Single-stranded DNA from abutilon mosaic virus is present in the plastids of infected Abutilon sellowianum

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ABSTRACT The leaf mosaic of Abutilon sellowianum is caused by abutilon mosaic virus (AbMV), a geminivirus that has a circular single-stranded DNA genome. DNA was isolated from intact plastids of AbMV-infected and noninfected plants. Plastids from infected plants were shown to contain the single-stranded AbMV DNA by Southern-blot hybridization experiments that used a probe made from highly purified AbMV DNA. The possibility of adsorption of AbMV virions or viral DNA onto the plastid envelope was ruled out by several in vitro experiments with DNase I and protease. Furthermore, the lamellar system of plastids from AbMV-infected plants was degenerated and substituted by amorphous electron-dense material. The transport of AbMV DNA across the plastid envelope has implications for the development of a chloroplast transformation vector.

Geminiviruses are plant viruses that contain single-stranded DNA (ssDNA) encapsidated in twin particles (1, 2). The genomes of some geminiviruses have been cloned and sequenced (for review, see refs. 3 and 4). Geminiviruses have been localized in the vascular-bundle area of infected plants in general (5) and, in one case (chloris striate mosaic virus), also in the remaining mesophyll (6). Virus-like twin particles have mainly been found in the nuclei (5) but, to our knowledge, never in plastids.

The whitefly-transmitted abutilon mosaic virus (AbMV) is classified as a geminivirus (7). The disease is transmitted by the whitefly Bemisia tabaci, which is not indigenous to Europe; therefore, cuttings were used for propagation of infected plants. The corresponding virus-like twin particles had been found (8, 9) in the nuclei of the phloem-associated cells of various host plants. Structures called "chains of pearls" that contain the AbMV DNA and transmit the disease can be isolated from AbMV-infected plants (10). After infecting Malva parviflora, comparable filaments could be detected in the chloroplasts of mesophyll cells, because they form paracrystalline inclusions (8, 9).

The appearance of virus-associated structures in plastids could explain why cytological symptoms of the disease are predominant in the mesophyll chloroplasts, whereas they are generally absent from the plastids of the vascular-bundle area (11). Therefore, the question arises whether AbMV DNA is present in the plastids. Our analysis has shown that it is. The tissue and organelle specificity of the virus are of central importance if geminiviruses are to be used as genetic vectors.

MATERIALS AND METHODS

Isolation and Purification of Plastids. Plant material from uninfected Abutilon sellowianum Regal and AbMV-infected Abutilon sellowianum var. marmorata Regal was homogenized in an isotonic medium of 0.4 M sorbitol/44 mM Mes, pH 6.1/10 mM NaCl/8 mM EDTA/1 mM MnCl₂/1 mM MgCl₂/0.5 mM KH₂PO₄/2 mM sodium ascorbate/2 mM cysteine/3 mM dithioerythritol/10% bovine serum albumin/0.01% polyvinylpyrrolidone 10 in a Waring blender at 0–2°C and filtered through 100-µm and 20-µm nylon gauze. The plastids were pelleted by centrifugation in a swinging-bucket rotor (SN 2293, Kontron, Munich, 5 min, 4000 rpm, 1°C) and purified by two cycles of Percoll-gradient centrifugation in HAB (homogenization medium in which polyvinylpyrrolidone, dithioerythritol, cysteine, and sodium ascorbate were added and EDTA replaced by 10 mM). The first cycle [40% (vol/vol) Percoll, 40 min, 115.000 × g, 1°C] gave three chlorophyll-containing bands of increasing density, called bands A, B, and C. Fractions with intact plastids (bands B and C), as judged by interference microscopy, were subjected to a second cycle of centrifugation: fraction B in 30% (vol/vol) Percoll, and fraction C in 40% (vol/vol) Percoll. To examine the purity of the fractions, material from each fraction was fixed in 4% (vol/vol) formaldehyde in HAB and examined with an interference microscope (Dialux, Leitz) or with an epifluorescence microscope (BP 340-380, RKP 400, LP 430, Dialux 20, Leitz) after staining with 4',6'-diamidino-2-phenylindone (DAPI; Serva, Heidelberg, 18860) at 0.05 μg/ml for 10–15 min. Photographs were taken with Ektachrome tungsten-light film, Kodak. For electron microscopic analysis the fractions were fixed sequentially in 4% (vol/vol) glutaraldehyde in HAB and in 1% osmium tetroxide in 0.15 M sodium phosphate (pH 7.5), embedded in 1% agarose, and processed as described (9).

Isolation of Plastid DNA. Plastids were lysed in 10 mM Tris·HCl, pH 7.5/100 mM EDTA/1% N-lauroylsarcosine at 50°C for 10 min, treated with proteinase K at 100 μg/ml (Serva) overnight, and freed from proteins by phenol/chloroform, 1:1 (vol/vol), extraction. The aqueous phase was dialyzed against distilled water, and DNA was precipitated with ethanol. The nucleic acids were then digested with RNase A at 50 μg/ml (Sigma) in TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). The DNA was further purified by exclusion chromatography on a Sephadex LH-60 column (Pharmacia) in TE buffer at room temperature, precipitated with ethanol, resuspended in TE. Restriction endonuclease and S1-nuclease digestions were performed according to the recommendation of the manufacturers (Bethesda Research Laboratories). The samples were electrophoresed on a 0.5% or a 1% agarose gel in 2.5 mM sodium acetate, 20 mM Tris acetate, pH 7.8/0.1 mM EDTA at room temperature and 5 vol/cm. The gel was stained with ethidium bromide at 1 μg/ml, and the DNA was transferred to nitrocellulose (Schleicher & Schuell BAB5) according to Southern (12), with 20× SSPE (3.6 M NaCl/0.23 M sodium phosphate/0.21 M NaOH/0.02 M EDTA) as transfer buffer.

DNase Treatment of Plastids. Crude fractions of plastids were prepared by differential centrifugation as described but omitting polyvinylpyrrolidone in the homogenization medium. Pelleted plastids were resuspended in HAB, MgSO₄ was...

Abbreviations: AbMV, abutilon mosaic virus; ssDNA, single-stranded DNA; DAPI, 4',6'-diamidino-2-phenylindone.
added to 8 mM, and the plastids were treated with DNase I (50 μg/ml; Boehringer grade II) for 30 min on ice according to Herrmann (13). In some samples 1 μg of HindIII-digested phage λ DNA or 1 μg of phage M13 ssDNA was included to control the digestion efficiency. The reaction was stopped by washing the plastids two times with NE (0.15 M NaCl/100 mM EDTA, pH 8.0) before lysis and further DNA purification.

Alternatively plastids were purified by differential centrifugation, resuspended in HAB containing 8 mM MgSO4, and layered on top of the first Percoll gradient. DNase I was added as above, and the sample was incubated for 30 min at 0.5°C in the centrifuge rotor. After a subsequent centrifugation, fraction C was harvested, and DNA was purified.

**Thermolysin Treatment of Plastids.** Plastids of infected plants were prepared by differential centrifugation as described (omitting polyvinylpyrrolidone) and resuspended in 50 mM Hepes, pH 7.5/0.4 M sorbitol for treatment with thermolysin according to Cline et al. (14). Thermolysin concentrations from 20 to 500 μg/ml were tested. After stopping the reaction, the plastids were washed twice with HAB, treated with DNase I, and purified by Percoll-gradient centrifugation.

**Isolation of Virions and ssDNA of AbMV.** Virions from AbMV (7) were purified from infected Sida micrantha by two cycles of Cs2SO4-gradient centrifugation. As an internal standard for purity, 300 ng of λ DNA was added to each preparation. Then the virions were freed from any DNA or RNA possibly adsorbed to them by treatment sequentially with RNase A at 1 mg/ml of TE for 1 hr at 37°C and with DNase I at 100 μg/ml (Boehringer Mannheim) in 10 mM Tris-HCl, pH 7.5/10 mM MgCl2 for 1 hr at 37°C (7). The digestion efficiency was also controlled at 4°C. The reaction was stopped by adding NaDodSO4 to a final concentration of 0.1%, EDTA to 10 mM, ethidium bromide to 0.5 μg/ml, Ficoll to 2.5% (vol/vol), and bromphenol blue to 0.025% and heating the mixture to 95°C for 5 min. The incubation mixture was electrophoresed on a 1% agarose gel in 0.09 M Tris-HCl/0.09 M sodium borate, pH 8.0/2 mM EDTA including ethidium bromide at 0.5 μg/ml at 5 V/cm for 3 hr. AbMV DNA was then electrophoresed onto a NA45 filter (Schleicher & Schuell) and recovered from the filter as recommended by the manufacturer for ssDNA (15). Only full-length viral ssDNA was used for subsequent second-strand synthesis. This procedure with DNase I suggests that the ssDNA of virions is fairly well protected against nuclease attack.

**Hybridization.** Second-strand synthesis with this purified viral DNA was performed according to Maniatis et al. (16) in the presence of [32P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Labeled DNA (4 x 10⁶ dpm/ml) was used for molecular hybridization in 5x SSPE/50% (vol/vol) formamide at 42°C according to Maniatis et al. (16). The filters were washed four times in 2x SSPE/0.3% NaDodSO4 at 42°C for 1 hr. After drying at room temperature, Kodak X-Omat AR-5 film was exposed to the filter at −70°C.

**RESULTS**

To test for AbMV DNA in plastids these organelles were isolated under isonic conditions to maintain the envelope. Plastids were isolated from Abutilon sellovianum var. marmorata, which is an AbMV-infected ornamental Abutilon cultivar characterized by a yellow–green mosaic and from uninfected control plants. The plastid-damaging effect of tannins and polyphenoloxidases present in the plant homogenate was reduced by adding polyvinylpyrrolidone and dithioerythritol to the isolation medium. After differential centrifugation, purification of plastids was achieved by two cycles of Percoll-gradient centrifugation (13). In the first gradient usually three chlorophyll-containing bands of increasing density were formed, which are designated bands A, B, and C. Band A consisted of free nucleic acid and plastid debris, whereas band B contained slightly damaged, and band C contained intact plastids (Fig. 1). The latter two types of plastids were further purified by a second cycle of gradient centrifugation. Light micrographs of fraction C purified in this way are shown in Fig. 1. The plastids of uninfected plants have higher amounts of starch and chlorophyll than those of infected plants. On the other hand the plastids of infected plants contain a larger amount of DNA indicated by the enhanced DAPI fluorescence. The quenching by chlorophyll.

![Fig. 1. Light micrographs of plastids after purification through two Percoll-gradient centrifugations (fraction C). (Upper) Uninfected plants. (Lower) Infected plants. (Bar = 5 μM.) (Left) Interference micrographs. (Right) DAPI fluorescence.](image-url)
in control plants had a minor effect, since, after prolonged extraction with methanol, the differences in staining behavior remained unchanged (data not shown). Both bands were free of nuclei, nuclear fragments, or contaminating exogenous DNA as far as could be inferred from DAPI fluorescence.

Moreover, electron microscopic examination (Fig. 2) revealed that these fractions were free of mitochondria, and there were no virus-related structures that copurified with plastids. The ultrastructure of plastids from infected plants present in band C was similar to that of degenerated plastids, which are generally found in the yellow mosaic regions of the mesophyll (11, 17) (Fig. 2B). An accumulation of electron-dense material in these plastids was visualized by electron microscopy of thin sections stained with uranyl acetate and lead citrate (Fig. 2B Inset). However, the paracrystalline inclusions, which are typical of infected Malva parviflora (9), were not detected in Abutilon sellovianum var. marmorata.

The plastids from band B and C were lysed separately for DNA purification. The DNA was then electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized to the radioactively labeled complementary strand of AbMV DNA.

Virions of AbMV were purified by two cycles of CsSO₄-gradient centrifugation. To exclude the copurification of cellular DNA bound to virus particles, λ DNA was added to the virus preparation before DNase I and RNase A digestion. In this way it was possible to estimate the amount of contaminating exogenous DNA in the AbMV preparation after hybridization since λ DNA was also used as size standard in gel electrophoresis.

The AbMV DNA probe exclusively hybridized with a single DNA band of plastid fraction C (Fig. 3) that corre-
sponds to that of isolated ssDNA of AbMV. Neither control plastids from healthy plants (Fig. 3A) nor plastids of fraction B of infected plants (Fig. 3B) gave a hybridization signal under these conditions. We, therefore, conclude that only the intact plastids of fraction C from infected plants contain viral DNA.

The hybridizing DNA band was not digested by the restriction endonucleases EcoRI, Pst I, or BamHI but was by Hae III, which cuts ssDNA (Fig. 4). S1-nuclease treatment completely extinguished the hybridization signal. From these results we conclude that viral ssDNA was present in the plastids, whereas free viral double-stranded DNA was not detected in them.

Adsorption of naked AbMV DNA or AbMV virions to intact plastids was excluded by the results of the following experiments as well as by the fact that the hybridization signal was only found in a particular plastid fraction and was comparably low in the bulk DNA of fraction A. Virions or ssDNA adsorbed to the plastid envelope in vivo should have shown an AbMV DNA signal in plastid fractions A and B, which contain envelope fragments and plastids with broken envelopes, respectively. Viral DNA was found only in intact plastids (fraction C, Fig. 3B).

Adsorption of viral ssDNA to plastids was ruled out by DNase I treatment of plastids purified by differential centrifugation with or without subsequent Percoll-gradient purification (Figs. 5 and 6). In this partially purified preparation most of the AbMV-specific DNA was protected from degradation by DNase I (Fig. 5). The DNase I digestion was controlled by adding λ DNA to the plastids (Fig. 5).

| INFECTED |  
|--------|--------|--------|--------|--------|--------|
| S | EcoRI | Pst I | Bam HI | Hae III | S1 |
| C |        |        |        |        |      |

Fig. 4. Southern-blot hybridization of plastid DNA from plastid fraction C, separated on a 1% agarose gel after treatment with various restriction endonucleases and S1 nuclease. S, hybridization standard as in Fig. 3. S1-nuclease digestion confirmed the single-stranded conformation of the hybridizing DNA. Correspondingly, the DNA is only cut by Hae III. There is no indication that double-stranded DNA homologous to viral genomes is present in the plastids under these conditions.

Fig. 5. Southern-blot hybridization of a 1% agarose gel of partially purified plastid DNA after treatment with DNase I (lanes D) (procedure a) in the presence and absence of added λ DNA HindIII digest (lanes λ). The radioactive probe was viral second-strand DNA (virus probe), and the same filter was rehybridized with radioactive λ DNA (λ probe). λ, Unprotected viral DNA unspecifically adsorbed to chromosomal DNA; ss, viral ssDNA; kb, kilobase.

Rehybridization of the same blot with λ DNA further showed that the digestion of exogenous viral DNA was complete (Fig. 5). Mixing plastids with M13 ssDNA gave the same results. We have shown (7) that isolated AbMV ssDNA is degraded by DNase I.

We tested whether adsorption of virus particles or viral ssDNA to plastid membranes could occur by mixing plastids from uninfected plants with purified virions or viral ssDNA,
subsequently treating plastids with DNase I and purifying plastids by Percoll-gradient centrifugation. The Southern-blot hybridization using a viral DNA probe showed no signals from plastids purified as described (Fig. 6).

DNase I experiments have shown that there is no adsorption of viral ssDNA. Results of the mixing experiments were used to reject the possibility of adsorption of virions during the purification of plastids. The attachment of virions to plastid envelopes in vivo was probed with thermolysin, a protease that affects only the outer envelope membrane (14). Purified plastids from infected plants were treated with thermolysin, washed twice, incubated with DNase I, in case of released ssDNA, and purified by Percoll-gradient centrifugation (Fig. 6). There was no reduction in the ssDNA signal of plastids (fraction C) compared to plastids prepared in the same manner without thermolysin. The efficiency of DNase I digestion after thermolysin treatment was controlled with M13 ssDNA that was added to some plastid samples. Controls with partially purified virions confirmed that thermolysin digested viral coat protein effectively (data not shown).

**DISCUSSION**

From our hybridization experiments, we conclude that in infected plants AbMV DNA is present specifically in intact plastids (fraction C). Hybridization signals were not detected in fraction-B chloroplasts from infected plants or in fraction-B and -C chloroplasts from uninfected plants (Fig. 3). The hybridizing DNA has the same migration behavior as intact AbMV DNA; thus the DNA is protected against nuclease attack during plastid purification. The specificity of the hybridization probe was confirmed by Southern-blot experiments.

We were not able to show any attachment of virions or viral DNA to the plastid envelope. Adsorption of viral ssDNA to the envelope membrane in the leaf (in vivo) or during purification (in vitro) was ruled out by DNase I treatment of plastids from infected plants or mixing of viral ssDNA with uninfected plastids and subsequent DNase I digestion. The possibility of virions attachment in vitro was rejected as a result of the mixing experiment with plastids of control plants and virions. Finally an in vivo adsorption of virions to the plastid envelope was probed with the thermolysin experiment. ssDNA was detected at the same level as without thermolysin treatment. Therefore, we conclude that the AbMV ssDNA is present inside the plastid.

The intact fraction-C plastids of infected plants were also characterized by enhanced DAPI staining and a changed morphology. An increased DAPI binding was also found in infected mesophyll cells in situ, whereas it was absent from healthy control plants even in senescent leaves (18).

A central part of the reported work was the isolation of intact degenerated plastids. Ultrathin sections of leaf tissue had shown that plastids lose their lamellar structure and part of their stability (11, 17). This could explain why DNA from other geminiviruses was not found in these organelles, unless the AbMV is an exception.

The results support the report that intermediate nucleo-protein filaments, called chains of pearls, are localized in the plastids (8, 9). These structures accumulate and hence become detectable only in the plastids of *Malva parviflora*. We have now been able to localize the AbMV DNA in *Abutilon sellowianum* where the filaments do not form paracrystals (9). It is not surprising that neither gemini particles nor single filaments were recognized in the plastids of *Abutilon*, because they are easily confused with ribosomes or normal fibrillar matrix material. On the other hand, it is possible that different AbMV nucleoprotein complexes are generally formed in plastids and nuclei (9). Assuming that plastids are prokaryotic-like endosymbionts in the eukaryotic plant cell, the localization of AbMV DNA suggests similarities with f phages (19, 20), which are known to form complexes of phage DNA and single-stranded binding protein inside the cell and coated phage particles outside of the bacterial cell.

So far no nucleic acid from other viruses has been found in plastids, although Shikata (21) reported tobacco mosaic virus-like structures in these organelles. This result, however, was questioned by Rochon and Siegel (22) and Reiner and Beachy (23). Thus the present communication provides, to our knowledge, the first evidence of single-stranded viral DNA in plastids, which could be of central importance for the evolutionary understanding of the interaction of the genomes of the various compartments of the plant cell. Although there are difficulties in using AbMV as a vector (1), elucidation of the mechanism by which AbMV DNA crosses the plastid envelope will assist the development of a chloroplast transformation system.

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