

Stability of *Rice yellow mottle virus* and Cellular Compartmentalization during the Infection Process in *Oryza sativa* (L.)

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Rice yellow mottle virus (RYMV) is icosahedral in morphology and known to swell *in vitro*, but the biological function of swollen particles remains unknown. Anion-exchange chromatography was used to identify three markedly stable forms of RYMV particles from infected plants: (1) an unstable swollen form lacking Ca^{2+} and dependent upon basic pH; (2) a more stable transitional form lacking Ca^{2+} but dependent upon acidic pH; and (3) a pH-independent, stable, compact form containing Ca^{2+} . Particle stability increased over the time course of infection in rice plants: transitional and swollen forms were abundant during early infection (2 weeks postinfection), whereas compact forms increased during later stages of infection. Electron microscopy of infected tissue revealed virus particles in vacuoles of xylem parenchyma and mesophyll cells early in the time course of infection and suggested that vacuoles and other vesicles were the major storage compartments for virus particles. We propose a model in which virus maturation is associated with the virus accumulation in vacuoles. In this acidic compartment, virus particles may bind Ca^{2+} to produce a highly stable, compact form of the virus. The localization of subcellular RYMV isoforms in infected cells and the corresponding biological properties of the virus are discussed. © 2002 Elsevier Science (USA)

Key Words: rice yellow mottle virus; sobemovirus; calcium; particle stability; vascular and mesophyll cells; vacuole; vesicles; electron microscopy.

INTRODUCTION

Rice yellow mottle virus (RYMV) is an icosahedral virus with $T = 3$ lattice symmetry (Opalka *et al.*, 2000) belonging to the genus *Sobemovirus*. RYMV is responsible for substantial economic losses in rice production throughout eastern and western Africa (Bakker, 1970; Awoderu, 1991; Abo *et al.*, 1998). Infection symptoms consist of yellow or discolored spots that appear at the base of the youngest leaves and expand parallel to the veins during late stages of infection. Plants are stunted during advanced infection, and flowers are frequently sterile (Bakker, 1974). Several species of chrysomelid beetles are known to transmit RYMV, and mechanical transmission has also been demonstrated (Bakker, 1970, 1971).

RYMV contains a single-stranded positive-sense RNA of 4450 nucleotides encoding four open reading frames (ORFs) (Ngon a Yassi *et al.*, 1994). Frameshift and deletion mutations in the coat protein (CP) demonstrated that the CP is dispensable for viral RNA replication but required for cell-to-cell and long-distance movement

(Brugidou *et al.*, 1995). In addition, virus encapsidation is necessary for long-distance spread of infection (C. Brugidou, unpublished results), and the P1 protein is required for cell-to-cell spread of the virus (Bonneau *et al.*, 1998). In susceptible rice varieties, mechanical inoculation results in systemic spread after 6 days, at which time there is an abundance of virus particles in mesophyll and xylem cells (Opalka *et al.*, 1998). In this model, RYMV particles accumulate in the vacuoles of xylem parenchyma cells. Invasion of mature vessel elements during xylem maturation may provide a crucial pathway for cell-to-cell movement through the pit membranes (Opalka *et al.*, 1998).

The RYMV particle is 28 nm in diameter with 180 identical CP subunits assembled on a $T = 3$ icosahedral lattice (Opalka *et al.*, 2000). Sobemovirus particles are stabilized by divalent cations, pH-dependent protein-protein interactions, and salt bridges between protein and RNA (Hull, 1988; Seghal, 1995). Sobemovirus, bromovirus, carmovirus, and tombusvirus particles swell *in vitro* if divalent cations are removed or if the pH is shifted from slightly acidic to basic (Incardona and Kaesberg, 1964; Hull, 1977; Adolph, 1978; Golden and Harrison, 1982; Robinson and Harrison, 1982; Heaton, 1992). The swelling process is reversible (Hsu *et al.*, 1977) and particles undergo cotranslational disassembly *in vitro* (Brisco *et al.*, 1985, 1986; Shields *et al.*, 1989). However,

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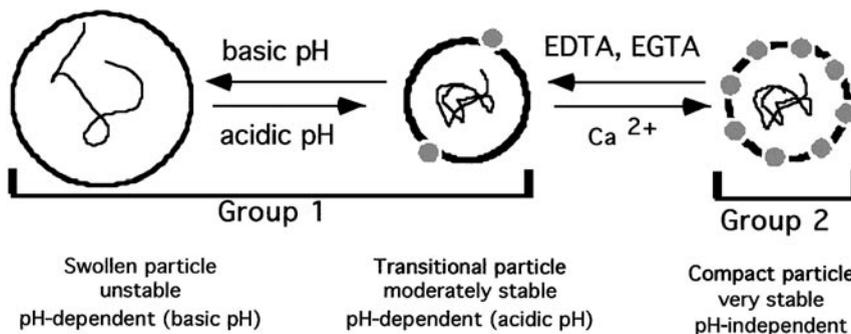


FIG. 1. A schematic model for relationships between compact, transitional, and swollen RYMV particles. Group 1 (swollen and transitional) and 2 (compact) particles are separated by anion-exchange chromatography (see Fig. 2) and can be interconverted by changes in pH and Ca²⁺.

in the case of *Cowpea chlorotic mosaic virus* (CCMV), cotranslational disassembly occurs without particle swelling (Albert *et al.*, 1997). Therefore it is unclear whether particle swelling has a function *in vivo*.

Particles of various plant viruses have already been localized *in situ*, especially inside the vacuole (Francki *et al.*, 1985a,b,c). However, nothing is known about virus assembly and the roles of cellular compartmentalization in viral maturation during infection.

Here we demonstrated the presence of virus isoforms *in planta* for the first time. Indeed, we describe the isolation of three forms of RYMV particles from infected plants that were distinguished by their stability: (1) an unstable swollen form dependent on basic pH but lacking Ca²⁺; (2) a transitional compact form dependent on acidic pH, but also lacking Ca²⁺; (3) and a stable compact form that is pH independent and contains Ca²⁺. A possible *in vivo* role for particle swelling, transition, and compaction is proposed on the basis of two types of studies: (1) analysis of virus particle stability using anion-exchange chromatography, and (2) electron microscopic localization of virus particles in infected leaves. The overall results allowed us to develop a model highlighting the role of cellular compartmentalization on virus stability and the consequences on the pathogeny and biological properties of RYMV. To our knowledge, this is also the first report documenting increased stability of virus particles in plants during the course of infection.

RESULTS

Selection of stable RYMV particles by anion-exchange chromatography

RYMV was purified from infected plants on density gradients and the virions were subjected to anion-exchange chromatography under conditions that would discriminate between group 1 (swollen and transitional particles) and group 2 (compact particles) as described in Fig. 1. Elution profiles displayed three peaks (Fig. 2A). Electron microscopy showed that peak 1 contained highly purified intact virus particles without aggregates

or particle deformation (Fig. 2C), similar to the purified virus samples prior to chromatography (Fig. 2B). Peaks 2 and 3 were eluted over the salt gradient at NaCl concentrations of 0.9 and 1.8 M, respectively (Fig. 2A). Although peaks 2 and 3 displayed reactivity with RYMV antibodies (data not shown), electron microscopy revealed amorphous material and disrupted virus particles (Fig. 2D). Peak 1 (Figs. 2 and 3A) contained the most stable particles with compact and transitional forms. The presence of both forms is demonstrated in Fig. 3B by shifting the pH of the elution buffer from 6.0 to 8.5. The height of peak 1 decreased corresponding to disrupted transitional particles (transitional shifted to swollen particles at pH 8.5, Fig. 3A vs 3B), as well as after dialysis against EDTA (Fig. 3C vs 3B) eluted at pH 6.0. The presence of compact particles was demonstrated after dialysis at pH 8.0 against EDTA prior to chromatography and visualized by the absence of peak 1 (Fig. 3C vs 3D). There was a concomitant increase in peaks 2 and 3 when peak 1 decreased (Figs. 3B, 3C, and 3D), and a sizable fraction of particles formed insoluble aggregates and failed to enter the column (data not shown). In addition, the absence of particles (Fig. 3D) demonstrated that swollen particles were not able to be eluted at pH 8.5 and could be eluted only in transitional form at pH 6.0. Hence, the amount of intact virus particles (as indicated by the height of peak 1) was dependent on the pH and whether the particles were exposed to EDTA. Since pH plays a crucial role in the conversion of RYMV particles and has an instantaneous effect, we further analyzed the influence of pH on RYMV particle stability by chromatography.

RYMV stability *in vitro*

The *in vitro* stability of virus particles was dependent on the pH at which anion exchange chromatography was conducted and whether samples were dialyzed in EDTA, EGTA, or calcium (Fig. 4). At basic pH virus particles were more stable in the presence than the absence of CaCl₂. For example, when purified virus was dialyzed at pH 5 in the presence of 3 mM CaCl₂ (condition 5, Fig. 4)

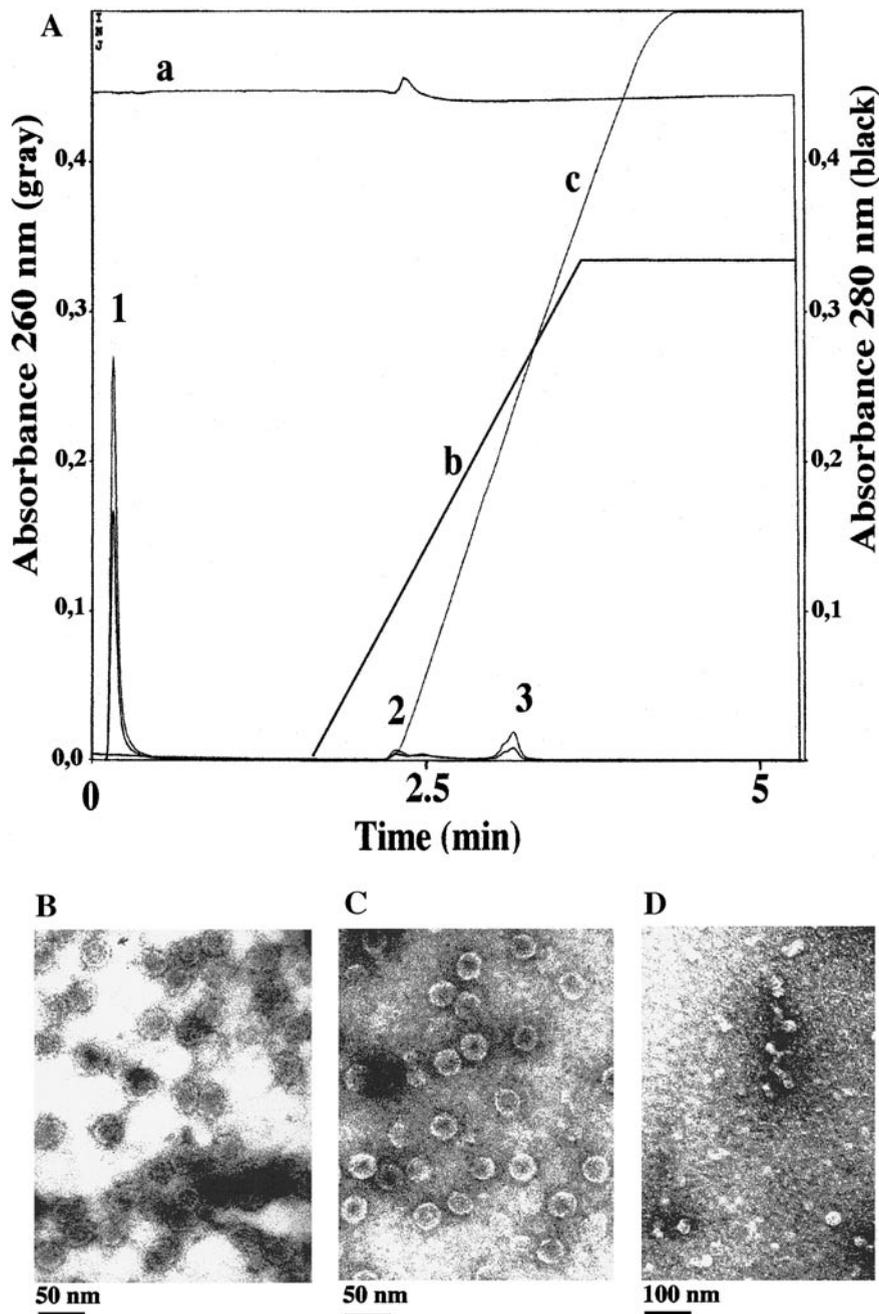


FIG. 2. (A) Anion exchange chromatography of a 100- μ l purified RYMV (0.5 mg/ml). Peak 1 had a retention time of 0.15 min and an A260/A280 ratio of \sim 1.5. Peaks 2 and 3 had retention times of 2 and 3 min and eluted at 0.9 and 1.8 M NaCl, respectively. Peak 1 corresponds to intact virus particles; peaks 2 and 3 correspond to RYMV-CP and viral RNA (a, pH; b, NaCl gradient from 0 to 2.55 M; c, conductivity). (B) Electron microscopy of negatively stained RYMV samples prior to chromatography (A), peak 1 (C), and peaks 2 and 3 (D) after overnight dialysis in 0.02 M phosphate buffer, pH 5.0.

and subjected to anion exchange chromatography, 57% of the virus particles remained intact and passed through the column at pH 9 compared to 100% at pH 6. However, when virus was dialyzed in the presence of 20 mM EDTA at pH 5 (condition 4, Fig. 4), the amount of intact particles decreased compared to treatment with CaCl_2 . Indeed, at pH 9, no intact virus particles were recovered, and at pH 8 76% were recovered compared to 90% when virus was dialyzed without EDTA (Fig. 4). Dialysis of

samples at pH 8.5 in the presence of 20 mM EDTA or EGTA yielded almost no intact virus, even when chromatography was performed at pH 6 (conditions 1 and 2, Fig. 4). Finally, dialysis for only 2 h (instead of overnight) with 20 mM EDTA at pH 8.5 (condition 3, Fig. 4) resulted in virus particles that were still highly sensitive to the pH of the chromatography conditions. Hence, the virus particles were most unstable when maintained at basic pH in the absence of CaCl_2 .

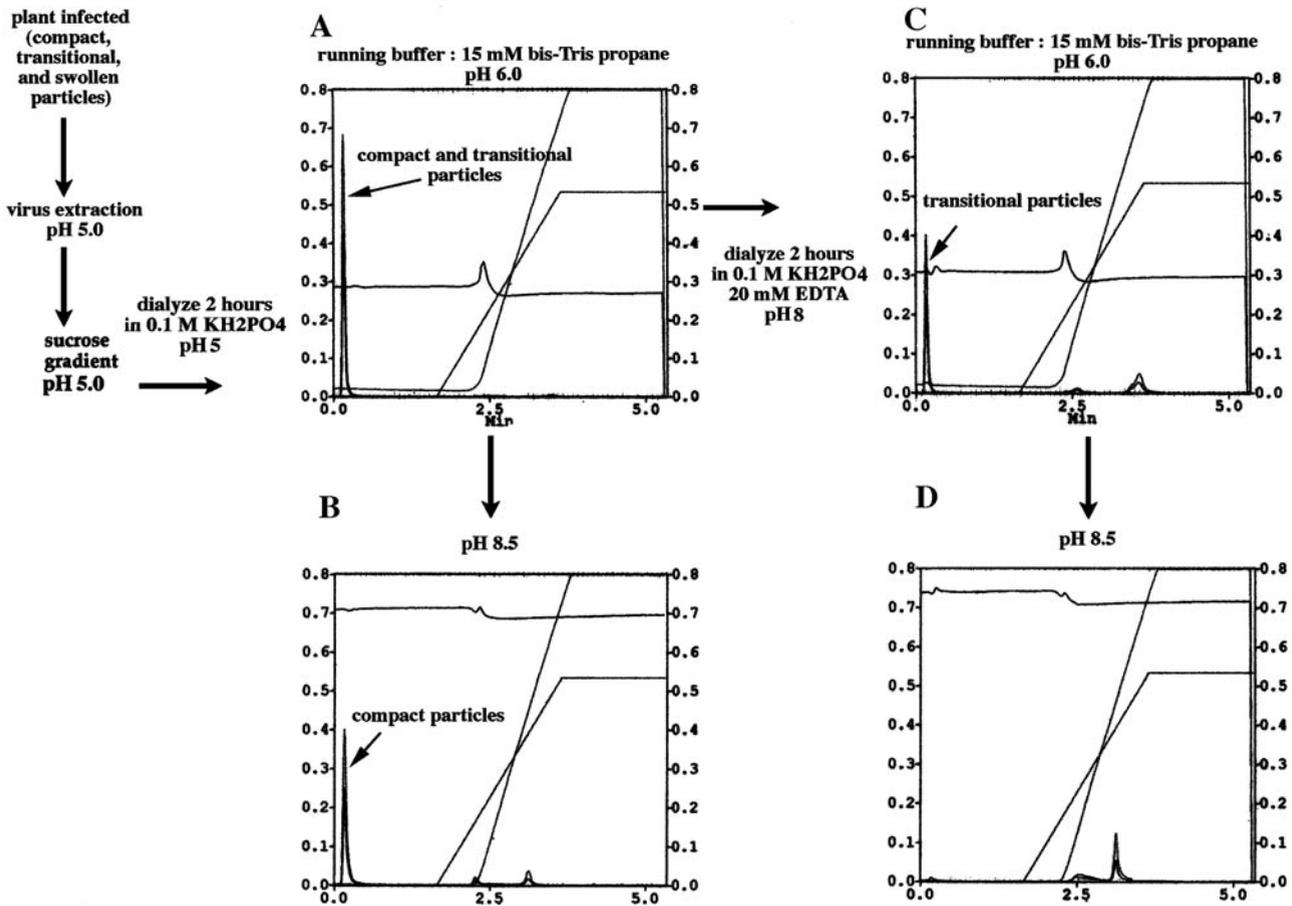


FIG. 3. Anion-exchange chromatography of RYMV samples pretreated under different dialysis conditions. (A, B) Two hours dialysis in 0.1 M KH_2PO_4 , pH 5.0. (A) Elution buffer at pH 6; (B) elution buffer at pH 8.5; (C, D) 2 h dialysis in 0.1 M KH_2PO_4 with EDTA 20 mM, pH 8.0; (C) elution buffer at pH 6.0; (D) elution buffer at pH 8.5. Aliquots of the same virus sample were used for each experiment.

RYMV stability *in vivo*

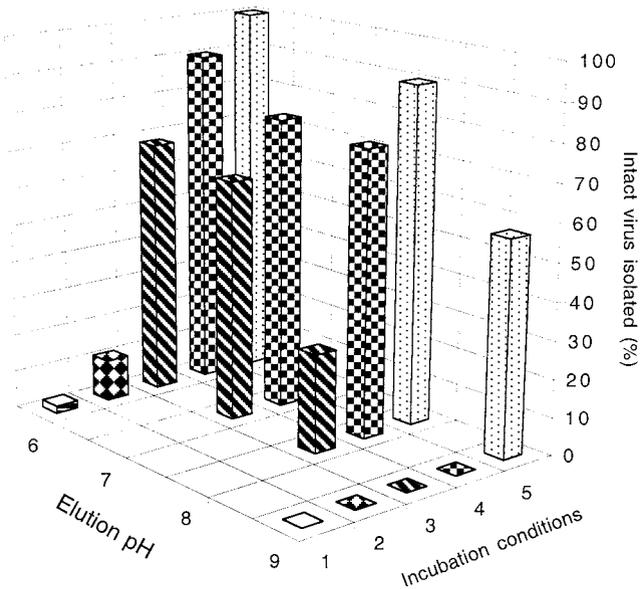
Virus extraction was performed at pH 5 to demonstrate that the ratios between compact (pH-independent), transitional (pH-dependent), and swollen (pH-dependent) particles changed over the time course of RYMV infection. Indeed, at this pH, swollen particles can apparently be converted into transitional particles. To explore the stability of virus particles during infection, virus particles were extracted at different times following infection and analyzed by anion-exchange chromatography (Fig. 5). Regardless of the pretreatments, a lower percentage of intact virus particles was recovered at pH 8.5 (Fig. 5B) vs pH 6.0 (Fig. 5A).

Virus particles isolated early in infection (2 weeks postinoculation (w.p.i.)) were especially unstable at basic elution pH and when pretreated with EDTA (Fig. 5B) vs pH 6.0 (Fig. 5A). In contrast, particles isolated later in infection (6 w.p.i.) were more stable, even when samples were pretreated with EDTA and the elution pH was 8.5. Indeed, no intact virus particles could be isolated from 2 and 4 w.p.i. plants at pH 8.5 in 20 mM EDTA, whereas at least half of the particles (30% in Fig. 5b vs 60% in Fig. 5a) remained intact when extracted from 6 w.p.i. plants.

Electron microscopy of infected leaves

Electron microscopy of systemically infected leaves sampled from 10 to 28 days postinoculation (d.p.i.) showed a large amount of virus particles (Vps) in xylem parenchyma and mesophyll cells. Most of the Vps were localized in the cytosol or in vacuoles. During the early stages of infection (10 d.p.i.), Vps were detected inside the vacuoles of xylem parenchyma cells (Fig. 6A). Later in infection (21 d.p.i.) a large amount of Vps were often observed inside vacuoles (Figs. 6B and 6C). Although the amount of Vps was high at 21 d.p.i., cytoplasmic integrity and mitochondrial structure were apparently not affected (Figs. 6B and 6C). In addition to virus-filled vacuoles, smaller vesicles that contained Vps (Figs. 6B and 6C) and virus-particle aggregates (Fig. 6B) were frequently observed in xylem parenchyma cells from 21 d.p.i. In the phloem or xylem parenchyma cells, Vps were organized as paracrystalline arrays surrounded by a membrane-like structure (Fig. 6D).

Vps accumulated differently in infected mesophyll cells. Vps were found in the cytoplasm and were associated with fibrils and electron-dense aggregates (Figs. 7A and 7B). In these cells, chloroplasts were smaller in



- 1 : overnight, 20 mM EGTA, pH 8.5
- 2 : overnight, 20 mM EDTA, pH 8.5
- 3 : 2hrs, 20 mM EDTA, pH 8.5
- 4 : overnight, 20 mM EDTA, pH 5
- 5 : overnight, 3 mM CaCl₂, pH 5

FIG. 4. Effect of different pretreatments and elution buffer pHs on RYMV stability. After different dialysis treatments with the same virus sample in conditions (1, 2, 3, 4, or 5), a virus aliquot (100 μ l) was separated using anion exchange chromatography. A 100% reference yield was designated by the amount of virus in peak 1 (Fig. 2A) using an elution buffer of 15 mM of bis-tris-propane:tris base (50:50) at pH 6.0 and with pretreatment 5. The other results are expressed as a percentage of this reference yield.

size and contained reduced numbers of starch grains (not shown). Degenerative changes within chloroplasts were observed, including a poorly differentiated grana-intergrana lamellae system and disorientation of the grana and dense stroma (Figs. 7A and 7B). Vps and fibrils were also visible within chloroplasts (arrow in Fig. 7B). In some cells Vps were concentrated inside vacuoles (Fig. 7C). Infected mesophyll cells (Fig. 7C) displayed chloroplasts and mitochondria that were less altered compared to those in Fig. 7B, with large amounts of Vps in the cytoplasm. Crystalline arrays of Vps were not detected in mesophyll cells. However, vesicles containing Vps were observed in the cytoplasm and in the vacuoles with electron-dense globules and cellular material (Figs. 7D and 7E).

DISCUSSION

Swollen, transitional, and compact forms of RYMV detected *in vivo*

In this study, we used anion exchange chromatography and electron microscopy to explore the stability of

swollen and native RYMV particles after different treatments and at different times during infection. The purification methods did not affect the infectivity of RYMV, as intact particles recovered following chromatography were infectious (data not shown). Similar methods have been used to purify infectious and noninfectious virus particles from plants and animals (Smith, 1987; Walin *et al.*, 1994; Carlsson *et al.*, 1994; Richieri *et al.*, 1998; Wellington-Wester *et al.*, 1998).

Since anion-exchange chromatography allowed rapid adjustment of the elution pH, compact and transitional particles could be easily separated. Both forms were eluted at pH 6.0, but only the compact form was eluted at pH 8.5. Swollen particles were generated from transitional particles at basic pH or from compact particles in the presence of chelators of cations in phosphate buffer (EDTA or EGTA) at pH 8.5. Electron cryomicroscopy and

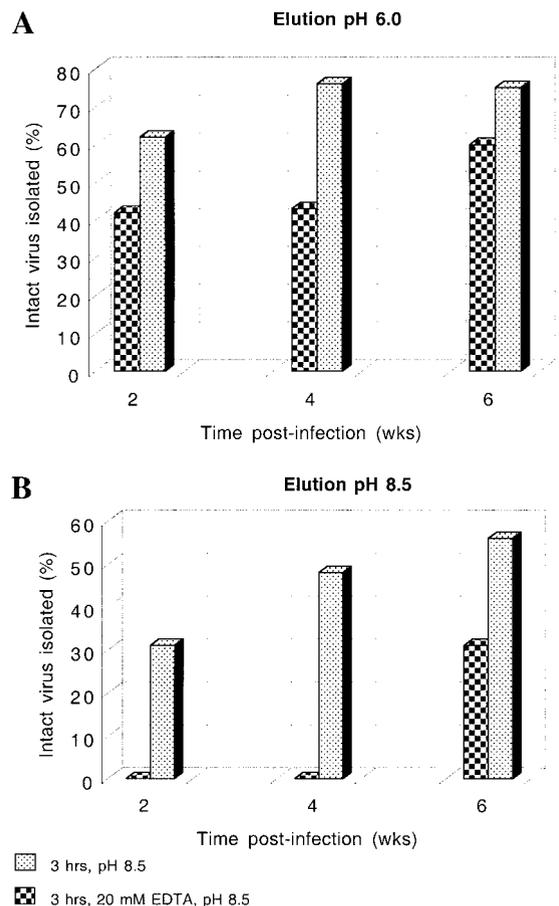


FIG. 5. Analysis of postinfection RYMV particle stability. Virus extraction was performed using plants harvested at 2, 4, and 6 w.p.i. in 0.1 M phosphate buffer at pH 5.0. After different dialysis treatments (3 h pH 8.5 with or without 20 mM EDTA) with the same virus sample, a virus aliquot (50 μ l) was separated using anion exchange chromatography. The running buffer was 15 mM of bis-tris-propane:tris base (50:50), and the elution pH was either pH 6.0 (A) or pH 8.5 (B). The results are expressed as a percentage of intact virus recovered in peak one (Fig. 2A). The variation between three independent runs from the same sample did not exceed 3%.

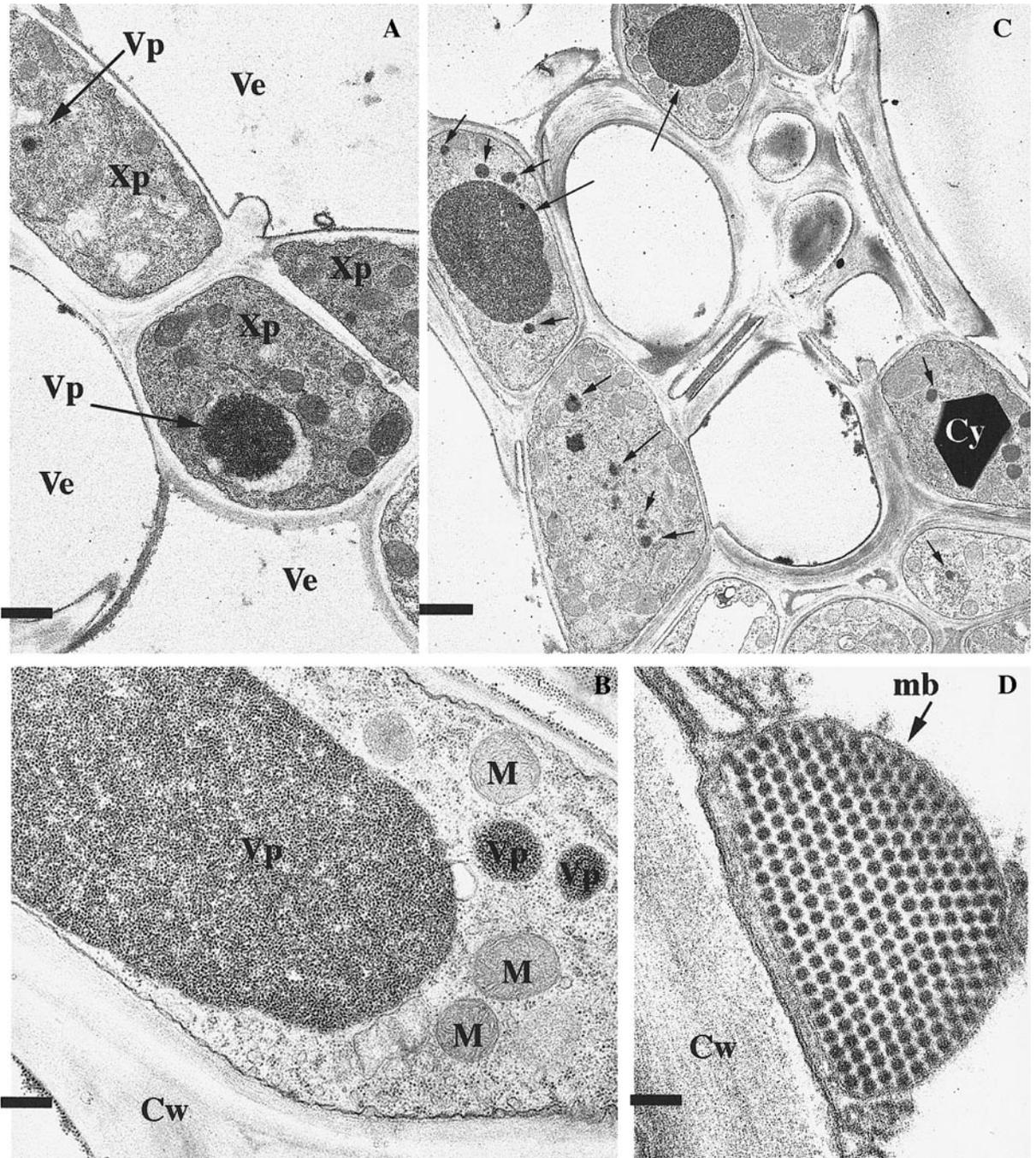


FIG. 6. Transmission electron micrographs of systemically infected rice leaves. (A) Section of the vascular bundle showing infected xylem cells at 10 d.p.i. (Xp, xylem parenchyma; Ve, vessel) showing virus particles (Vp; arrowheads) located within the vacuole and in vesicles. The cytoplasm is apparently unaffected and contains intact mitochondria (M) and rough endoplasmic reticulum (R). Virus particles (Vp) can be seen adjacent to the secondary cell wall (Cw) (bar: 0.75 μm). (B) Xylem parenchyma cell infected at 21 d.p.i. showing virus particles (Vp; arrowheads) located within the vacuole and in vesicles. The cytoplasm is apparently unaffected and contains intact mitochondria (M) and rough endoplasmic reticulum (R). Virus particles (Vp) can be seen adjacent to the secondary cell wall (Cw) (bar: 0.75 μm). (C) Infected xylem parenchyma cells at 21 d.p.i. showing virus particles (Vp; arrowheads) within the vacuole and vesicles and in a large crystal (Cy; arrowhead) (bar: 1.2 μm). (D) An infected sieve tube at 21 d.p.i., containing a virus crystal located close to the cell wall (Cw). The crystal appears to be enveloped by membrane (mb; arrowhead), (bar: 120 nm).

image analysis of native and swollen particles has shown that the expanded form is $\approx 8.5\%$ larger in volume compared to the compact form (Opalka *et al.*, 2000). However, swollen particles were not eluted from the column under the conditions used. At pH 6.0, some particles were probably eluted because swollen parti-

cles were rapidly converted to transitional particles (which were eluted from the column). When the pH was shifted to 8.5 (transitional particles were converted to swollen particles), no intact particles were recovered, and CP with viral RNA were eluted separately over a sodium chloride gradient.

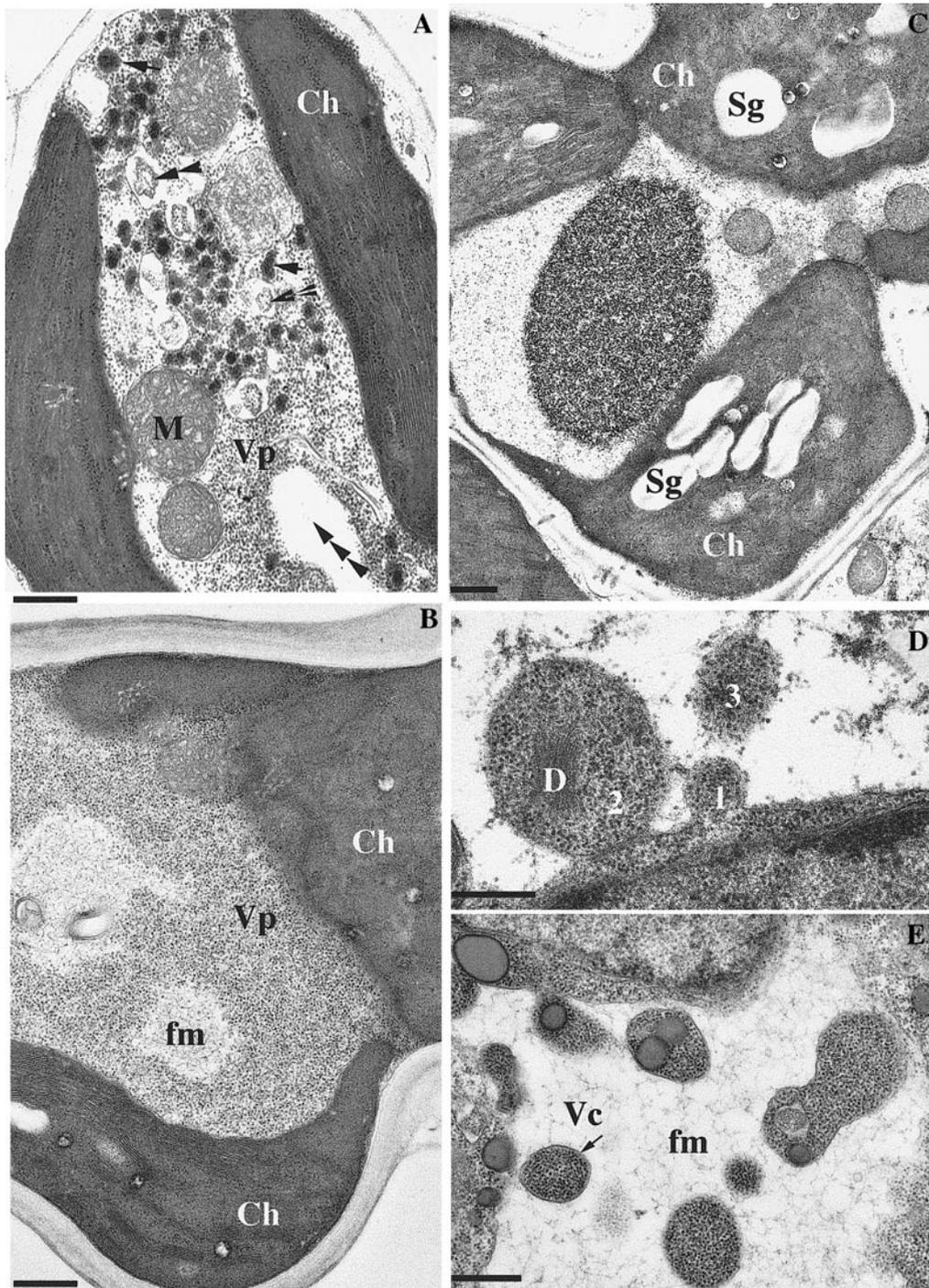


FIG. 7. Transmission electron micrographs of systemically infected rice leaves at 21 d.p.i. (A) An infected mesophyll cell containing chloroplast (Ch) and mitochondria (M) with virus particles (Vp) scattered throughout the cytoplasm. Electron-dense material (arrows) is adjacent to vesicles which contain electron dense material (double arrows) and fibrillar material (triple arrows) (bar, 0.6 μm). (B) An infected mesophyll cell showing virus particles (Vp) filling the cytoplasmic compartment. Fibrillar material (fm) is associated with virus particles (bar, 0.6 μm). (C) An infected mesophyll cell with starch grain (Sg) within a chloroplast (Ch). There are numerous virus particles within the vacuole (bar, 0.6 μm). (D) An infected mesophyll cell showing the following: (1) an invaginated tonoplast containing virus particles and cytoplasmic material pinched off from the tonoplast; (2) a vesicle containing virus particles and dictyosome (D); and (3) virus particles released into the vacuole (bar, 0.6 μm). (E) Vacuole of the same mesophyll cell containing fibrillar material (fm) and several virus-filled vesicles (Vc). The osmiophilic globules (Og) within the vesicles and the fibrillar material were not seen in uninfected cells (bar, 0.6 μm).

In vitro swelling of sobemoviruses has been demonstrated for *Southern bean mosaic virus* (SBMV) (Wells and Sisler, 1969; Hsu *et al.*, 1976; Brisco *et al.*, 1986) and *Turnip*

rosette virus (Hull, 1977, 1988) and is a common feature for other icosahedral plant viruses (reviewed by Heaton and Morris, 1992). For several spherical viruses, *in vitro* conver-

sion between native and swollen forms is regulated by a shift to basic pH and chelation of divalent cations (Heaton and Morris, 1992). In our study, transitional particles (pH-dependent), including swollen particles transformed to transitional particles and compact particles (very stable, pH-independent), were recovered from infected plants (see summary in Fig. 1). The transitional particles were only stable at a pH ranging from 6 to 8.

Dialysis of compact particles in buffers containing EDTA or EGTA at pH 8.5 was sufficient to convert compact particles to transitional particles at pH 6.0 or to swollen particles at pH 8.5. We thus propose that RYMV is similar to *Turnip rosette virus* (TRoV) and SBMV (Hull, 1978) in that Ca^{2+} is the major stabilizing cation. This conclusion is in agreement with the structure of RYMV, which was recently resolved to 2.8 Å by X-ray crystallography (Qu *et al.*, 2000). Similar to *Southern cowpea mosaic virus* (SCPMV, previously known as cowpea strain SBMV) (Hull *et al.*, 2000), each RYMV particle has 180 Ca^{2+} binding sites localized at subunit interfaces near the quasi-threefold positions (Abdel-Meguid *et al.*, 1981). As proposed for *Human rhinovirus* (Zhao *et al.*, 1997), Ca^{2+} may play a role in regulating the stability of RYMV between transitional and compact particles.

It appears difficult to accurately determine the proportion of swollen particles in infected tissues. Indeed, most of the swollen particles (which are very unstable) were converted into transitional particles during the isolation procedure. Nevertheless, the proportion of compact particles increased during infection relative to transitional particles. Even though some or all of the swollen particles were disrupted during the purification process, transitional particles could be the dominant form early in infection, with the compact form becoming predominant late in infection.

RYMV localization in cell compartments

Electron microscopy of infected tissues showed that virus particles are abundant in the cytoplasm, vacuoles, and in association with the cell wall (Baker, 1974; Opalka *et al.*, 1998). In this study, we focused on the vacuole and its involvement in the localization of Vps in mesophyll or xylem parenchyma cells. In xylem parenchyma cells, virus particles were localized in vacuoles and vesicles. Despite the large numbers of virus particles, cellular organelles were not disrupted, and virus crystals were frequently observed inside the vacuoles and vesicles. In mesophyll cells, virus particles were observed in the cytoplasm and vacuoles, but no crystals were observed.

As infection progressed, we observed that (1) Vps accumulated inside the vacuoles and in cytoplasmic vesicles; (2) virus crystals surrounded by a membrane were visible only in vascular cells at late infection times; and (3) host cell integrity was well preserved when virus particles accumulated within the vacuoles. The presence of Vps in vesicles

and vacuoles is a common feature in plant virus infection (Francki *et al.*, 1985a,b), but nothing is known about the role of compartmentalization during the infection cycle in xylem parenchyma cells. Our analyses suggest that RYMV particles may accumulate in the vacuole in two different ways. Virus particles were rarely observed in the cytoplasm, compared to a high level observed in vacuoles of xylem parenchyma cells. This suggests that Vps might accumulate in a fashion similar to storage proteins. Reserve proteins are synthesized on the endoplasmic reticulum, pass through the Golgi apparatus, *trans*-Golgi network, and are then targeted to the vacuole (Chrispeels and Staehelin, 1992; Hohl *et al.*, 1996). Alternatively, the presence of large numbers of virions in vacuoles may be due to invagination of the tonoplast, which is filled with Vps and cytosol, suggesting that the host responds to RYMV by compartmentalizing the particles in the vacuole. Autophagy processes mediate the uptake of proteins into vacuoles (Chrispeels and Staehelin, 1992). Similar observations were reported for plant viruses such as tombusviruses, necroviruses (Francki *et al.*, 1985a,b), sobemoviruses (Francki *et al.*, 1985a), Colopo yellow mosaic virus (Morales *et al.*, 1995), and *Velvet tobacco mottle virus* (VTMoV) (Francki *et al.*, 1985c). Our observations suggest that RYMV accumulates in vacuoles early in the infection process. Later in the infection process, virus crystals are found within the vacuole, suggesting that vacuoles may be a storage compartment for RYMV.

Based on our results, we propose a model for the cellular localization of RYMV (Fig. 8). Since the pH of different cellular compartments varies (Fig. 8), and as pH affects particle stability (Fig. 1), it is reasonable to suggest that swollen and at a lower level compact particles coexist in the cytoplasm. Since the vacuolar pH of plant cells is typically 5.0 (Kurkdjian and Guern, 1989), RYMV may coexist as transitional (pH-dependent) and compact particles (pH-independent) within vesicles or vacuoles (Fig. 8). The presence of dense electron material immunolabeled by RYMV-CP antibody (Fig. 8) within the cytoplasm and nucleus (without any virus particles observed in the nucleus) suggests transport of the replicative viral complex between the cytoplasm and nucleus. This possible subcellular transport is in agreement with the presence of a putative bipartite nuclear localization signal (KK(x)₁₀KRKxRR) in the N-terminal basic domain of CP (Ngon Yassi *et al.*, 1994) and its RNA binding properties (Lee and Hacker, 2001).

The biological functions of these three forms should differ. Group I (Fig. 1) includes unlocked forms (transitional and swollen particles) which are only pH dependent. These two forms are probably active for replication and subcellular transport. Conversely, since Ca^{2+} ions saturated the 180 binding sites, group II includes locked forms, i.e., probably for storage of the virus for subsequent long distance movement, vector transport, or mechanical transmission by leaf contact. The inoculum infectivity of compact particles thus has a dilution end

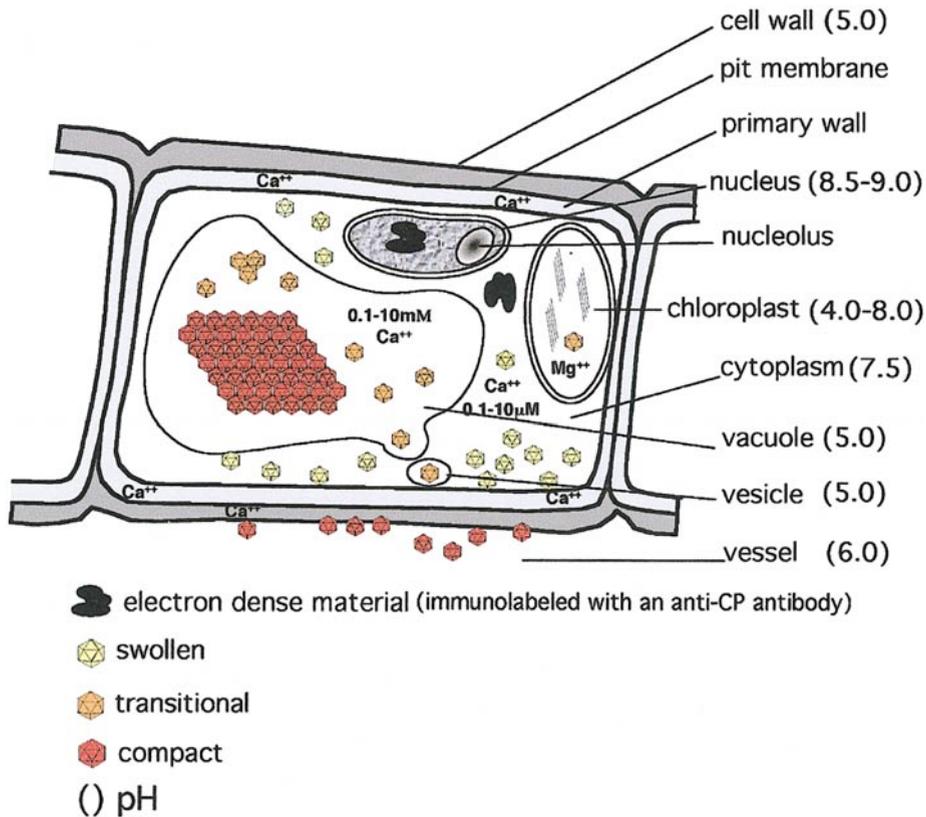


FIG. 8. A schematic model for the compartmentalization of compact, transitional, and swollen particles during maturation within infected rice cells according to subcellular pHs (Raven, 1985; Kurkdjian and Guern, 1989; De Zoeten, 1995). Electron dense materials (represented by black spots) were immunolabeled with anti-RYMV antibody and localized within cytoplasm and nucleus. Virions were localized by electron microscopy within cytoplasm, vacuoles, vesicles, chloroplasts (a few), and cell walls (Opalka *et al.*, 1998; this paper). Calcium concentrations are indicated according to Sze *et al.* (1992); vacuoles and cell walls are the major calcium reservoirs in cells.

point at 10^{-8} , whereas it dropped down to 10^{-4} for swollen particles (data not shown).

RYMV maturation occurs by cell compartmentalization.

Vacuoles are known to be a major Ca^{2+} reservoir (Sze *et al.*, 1992), and the H^{+} -ATPase and H^{+} -PPase pumps (Rea and Sanders, 1987) maintain a *trans*-tonoplast H^{+} gradient and transport ions, including Ca^{2+} , into vacuoles (Sze *et al.*, 1992, Wink, 1993). Therefore, maturation of RYMV particles to compact forms could occur in vesicles or vacuoles. The acidic pH of vacuoles and the presence of Ca^{2+} could stabilize viral particles by producing the compact form and by facilitating RYMV crystal formation. This would allow the virus to accumulate to high levels without having deleterious effects on cellular viability and integrity. High levels of virus stored within the vacuole could be the major reason for the absence of symptoms and high virus accumulation levels in tolerant plants (C. Brugidou, unpublished result). We thus propose that most virus particles released from the vacuole during the differentiation process of vessel cells could be highly stable, compact particles, which are adapted to

allow long distance movement in the vessels (Opalka *et al.*, 1998). Indeed, in this drastic environment, virus particles move up passively with water and bind the cell wall. Since virus particles lose Ca^{2+} in water, we suggested that Ca^{2+} transfer occurs from the pit membrane to maintain particle stability (Opalka *et al.*, 1998). This model is supported by a mutational analysis of *Turnip crinkle virus* (Lin and Heaton, 1999). Mutations in the putative calcium binding site prevent cell-to-cell spread and long-distance movement.

MATERIALS AND METHODS

Virus isolation and plant inoculation

The RYMV isolate used in this study was collected from rice fields in Côte d'Ivoire and was propagated in a susceptible rice variety IR8 (*Oryza sativa* L.). The inoculum was prepared from 1 g of infected leaves ground in 3 ml of 0.01 M phosphate buffer, pH 7. Mechanical inoculation of 2-week-old rice plants was accomplished by abrading the upper leaf surface with Carborundum (330 grit, Fisher Scientific) before inoculation. Plants were grown at 30°C and 80% relative humidity with a photo-

period of 16 h light/8 h dark, which was provided by 4 × 30 W Sylvania cool white lights supplemented by 60 W incandescent bulbs.

Virus purification

RYMV was purified using a modification of a previously published procedure (Bakker, 1974). Infected leaves from 75 plants were ground in liquid nitrogen at different times after infection. The ground material was suspended in 0.1 M phosphate buffer ($\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$), pH 5.0, containing 0.2% β -mercaptoethanol (100 ml per 10 g of fresh leaves), homogenized in a Waring blender for 1 min at full power, and then filtered through cheesecloth (Fisher Scientific). An equal volume of chloroform was added, and the suspension was homogenized for an additional 15 s. The mixture was centrifuged at 9800 *g* for 15 min, and the aqueous phase was recovered and stirred for 2 h at 4°C with 0.3 M NaCl and 6% of polyethylene glycol (PEG) 8000 (w/v). The suspension was centrifuged at 4°C for 30 min at 22,000 *g*, and the pellets were resuspended in 1–5 ml of 0.1 M phosphate buffer, pH 5. The suspension was applied to 10–40% (N/V) continuous sucrose gradient in phosphate buffer at pH 5. After ultracentrifugation at 180,000 *g* in a Beckman SW 50.1 rotor for 1 h at 4°C, purified virus was collected at the 20–30% sucrose gradient interface and stored at 4°C. The virus concentration was estimated by absorption spectroscopy at 260 nm (A_{260}). A virus suspension at 1 mg/ml in a 1-cm path length has an $A_{260} = 6.5$ (Bakker, 1970, 1974). A typical $A_{260}:A_{280}$ ratio for purified virus was ~1.5.

Stability during virus precipitation with PEG (2 h at 4°C) was demonstrated by injecting crude extract in the Biocad 700 E (PerSeptive Biosystems, Framingham, MA) with comparison to precipitated virus. In addition, subsequent virus purification steps were also checked and demonstrated, in our conditions, that virus particles remained stable throughout the extraction and purification process.

Virus treatments

Aliquots of purified virus (0.5–1.0 ml at 0.5 $\mu\text{g}/\mu\text{l}$) from the sucrose gradient were dialyzed at 4°C against 0.05 M phosphate buffer at varying pH for different times and under magnetic stirring at low speed. All buffers were prepared with water treated in diethyl-pyrocabonate (DEPC, Sigma). Prior to analysis, each virus preparation was dialyzed for 20 min against two changes of 0.05 M phosphate buffer at pH 5. Each experiment was repeated at least three times using three different purified virus preparations.

Electron microscopy

Leaf samples from infected or noninfected rice were harvested at 10, 15, 17, 21, 24, and 28 days postinoculation and were prepared for electron microscopy as pre-

viously described (Opalka *et al.*, 1998). Virus particles stained with 1% uranyl acetate were visualized by transmission electron microscopy using a Philips CM12 operating at 100 kV, at magnifications of ×80,000 and ×150,000. Immunostaining with polyclonal CP antibodies has been previously used to confirm that Vps is a virus particle (Opalka *et al.*, 1998).

Anion exchange chromatography

Chromatography was performed with a PerSeptive Biosystems Biocad 700E connected to a Gilson fraction collector F-250. One hundred microliters of virus samples was injected and purified on a prepacked POROS anion exchange column (quaternized polyethyleneimine matrix (HQ/H), 4.6 mm D/100 mm L, CV = 1.7 ml) containing 10 μm POROS particles using a running buffer of 15 mM Bis-Tris propane: Tris base (50/50, Sigma) at pH 6 or 8.5, a flow rate of 6 ml/min, and a pressure of 1500 psi. After equilibration of the column with running buffer, the virus sample was injected, and the column was washed with five column volumes (CV) of running buffer, followed by a linear NaCl gradient up to 2.55 M over 3 min (11 CV), concluding with five column volumes of running buffer and 2.55 M NaCl. Three injections of the same virus sample yielded a maximum variation of around 3% in the absorbance at 260 and 280 nm. The lower limit for virus detection was ~7 ng/ μl . Fractions of 1 ml were collected and dialyzed overnight in 20 mM phosphate buffer, pH 5 at 4°C. In addition to electron microscopy, samples were analyzed by Western immunoblot analysis using polyclonal RYMV antibodies (Brugidou *et al.*, 1995).

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