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Different *in Vitro* and *in Vivo* Targeting Properties of the Transit Peptide of a Chloroplast Envelope Inner Membrane Protein*

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The triose phosphate 3-phosphoglycerate phosphate translocator (TPT) is a chloroplast envelope inner membrane protein whose transit peptide has structural properties typical of a mitochondrial presequence. To study the TPT transit peptide in more detail, we constructed two chimeric genes encompassing the TPT transit peptide and either 5 or 23 amino-terminal residues of the mature TPT, both linked to the reporter chloramphenicol acetyltransferase (*cat*) gene. The precursors were synthesized *in vitro* and translocated to and processed in purified plant mitochondria. However, this import was not specific since both precursors were also imported into isolated chloroplasts. To extend this analysis *in vivo*, the chimeric genes were introduced into tobacco by genetic transformation. Analysis of CAT distribution in subcellular fractions of transgenic plants did not confirm the data obtained *in vitro*. With the construct retaining only 5 residues of the mature TPT, CAT was found in the cytosolic fraction. Extension of the TPT transit peptide to 23 residues of the mature TPT allowed specific import and processing of CAT into chloroplasts. These results indicate that, despite its unusual structure, the TPT transit peptide is able to target a passenger protein specifically into chloroplasts, provided that NH_2-terminal residues of the mature TPT are still present. The discrepancy between the *in vitro* and *in vivo* data suggests that the translocation machinery is more stringent in the latter case and that sorting of proteins might not be addressed adequately by *in vitro* experiments.

Mitochondria and chloroplasts have their own genetic information. However, these endosymbionte-derived genomes have a limited coding capacity, as most of their genes were transferred to the nucleus during evolution. The majority of the organellar proteins are thus nuclear-encoded and synthesized in the cytosol as larger precursor forms. The latter usually have an amino-terminal targeting sequence (called presequence for the mitochondria and transit peptide for the chloroplast), which is responsible for directing transport of the precursors to their respective organelles (1–3).

The mitochondrial and chloroplast targeting signals have some intriguing structural similarities. Both are rich in basic and hydroxylated amino acids and usually lack acid and apolar amino acid residues (4, 5). However, their predicted secondary structures differ. Whereas mitochondrial presequences are predicted to form an amphiphilic α-helix, chloroplast transit peptides display a random coil structure (6, 7). As a consequence, the *in vivo* import process is assumed to be specific to each organelle. This has been observed experimentally (2, 8–10), indicating that protein import into mitochondria and chloroplasts follows different routes.

An unusual situation is observed with the chloroplast triose phosphate 3-phosphoglycerate-phosphate translocator (TPT),[1] a protein of the chloroplast envelope inner membrane. This transporter is encoded in the nucleus and synthesized as a higher molecular weight precursor in the cytosol. It bears an NH_2-terminal transit peptide, which is assumed to direct transport across the envelope membrane (11). Recently it was shown by *in vitro* import studies that the transit sequence contains only stromal targeting information and that a hydrophobic region of the NH_2-terminal of the mature TPT might be responsible for directing the protein to the inner envelope membrane (12, 13). Several phosphate translocator genes have been isolated from various species. Analysis of the structures predicted for the various transit peptides reveals, in the majority of cases, the presence of a positively charged amphiphilic α-helix in the NH_2-terminal region (14, 15). Interestingly, this feature is typical of mitochondrial presequences. Consistent with this observation is the report that the phosphate translocator is imported and processed in mitochondria isolated from yeast and broad bean (16). However, these data did not prove that the TPT transit peptide was sufficient for *in vitro* mitochondrial uptake. Moreover, the hydrophobic nature of TPT made its analysis more difficult as, in *in vitro* uptake assays, the mitochondrial outer membrane is the only hydrophobic environment offered to the TPT precursor. The paradox is thus that the TPT is imported *in vitro* both in chloroplasts and in mitochondria, but that, *in vivo*, the TPT localization is the chloroplast only. This suggests that *in vivo* either TPT is only targeted to chloroplasts or that it is targeted to both organelles and destroyed in mitochondria. To resolve this conflict, we

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[1] The abbreviations used are: TPT, triose phosphate 3-phosphoglycerate-phosphate translocator; CAT, chloramphenicol acetyltransferase; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

prepared two chimeric genes linking the TPT translocator transit peptide and either 5 or 23 residues of the NH_2-terminal mature protein, to chloramphenicol acetyltransferase (CAT), a soluble reporter protein. We show here that the TPT transit peptide can direct transport of CAT into both isolated chloroplasts and mitochondria. However, when the same constructs were tested in transgenic plants, CAT was found mainly in the cytosol or in the chloroplast depending on whether the TPT transit peptide was followed by 5 or 23 residues of the mature TPT. These results question the *in vitro* approach to studying specificity of targeting. They also indicate that the TPT transit peptide requires residues of the amino part of the mature TPT for specific *in vivo* targeting to the chloroplast.

EXPERIMENTAL PROCEDURES

Gene Construction—Standard procedures were used for DNA manipulations (17). The constructs assembling the TPT transit peptide and CAT were made as follows. The pSP72 plasmid carries a cDNA fragment corresponding to the 5′-noncoding region, the entire transit peptide, and 42 amino acids from the mature spinach phosphate translocator (11). This fragment was released by *Hin*dIII and *Bgl*II digestion. Two 3′-shortened fragments were obtained by polymerase chain reaction. The first fragment (TPT5) corresponds to the entire TPT transit peptide and 5 amino acids from mature TPT; the second (TPT23) comprises the whole TPT transit peptide and 23 amino acids from mature TPT. Synthetic primers provided with flanking *Hin*dIII sites were as follows.

The TPT5 and TPT23 upstream primer was 5′- CCCCCAACTTCTTCCGACAATGGAG.

The TPT5 downstream primer was 5′-CCCCCAAGCTTCCAGAGCCACTAGCGGC.

The TPT23 downstream primer was 5′-CCCCCAAGCTTCCGCCAGTGACAAGAGC.

After polymerase chain reaction amplification, the fragments were digested with *Hin*dIII and cloned into the *Hin*dIII site of the polylinker region of SK(+) Bluescript (Stratagene), resulting in the TPT5 and TPT23 plasmids, respectively. Both constructs were sequenced.

The CAT encoding sequence was isolated by *Hin*dIII and *Bam*HI digestion of plasmid pBin35ScatE9′ (18) and inserted into the corresponding sites of SK(+) Bluescript, resulting in the CAT plasmid. The *Hin*dIII site previously used for inserting targeting sequences (8, 18) is localized 75 nucleotides upstream of the CAT translation initiation codon. When translated, this region encodes a cryptic mitochondrial cleavage site (18). Using polymerase chain reaction, therefore, we engineered a new CAT gene provided with a *Hin*dIII site 10 nucleotides upstream of the CAT translation initiation codon. Primers were as follows.

The upstream primer was 5′-CCCCCAAGCTTGGAAGCTAAAATGGAG.

The downstream primer was 5′-CCATACGGAATTCCGG.

This new construct was checked by sequencing.

To prepare the TPT-CAT constructs, the TPT5 and TPT23 fragments were obtained by *Hin*dIII digestion and cloned at the *Hin*dIII site of the modified CAT plasmid digested previously with *Hin*dIII, resulting in TPT5-CAT and TPT23-CAT.

TPT-CAT constructs for tobacco transformation were prepared as follows. The TPT5-CAT and TPT23-CAT plasmids were digested with *Bam*HI and partial *Hin*dIII, releasing the fragments TPT5-CAT and TPT23-CAT. These fragments were inserted into the corresponding sites of Bin35ScatE9′ (18), digested previously with *Hin*dIII and *Bam*HI, producing the plant transformation vectors Bin35S-TPT5-CAT and Bin35S-TPT23-CAT, respectively.

In Vitro Transcription-Translation—The plasmids TPT5-CAT and TPT23-CAT were linearized with *Bam*HI. Transcription was carried out in the presence of 7mGpppG using T7 RNA polymerase (19). Translation was performed in a reticulocyte lysate system (Boehringer Mannhein) in the presence of [^{35}S]methionine (Amersham).

In Organello Import—In vitro import reactions were performed with broad bean mitochondria isolated from dark-grown hypocotyls as described previously (19), except that 1% bovine serum albumin was added to the import medium. *In vitro* import in spinach chloroplasts was performed as described by Bartlett *et al.* (20).

Plant Transformation—Plant expression vectors were mobilized in the *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating. Helper plasmid pRK 2013 (21) was used. Leaf discs of *Nicotiana tabacum* cv SR1 were infected, and transgenic plants were raised as described (22). All the experiments reported here were carried out on F1 plants obtained after self-crossing.

Fractionation of Tobacco Cells and Protein Quantitation—Subcellular fractions were obtained from 10 g of leaves as described previously (18), except that homogenization was performed in 50 ml of homogenization buffer and that 0.2% (w/v) polyvinylpyrrolidone was added to the buffer.

Purification of chloroplasts and thykaloids on a continuous Percoll gradient was performed as described (23).

Protein concentration was determined by the enhanced alkaline copper assay (24) using bovine serum albumin as a standard.

Western Blot Analysis—After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane and immunodetected with antibodies raised against purified CAT (1/1,000), lipoamide dehydrogenase (1/8,000), ribulose 1,5-bisphosphate carboxylase (1/20,000) followed by ^{125}I-protein A (see Fig. 4, *A* and *C* and Fig. 5) or chemiluminescence (see Fig. 4*B*) detection. Signals were quantitated using an Image Master densitometer (Pharmacia Biotech Inc.).

RESULTS

The TPT Transit Peptide Addresses a Reporter Protein into Isolated Chloroplasts—The TPT has been shown to be imported *in vitro* into isolated chloroplasts (11). We replaced the mature TPT by the CAT reporter protein for a double purpose. First, it exchanged a hydrophobic protein for a soluble passenger protein and therefore avoided the problem of unspecific binding of the precursor to the outer chloroplast or mitochondrial membrane through hydrophobic interactions. Second, it enabled us to test directly the ability of the transit peptide on its own to target a protein to either chloroplasts or mitochondria. CAT was chosen because it had previously been shown to be targeted to either organelle according to the targeting sequence (8). Two constructs were prepared (Fig. 1). One, TPT5-CAT, retained 5 residues of the mature TPT to still allow cleavage of the transit peptide which might have required surrounding residues. The second construct, TPT23-CAT, included 23 residues of the mature TPT, in case the amino-terminal part of the mature TPT was also involved in targeting, as this had already been shown for the chlorophyll *a/b*-binding protein (25, 26).

Both chimeric genes were placed on an *Escherichia coli* plasmid, under the control of the T7 RNA polymerase promoter. RNA transcribed *in vitro* was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The precursors (Fig. 2, *lanes 1* and *6*) were imported into spinach chloroplasts and processed to a mature protein (*lanes 2* and *7*), whose size, compared with that of CAT without a transit sequence (*lane 5*), was compatible with the extra 11 (TPT5-CAT) or 29 (TPT23-CAT) residues remaining upstream of the CAT initiation codon (Fig. 1). The mature CAT was inside the chloroplast, as a brief sonication released it into the soluble fraction (*lanes 3* and *8*). We therefore concluded that the TPT transit peptide with 5 or 23 residues of the mature protein was sufficient to target a foreign protein *in vitro* into chloroplasts. No further analysis was performed with chloroplast import as our results were in agreement with the data showing that the TPT transit peptide is a signal for *in vitro* stromal targeting (12, 13).

The TPT Transit Peptide Addresses a Reporter Protein into Isolated Mitochondria—The spinach TPT precursor has been shown to be imported *in vitro* into plant or yeast mitochondria (16). *In vitro* mitochondrial uptake of the TPT-CAT precursors was therefore performed with mitochondria isolated from etiolated seedlings of broad bean. This material, which circumvents the problem of contaminating chloroplasts, was shown previously to allow the uptake of mitochondrial, and not chloroplast, precursors (19). Incubation of the labeled precursors (Fig. 3*A*, *lanes 2* and *6*) with purified mitochondria produced a cleaved mature form (*lanes 3* and *7*), whose size, compared with that of CAT without the additional sequence (*lanes 1* and *5*), suggests that cleavage occurred between the transit peptide

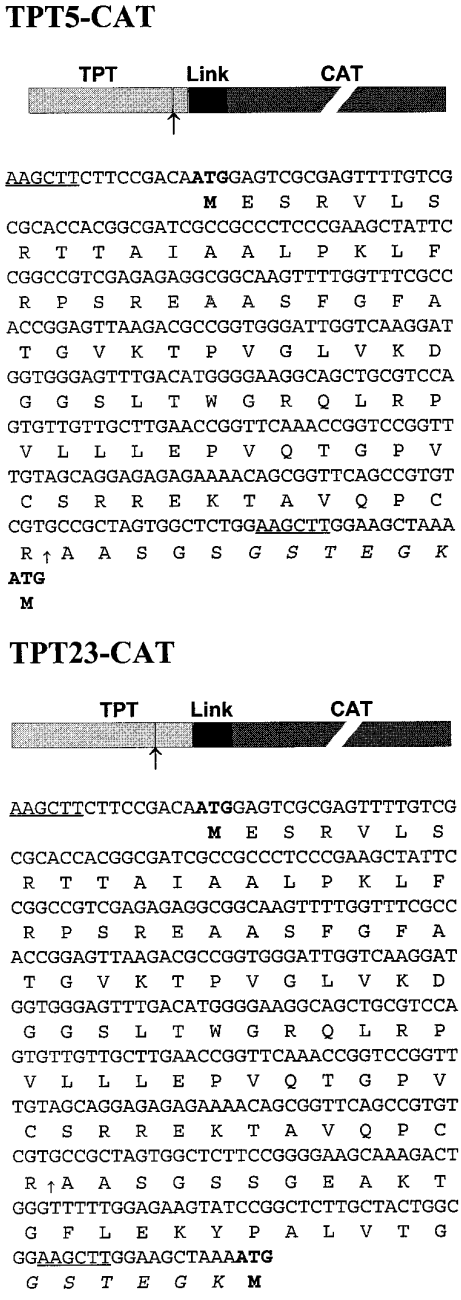

FIG. 1. **Chimeric TPT5-CAT and TPT23-CAT gene constructs.** Below the scheme of the TPT5-CAT and the TPT23-CAT constructs are shown the nucleotide and amino acid sequences from the TPT transit peptide, the kept mature TPT, as well as the linker region upstream of the CAT initiation codon. Linker amino acid residues are in *italics*. *Hin*dIII restriction sites are *underlined*. The CAT and TPT-CAT initiation codons are in *bold*. The *vertical arrowhead* represents the cleavage point between the TPT transit peptide and the mature protein. Drawing is not to scale.

and the sequence kept from the mature TPT (Fig. 1). The mature proteins were protected against externally added proteinase K, indicating that they were effectively imported into the organelle (Fig. 3*A*, *lanes 4* and *8*). Contrary to the import experiment performed with chloroplasts (Fig. 2), a significant amount of unprocessed precursor was found bound to the mitochondria but was degraded upon proteinase K digestion. A similar observation has been reported for a truly mitochondrial precursor (19).

As 5TPT-CAT and 23TPT-CAT behaved similarly, the next experiments were performed using the shortest construct only. To show the dependence of TPT5-CAT mitochondrial uptake on

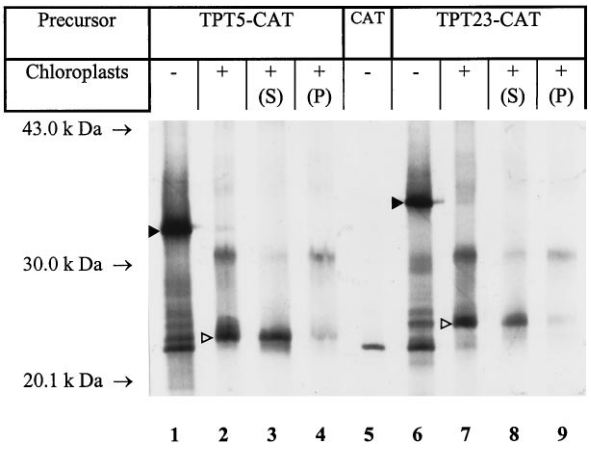

FIG. 2. **Import of TPT5-CAT and TPT23-CAT into isolated chloroplasts.** The reticulocyte lysate containing labeled TPT5-CAT (*lanes 1–4*) or TPT23-CAT (*lanes 6–9*) was incubated with spinach chloroplasts (*lanes 2–4* for TPT5-CAT and *lanes 7–9* for TPT23-CAT) as described under "Experimental Procedures." A sample was then briefly sonicated and centrifuged for 10 min at 20,000 × *g* to give a supernatant (*S*, *lanes 3* and *8*) and a pellet (*P*, *lanes 4* and *9*). In *lane 5*, CAT was synthesized in a reticulocyte lysate. Molecular mass standards (kDa) are indicated on the *left* side. *Filled* and *open arrowheads* indicate the precursor and mature forms, respectively.

import machinery, we performed *in vitro* uptake experiments in the presence of valinomycin, a potassium ionophore and an uncoupler of oxidative phosphorylation (Fig. 3*B*). Mitochondrial import of TPT5-CAT was prevented (*lanes 4* and *5*), indicating that uptake depends on an electrochemical potential across the inner mitochondrial membrane. Import was also prevented when oligomycin, a specific inhibitor of the mitochondrial ATP synthase, was added to the import medium (Fig. 3*B*, *lanes 6* and *7*). Import thus requires internal ATP. Other import parameters were found to be typical of mitochondrial import: requirement of external ATP, absence of import at 0 °C or with trypsin-treated mitochondria (data not shown). In conclusion, properties of the *in vitro* import of TPT-CAT precursors into mitochondria are similar to those of the import of a truly mitochondrial protein.

In Vivo Targeting Properties of the TPT Transit Peptide—The results reported so far support the conclusion that the TPT transit peptide is capable of addressing a passenger protein *in vitro* into both isolated chloroplast and mitochondria. Moreover, this import displays the expected properties of a process that depends on a functional translocation machinery. Since TPT has never been found *in vivo* in plant mitochondria, it could be hypothesized that sequences within the mature TPT and absent in the chimeric proteins analyzed here are involved in specifically directing the protein to chloroplasts. An alternative explanation would be to consider that *in vitro* uptake does not reflect what occurs *in vivo*, possibly due to a lack of the cytosolic factors involved in targeting specificity. Finally, we also have to consider the possibility that *in vivo* TPT is also targeted to mitochondria but rapidly degraded. To evaluate these hypotheses, we equipped the TPT5-CAT and TPT23-CAT constructs with the 35S transcription promoter of cauliflower mosaic virus and the 3′-noncoding region of a pea Rubisco small subunit gene. The chimeric genes were introduced into tobacco, using an *A. tumefaciens* Ti plasmid-derived vector. Transgenic plants were selfed, and several independent F1 plants were characterized for both constructs.

Subcellular fractionation of the plant material was performed by centrifugation to obtain a crude cytosolic supernatant and a crude organellar pellet. Chloroplast- and mitochondria-enriched fractions were also obtained by differential

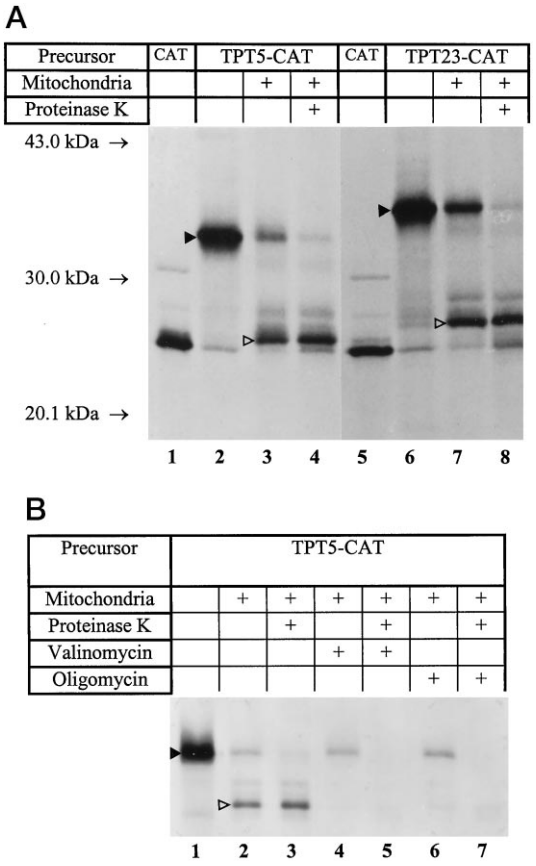

FIG. 3. **Import of TPT5-CAT and TPT23-CAT into isolated mitochondria.** *Panel A*, imported CAT is resistant to proteinase K. The reticulocyte lysate containing labeled TPT5-CAT (*lane 2*) or TPT23-CAT (*lane 6*) was incubated with broad bean mitochondria (*lanes 3* and *4* for TPT5-CAT and *lanes 7* and *8* for TPT23-CAT) as described under "Experimental Procedures." The reaction was then incubated for 20 min at 0 °C with 75 μg/ml proteinase K (*lanes 4* and *8*). In *lanes 1* and *5*, CAT was synthesized in a reticulocyte lysate. Electrophoresis was performed in a 12–16% polyacrylamide gel. Molecular mass standards (kDa) are indicated on the *left* side. *Filled* and *open arrowheads* indicate the precursor and mature forms, respectively. *Panel B*, import of CAT is sensitive to valinomycin and oligomycin. The reticulocyte lysate containing labeled TPT5-CAT (*lane 1*) was incubated with broad bean mitochondria (*lanes 2–7*) in the absence of drugs (*lanes 2* and *3*) or in the presence of 20 μM valinomycin (*lanes 4* and *5*) or 20 μM oligomycin (*lanes 6* and *7*). After incubation, proteinase K treatment was performed (*lanes 3*, *5*, and *7*). Electrophoresis was performed in a 12% polyacrylamide minigel.

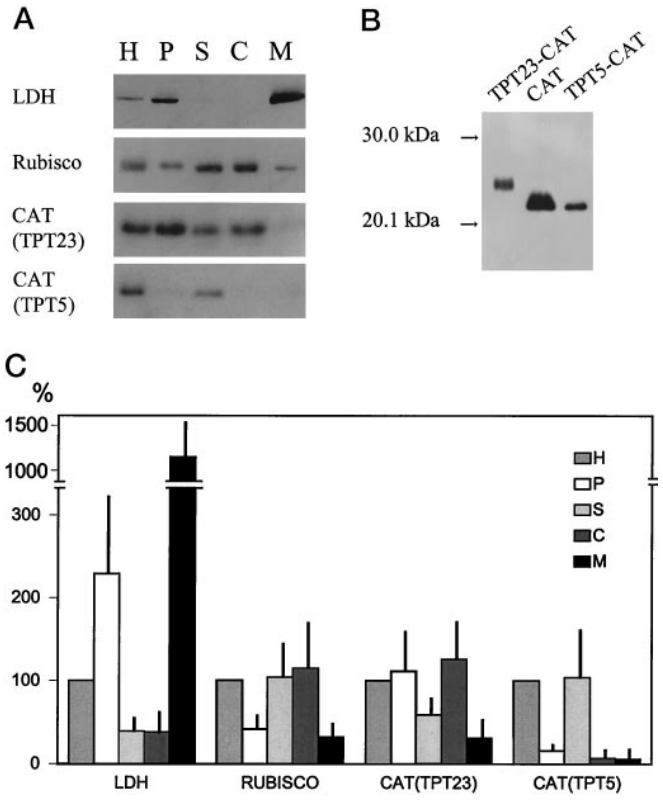

FIG. 4. **Immunodetection of CAT and markers in subcellular fractions of transgenic plants.** Homogenization and subcellular fractionation of TPT23-CAT and TPT5-CAT transgenic plants were carried out as described under "Experimental Procedures." Western blot analysis was carried out on 5- (Rubisco) or 40- (lipoamide dehydrogenase and CAT) μg proteins of the homogenate (*H*), the crude organellar pellet (*P*), the crude cytosolic supernantant (*S*) and the chloroplast (*C*)- and the mitochondria (*M*)-enriched fractions. *Panel A*, Western blot analysis of lipoamide dehydrogenase (*top row*) and Rubisco (*second row*), a mitochondrial or chloroplast marker, respectively, and of CAT in a PT23-CAT and a TPT5-CAT plant. *Panel B*, size comparison of the mature TPT23-CAT, TPT5-CAT, and CAT products. Western blot analysis of CAT was carried out on a chloroplast fraction of a TPT23-CAT plant and a cytosolic fraction of a TPT5-CAT and a CAT plant. *Panel C*, Western blot signals obtained for lipoamide dehydrogenase, Rubisco, and CAT in the various fractions were quantitated for four to seven independent plants. Values represent the mean of the ratios of the signal in the indicated fraction *versus* the signal in the homogenate. The *bar* represents the S.D.

centrifugation and subsequent purification of the organelles on Percoll gradients. To evaluate organelle enrichment, we immunodetected in the various fractions the large subunit of Rubisco, a soluble marker of the chloroplastic stroma, and the lipoamide dehydrogenase, a soluble mitochondrial marker found in several mitochondrial matrix enzymes (27). Distribution of these markers among the various fractions was analyzed by Western blot (an example is shown in Fig. 4*A*) and quantitated by image analysis (Fig. 4*C*). The majority of the Rubisco was released into the supernatant, confirming the high sensitivity of tobacco chloroplasts to mechanical grinding (26). Enrichment in the chloroplast fraction was low, but this has to be expected since chloroplast proteins of mesophyll cells represent up to 50% of total proteins and the highest enrichment can therefore be only 2-fold. In contrast, the majority of the mitochondrial marker was found in the crude organellar pellet and was enriched about 10-fold in the mitochondrial fraction. A certain amount of Rubisco was found in the mitochondria-enriched fraction. As discussed previously (26), it is not clear whether this stems from the presence of intact chloroplasts in the mitochondria-enriched fraction or from the binding to mitochondria of Rubisco released during grinding.

Western blot (or enzyme activity, not shown) analysis of CAT in a TPT23-CAT plant shows that the reporter protein was addressed to chloroplasts since CAT and Rubisco enrichment in the chloroplast fraction was similar (Fig. 4*A*). There was no enrichment in the mitochondrial fraction. As expected, the size of the mature CAT observed for TPT23-CAT in the chloroplast fraction (Fig. 4*B*) was larger than that of a control cytosolic CAT without any targeting sequence. This difference accounts for the 23 residues of mature TPT and 6 residues from the linker region.

Quantification of Western blot data from independent TPT23-CAT transgenic plants confirmed the presence of CAT within the chloroplast (Fig. 4*C*). However, we observed that CAT enrichment in the crude organellar fraction was higher than enrichment of Rubisco in the same fraction, suggesting that the processed TPT23-CAT somehow binds to chloroplast membranes *in vivo* or after homogenization. To analyze this point, we centrifuged a chloroplast fraction on a self-generated Percoll gradient. As expected, two main green bands were

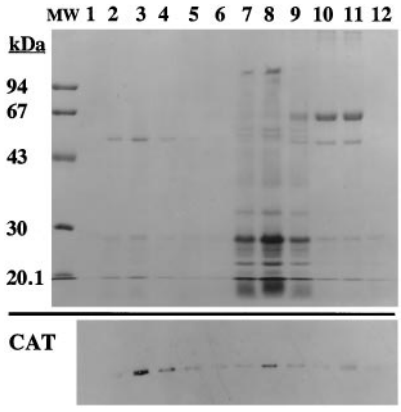

FIG. 5. **Subcellular fractionation of CAT from a TPT23-CAT plant.** A crude chloroplast fraction from a TPT23-CAT plant was centrifuged on a continuous Percoll gradient. The various fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*left* to *right*, *bottom* to *top*). The *upper panel* shows the Coomassie Blue staining. The *lower panel* displays immunodetection of CAT.

obtained, corresponding to the intact chloroplasts (heavier band) and thykaloids (lighter band). This was confirmed by gel electrophoresis analysis (Fig. 5), showing in fraction 3, the heaviest green peak, the bands corresponding to the large subunit of Rubisco and the chlorophyll *a/b*-binding protein, the two major soluble and membrane chloroplast proteins, respectively. Fraction 7, the other green peak, still contains the chlorophyll *a/b*-binding protein but not Rubisco, confirming that it corresponds to thykaloids. CAT was found to peak with intact chloroplasts (fraction 3), thykaloids (fraction 8), and a top fraction (11) where bovine serum albumin (67 kDa) could be detected, thus corresponding to the soluble fraction. These observations therefore suggest that part of the CAT within the TPT23-CAT plant is in the chloroplast stroma, and part binds to the thykaloids. This was confirmed by showing the sensitivity of CAT to thermolysin in the thykaloid and soluble fractions but not in the intact chloroplast fraction (data not shown).

For the TPT5-CAT plant, however, CAT remained essentially in the supernatant (Fig. 4, *A* and *C*). Little, if any, activity was found in the chloroplast or mitochondrial fractions. We therefore conclude that *in vivo* the TPT transit peptide together with 5 residues of the mature TPT is not sufficient to address CAT into chloroplasts.

The size of CAT within the TPT5-CAT plant was found to be similar to that of control cytosolic CAT (Fig. 4*B*), suggesting that TPT5-CAT was nevertheless processed in the cell by some kind of cytosolic proteolytic activity or, after homogenization, by a protease released from an organelle. Homogenization of the plant material in the presence of phenol gave similar results (data not shown), suggesting that processing probably occurs *in vivo*. To exclude any DNA rearrangement during *A. tumefaciens* or plant transformation which could have removed the TPT transit peptide initiation codon, we used polymerase chain reaction to retrieve a DNA fragment encompassing the NH_2-terminal region of TPT5-CAT from a TPT5-CAT-transformed plant. We then checked by sequencing that the sequence was not modified.

DISCUSSION

TPT is a chloroplast protein and has never been detected in mitochondria. Consistent with this is the observation that the TPT precursor is addressed to, and processed in, isolated chloroplasts (11). Recent reports (12, 13) showed that the TPT transit peptide serves *in vitro* as a targeting signal to the chloroplast stroma. Here we showed that the TPT transit peptide is able to address a foreign protein to chloroplasts. The TPT precursor, however, is also imported *in vitro* into mitochondria isolated from yeast and plants, and this process has shown the typical features of specific protein import such as energy requirement and protease-sensitive binding of the precursor to the yeast MOM19 receptor (16). The data reported here with two precursors retaining the transit peptide and 5 or 23 residues of mature TPT delimit the sequence involved in *in vitro* mitochondrial targeting within a region spanning the 80 residues of the transit peptide and the 5 NH_2-terminal residues of the mature TPT. This transport requires a membrane potential and external ATP. These observations strongly suggest that the mitochondrial import of the TPT-CAT precursors occurs via a normal import route. Examination of the primary structure of the TPT transit peptide from various sources suggests the presence of a putative positively charged amphiphilic α-helix (14, 15). This feature has been shown to be important for membrane insertion and for the translocation of imported mitochondrial precursors (4) and could account for the TPT-CAT mitochondrial import.

In both *in vitro* chloroplast and mitochondrial import, the apparent size of the mature TPT5-CAT and TPT23-CAT is compatible with processing of the precursors at the expected cleavage site. As suggested previously (16, 26), this implies that proteases recognizing the same target are present in both mitochondria and chloroplasts.

A major conclusion of the data reported here is that the TPT transit peptide behaves differently *in vitro* and *in vivo*. In the latter case, indeed, the shortest construct (TPT5-CAT) remained in the cytosol. It could be argued that the targeting peptide of TPT5-CAT is degraded rapidly by a cytosolic protease, therefore preventing chloroplast import. This is, however, unlikely since it would imply that the additional 18 internal residues of TPT23-CAT confer protease resistance in the cytosol but not in the chloroplast. Another hypothesis would be that the NH_2-terminal region of TPT5-CAT is engaged in the chloroplast import machinery, processed within the stroma, and, because of a failure of complete translocation, released back into the cytosol. This hypothesis, however, is not in agreement with the observation that *in vivo* cleavage of TPT5-CAT does not occur at the expected site as observed after *in vitro* targeting (see Fig. 2) but probably at, or very close to, the CAT initiation codon (see Fig. 4*B*). Moreover, interfering sequences within the CAT reporter are unlikely to be present since this protein has been used previously as a passenger for either mitochondrial or chloroplast import (8, 26). A more likely hypothesis would be that structural features required for *in vivo* mitochondrial or chloroplast uptake are missing in the TPT transit peptide. Failure of import could occur due to lack of interaction of the precursor either with specific chaperones involved in organellar targeting (28) or with receptors at the organelle outer membrane. The precursor would then be processed by a cytosolic protease. Cleavage of a chloroplast or mitochondrial targeting peptide of a chimeric protein which was not imported either has been observed before (26, 29).

When the TPT transit peptide was followed by 23 residues of mature TPT, CAT was efficiently targeted *in vivo* to chloroplasts but not to mitochondria. Since CAT stably accumulates in mitochondria when linked to a truly mitochondrial presequence (8), we can conclude that *in vivo* the TPT transit peptide together with 23 residues of the mature protein does not constitute an *in vivo* mitochondrial targeting sequence. This weakens the hypothesis that TPT would also be addressed *in vivo* to mitochondria and then destroyed rapidly. The requirement of sequences within the mature TPT for import is not unexpected in itself. Similarly, the transit peptide of the chlorophyll *a/b*-

binding protein was unable to direct either CAT or the β-glucuronidase reporter protein into chloroplasts unless sequences of the mature protein (25) or surrogate sequences (26) were present. The similarity between TPT and the chlorophyll *a*/*b*-binding protein goes no further since the latter has a classical transit peptide and has been shown previously to be addressed under the same experimental conditions *in vitro* to chloroplasts and not to mitochondria (19). The nature of the additional residues required for *in vivo* chloroplast import is not clear. The amino-terminal part of the mature TPT is not well conserved in various species. We could thus hypothesize that secondary, instead of primary, structures might be involved.

A last conclusion from this work is that, as far as sorting is concerned, caution is necessary when analyzing *in vitro* import data. For instance, we found similar rates of *in vitro* mitochondrial import for TPT5-CAT or TPT23-CAT as for a construct linking CAT to a truly mitochondrial presequence, that of the mitochondrial ATPase β subunit, and yet *in vivo* the latter construct was imported efficiently into mitochondria (18).

What could the explanation be for the different observations *in vivo* and *in vitro*? Since in the latter case, the precursors are radioactively labeled, the number of precursor molecules that are targeted to mitochondria is probably small compared with the number of mitochondria present in the assay. On a quantitative basis, the process is probably not very efficient. *In vivo*, the targeting machinery is expected to be very efficient (to avoid the accumulation of precursors into the cytosol) and specific (to avoid mistargeting). Concerning this last point, it could be argued that the *in vitro* uptake experiments are not very stringent since there is no competition for various subcellular compartments. Performing *in vitro* import in the presence of both mitochondria and chloroplasts would address this point but would mean first finding conditions that are appropriate for both types of import. *In vivo* specificity might be achieved by the affinity of the various precursors for their respective receptors localized at the outer face of organelles. However, specificity might occur at an earlier step, since precursors may bind to cytosolic or membrane chaperones after, or even during, their translation (28, 30, 31). In the framework of this hypothesis, our data suggest that *in vivo* binding to chaperones and/or to chloroplast receptors requires, in addition to the transit peptide, residues of the mature TPT protein.

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