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Import of Tobacco Mosaic Virus Coat Protein into Intact Chloroplasts *In Vitro*

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Tobacco mosaic virus coat protein (TMV CP) has been found in chloroplasts. However, there is evidence that chloroplasts from infected plants do not synthesize CP under conditions in which active protein synthesis can be shown *in organello* (A. Reinero and R. N. Beachy, Plant Physiol. 89:111-116, 1989). Here, *in vitro*-labeled TMV CP is shown to be imported into isolated, intact chloroplasts even though it does not have a recognizable transit sequence. Within the chloroplast, some of the labeled CP is found as higher molecular weight forms. The import is not dependent on protease-sensitive outer membrane receptors, and it does not require ATP. The CP is imported very rapidly and is found almost exclusively in the thylakoid membrane fraction of the chloroplast.

Additional keyword: *Nicotiana tabacum*.

Tobacco mosaic virus (TMV) is a single-stranded plus-sense RNA plant virus. The 6,395-base U1 strain RNA molecule is encapsidated by about 2,130 copies of a single 17.4-kDa coat protein (CP) forming a helical virus particle. TMV-infected plants develop chlorotic symptoms that are the outward manifestation of unhealthy chloroplasts. TMV particles are known to be associated with chloroplasts. They have been observed in chloroplasts by electron microscopy (Shalla 1964; Milne 1966; Esau and Cronshaw 1967), and virions have been isolated from a chloroplast fraction by Zaitlin and Boardman (1958). However, Shalla *et al.* (1975) showed that most of the viruslike particles within chloroplasts are only one-third the length of the TMV particle; these viruslike particles are pseudovirions composed of host chloroplast transcripts encapsidated in CP (Siegel 1971; Rochon and Siegel 1984).

Reinero and Beachy (1986) demonstrated the presence of TMV CP in infected tobacco chloroplasts; most of the TMV CP was associated with the thylakoid membranes. They also showed that about 10- to 50-fold more CP accumulated in the chloroplasts of plants infected by a severe TMV strain than in plants infected by mild strains and postulated that this might have a bearing on the symptomatology. We have confirmed this correlation by using TMV strain U1 and mutant YSI/1 (mutant b6 in Garcia-Arenal *et al.* 1984), which induces a severe yellow mosaic. Recently, Reinero and Beachy (1989) measured electron transport rates in chloroplasts containing different levels of TMV CP and reported that photosystem II activity was inhibited in plants infected by a severe strain, but that no reduction was observed in plants infected by a mild strain.

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Using a temperature-sensitive mutant of TMV that does not form virions at a restrictive temperature, Schoelz and Zaitlin (1989) found genomic TMV RNA in the chloroplasts of infected plants. They were unable to show the presence of subgenomic viral RNAs. This implied that only the full-length genomic TMV RNA could enter the chloroplasts *in vivo* and raised the question of the site of synthesis of the chloroplast-associated CP, because CP is normally synthesized on a subgenomic mRNA (reviewed by Palukaitis and Zaitlin 1986). One possibility is that it is synthesized on chloroplast 70S ribosomes. Glover and Wilson (1982) pointed out that TMV RNA contains a Shine-Dalgarno-like sequence for prokaryotic ribosome association upstream from the start of the CP cistron. It has also been demonstrated that TMV CP can be synthesized on 70S ribosomes prepared from *Escherichia coli* (Migula) Castellani and Chalmers (Glover and Wilson 1982) or from spinach chloroplasts (Camerino *et al.* 1982) by using genomic TMV RNA as messenger. This would suggest that it is possible for TMV CP to be synthesized in the chloroplast from the genomic RNA. However, Reinero and Beachy (1989) failed to detect CP synthesis in chloroplasts prepared from virus-infected plants. We have also failed to detect CP synthesis *in organello*, although the chloroplasts were active in protein synthesis (unpublished).

The alternative explanation is that TMV CP is synthesized in the cytoplasm and imported into the chloroplast. Evidence supporting this hypothesis is provided by Osbourn *et al.* (1989). They found that most of the immunoreactive CP synthesized in tobacco plants transformed with the TMV coat protein gene was located in the chloroplast in electron-dense bodies. Because TMV RNA was absent, one can only conclude that TMV CP is imported into the chloroplasts post-translationally. This is unexpected, because TMV CP lacks a recognizable transit peptide normally found in most proteins imported into the chloroplast (Karlin-Neumann and Tobin 1986; Flügge 1990). There are only two known exceptions. Salomon *et al.* (1990) have shown import of a 6.7-kDa chloroplast outer envelope poly-

peptide that lacks a cleavable transit sequence. More recently, Li *et al.* (1991) have shown that a 14-kDa outer envelope polypeptide also lacks a cleavable transit sequence and is imported into isolated chloroplasts.

Our *in vitro* studies show that TMV CP is rapidly imported into isolated chloroplasts and is found almost entirely in the thylakoid membrane fraction. This supports the hypothesis of *in vivo* import from the cytosol as opposed to synthesis within the chloroplast.

MATERIALS AND METHODS

Materials. The common (U1) strain and strain U2 (also known as tobacco mild green mosaic virus) of TMV were used in this study (Siegel and Wildman 1954). Chloroplasts were isolated from *Nicotiana tabacum* L. 'Turkish Samsun'. The cDNA clone for pea ribulose bisphosphate carboxylase pre-SSU, pSP64/SSU (termed pre-SSU in this report), was provided by Catherine Wasmann (University of Arizona, Tucson). A cDNA clone containing the chloramphenicol acetyl transferase (CAT) gene was provided by David Sleat (Cleveland Clinic, Ohio).

***In vitro* transcription-translation.** For *in vitro* transcription of linearized plasmids, we utilized SP6 polymerase according to the instructions of the manufacturer (Promega, Madison, WI). *In vitro* translations of the transcribed RNA were performed in a wheat germ cell-free extract, according to the instructions of the manufacturer (Promega). Translations were carried out for 60 min at 40° C. About 30 μCi of [^{3}H]leucine at a specific activity of 143 Ci/mmole (obtained from Amersham, Arlington Heights, IL) was used in a reaction volume of 50 μl. We stopped incorporation of the [^{3}H]leucine by chilling the reaction mixture on ice and adding an equal volume of 2× import buffer (0.1 M HEPES-KOH, pH 8.0, containing 0.66 M sorbitol) and unlabeled leucine to a final concentration of 30 mM.

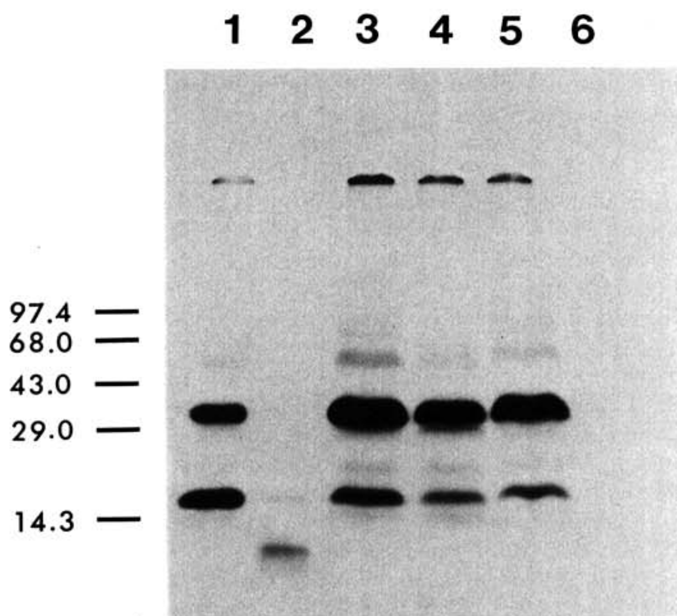

Fig. 1. Import of labeled tobacco mosaic virus coat protein (TMV CP) into intact or broken chloroplasts. Radiolabeled TMV CP (10^6 cpm) was incubated with isolated tobacco chloroplasts (100 μg of chlorophyll) for 5 min. After incubation, the chloroplasts were reisolated and either treated or not treated with thermolysin. The imported polypeptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, *in vivo*-labeled CP used in this import assay; lane 2, labeled CP digested with thermolysin; lanes 3 and 4, proteins from intact chloroplasts incubated with CP and, after reisolation, either not treated (lane 3) or treated with thermolysin (lane 4); lanes 5 and 6, proteins from broken chloroplasts incubated with CP and, after reisolation, either not treated (lane 5) or treated with thermolysin (lane 6). The positions of molecular weight markers (in kilodaltons) are indicated. Proteins from chloroplasts equivalent to 10 μg of chlorophyll were loaded on each lane.

Preparation of *in vitro*-labeled CP. Tobacco plants were inoculated on all leaves, on both sides, with TMV (U1) at a concentration of 0.1 mg/ml in 50 mM phosphate buffer, pH 7.0, containing Celite as an abrasive. The plants were grown for an additional 2 days at 25° C in a growth chamber. About 3 g of the directly inoculated leaves was cut laterally into 1-mm slices, and a solution containing 0.1 mCi of a [^{14}C]amino acid mixture in 3.0 ml of 50 mM KH_2PO_4 was vacuum-infiltrated into the leaves. This mixture of 15 amino acids, each ranging from 111 to 512 mCi/mmol, was obtained from New England Nuclear, Boston, MA (NEC-445E). The leaves were incubated at 23° C under dim light for 1 day before being harvested, and virus was prepared by a conventional differential centrifugation procedure. The virus was degraded with acetic acid, and the CP was isolated as described by Fraenkel-Conrat (1957). The ^{14}C-CP (at 10 mg/ml) was dialyzed against sodium phosphate buffer (pH 7.0, ionic strength 0.1) at 4° C and then stored at 4° C for a minimum of 2 days before use. Under these conditions, the CP was principally in the form of the 4S A-protein (Butler 1974). When analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), initially the CP migrates to the position of the CP monomer. But as the CP preparation ages, the proportion of CP migrating as dimers and trimers increases (see Fig. 1, lane 1 compared with Fig. 2, lane 1). This phenomenon is also regularly observed with stored *in vitro*-translated TMV CP.

Isolation of chloroplasts and determination of protein import. Chloroplasts were isolated, and intact chloroplasts were purified on a Percoll gradient as described by Fish and Jagendorf (1982). The intact chloroplasts were washed with 3 vol of import buffer and then were resuspended in import buffer at a concentration of 1 mg/ml of chlorophyll. Chloroplasts that were treated with nigericin before protein import were prepared as described by Cline *et al.* (1985). The import of pea pre-SSU into tobacco chloroplasts was performed as described by Cline *et al.* (1985) at 23° C under 550 $\mu E \cdot m^{-2} s^{-1}$ incandescent white light. In preliminary experiments, the import of TMV CP was performed under the same light conditions, but after light was found to be nonessential, subsequent import reactions (reported herein) were carried out on the lab bench. Each import reaction contained chloroplasts equivalent to 100 μg of chlorophyll, import buffer, and 10^6 cpm of labeled protein. For reactions with broken chloroplasts, we broke intact chloroplasts by vortexing them vigorously. Repurified chloroplasts from the import of pre-SSU were treated with thermolysin as described (Cline *et al.* 1985). Because TMV CP in the form of A-protein was found to be insen-

sitive to digestion by thermolysin at 4° C, this post-import step was performed for 15 min at 23° C for chloroplasts incubated with CP (Fig. 1). This step was omitted in the experiments depicted in the remaining figures. For analysis of the imported proteins, the chloroplasts were washed twice with import buffer containing 5 mM EDTA, centrifuged in a microfuge at 15,500 × *g* for 10 s, and dissolved in SDS gel-loading buffer containing 10 mM EDTA. The *in vitro* translation products, the *in vivo*-labeled proteins, and the imported proteins were analyzed by gel SDS-PAGE through 15% gels with a 5% stacking gel and the buffer system described by Laemmli (1970). Aliquots of the import reaction equivalent to 10 μg of chlorophyll were loaded on the gel. The samples were boiled for 5 min before loading. Fluorography was performed as described in Dunigan and Zaitlin (1990). Preflashed X-ray films were used.

Chloroplast membrane fractionation. Fractionation of chloroplasts into inner envelope, outer envelope, and thylakoid membranes was as described by Salomon *et al.* (1990). The chloroplasts were incubated on ice for 10 min in 0.6 M sucrose and then subjected to three freeze-thaw cycles. The membrane fractions were collected from a sucrose-density step gradient as described by Block *et al.* (1983).

RESULTS

Rapid import of TMV CP into isolated chloroplasts. The first question was whether TMV CP could be imported into isolated chloroplasts *in vitro*. *In vivo*-labeled TMV CP was incubated with intact or broken chloroplasts in a standard protein import assay at 23° C for 5 min. We stopped the reaction by transferring the reaction tubes to ice and diluting the mixture with cold import buffer. The

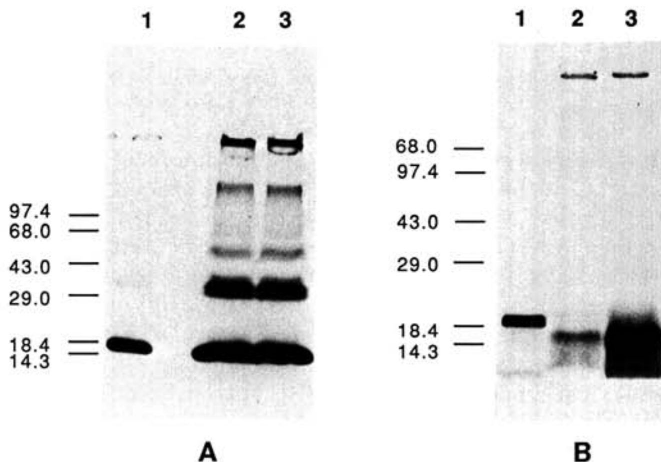

Fig. 2. Time points of import of radiolabeled tobacco mosaic virus coat protein (TMV CP) and pre-SSU. Radiolabeled TMV CP or pre-SSU (10^6 cpm) was incubated with isolated chloroplasts (100 μg of chlorophyll) for either 1 min or 60 min. The import reactions were stopped as described (see text), and the imported proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1 in **A** and **B**, *in vivo*-labeled CP and *in vitro*-labeled pre-SSU, respectively, that were used in the import assay; lane 2 in A and B, proteins from chloroplasts after 1 min of import in the presence of CP and pre-SSU, respectively; lane 3 in A and B, proteins from chloroplasts after 60 min of import in the presence of CP and pre-SSU, respectively. The positions of molecular weight markers (in kilodaltons) are indicated. Proteins from chloroplasts equivalent to 10 μg of chlorophyll were loaded on each lane.

chloroplasts were recollected by centrifugation and either treated or not treated with thermolysin. The imported polypeptides were then analyzed by SDS-PAGE (Fig. 1).

Lane 1 in Figure 1 shows the *in vivo*-labeled, purified CP used in the experiment. The monomer of 17.4 kDa can be clearly seen. In addition, higher molecular weight polypeptides are present. These are oligomers of TMV CP, analogous to those observed in purified TMV CP by Hodgson *et al.* (1989), both by a gel stained with Coomassie blue and by Western blotting with antibodies raised against purified TMV CP. It is unclear why these higher aggregation states are not dissociated with SDS and heat. The CP polypeptides are digested on incubation with thermolysin (Fig. 1, lane 2). Lanes 3 and 5 in Figure 1 show that the CP polypeptides are associated with intact and broken chloroplasts, respectively. These are the same size as the *in vivo*-labeled, purified CP (Fig. 1, lane 1). The CP polypeptides that are associated with intact chloroplasts are resistant to thermolysin treatment (Fig. 1, lane 4), whereas the polypeptides that are associated with broken chloroplasts are completely digested by thermolysin (Fig. 1, lane 6). Further, on association with chloroplasts, there is an enrichment of the CP dimer with respect to the monomer.

To assess the time required for import of TMV CP, we took two time points of the import reaction. We preincubated isolated chloroplasts at 23° C for 15 min and then added radiolabeled ^{14}C-CP or ^{3}H-pre-SSU. We stopped the incubation reactions after 1 min (Fig. 2A, lane 2; 2B, lane 2) or 60 min (Fig. 2A, lane 3; 2B, lane 3) by transferring the reaction tubes to ice and diluting them with import buffer. The imported polypeptides were again analyzed by SDS-PAGE. It can be seen that TMV CP is imported very quickly. The intensity of the various CP-related bands is the same for the 1-min time point (Fig. 2A, lane 2) as for the 60-min time point (Fig. 2A, lane 3). This is not so for the pre-SSU, which is imported much more slowly (Fig. 2B, lanes 2 and 3). In contrast, the imported SSU (Fig. 2B, lanes 2 and 3) is smaller than the *in vitro*-labeled pre-SSU (Fig. 2B, lane 1), indicating the expected cleavage of the transit peptide from pre-SSU (Chua and Schmidt 1978). Finally, the *in vitro*-radiolabeled protein product of the CAT gene transcripts was not imported into isolated chloroplasts under identical conditions, confirming the specificity of import (data not shown).

The results presented in Figures 1 and 2 show that TMV CP is rapidly imported into isolated chloroplasts without the cleavage of a transit peptide. This is in contrast to the slower import of pre-SSU, which involves the cleavage of a transit peptide.

To demonstrate that the signal was not due to some CP aggregating and pelleting with the chloroplasts, we repurified the intact chloroplasts on a 40–85% Percoll gradient after import. The radiolabeled CP co-purified with the chloroplasts at the expected 40–85% Percoll interface (data not shown). When radiolabeled CP alone was loaded, all the counts were found in the buffer above the 40% Percoll, and none were found at the 40–85% Percoll interface (data not shown).

Imported TMV CP associated with thylakoid membranes. A preliminary experiment (not shown) indicated

that most of the imported radiolabeled CP went to the chloroplast membrane fraction. To determine which membrane component the imported CP associates with in the chloroplast, we incubated radiolabeled CP with isolated chloroplasts under standard conditions of protein import. The chloroplasts were subsequently incubated on ice for 10 min in 0.6 M sucrose, subjected to three freeze-thaw cycles, and the membranes were fractionated on a sucrose-density gradient (Salomon *et al.* 1990). The imported CP was located exclusively in the thylakoid membrane fraction (Fig. 3A, lane 4). No CP was found in the inner or outer envelope membrane fractions (Fig. 3A, lanes 2 and 3, respectively). Lane 1 in Figure 3A shows the polypeptides imported into unfractionated chloroplasts. To show that the protocol we used fractionated the chloroplast membranes successfully, we repeated the experiment and ran aliquots of the different membrane fractions on a gel and stained with Coomassie brilliant blue R (Fig. 3B). About 40 μg of protein was loaded in each lane except for lane 2, in which the entire outer envelope fraction was loaded. Protein patterns diagnostic for the different membrane fractions (Cline *et al.* 1981; Cline *et al.* 1984) were obtained (Fig. 3B, lanes 1–4).

Further, to show that TMV CP is not nonspecifically associated with any membrane, radiolabeled CP was incubated with isolated tobacco protoplasts for 30 min at 23° C under standard conditions for import. We stopped the incubation reactions by diluting them with import buffer. The protoplasts were reisolated by centrifugation, and the polypeptides associated with protoplasts were analyzed by SDS-PAGE. No CP was associated with the protoplasts (data not shown).

To determine whether the import of TMV CP was mediated via protease-sensitive receptors in the outer envelope, we pretreated isolated chloroplasts with 100 μg/ml of thermolysin at 4° C. The chloroplasts were pelleted, washed, and then incubated with radiolabeled CP. No effect on CP import was noticed (Fig. 4, lane 5).

To determine whether ATP was required for import, we preincubated the isolated chloroplasts either in the dark (Fig. 4, lane 3) or in the light (Fig. 4, lane 2) at room temperature for 30 min. Radiolabeled CP was then incubated with the chloroplasts in a standard import assay in the dark (lane 3) or light (lane 2). The chloroplasts were subsequently pelleted and assayed for import as described in Materials and Methods. Again, no effect on CP import was noticed. Because nigericin is an uncoupler of photophosphorylation, it inhibits light-driven (ATP-requiring) import of proteins such as pre-SSU into isolated chloroplasts (Cline *et al.* 1985). But it has no effect on TMV CP import (Fig. 4, lane 4), indicating that the import of TMV CP does not require ATP.

DISCUSSION

In this study, we have demonstrated that TMV CP is imported very rapidly into isolated chloroplasts *in vitro*. The results show that the imported CP is located almost

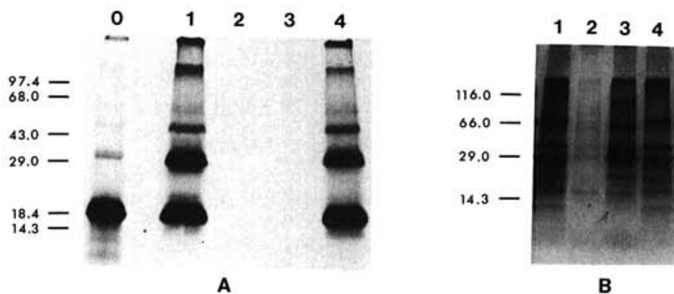

Fig. 3. Location of tobacco mosaic virus coat protein (TMV CP) in the thylakoid membrane fraction. Radiolabeled CP (10^6 cpm) was incubated with isolated chloroplasts (100 μg of chlorophyll) for 5 min. The chloroplasts were pelleted, subjected to three freeze-thaw cycles in 0.6 M sucrose solution, and the membranes were fractionated on a sucrose-density gradient. The different membrane fractions collected from the gradient were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was either **A**, dried and subjected to autoradiography or **B**, stained with Coomassie blue. A, Lane 0 contains *in vivo*-labeled CP used in these assays; lane 1 in A and B, total polypeptides from unfractionated chloroplasts; lane 2 in A and B, proteins from the outer envelope fraction; lane 3 in A and B, proteins from the inner envelope fraction; and lane 4 in A and B, proteins from the thylakoid membrane fraction. The positions of molecular weight markers (in kilodaltons) are indicated. Proteins from chloroplasts equivalent to 10 μg of chlorophyll were loaded on each lane of A.

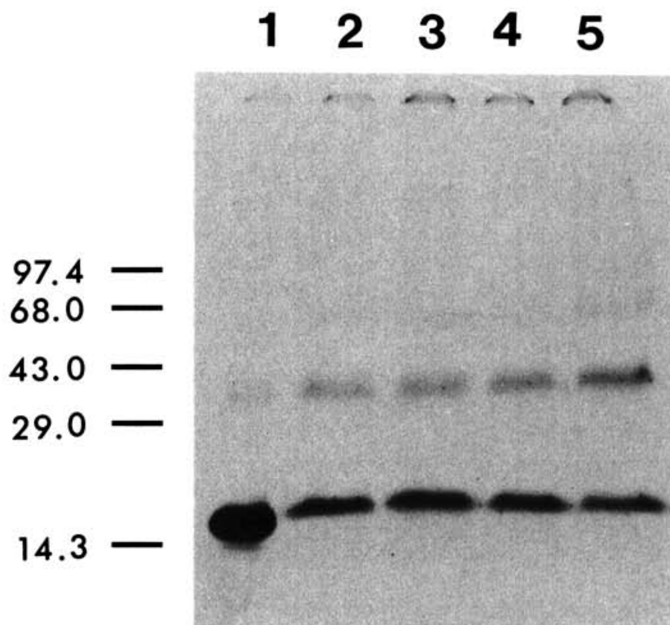

Fig. 4. The effects of light, dark, nigericin, and protease pretreatment on the ability of isolated chloroplasts to import tobacco mosaic virus coat protein (TMV CP). Isolated tobacco chloroplasts (100 μg of chlorophyll) were pretreated under different conditions (see below) for 30 min. Radiolabeled TMV CP (10^6 cpm) was then incubated with the chloroplasts for 5 min in the light (lanes 2, 4, and 5) or in the dark (lane 3). The import reactions were stopped as described (see text), and the imported proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, *in vivo*-labeled CP used in this import assay; lane 2, proteins from chloroplasts preincubated in the light; lane 3, proteins from chloroplasts preincubated in the dark; lane 4, proteins from chloroplasts preincubated in the light in the presence of 400 nM nigericin; lane 5, proteins from chloroplasts preincubated in the light in the presence of 100 μg/ml of thermolysin. The positions of molecular weight markers (in kilodaltons) are indicated. Proteins from chloroplasts equivalent to 10 μg of chlorophyll were loaded on each lane.

entirely in the thylakoid membranes of the chloroplasts, indicating that the *in vitro* import behavior corresponds to that found *in vivo* in infected plants. Reinero and Beachy (1986) showed that *in vivo*, the majority of the CP in chloroplasts from infected plants was associated with the thylakoid membranes. They also demonstrated the presence of several high molecular weight polypeptides in the thylakoid membranes which, in addition to the CP monomer, immunoreacted with CP antibodies. These were thought to be oligomers of CP because of their appropriate molecular weights and also because analogous bands were found in CP prepared from purified TMV. We have found similar high molecular weight polypeptides in addition to the CP monomer present in the thylakoid membrane fraction after import into isolated chloroplasts *in vitro*. Furthermore, the degradation products of the *in vivo*-labeled CP are not imported (faint bands below the principal CP band in Fig. 3, lane 0 compared with the import in lanes 1 and 4). This lack of import of the degradation products is reproducible and has been observed several times very clearly.

CP from TMV strain U2 was also rapidly imported into isolated chloroplasts (data not shown). Again, higher molecular weight polypeptides in addition to the CP monomer were associated with the chloroplasts.

TMV CP appears to follow a different import pathway into chloroplasts than the one taken by most chloroplast-targeted proteins, of which the pre-SSU is an example. Typically, the precursor first binds to a protease-sensitive receptor on the outer membrane. It is then translocated across the membrane, and the N-terminal transit sequence is cleaved by a stromal peptidase. This translocation step depends on ATP hydrolysis and takes place either in the light or in the dark in the presence of ATP. TMV CP has no cleavable transit sequence because the molecular weight of the CP isolated from chloroplasts and cytoplasm is the same. It does not require a protease-sensitive receptor for import into chloroplasts. The import is also independent of ATP. These import properties resemble those of the 6.7- and 14-kDa outer chloroplast envelope polypeptides studied by Salomon *et al.* (1990) and Li *et al.* (1991), respectively. Neither has a cleavable transit sequence, and both are inserted into the outer chloroplast envelope in the absence of ATP. Further, their import does not require a thermolysin-sensitive component on the chloroplast surface. However, TMV CP is the first example of a protein being imported into the thylakoid membrane in the absence of a cleavable transit sequence and of ATP hydrolysis.

The rapid import of TMV CP into isolated chloroplasts is consistent with the fact that it does not require protease-sensitive receptors in the outer envelope. Receptor-mediated import of proteins such as pre-SSU occurs much more slowly. Further, TMV CP is imported into broken chloroplasts. This would suggest that its uptake is a passive phenomenon involving some kind of CP-membrane interaction and is consistent with the fact that no ATP is required. The mechanism of this interaction should prove interesting because TMV CP is a predominantly hydrophilic protein with no major hydrophobic regions capable of traversing a membrane. This is unlike the 6.7- and 14-kDa outer chloroplast envelope proteins, both of which are highly hydrophobic proteins capable of transversing membranes. That neither the translation product of the CAT gene nor the degradation products of the CP are imported into chloroplasts and the CP does not associate with the plasma membrane of protoplasts suggest there is some specificity to the import of TMV CP into the chloroplasts. Finally, it is unclear whether the enhanced proportion of the higher molecular weight CP polypeptides associated with chloroplasts is due to more efficient import of higher aggregation states of CP or due to protein-protein interactions between large levels of CP monomer being imported and accumulating at the thylakoid membrane.

It has been suggested that TMV CP plays a role in the development of disease symptoms. Lindbeck *et al.* (1991) showed that mutants of TMV with nonfunctional CP that cause weak yellowing symptoms had no observable effect on chloroplasts, whereas CP mutants that induce chlorosis caused significant degradation. Immunocytochemical localization suggested that the altered CP was located outside the chloroplasts. However, in nonmutant TMV, virions have been found to be associated within the chloroplast (Esau and Cronshaw 1976). Most of these are actually pseudovirions. Different TMV strains differ in the amount of pseudovirions found in chloroplasts. Many more pseudovirions are found in the mild strains U5 and U2 than are found with severe strains. So, it could be argued that the mere presence of CP inside the chloroplasts is not related to symptom expression. However, the crucial point for symptomatology may be how much CP is associated with the thylakoid membranes rather than how many pseudovirions are present in the stroma.

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