Tobacco mosaic virus RNA enters chloroplasts in vivo
(stress/RNA transport)

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ABSTRACT Several lines of evidence are presented to allow us to conclude that tobacco mosaic virus (TMV) RNA enters the chloroplast in vivo. Chloroplasts were prepared from either directly inoculated or systemically infected leaves of tobacco plants inoculated with one of several strains of the virus and from uninfected control plants. Intact chloroplasts were isolated on Percoll gradients and treated with pancreatic RNase and thermolysin to destroy potential TMV virions and RNA on the outside or bound to their surfaces. Northern blot analysis of RNA extracted from these chloroplasts demonstrated that full-length TMV RNA was present within the chloroplasts prepared from both directly inoculated and systemically infected leaves. Only genomic length, but not subgenomic length, RNA was found in the chloroplast extracts, indicating a selectivity of the transport of the viral RNA into the chloroplast. A temperature-sensitive TMV mutant (Ts 38), in which no virions are formed at 35°C, was used to demonstrate that at this restrictive temperature viral RNA is detected in the chloroplast, indicating that free viral RNA can enter the chloroplast rather than intact virions. To our knowledge, the transport of a foreign RNA species into chloroplasts has not been reported previously.

Over many years, and in many laboratories, tobacco mosaic virus (TMV) has been shown to have an association with chloroplasts. As early as 1940, Kausche and Ruska (1) presented evidence that suggested that TMV virions were present within chloroplasts of infected plants. In 1958, Zaitlin and Boardman (2) isolated TMV virions from the chloroplast fraction of homogenates prepared from infected plants. Using the electron microscope, Esau and Cronshaw (3) published micrographs that showed that virus-like particles were present in chloroplasts of TMV-infected plants. They suggested that the particles had been produced in the plastids because the virus-like aggregates were free of host-cell components and were not surrounded by a membrane. In later studies, Shalla and coworkers (4, 5) confirmed that virus-like particles could be found in chloroplasts of both directly inoculated and systemically infected leaves. When tobacco plants were inoculated with TMV strain U1, 1% of the chloroplasts in inoculated leaves and 12% of the chloroplasts in systemically infected leaves contained virus-like particles (5).

Not all of the virus-like particles within chloroplasts contain TMV RNA, however. Shalla et al. (6) showed that most virus-like particles within chloroplasts are 100 nm in length, which is one-third the length of the TMV virion. Earlier Siegel (7) had demonstrated that host RNAs of chloroplast origin could be encapsidated with TMV coat protein to form pseudovirions. He has also shown that chloroplast DNA transcripts are preferentially encapsidated over nuclear DNA transcripts. Rochon and Siegel (8) have suggested that the encapsidation of chloroplast transcripts by TMV coat protein occurs within the chloroplast. It is therefore probable that the 100-nm rods observed by Shalla (6) in the chloroplast are pseudovirions. This is strengthened by the observation that TMV coat protein is present in chloroplasts (9).

Although it has been established that TMV coat protein (9) and virus-like rods (3) are present within chloroplasts of TMV-infected plants, the presence or absence of TMV RNA within chloroplasts has not been investigated. In this study, we demonstrate that TMV RNA is present in chloroplasts of infected plants. Our evidence also indicates that encapsidated TMV RNA, rather than whole virions, can enter chloroplasts. The DNA of abutlon mosaic virus, a geminivirus, has recently been isolated from intact chloroplasts (10), representing the only other example of a viral nucleic acid in chloroplasts.

MATERIALS AND METHODS

Inoculation of Plants. When working with either the cowpea (Cv.), U2, or the common (U1) strain of TMV, tobacco leaves (Nicotiana tabacum cv. Turkish Samsun) were inoculated with a 0.1 mg/ml solution of TMV and placed in a greenhouse. Six days after inoculation the plants were placed in the dark in order to reduce the amount of starch in the leaves. Chloroplasts were isolated 8 days after inoculation from either the systemically infected or directly inoculated leaves, as indicated. When working with the temperature-sensitive strain Ts 38 (11), chloroplasts were isolated 2 days after inoculation. In this case, plants were inoculated with a 0.1 mg/ml solution of either Ts 38 or U1 and then placed in a growth chamber at 35°C. One day after inoculation, the lights were turned off. Chloroplasts were then isolated 2 days after inoculation from the directly inoculated leaves.

Isolation of Chloroplasts and Extraction of Nucleic Acid. Chloroplasts were isolated following the procedure of Nivison et al. (12). Fifteen grams of tobacco tissue was chopped into small pieces with a razor blade and then transferred into 100 ml of cold grinding buffer (0.35 M sorbitol/0.05 M Hepes-KOH, pH 7.5/2.0 mM EGTA, pH 7.5/0.5 mM MgCl2/1 mM dithiothreitol/10 mg of bovine serum albumin per ml). The tissue was homogenized with a Polytron equipped with a PT 10 generator (Brinkmann) set at low speed for 20–30 sec and filtered through eight layers of cheesecloth. The filtrate was spun for 3 min at 1000 × g, the supernatant was discarded, and the chloroplasts were gently resuspended in 1 ml of resuspension buffer (0.35 M sorbitol/35 mM Hepes-KOH, pH 8.3/10 mM K2HPO4/0.5 mM MgCl2/1 mM dithiothreitol). The suspension was then layered on a 40%:85% (vol/vol) Percoll step gradient. The Percoll was suspended in 40 mM Hepes-KOH, pH 7.5/0.05 mM MgCl2/0.35 M sorbitol/1 mM dithiothreitol. The gradient was spun for 7 min at 13,000 × g. The top band of broken chloroplasts and the

Abbreviations: TMV, tobacco mosaic virus; L-RNA, genome length RNA; S-RNA, coat protein subgenomic RNA; L2-RNA, subgenomic for the 30-kDa transport protein.

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intervening Percoll layer were removed with a Pasteur pipet. The intact chloroplasts were collected, washed in 20 ml of resuspension buffer, and subsequently resuspended in 1.0 ml of resuspension buffer.

For extraction of nucleic acid from chloroplasts, the purified chloroplasts were incubated for 1 hr with 10 μg of RNase A per ml on ice and then spun at 1000 × g for 3 min. The supernatant was discarded and the pellet was gently resuspended in 2.2 ml of resuspension buffer. CaCl₂ was added to 0.5 mM and thermolysin (protease type X, Sigma) was added to 200 μg/ml (13). The solution was incubated on ice for 30 min and then EDTA was added to 1 mM. The chloroplasts were washed twice with 5 ml of resuspension buffer plus 1 mM EDTA. After the final washing, the chloroplasts were lysed by resuspension in 4.0 ml of 50 mM Tris-HCl, pH 7.6/2% SDS/2 mM EDTA. Nucleic acid was extracted with an equal volume of 1:1 (vol/vol) phenol/chloroform and then precipitated by addition of 0.1 volume of 3.0 M sodium acetate and 2 volumes of ethanol. The nucleic acid was then resuspended in 10 mM Tris-HCl/1 mM EDTA, pH 8.0.

Extraction of Nucleic Acid from Whole Plants. Nucleic acid was isolated from whole plants following the procedure of Dellaporta et al. (14).

Analysis of RNA Samples. RNA samples were electrophoresed in either a nondenaturing 1.8% agarose gel system following the TAE procedure of Palukaitis et al. in the experiment depicted in Fig. 1 (15) or a modified, formaldehyde denaturing gel system in the other experiments. In the modified, formaldehyde denaturing gel system, RNA samples (up to 3 μg in 10 μl) were first denatured by adding 10 μl of denaturing sample buffer (20 mM Mops, pH 7.0/5 mM sodium acetate/1 mM EDTA/50% deionized formamide/65% formaldehyde) and heating the sample for 10 min at 65°C. After addition of tracking dye, the samples were loaded onto a 1.2% agarose gel in 20 mM Mops-NaOH, pH 7.0/5 mM sodium acetate/1 mM EDTA and electrophoresed in the same buffer at 120 volts for ~1 hr. The RNA was transferred to nitrocellulose or to GeneScreen (DuPont), baked, prehybridized, and hybridized as described by Maniatis et al. (16). After hybridization, the filter was washed and exposed to Kodak XAR x-ray film as described by Palukaitis et al. (15).

Preparation of Probes. Random-primed 32P-labeled complementary DNA (cDNA) to TMV RNA was prepared by the method of Taylor et al. (17).

RESULTS

Full-Length TMV RNA Is Present Within Chloroplasts of Directly Inoculated and Systemically Infected Leaves. The first question that we wanted to investigate was whether TMV RNA could be isolated from chloroplasts of tobacco leaves systemically infected with TMV. Accordingly, we isolated total nucleic acid from three different chloroplast preparations. The first preparation consisted of nucleic acid from chloroplasts of tobacco leaves systemically infected with U2 TMV, the second consisted of nucleic acid from healthy leaf chloroplasts, and the third was a reconstruction control in which healthy chloroplasts were resuspended in a chloroplast free leaf homogenate from a TMV-infected leaf just before the chloroplasts were loaded onto the Percoll gradient. The reconstruction was performed as follows: after the initial low-speed centrifugation step the healthy chloroplasts were resuspended in the chloroplast-free, 1000 × g supernatant of the TMV-infected leaf homogenate and collected at 1000 × g. They were loaded immediately onto the Percoll step gradient, the resultant intact chloroplasts were treated with thermolysin and RNase, and RNA was extracted as described in Materials and Methods. The RNAs were separated on a 1.8% nondenaturing agarose gel for further analysis.

In the ethidium-stained gel, the TMV genomic length RNA (L-RNA) could be clearly seen in the total nucleic acid preparation from chloroplasts of systemically infected leaves (Fig. 1, lane 5). In contrast, no TMV RNA could be seen in either the healthy chloroplast preparation or the reconstruction control (Fig. 1, lanes 3 and 4). When the nucleic acids in the gel were transferred to nitrocellulose and probed with radiolabeled U2 cDNA, only TMV L-RNA was present in the chloroplast preparation from infected leaves (Fig. 1, lane 5'). None of the TMV subgenomic RNAs were present in the preparation. The Northern blot also confirmed that no TMV RNA was present in the healthy chloroplast preparation or in the reconstruction control (Fig. 1, lanes 3' and 4').

To demonstrate that TMV RNA was present within chloroplasts of directly inoculated leaves, we isolated total nucleic acid from U1-inoculated tobacco leaves and total nucleic acid from chloroplasts of directly inoculated leaves and healthy leaves. We also included a reconstruction control in which U1 virions and U1 RNA were added to healthy chloroplasts just before the chloroplasts were loaded onto the Percoll step gradient. The reconstruction was performed as follows: after the initial low-speed centrifugation step, the healthy chloroplasts were resuspended in 1.0 ml of resuspension buffer that contained 10 mg of TMV virions and 10 μg of TMV RNA. The chloroplasts were loaded immediately onto the Percoll step gradient, the resultant intact chloroplasts were treated with thermolysin and RNase, and RNA was extracted as described in Materials and Methods. The RNAs were separated on a 1.2% denaturing agarose gel for further analysis.

In this experiment the TMV L-RNA could not be detected in the ethidium-stained gel (data not shown). However, when the nucleic acids in the gel were transferred to GeneScreen and probed with radiolabeled U1 cDNA, TMV L-RNA could be detected in the chloroplast preparation from U1-inoculated leaves (Fig. 2, lane 2). Only the TMV L-RNA could be clearly identified. The coat protein subgenomic RNA (S-RNA) was not present; the other bands that appeared were electrophoro-

FIG. 1. Analysis of RNAs extracted from leaves and chloroplasts of leaves systemically infected with TMV strain U2. (Left) Ethidium bromide-stained nondenaturing 1.8% agarose gel. (Right) Northern blot of the transfer of the nucleic acids from the gel to nitrocellulose and probed with 32P-labeled cDNA prepared to U2 RNA. Lanes 1 and 1', RNA from strain U2 virions; lanes 2 and 2', RNA from virions of strain C (the arrow marks the position of migration of the subgenic coat protein mRNA); lanes 3 and 3', nucleic acid from chloroplasts of an infected leaf; lanes 4 and 4', chloroplast nucleic acid from reconstruction experiment; lanes 5 and 5', chloroplast nucleic acid from U2-infected leaves; lanes 6 and 6', nucleic acid from post-chloroplast supernatant of uninfected leaves; lanes 7 and 7', nucleic acid from post-chloroplast supernatant of U2-infected leaves.
Fig. 2. Northern blot of total nucleic acid from leaves and chloroplasts of U1-inoculated and healthy tobacco plants. RNAs were separated on a 1.2% denaturing agarose gel. The RNAs were subsequently transferred to GeneScreen and probed with randomly primed 32P-labeled cDNA made from U1 RNA. Lane 1, nucleic acid from a U1-inoculated leaf; lane 2, chloroplast nucleic acid isolated from a U1-inoculated leaf; lane 3, chloroplast nucleic acid isolated from a healthy leaf; lane 4, chloroplast nucleic acid isolated from a reconstruction control. Healthy chloroplasts were resuspended in resuspension buffer that contained 10 mg of TMV virions per ml and 10 μg of TMV RNA per ml prior to the Percoll gradient step.

Artifactual artifacts. It has been shown that artifactual hybridization bands will appear when partially degraded viral RNA is electrophoresed in the presence of plant rRNAs (15, 18). No TMV RNA could be detected in the healthy chloroplast preparation or in the reconstruction control (Fig. 2, lanes 3 and 4).

The results presented in Figs. 1 and 2 demonstrate that a small amount of full-length TMV RNA may be isolated from chloroplasts of directly inoculated and systemically infected leaves. The reconstruction controls included in each experiment rule out the possibility that TMV virions or RNA may co-purify with chloroplasts or that they might be attached to the surface of chloroplasts as a result of the isolation procedure.

Unencapsidated TMV RNA Is Transported into Tobacco Chloroplasts. The results given above demonstrate that TMV RNA is present in chloroplasts of infected leaves. A second question of importance was to ascertain if TMV RNA was being transported into chloroplasts as unencapsidated RNA or as RNA encapsidated in virions. For these experiments we used TMV mutant Ts 38, which is temperature sensitive for encapsidation. When Ts 38-inoculated plants are held at 20°C, the virus can encapsidate and move systemically. On the other hand, when plants are inoculated at 35°C, the virus will not encapsidate and systemic movement is blocked. The wild-type strain, U1, can encapsidate and move systemically at both temperatures.

Tobacco plants were inoculated with either Ts 38 or U1 and placed immediately in a plant growth chamber at the restrictive temperature of 35°C. Chloroplasts were isolated 2 days after inoculation rather than at 7 days because chloroplasts could not be isolated from leaves 7 days after inoculation with Ts 38. (Tobacco leaves inoculated with Ts 38 developed chlorotic lesions in 2–3 days and the chlorotic lesions gradually turned necrotic by 7 days. Chloroplast yields started to decrease at 3 days and by 7 days, no intact chloroplasts could be isolated from Ts 38-inoculated leaves. The necrosis induced by Ts 38 must be related to the temperature-sensitive defect because U1-inoculated leaves do not develop necrosis under high temperatures and Ts 38-inoculated leaves do not develop necrosis at the permissive temperature.) Fig. 3 is an autoradiogram that compares the amount of TMV RNA detected in chloroplast preparations of U1- and Ts 38-inoculated leaves to the amount of TMV RNA present in whole leaf extracts. The blot was hybridized with 32P-labeled randomly primed cDNA to U1 RNA. Small amounts of genome length U1 RNA and Ts 38 RNA could be detected in chloroplast preparations that were isolated 2 days after inoculation (Fig. 3, lanes 2 and 3). Total RNA extracts from U1- and Ts 38-inoculated leaves were included on the blot (Fig. 3, lanes 1 and 4). Although only a very small amount of viral RNA was detected in the chloroplast preparations at 2 days, Fig. 3 suggests that unencapsidated TMV RNA is transported into chloroplasts of infected leaves.

To confirm that Ts 38 does not form virions at 35°C, we tried to isolate virions from Ts 38-inoculated plants grown at the restrictive temperature. After the Ts 38-inoculated leaves were homogenized and the chloroplasts and nuclei were removed by low-speed centrifugation, a portion of the supernatant was subjected to ultracentrifugation at 200,000 × g for 1 hr to pellet any virions in the solution. The resultant pellet was resuspended in 0.5 ml of H2O and a sample was examined with an electron microscope. No virions were found, demonstrating that Ts 38 was temperature sensitive for encapsidation. A small amount of the resuspended pellet was also assayed for infectivity on Xanthi nc tobacco. Surprisingly, a few lesions formed on the inoculated leaves, indicating that there was a small proportion of some unexplained non-virion-associated infectivity in the preparation.

TMV RNA Encapsidated into Virions Is Not Transported into Tobacco Chloroplasts. To investigate the possibility that encapsidated TMV RNA may be transported into chloroplasts, we inoculated the C5 strain of TMV to tobacco, isolated chloroplasts from inoculated leaves and then determined the sizes of viral RNA present within the chloroplast. With most strains of TMV, only the full-length RNA and the larger subgenomic RNAs are encapsidated; the smallest subgenomic mRNA that codes for the viral coat protein is not encapsidated. On the other hand, the C5 strain of TMV differs from most other TMV strains in that all subgenomic RNAs are encapsidated during the replication process. In addition to the L-RNA, the S-RNA is encapsidated to form the S-rods, and the subgenomic RNA for the 30-kDa transport protein (I2-RNA) is encapsidated to form the I2-rod (19, 20). If it is the encapsidated form of the virus that enters the chloroplast,
then we would expect to find the S-RNA and I₂-RNA in chloroplasts in addition to the genome length RNA.

Fig. 4 is an autoradiogram that compares hybridization of cDNA prepared to Cₑ RNA to total RNA from a Cₑ-inoculated tobacco leaf and to total RNA from chloroplasts isolated from a Cₑ-inoculated leaf. The RNA blot was probed with ³²P-labeled randomly primed cDNA to Cₑ RNA. Two distinct viral bands were present in the preparation from whole leaves. The larger viral RNA band was the L-RNA, which was encapsidated to form the L-rod. The smaller viral RNA was the S-RNA, which was encapsidated to form the S-rod. The S-RNA could be detected with approximately the same sensitivity as the L-RNA in tobacco leaves. Other bands present in lane 1 are artifacts that form when partially degraded viral L-RNA is run in the presence of leaf rRNAs. The I₂-RNA cannot be identified in this blot because it comigrates with one of the artifactual bands. In contrast to results obtained with total RNA from whole leaves, only viral L-RNA is present in the total RNA preparation from purified chloroplasts. The S-RNA was not present in chloroplasts isolated from Cₑ-inoculated leaves, indicating that the encapsidated form of TMV does not enter chloroplasts.

**DISCUSSION**

In this study we have demonstrated that TMV L-RNA is present within chloroplasts. We could consistently isolate full-length TMV RNA from chloroplasts of infected leaves. A reconstruction control eliminated the possibility that the TMV RNA we detected was bound to the surface of the chloroplast as a result of the isolation procedure. A similar reconstruction experiment has been used to demonstrate that TMV coat protein is present within chloroplasts (9).

Results obtained with the mutant TMV isolate Ts 38 and the Cₑ strain of TMV indicate that unencapsidated TMV RNA is transported into chloroplasts. The mutant Ts 38 demonstrated directly that unencapsidated TMV RNA could be transported into chloroplasts. Conversely, results obtained with the Cₑ strain of TMV suggested that the encapsidated form of the virus does not enter chloroplasts. If the mechanism of transport involved whole virions, then both L- and S-RNAs would be expected to be present in chloroplast preparations. The fact that only the L-RNA was found suggests that virions are not transported and that chloroplasts may actually discriminate between L- and S-RNAs. Thus, S-RNA may lack the sequences required for transport into chloroplasts. An alternative explanation is that chloroplasts can distinguish between long and short rods and that a minimum length is required for transport into chloroplasts. We do not favor this explanation as it seems less likely to us that chloroplasts could distinguish between different sizes of virus rods.

The results in Fig. 4 also validate the purity of our chloroplast preparations. S-RNA could be detected in leaves with approximately the same sensitivity as the L-RNA. However, at the end of the chloroplast isolation procedure chloroplasts contained L-RNA were detected which, S-RNA, indicating that we had discriminated the L-RNA from the S-RNA.

The observation that TMV RNA is transported into chloroplasts may explain the presence of TMV coat protein in chloroplasts. Reinero and Beachy (9) have demonstrated that TMV coat protein could be isolated from chloroplasts of systemically infected leaves. However, they could not explain how the coat protein entered the chloroplast. The TMV coat protein probably is not translocated from the cytoplasm into chloroplasts in a manner similar to that known for the small subunit of ribulose bisphosphate carboxylase because it does not have a transit peptide sequence. Moreover, in preliminary experiments patterned on the optimal conditions established for the uptake of several proteins into isolated chloroplasts (21), we were unable to demonstrate that TMV coat protein was taken up by isolated chloroplasts. More experiments would be needed to establish this negative finding, but we consider it more likely that the coat protein in the chloroplast is translated from a TMV RNA template by chloroplast ribosomes.

The results of studies in two laboratories indicate that chloroplast ribosomes might efficiently translate the coat protein from the TMV RNA. Glover and Wilson (22) have demonstrated that the 17.5-kDa coat protein was produced from a TMV RNA template in a cell-free translational system derived from *Escherichia coli*. A search of the TMV RNA sequence showed that an idealized 70S ribosome-binding site (23, 24) occurred just upstream from the coat protein initiation codon. Camerino *et al.* (25) have shown that an in *vitro* translational system prepared from spinach chloroplasts employing TMV RNA as a messenger RNA will form a product that is similar in size to the coat protein. This important finding has not been confirmed to our knowledge, but it suggests that TMV coat protein could be synthesized on chloroplast ribosomes without the need for a subgenomic RNA as a messenger.

The possibility that the coat protein may be translated from TMV RNA within the chloroplast is also consistent with an observation made by Esau and Cronshaw (3). They stated that the aggregates of virus-like particles in their electron micrographs appeared to have been produced within the chloroplast because the grana surrounding the particles had been displaced. They suggested that this might be evidence of TMV replication in the chloroplast. Subsequent to their work it has been shown that TMV RNA is synthesized in the cytoplasm. The possibility that small amounts of TMV RNA could be replicated in the chloroplast cannot be completely ruled out, but the absence of subgenomic RNAs as demonstrated here would make that unlikely. However, part of the hypothesis of Esau and Cronshaw may still be valid; we have shown that unencapsidated TMV RNA can enter the chloroplast, and conceivably coat protein could be synthesized on chloroplast ribosomes. The coat protein may then encapsidate either TMV RNA to form virions or chloroplast transcripts to form pseudovirions. Attempts to synthesize coat protein "in organello" in chloroplasts from virus-infected leaves have been unsuccessful, however (M. Z., unpublished; ref. 26).

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