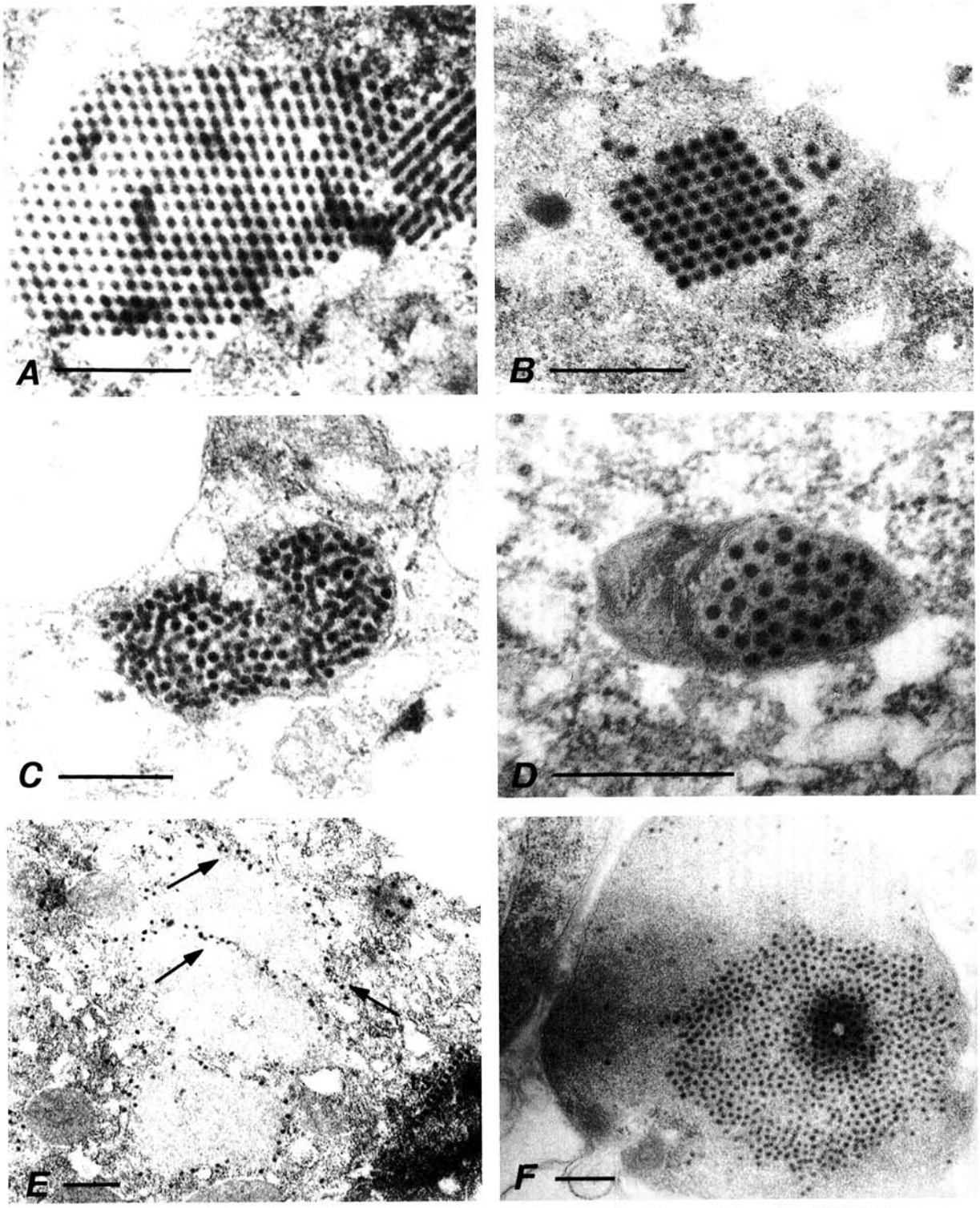

Fig. 3. Electron micrographs of ultrathin sections of *Sogatella longifurcifera* viruliferous for Echinochloa ragged stunt virus (ERSV). Bars represent 500 nm. **A and B,** Particles arranged in a crystalline array in salivary gland cells. **C,** Virus particles embedded in the capsulelike structure of the fat body. **D,** Immature particles about 40–45 nm in diameter are present in the capsulelike structure of gastric caeca cell. **E,** Virus particles in the cells of the Malpighian tubule. Some immature particles (arrows) about 40–45 nm in diameter are arranged around the viroplasmlike structure, and virion about 60–65 nm in diameter are arranged in the tubular structure (ts) outside the viroplasmlike inclusion. **F,** Virus particles in the viroplasmlike inclusion in the ommatidia of a compound eye.

occurred in the cytoplasm of the phloem cells (Fig. 2E and F). These particles usually were scattered in viroplasms in the phloem parenchyma cells. In contrast to ERSV preparations, RRSV particles were observed at a lower frequency in infected cells.

Ultrathin sections of viruliferous insect vectors. In ultrathin sections of the various organs of ERSV-viruliferous *S. longifurcifera*, virus particles approximately 55–75 nm in diameter were scattered or arranged in crystalline arrays in the cytoplasm of cells of the salivary gland, gut, gastric caeca, brain, and ommatidia cornea of the compound eye (Fig. 3). They were numerous in the salivary gland cells. Aggregates of virus particles occurred only in the cytoplasm and were not present in either nuclei or mitochondria. In general, particles in large clusters were arranged in a regular pattern (Fig. 3A and B). Sometimes virus particles

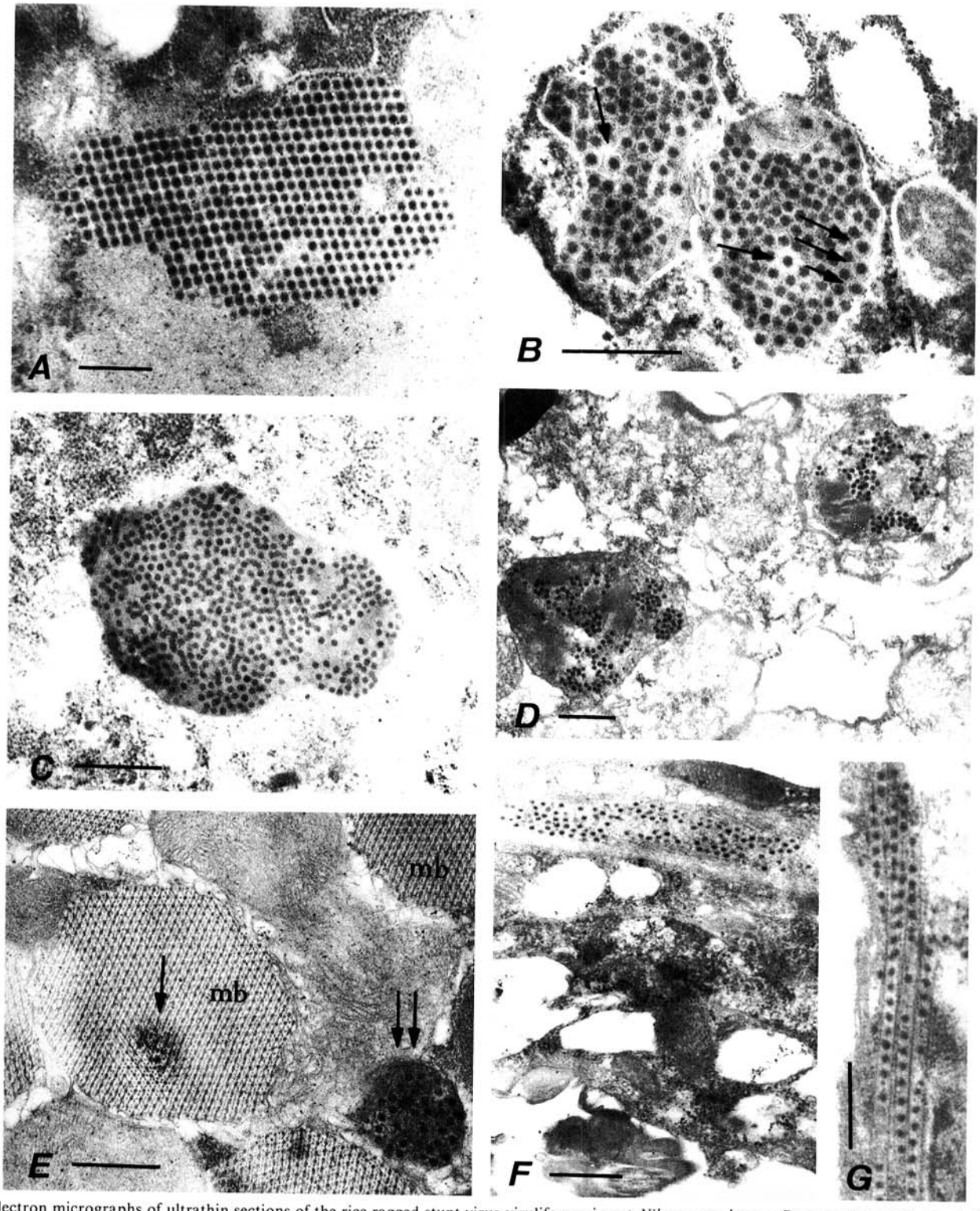

Fig. 4. Electron micrographs of ultrathin sections of the rice ragged stunt virus-viruliferous insect *Nilaparvata lugens*. Bars represent 500 nm. **A,** Virus particles arranged in a crystalline array in a salivary gland cell. **B,** Particles embedded in the viroplasmlike inclusions of fat body. Some particles with clear resolution (arrows) were about 75 nm in diameter. **C and D,** Immature particles about 40–45 nm in diameter scattered in the viroplasm of hindgut and foregut cells. **E,** Particles in the muscle of a viruliferous insect. Particles are embedded in the viroplasm either in (single arrow) or outside (double arrow) the muscle bundles (mb). **F,** Particles scattered in the cuticle of the seminal vesicle. **G,** An ultrathin section of thorax showing tubular structures containing virus particles.

occurred in a capsulelike structure in the fat body (Fig. 3C). Particles about 40–45 nm in diameter were present in the capsulelike structure of the gastric caeca cells (Fig. 3D). Such particles also were noted in the periphery of a viroplasm (Fig. 3E). Larger particles approximately 65 nm in diameter were enclosed in tubular structures outside the viroplasm (Fig. 3E). Virus particles were present in the viroplasmlike inclusion in the ommatidia of the compound eye of viruliferous insects (Fig. 3F). These particles were not found in sections of nonviruliferous *S. longifurcifera*.

Particles of 60–75 nm is diameter were found in various organs of RRSV-viruliferus *N. lugens*. These particles were found aggregated or scattered in the cytoplasm of cells of the salivary gland, fat body, gut, seminal vesicle, and muscles (Fig. 4). In salivary glands, virus particles were arranged in crystalline arrays (Fig. 4A). In the fat body, particles approximately 75 nm in diameter having an electron dense core of about 40–45 nm were surrounded by a lightly stained layer (Fig. 4B). In addition, immature particles about 40–50 nm were found in the electron-dense viroplasm in the cytoplasm of gut cells (Fig. 4C and D). Viroplasms containing particles were found both inside and outside of muscle bundles of viruliferous insects (Fig. 4E). Particles also were scattered in the cuticle of the seminal cell (Fig. 4F). In the cytoplasm of the thorax cells, tubular structures containing particles were arranged in rows (Fig. 4G). No particles of any size were observed in thin sections prepared from healthy *N. lugens*.

DISCUSSION

ERSV and RRSV in dip preparations are morphologically distinct. In PTA-treated crude sap preparations from glutaraldehyde-fixed plant tissues, ERSV particles were mostly subviral, 54–58 nm in diameter, but some were double-shelled particles about 75–80 nm in diameter. There were A-spiked projections sparsely distributed on the surface of the 80-nm-diameter particles (Fig. 1C and D). These double-shelled particles are similar to virions of fijiviruses (3,20,21). On the other hand, the preparations from RRSV-infected plants contained only B-spiked subviral particles about 65–70 nm in diameter and had no outer shell (Fig. 1H). Our observations of the B-spiked subviral particles of RRSV are consistent with those made by Milne (19) and Kawano et al (16). No similar particles were seen in ERSV preparations.

Plant reoviruses are restricted, for the most part, to neoplastic tissues derived from the phloem (2,11,12,17,23). The only exception is rice dwarf phytoreovirus which invades mesophyll cells adjacent to the veins as well as the vascular tissue and does not cause hyperplasia (15). ERSV particles occurred principally in vein swellings. They also were present in mesophyll cells (Fig. 2A) but less frequently.

ERSV and RRSV particles in thin sections prepared from their respective vectors or their respective host plant tissues were very similar in appearance. Particles usually accumulated in viroplasms in the cytoplasm. Similar structures have been reported for other plant reoviruses. It has been suggested that the viroplasm serves as the site of virus assembly (1,2,10,11,14,22,24). In this study, tubular structures containing virus particles occurred in thin sections of insect cells but not in infected plant tissues for both ERSV and RRSV.

Immature plant reovirus particles appear primarily within viroplasms in the cytoplasm, whereas mature particles are enclosed in tubular structures or in crystalline arrangements (1,2,8,10,22,24). In this study, the particles 40–45 nm in diameter were found mainly in the densely stained viroplasms which contained matrix material in cells infected with either ERSV or RRSV (Figs. 3D and 4C). Mature virus particles were mostly in the lightly stained viroplasmlike structures which have a less-defined matrix and well-defined borderline (Figs. 3A and F, 4A and B). The filamentous structures extending from the particles were in thin sections of the phloem cells of ERSV-infected plants (Fig. 2C). Similar structures were previously observed in dip preparations of RRSV, pangola stunt virus, and Fiji disease virus (10,11,14). In the phloem cell undergoing plasmolysis, large masses of virus particles were found enclosed by a membrane with threadlike material connecting the plasma membrane and the cell wall (Fig. 2D). Similar structures have not been reported for other viruses belonging to the plant reovirus group.

Diameters of particles with visible outer shells were difficult to measure because the shells could not be differentiated from the surrounding cytoplasm (9). Variation in particle size might also be the result of different procecures of specimen preparation (21). Thus, in Figure 4A, particles of poor resolution measured approximately 60 nm in diameter, whereas those of good resolution measured approximately 75 nm. In addition, an average particle size of about 70 nm in Figures 3A and 4A was based on measurements of single particles. Yet, an average particle size of about 75–80 nm was obtained from length measurements of a whole array of particles subsequently divided by the number of particles in the array. This latter means of size measurement minimizes the effect of different degrees of specimen resolution and provides a more accurate measurement for viruses that form crystalline structures.

Plant reoviruses fall into two subgroups: phytoreovirus and fijivirus (18). Genomes of fijiviruses contain 10 segments of dsRNA. Fijiviruses further divide into three groups according to serological relationships. Electrophoresis of genomic RNA on 10% polyacrylamide gels revealed the presence of 10 dsRNA segments for both ERSV (4) and RRSV (4,16,22). Neither ERSV nor RRSV has been assigned to one of the three groups in fijivirus. ERSV is typical of the fijiviruses in symptomatology, host range, and vector relationships (4,5). In common with fijiviruses, ERSV particles have the properties of a double shell. Both ERSV and RRSV are similar in pattern of genome segments. In addition, they are serologically related (4). Analysis of ERSV genomic composition and base sequence would provide information for its precise association with the subgroup of fijivirus.

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