Disassembly of African cassava mosaic virus

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The plant-infecting geminiviruses encapsidate their single-stranded DNA genome in characteristic twinned particles that are unique among viruses. These particles are formed by joining two incomplete T=1 icosahedra. African cassava mosaic virions were purified by density-gradient centrifugation from infected Nicotiana benthamiana plants and analysed for their stability with respect to pH changes and heat treatment by using electron microscopy. Negative staining and rotary shadowing revealed stable virions as well as isolated capsomeres between pH 4.0 and 8.5. At pH 9.0 and above, particles disintegrated, whereas they mainly aggregated at a pH below 6.0. Heating the preparations to 55 °C and above resulted in the complete loss of any discernible structure. A low proportion (approx. 10 %) of particles ejected their DNA within the pH range of 6.0–8.5. Most virions released their DNA at the top (15.9 %) or the shoulder (71.4 %) of the twin particles and only 12.7 % at the waist. Compared with the expected numbers of pentameric capsomeres at the top (9 %), the shoulder (45.5 %) or the waist (45.5 %), the results revealed a preferential DNA release from the top and shoulder of the geminate particle.

INTRODUCTION

The small plant pathogenic geminiviruses are distributed all over the world and have caused severe economic damages in tropical and subtropical countries (Moffat, 1999). African cassava mosaic virus (ACMV) is the most prevalent pathogen of cassava in Africa (Fauquet & Fargette, 1990); it belongs to the genus Begomovirus (Stanley et al., 2005), and is transmitted by the whitefly Bemisia tabaci (Gennadius). Its bipartite genome consists of two circular single-stranded DNA molecules (DNA A and DNA B). The three-dimensional structure of the geminate (twin) ACMV particles has been resolved by cryo-electron microscopy (EM) and image reconstruction (Böttcher et al., 2004). The two incomplete T=1 icosahedra of the virion are formed by 110 copies of a single capsid protein (CP) with pentameric capsomere organization. Each half of the geminate particle is built up by 11 pentameric capsomeres, one pentamer missing to complete a T=1 icosahedron. The two halves are joined at the place of the missing capsomeres, forming the waist of the particle (see Fig. 7). The two capsomeres at both ends of the particle with strict fivefold symmetry will be called the ‘top capsomeres’, the adjacent five capsomeres on each side of the virion with local fivefold symmetry will be called the ‘shoulder capsomeres’ and the five remaining capsomeres at the waist will be called the ‘waist capsomeres’ (see Fig. 7). The single CP possesses a molecular mass of 30.1 kDa and a calculated pI of 10.0. Each geminate particle encapsidates one circular DNA molecule so that two virions are needed for infection in case of bipartite geminiviruses.

Encapsidating the viral genome, the gemivirus CP is important for insect transmission (Briddon et al., 1990; Höfer et al., 1997; Höhnle et al., 2001; Kheyry-Pour et al., 2000; Noris et al., 1998) and transport of the viral DNA in and out of the nucleus (Kotlízky et al., 2000; Kunik et al., 1998, 1999; Liu et al., 1999; Unseld et al., 2001, 2004). For the ACMV CP, three regions have been determined that mediate nuclear import (Unseld et al., 2001), whereby the central region is not only important for nuclear import but also for export. Deletions within these regions, which may overlap with DNA binding and multimerization regions, inhibited the formation of virus particles (Unseld et al., 2004). Upon infection of a plant by the vector, the CP is the only viral protein present in the newly infected cell. Localization of the virus particle to the nucleus is therefore dependent on the CP and on utilization of host transport mechanisms (Gafni & Epel, 2002). Whether particle disassembly takes place at the nuclear pore complex and, as a consequence, only the genome is transferred into the nucleus, or whether the whole particle is transported through the nuclear pore complex is not known, yet. Likewise, the in vitro disassembly mechanism has still to be explored.

The stability of geminivirus particles depends on several environmental factors. At the beginning of geminivirus research, most purification protocols used buffers with a pH of 7.0–8.0 (Bock et al., 1978; Dollet et al., 1986; Hamilton et al., 1981; Ikegami et al., 1985). A pH of 8.0 has also been chosen for ACMV purification for cryo-EM
(Böttcher et al., 2004) under the assumption that this pH is close to that of phloem sap and insect gut (Fife & Frampton, 1936; Hocking, 1980). In contrast, the structure of maize streak virus (MSV), which belongs to the genus Mastrevirus, was determined from a preparation purified at pH 4.8 (Zhang et al., 2001). So far, only one systematic investigation of the pH-dependent stability of gemini-viruses has been accomplished (Matysis et al., 1975). The analysis of particle stability with respect to pH- and temperature-induced changes may help to understand the life cycle of the virus in greater detail, especially the assembly/disassembly mechanism.

In the following study, we have investigated the effects of pH and temperature on ACMV particles by using EM.Particles extruding their DNA were further examined for the releasing site within the germinate capsid. The analysis revealed a preference for DNA release at the top and shoulder of the particles.

**METHODS**

**Virus propagation.** Initially, *Nicotiana benthamiana* DOMIN plants were inoculated with plasmids containing an infectious DNA clone of the Kenyan strain of African cassava mosaic virus [ACMV-(K)] formerly known as cassava latent virus (Klinkenberg et al., 1989; Stanley & Gay, 1983), kindly provided by John Stanley. Further *N. benthamiana* plants at the seven- to eight-leaf stage were mechanically inoculated with the sap of those plants: small leaves showing strong symptoms were homogenized in water, and 10 μl of the resultant sap was used as inoculum on two not completely developed carborundum-dusted leaves.

**Virus purification.** Young leaves of ACMV-infected *N. benthamiana* plants were harvested 20–25 days post-inoculation, ground at 4 °C in 10 ml per gram leaf material of 100 mM NaH2PO4 pH 7.0, 2 mM EDTA, 10 mM Na2SO4, 10 mM NaN3 and 1 % poly(vinylpolypyrrolidone) (Sigma; P6735), and filtered through four layers of gauze. After centrifuging the filtrate for 20 min at 4 °C and 16 344 g, the supernatant was stirred with 1/2 volume chloroform for 1.5 h at 4 °C, and phases were separated by centrifugation (15 min, 4 °C. 4086 g). Virus particles were precipitated from the aqueous phase overnight at 4 °C by adding polyethylene glycol (PEG 6000) and sodium chloride (final concentration 4 % and 0.2 M, respectively) and sedimented (20 min, 4 °C, 16 344 g). The resultant pellet was suspended in 0.1 M sodium borate buffer pH 8.0 containing 35 % (w/v) Cs2SO4 and 19–22 were also dialysed against borate buffer. A volume of 7 2030 Journal of General Virology m pooled and chosen for analysing the stability of ACMV virions because the other fractions with higher density contained increasing amounts of contaminating nucleic acids besides viral particles as observed by transmission EM of rotary shadowed samples (data not shown). The fractions with lower density harboured several host proteins, which resulted in additional bands on silver-stained SDS-PAGE (Fig. 1b). The pool of purified particles was dialysed against buffers at different pH values ranging from pH 4.0 to 10.0. To exclude an influence of the buffer components, different buffers at the same pH values were compared. Using both acetate and phosphate at pH 6.5 and phosphate as well as borate buffer at pH 7.5, no differences in the samples with the same pH were discernible. At pH 4.0 and 5.0, almost no viral particles were observed in the shadowed samples (Fig. 2a and b), and aggregates were perceived only rarely. However, the

**EM.** For shadowing, samples of 20–100 μg ml⁻¹ were applied to parlodion-coated copper grids. The grids were washed for 30 s on a drop of water, stained for 30 s with uranyl acetate (1 μl 50 mM acid uranyl acetate in 1 ml 90 % ethanol), washed for 30 s on a drop of 90 % ethanol and rotary shadowed with platinum at an angle of 7.5° in a coating unit (Bal-tec MED200). A supporting carbon film was evaporated at an angle of 90°.

For negative staining, samples of 0.5–1 mg ml⁻¹ were subjected to glow-discharged Formvar- and carbon-coated copper grids, washed with water and stained with 2 % uranyl acetate for 5 min.

Samples were analysed with a FEI Tecnai G² operated at 80 kV for rotary shadowed and at 200 kV for negatively stained specimens. Images were taken with a Tietz F214 CCD camera (TVIPS).

**Chemical cross-linking.** Aliquots of purified virus particles (200–300 ng) either at pH 8.0 or after dialysis against 0.1 M sodium phosphate pH 7.5 were cross-linked with different glutaraldehyde concentrations in a total volume of 8 μl buffer (20 mM triethanolamine containing 50 mM sodium chloride at pH 7.5) for 30 min at room temperature. For some experiments, samples were digested with DNaseI (5 U; Roche) prior to cross-linking for 30 min at 37 °C. Cross-linking reactions were terminated by adding 8 μl 2 × gel loading buffer (Laemmli, 1970) and heating at 95 °C for 5 min. The samples were analysed on 8 % SDS-PAGE (Laemmli, 1970), and gels were either silver stained or semi-dry blotted on to nitrocellulose membranes. CP on membranes was detected using raw polyclonal antiserum against ACMV (AS0421, kindly provided by Dr Stephan Winter, DSMZ Braunschweig, Germany), alkaline phosphatase conjugated goat anti-rabbit antibodies (Rockland Immunochemicals) and nitro blue tetrazolium/5-bromo-4-chloro-3′-indolyl phosphate (NBT/BCIP).

**RESULTS**

Viral particles were isolated from *N. benthamiana* plants previously inoculated with the sap of ACMV-infected plants. Using density-gradient centrifugation, fractions with a high amount of purified viral particles were obtained as seen by negative staining (Fig. 1a). Fractions corresponding to a density of 1.29–1.33 g cm⁻³ were pooled and chosen for analysing the stability of ACMV virions because the other fractions with higher density contained increasing amounts of contaminating nucleic acids besides viral particles as observed by transmission EM of rotary shadowed samples (data not shown). The fractions with lower density harboured several host proteins, which resulted in additional bands on silver-stained SDS-PAGE (Fig. 1b). The pool of purified particles was dialysed against buffers at different pH values ranging from pH 4.0 to 10.0. To exclude an influence of the buffer components, different buffers at the same pH values were compared. Using both acetate and phosphate at pH 6.5 and phosphate as well as borate buffer at pH 7.5, no differences in the samples with the same pH were discernible. At pH 4.0 and 5.0, almost no viral particles were observed in the shadowed samples (Fig. 2a and b), and aggregates were perceived only rarely. However, the
failure to detect particles was obviously due to the preparation technique because the negatively stained samples showed large aggregates of particles and only a few well-separated particles (Fig. 3a and b). Larger aggregates presumably had caused the disruption of the more fragile parlodion film used for shadowing, so that they could not be examined with this preparation technique. At pH 6.0, large fields of well-shaped geminate particles were observed in the shadowed samples (Fig. 2c), and negatively stained samples revealed single (Fig. 3c) as well as aggregated virus particles (data not shown). In contrast to the situation at the more acidic pH, these aggregates were formed by only 4–20 particles. At the pH range of 7.0–8.5, mainly single viral twin particles were observed (Fig. 2d–g and Fig. 3d–g) with both preparation techniques.

As for pH-dependent changes, the stability and aggregation of ACMV particles were influenced by temperature at a constant pH of 8.0. At 4 and 37 °C for 5 min, similar results were obtained for the samples after shadowing as well as after negative staining (Fig. 4). Small aggregates of geminate particles were only rarely observed (data not shown). Treating virus particles at 55 or 65 °C, however, resulted in massive aggregation of the material (Fig. 4). Different from the negatively stained samples at acidic pHs, these aggregates did not display any discernible substructure.

Although most virions appeared intact at pH 4.0–8.5 and temperatures of 4–37 °C, all samples within these ranges showed smaller particles, in addition, which were visible after shadowing as well as after negative staining (Figs 2

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**Fig. 1.** ACMV particle isolation. (a) ACMV particles of fraction 16 of the density gradient stained with 2% uranyl acetate. Bar, 100 nm. (b) Silver-stained 12.5% SDS-PAGE of pooled and dialysed density-gradient fractions containing geminate particles. The single band in pool 8–13 corresponds to the CP. In pool 14–18, besides the monomeric CP band, multimers of the CP are also resolved. In the pool comprising fractions 19–22 several additional host-derived bands were present. Positions of marker proteins are indicated on the left.

**Fig. 2.** Rotary shadowed ACMV particle preparation after dialysis against buffers with different pH regimes. Purified ACMV particles at pH 8.0 (f) were dialysed against 0.1 M sodium acetate pH 4.0 (a), pH 5.0 (b), pH 6.0 (c), 0.1 M sodium phosphate pH 7.0 (d), pH 7.5 (e), 0.1 M sodium borate pH 8.5 (g), pH 9.0 (h) or 0.1 M sodium glycine pH 10.0 (i). Dialysed samples were applied to parlodion-coated copper grids, stained with uranyl acetate and rotary shadowed with platinum at 7.5°. The technique facilitates visualization of DNA, which is exemplary marked by arrowheads in (h). Arrows in (d) and (e) indicate two representative particles releasing their DNA. Capsomere-like structures are encircled. Arrangements of proteins comparable to Fig. 3(i) are indicated by ovals in (i). Bar (200 nm) in (a) is representative for all images.
and 3, circles). Their size fits to those of capsomeres, and some of them showed a pentameric outline (Fig. 5). Averaging over the shown capsomeres (Fig. 5) resulted in a pentagon (Fig. 5, image in the right lower corner). The proportion of the putative capsomeres was lowest at pH 8.0 (Fig. 3f) and increased at pH 7.0 (Fig. 3d) as well as under more alkaline conditions (Fig. 3h). For the shadowed samples, the capsomeres were observed in the

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**Fig. 3.** Negatively stained ACMV particles after dialysis against buffers with different pH regimes. Purified ACMV particles at pH 8.0 (f) were dialysed against 0.1 M sodium acetate pH 4.0 (a), pH 5.0 (b), pH 6.0 (c), 0.1 M sodium phosphate pH 7.0 (d), pH 7.5 (e), 0.1 M sodium borate pH 8.5 (g), pH 9.0 (h) or 0.1 M sodium glycine pH 10.0 (i) and stained with 2% uranyl acetate. Capsomeres are marked by circles, whereas in (i) characteristic arrangements of proteins are indicated by ovals. Bar (100 nm) in (a) is representative for all images.

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**Fig. 4.** ACMV particles after heat treatment. Purified ACMV particles were either kept on ice (a and e) or heated for 5 min to 37 °C (b and f), 55 °C (c and g) or 65 °C (d and h). Samples were prepared for EM by rotary shadowing (a–d) or negative staining (e–h). Bar (200 nm) in (a) is representative for images (a)–(d); bar (100 nm) in (e) is representative for images (e)–(h).
same pH range (Fig. 2c–h), and additionally and more prominent at pH 4.0 and 5.0 (Fig. 2a and b). Chemical cross-linking with glutaraldehyde (0.1–5 mM) confirmed the presence of pentameric capsomeres in samples at pH 7.5 at concentrations between 1 and 5 mM glutaraldehyde as determined in silver-stained SDS-PAGE (Fig. 6a). Even lower glutaraldehyde concentrations (0.1, 0.25 or 0.5 mM) were sufficient to detect these multimers on Western blots (Fig. 6b). In addition, size-exclusion chromatography after or without DNaseI treatment (Supplementary Figs S1 and S2 available in JGV Online) revealed a destabilization of virus particles at pH 7.5 as compared with pH 8.0. Upon chromatography at pH 7.5, CPs eluted at a volume comparable to alcohol dehydrogenase (*M* 150 kDa), consistent with a molecular mass of ACMV CP pentamers.

Corresponding to the occurrence of capsomeres, some particles started to disassemble as indicated by the appearance of extruded DNA in the shadowed samples (arrows in Fig. 2d and e). At pH 9.0 (Fig. 2h) and more pronounced at pH 9.5 (not shown), a higher amount of DNA became visible in the samples (arrowheads in Fig. 2h) although some intact twin particles were still present. At these pH values, DNA was attached to approximately 12% of particles. It was difficult to quantify this aspect accurately, because particles with extruding DNA tended to aggregate, and it was not possible to discriminate between aggregates of particles and aggregates of particles with ejected DNA. At the alkaline end of the analysed pH range (pH 10.0), only a few intact virus particles were left in the shadowed samples (Fig. 2i). Similarly, few particles with roughly the shape of geminiviruses (arrow in Fig. 3i) were seen after negative staining, but most of the virions were disintegrated. From some perspectives (oval in Fig. 3i), the disintegrated virions retained, nevertheless, a circular substructure on which capsomeres were distributed like beads on a string. This type of arrangement may also be inferred from the corresponding shadowed samples (ovals in Fig. 2i). In summary, we have found two types of disassembly, the ejection of DNA out of still intact twin particles within the pH range of 6.0–8.5 and a swelling and disruption of the particles at higher pH.

**Fig. 5.** Arrangement of negatively stained capsomeres with pentameric outline. Purified ACMV particles were dialysed against 0.1 M sodium phosphate pH 7.5 and stained with 2% uranyl acetate. Images of capsomeres with pentameric outline were grouped. The average of the selected capsomeres is shown in the lower right corner. Bar, 10 nm

The ejection of DNA at pH 6.0–8.5 (Fig. 2d and e, arrows) was further examined in order to find out whether a preferential extrusion site in the twin particle exists. Since in some cases a protein structure was found to be attached at the end of the extruding DNA (Figs 2e, 7b, upper row, second image from the left), corresponding to the size range of the capsomeres, we assume that a capsomere is also released upon ejection. Virus particles (553) with extruding DNAs were collected from all the samples from pH 6.0 to 9.5 and divided into three classes. In the first class, the DNA was released from the top of the particle (Fig. 7a), in the second class from the shoulder (Fig. 7b) and in the third class from the waist of the particle (Fig. 7c). Most of the analysed particles (71.4%) belonged to the second class (Table 1). Assuming that a capsomere is detached from the particle prior to DNA release, there are three different possibilities: two top capsomeres with strict fivefold symmetry at both ends of the particle, 10 capsomeres with local fivefold symmetry at the shoulder.

**Fig. 6.** Determination of CP oligomers by using cross-linking with glutaraldehyde and gel electrophoresis. (a) Silver-stained 8% SDS-PAGE and (b) Western blot analysis of cross-linked proteins. Purified virus particles either at pH 8.0 or after dialysis against 0.1 M sodium phosphate pH 7.5 were cross-linked using increasing amounts of glutaraldehyde (0.1, 0.25, 0.5, 1, 2 or 5 mM as indicated above the lanes) for 30 min at room temperature. For (b), samples were treated with DNaseI (+) prior to cross-linking. The positions of monomeric CP (30.1 kDa, 1×) and its oligomers (2×, 3×, 4× and 5×) as well as DNaseI (asterisk) are indicated. Marker proteins (M) with their molecular mass (kDa) are shown.
and 10 capsomeres at the waist of the particles may be displaced to allow ejection of DNA. If the capsomere site was chosen at random for DNA release, particles with extruded DNA are therefore expected with 9.0, 45.5 and 45.5 % probability for top, shoulder and waist attachments, respectively. Among the 553 analysed particles 88 showed DNA release at the top, 395 at the shoulder and 70 at the waist, corresponding to 16, 71 and 13 %, respectively (Table 1). Whereas the observed values for the top and shoulder ejection were 1.8- or 1.6-fold higher than expected, the observed value at the waist was 3.3-fold lower than expected. These results showed a clear preference for DNA to be released from the top or the shoulder of the particles.

**DISCUSSION**

ACMV showed stable virions between pH 4.0 and 8.5, although a certain number of particles started to disassemble in this pH range. In addition, capsomeres were always present. Since they were nearly absent from the starting material of isopycnically purified virions and the proportion increased after changing the pH, we believe that the capsomeres detached from the virions. Their size of approximately 12 nm was consistent with the predicted size of a pentamer in the structure of ACMV (Böttcher et al., 2004). Since purified virus particles were used, the analysed stability of virions solely relies upon the CP and the encapsidated DNA in vitro, and additional stabilizing/destabilizing host factors might be involved in vivo.

At pHs lower than 6.0, virus particles formed large aggregates but retained their geminate structure. In contrast, above pH 9.0, virus particles were completely deformed, but proteins still covered the viral DNA. At pH 9.0 and 9.5 some of the geminate particles were at least 10 % larger than normal, which might be an indication of swelling known for several plant viruses (Hsu et al., 1976; Incardona & Kaesberg, 1964; Krüse et al., 1982; Speir et al., 1995). In contrast to our findings, tomato golden mosaic virus (TGMV) particles were stable in the pH range of 4.0–7.0 and disintegrated by pH 8.0 (Matyis et al., 1975). The disparity of TGMV and ACMV may result from the different preparation techniques applied or might be virus-specific.

Different from the pH-dependent changes, no intermediate structures were found with various temperatures. The overall geminate structure was retained in samples heated to 37 °C, but only large aggregates with no internal structures were obtained at 55 °C or above. This behaviour corresponds to the thermal inactivation point of 55 °C for 10 min as reported by Bock et al. (1978). Whereas this value is typical for many geminiviruses, paroviruses that possess a single-stranded linear DNA genome in an icosahedral shell are more stable. The B19 capsid integrity is retained up to 60 °C (Ros et al., 2006), and virions of minute virus of mice and adeno-associated virus 2

<table>
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<tr>
<th>Site of DNA release</th>
<th>Count</th>
<th>Expected (%)</th>
<th>Observed (%)</th>
<th>Ratio (obs./exp.)</th>
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<td>9.0</td>
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<td>45.5</td>
<td>71.4</td>
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<td>70</td>
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Fig. 7. Viral DNA released from particles at different sites. Purified virus particles were rotary shadowed with platinum, and particles were classified according to the site of DNA release. In total, 553 particles were analysed of which 16 % released the DNA on one end of the particle (a, ‘top’), 71 % at the shoulder of the particle (b, ‘shoulder’) and only 13 % at the waist of the particle (c, ‘waist’). Bars (100 nm) in the upper left image of each section are representative for all images.

Table 1. Comparison of expected and observed occurrence of DNA release at the different sites of the particles

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disassemble at temperatures above 70–75 °C (Bleker et al., 2005; Carreira et al., 2004; Kronenberg et al., 2005).

DNA extruded at different pH values from a few particles, while most of the capsids remained intact. Comparing the different pH values, particles releasing DNA were more prevalent in the samples above pH 8.0. Under the assumption that a pentameric capsomere was detached from the particle, virions ejecting the DNA from the waist were clearly underrepresented. However, DNA release at the top or shoulder of the particles was more frequent. In a few cases, capsomere-like structures were still attached to the end of the released DNA, and pentameric capsomeres were delineated by EM. Chemical cross-linking (Fig. 6) as well as size-exclusion chromatography analysis (Supplementary Figs S1 and S2) supported this conclusion. A small amount of CP aggregates that resisted the detergent and the reducing conditions of the SDS-PAGE were already present in the samples prior to glutaraldehyde treatment. Similar to our findings, Sitharam & Agbandje-McKenna (2006) suggested pentameric capsomeres as subassemblies for MSV. Detaching a capsomere to release the genome would differ in the mechanism from paroviruses and other icosahedral viruses, where it is assumed that a pore at the fivefold axes serves for DNA externalization (Bleker et al., 2005), as it has been described for several other icosahedral viruses (Smyth & Martin, 2002). Similar to ACMV, turnip yellow mosaic virus (TYMV) releases its single-stranded RNA genome through a hole left by a departed capsomere of its icosahedral structure (Böttcher & Crowther, 1996; Canady et al., 1996; Kuznetsov & McPherson, 2006). Despite the similarity of releasing a capsomere, TYMV capsid stability is mainly driven by protein–protein interaction as inferred from the existence of empty shells. Geminivirus particle stability seems to be mainly the result of protein–DNA interaction. To our knowledge, no empty particles have been observed so far. Considering the circular form of the encapsidated DNA molecule, two stretches of the molecule have to pass through the particle waist. The interaction of the waist capsomeres might be particularly stabilized by interacting with the DNA moiety compared with the non-waist capsomeres that are preferentially released. On the other hand, for unknown reasons the interaction between capsomeres at the particle waist might be stronger in comparison to the interaction between the top and shoulder capsomeres.

The shadowing technique was utilized nearly 30 years ago to show the disassembly of cauliflower mosaic virus (CaMV) (Al Ani et al., 1979). Whereas almost all CaMV particles released their DNA at pH 11.25, only approximately 10% of ACMV particles ejected DNA in the analysed pH range. The length of the ejected DNA appearing relatively short is underestimated because the DNA portion was not completely spread under the applied experimental conditions and, therefore, cannot be measured accurately. The thickness of the DNA strand might indicate that it is still covered by some CPs.

In summary, we have shown that DNA release is not equally distributed between the three structurally different sites of the virion. The results provide valuable information for further elucidating the disassembly process of geminiviruses.

ACKNOWLEDGEMENTS

The authors would like to thank Drs Bettina Böttcher, Anan Kadri, Stefan Nußberger and Dipl. Biol. Fania Grimm for helpful discussions and for critically reading the manuscript, the gardeners Diether Gotthardt and Annika Allinger for taking care of the plants and DFG for financial support (DFG Je 116/11-1).

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