

Chloroplast import of an intermembrane space protein is facilitated by translocon components Toc75 and Tic236

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Abstract

Chloroplasts are divided into six subcompartments: the outer membrane, intermembrane space, and inner membrane of the envelope, the stroma, the thylakoid membrane, and the thylakoid lumen. Compared with our knowledge of protein import into other subcompartments, extremely little is known about how proteins are imported into the intermembrane space of the envelope. Tic22 was one of the first proteins identified as localizing to the intermembrane space and the only one for which import has been analyzed in some detail. However, conflicting results have been obtained concerning whether the general translocon is used to import Tic22 into the intermembrane space. Taking advantage of available translocon component mutants, we reanalyzed import of Tic22. We reveal reduced in vitro import of Tic22 preprotein (prTic22) into chloroplasts isolated from the Arabidopsis *mar1* and *tic236* mutants, which are functional knockdown mutants of the outer-membrane channel Toc75 and the intermembrane space linker Tic236, respectively. Import competition experiments also showed that prTic22 import was reduced by excess amounts of a stroma-targeted preprotein. Our results indicate that prTic22 uses at least part of the general translocon for import into the intermembrane space.

KEYWORDS

chloroplast envelope, intermembrane space, *mar1*, protein transport, Tic22, Tic236, Toc75

1 | INTRODUCTION

Chloroplasts display one of the most complex structures among organelles. They are divided into six subcompartments: the outer membrane, intermembrane space (IMS), and inner membrane of the envelope, the stroma, the thylakoid membrane, and the thylakoid lumen. Each subcompartment has a distinct set of proteins. Most proteins in chloroplasts are encoded by the nuclear genome and synthesized in the cytosol as a higher molecular mass preprotein with an N-terminal transit peptide for import into chloroplasts. Import is executed by translocons at the outer (TOC) and inner (TIC) chloroplast

envelope membranes. Proteins destined to the outer and inner membranes, the stroma, the thylakoid membrane, and the thylakoid lumen have all been shown to use all or part of this general translocon machinery for transport across the envelope. As core translocon components, Toc159 and Toc34 function as the initial receptors, Toc75 forms the channel for protein translocation across the outer membrane, Tic236 links the TOC and TIC complexes, and Tic20 forms the channel for protein translocation across the inner membrane (Richardson & Schnell, 2020).

Tic22 is a translocon component located in the IMS. It functions as a small chaperone facilitating preprotein transit across the IMS

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(Kasmati et al., 2013; Kouranov et al., 1998; Paila et al., 2016; Rudolf et al., 2013). In addition, Tic22 was one of the first proteins to be molecularly localized to the IMS. Therefore, it has been used to study how proteins are imported into the IMS. The molecular mass of the Tic22 preprotein (prTic22) is ~30 kD, and the N-terminal 54 amino acids of pea prTic22 have been shown to function as a transit peptide that is necessary and sufficient to direct protein import into the IMS (Kouranov et al., 1999). Two studies have analyzed the import requirements of prTic22 in more detail (Kouranov et al., 1999; Vojta et al., 2007). Both studies showed that import of prTic22 into the IMS does not require ATP but is stimulated by it. However, although both studies demonstrated that prTic22 import is facilitated by thermolysin-sensitive components on the chloroplast surface, Kouranov et al. found that prTic22 import was not inhibited by excess amounts of the preprotein to the small subunit of RuBP carboxylase (prRBCS), which uses the TOC-TIC translocon for import into chloroplasts. Accordingly, Kouranov et al. suggested that prTic22 is imported into the IMS via a novel import pathway distinct from known pathways that target proteins to other chloroplast sub-compartments. In contrast, Vojta et al. reported that import of prTic22 was inhibited by excess amounts of prOE33 (the preprotein of the 33-kD subunit of the oxygen evolving complex of the thylakoid lumen), which also employs the TOC-TIC translocon for its transport across the envelope. They further showed that prTic22 bound on chloroplasts could be coimmunoprecipitated by antibodies against Toc75 and Toc34, supporting that prTic22 associates with the TOC complex during import (Vojta et al., 2007).

One way to investigate if a translocon component is involved in an import process is to perform import experiments using chloroplasts isolated from *Arabidopsis* mutants defective in the translocon component. Although most core translocon components are essential and their genetic knockout usually results in embryonically lethal or albino phenotypes, viable mutant alleles harboring knockdown mutations are available for several components, including Toc75 and Tic236 (Chen et al., 2018; Stanga et al., 2009). In addition, Tic236 is required for import of inner envelope membrane, stromal, and thylakoid proteins (Chen et al., 2018). Whether Tic236 is required for the import of IMS proteins has not been studied. In this report, we used chloroplasts isolated from mutant *Arabidopsis* lines hosting knockdown alleles of Toc75 and Tic236 to investigate prTic22 import. Our results show that prTic22 import was reduced in these mutant lines, evidencing that the prTic22 import process employs at least part of the general TOC-TIC translocon.

2 | MATERIALS AND METHODS

2.1 | Plant materials, chloroplast isolation, plasmid constructs, and in vitro translation

Chloroplast isolation from 7- to 9-day-old pea seedlings (*Pisum sativum* Green Arrow) grown on vermiculite and 14-day-old *Arabidopsis* plants grown on MS synthetic agar medium with 2%

sucrose was performed as described previously (Chu & Li, 2011). The *tic236-2* and *tic236-3* mutants used were described previously (Chen et al., 2018). Briefly, *tic236-2* (SAIL104-F07, T-DNA insertion at -306 and -307, *Col* ecotype) was obtained from the Arabidopsis Biological Resource Center (ABRC). The *tic236-3* mutant (RIKEN PST00216) in the *No-0* ecotype was obtained from RIKEN BRC Experimental Plant Division. It has a Ds insertion in the same position in the 5' UTR at -276 and -277. A plasmid containing the full-length cDNA of *Arabidopsis* prTic22 (At4g33350) was obtained from ABRC (EST 144A17). The prTic22 insert was excised using PstI and XbaI and cloned into the PstI/XbaI site of pSP64. The resulting plasmid was named pSP64-prTic22, and it was used to produce in vitro-translated full-length prTic22 using the TNT reticulocyte lysate system (Promega) and [³⁵S]Met. To synthesize prTic22(1-242)MM preprotein, site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies Inc.) was performed on pSP64-prTic22 to change residues 243, 244, and 245 into Met, Met, and a stop codon, respectively.

2.2 | Protein import into chloroplasts and postimport treatments

Isolated chloroplasts were adjusted to 1-mg chlorophyll per ml in import buffer (330-mM sorbitol and 50-mM HEPES-KOH, pH 8.0), and the same volumes of mutant and wild-type chloroplasts were used for each experiment. For regular import, [³⁵S]Met-labeled preproteins were incubated with isolated chloroplasts in the presence of 5-mM ATP and 0.8-mM DTT in import buffer at room temperature for 30 min. The import reaction was stopped at desired time points by adding an excess amount of ice-cold import buffer. Treatment of chloroplasts with thermolysin (200 and 100 µg/ml for pea and *Arabidopsis* chloroplasts, respectively) after import was performed as described previously (Perry et al., 1991). Chloroplasts after thermolysin treatment were reisolated through a 40% Percoll cushion to ensure that only intact chloroplasts were analyzed (Cline et al., 1984). Chloroplasts were further washed and resuspended in import buffer. A small volume was removed for protein concentration determination using the Pierce BCA assay kit (Thermo Fisher). The remaining chloroplasts were mixed with an equal volume of 2× SDS-PAGE sample buffer, boiled, and then analyzed in 4-12% NuPAGE Bis-Tris gels (Thermo Fisher).

2.3 | Import competition experiments

Competition experiments using recombinant prRBCS and RBCS were performed as described previously (Tranel et al., 1995; Tu et al., 2004). In brief, import was performed with [³⁵S]Met-labeled preproteins and various concentrations of recombinant prRBCS or RBCS at room temperature for 30 min. All reactions had the same concentration of urea as the reaction with the highest amount of recombinant proteins. After import, chloroplasts were further treated

with 200- μ g/ml thermolysin. Intact chloroplasts were reisolated through a 40% Percoll cushion after the thermolysin treatment (Cline et al., 1984). Accession numbers or references for the preproteins used are prRBCS (Lubben & Keegstra, 1986), prHsp93 (L09547), and prTic22 (At4g33350).

3 | RESULTS

3.1 | Import time course of prTic22

In previous studies, a pea prTic22 cDNA (AF095284, Kouranov et al., 1999) transcribed from the T7 promoter and [35 S]-methionine ([35 S]Met)-radiolabeled preprotein, and an Arabidopsis cDNA (At4g33350, Vojta et al., 2007) transcribed from the SP6 promoter and [35 S]-cysteine-radiolabeled preprotein, were used to study the import of prTic22 into the IMS. Import efficiencies in both studies seemed to be low, and it was calculated that \sim 4% of the radiolabeled prTic22 added to the pea chloroplast import reactions was imported after 30 min (Kouranov et al., 1999), which may render quantifications difficult. To increase signal intensity, we subcloned the Arabidopsis prTic22 cDNA, so it is also transcribed by the SP6 promoter but used [35 S]Met for in vitro translation. For reasons not yet clear to us, the use of the SP6 promoter and [35 S]Met usually resulted in production of preproteins displaying enhanced radioactivity relative to use of the T7 promoter and [35 S]-cysteine. However, whether there are quantitative differences remains to be determined. We first performed import time-course experiments with isolated pea chloroplasts to gain an initial understanding of the import characteristics of prTic22. As shown in Figure 1, amounts of processed mature Tic22 increased with time. At the 30-min time point, \sim 25% of added prTic22 had been processed into

mature Tic22 (Figure 1, Lane 7; Lane 1 represents 50% of prTic22 added). Interestingly, similar to previous results (Kouranov et al., 1999; Vojta et al., 2007), most prTic22 was degraded after thermolysin treatments to remove proteins that were still exposed at the chloroplast surface, but a small proportion of prTic22 was thermolysin resistant (Figure 1, Lanes 8 to 12, prTic22). Control experiments in which prTic22 was directly treated with thermolysin without chloroplasts resulted in complete degradation (Figure 1, Lane 2), suggesting that the small amount of thermolysin-resistant prTic22 had been translocated across the outer membrane. Furthermore, some processed mature Tic22 was thermolysin sensitive, indicating that it had not been fully translocated across the outer membrane. Kouranov et al. (1999) have therefore suggested that processing of prTic22 is not tightly coupled to outer-membrane translocation. The transit peptide of prTic22 could be cleaved during or after translocation across the outer membrane, resulting in the populations of thermolysin-sensitive mature Tic22 and thermolysin-resistant prTic22, respectively.

3.2 | Import of prTic22 is competed by excess amounts of prRBCS

To investigate if import of prTic22 uses part of the general translocon, we performed import competition experiments using *Escherichia coli*-produced recombinant prRBCS. Recombinant mature RBCS without the transit peptide was used as a control. As shown in Figure 2, when we added 1- μ M recombinant prRBCS, import of [35 S]Met-labeled prTic22 and prRBCS was reduced by \sim 40%. This reduction was not seen when recombinant mature RBCS was added. Thus, the prTic22 import process may operate via components shared with that of prRBCS.

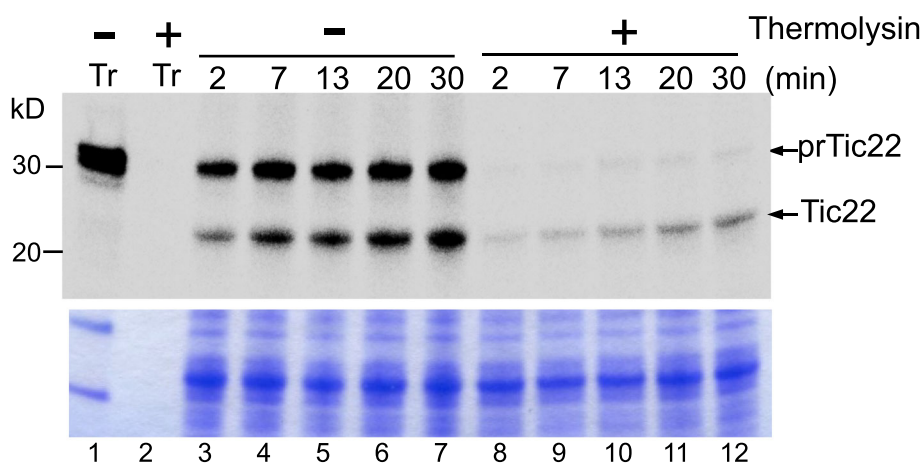


FIGURE 1 Import time course of prTic22 into isolated pea chloroplasts. [35 S]Met-prTic22 produced by in vitro translation (Tr, Lanes 1 and 2) was incubated with isolated pea chloroplasts under conditions of 5-mM ATP and room temperature for various time frames as indicated at the top (Lanes 3 to 7). Half of the chloroplasts were further treated with 200- μ g/ml thermolysin (Lanes 8 to 12). Intact chloroplasts were reisolated and analyzed by SDS-PAGE. An equal amount of protein was loaded in all lanes, except for the Tr lanes that represent 50% of [35 S]-prTic22 added to the import samples. The gels were stained with Coomassie blue and dried for fluorography. The region around endogenous CAB is shown below the fluorograph of the same gel

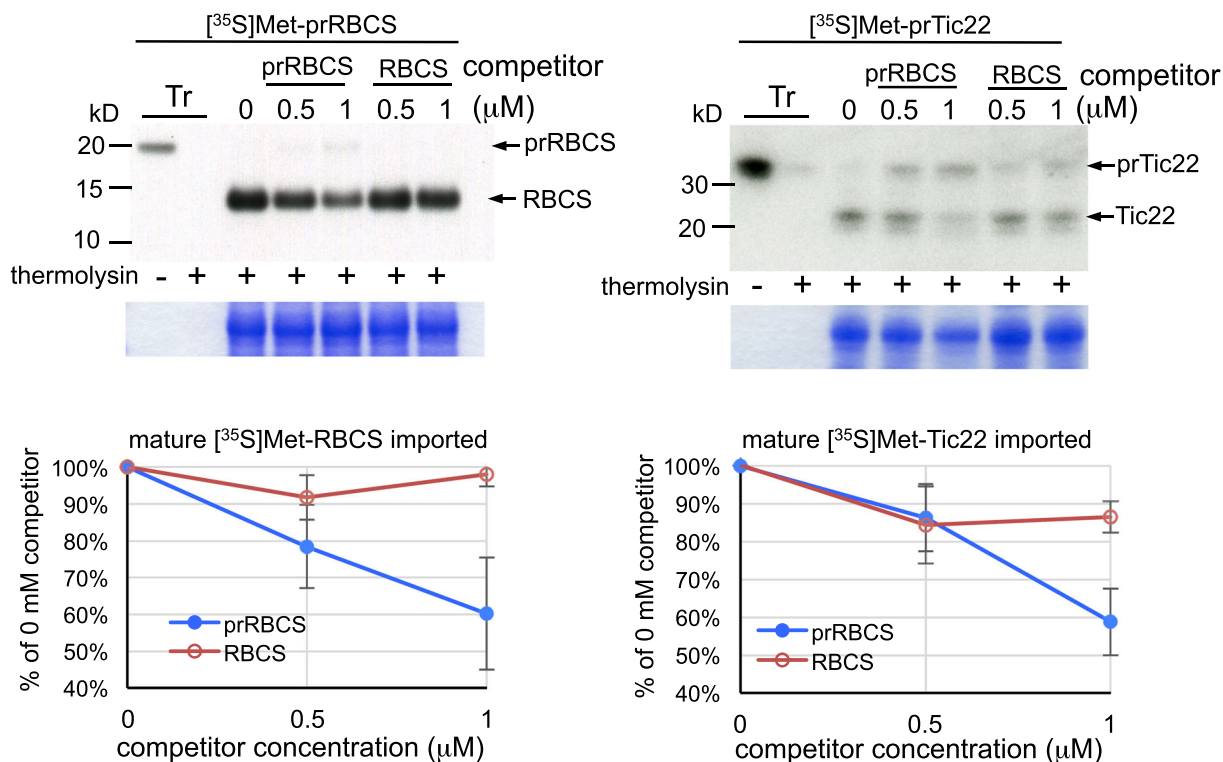


FIGURE 2 Import competition of prTic22 with prRBCS and RBCS. $[^{35}\text{S}]\text{Met-prRBCS}$ or $[^{35}\text{S}]\text{Met-prTic22}$ produced by in vitro translation (Tr) was imported into isolated pea chloroplasts for 30 min in 5-mM ATP with 0- to 1- μM unlabeled recombinant prRBCS or RBCS competitor, as indicated on top of the gel. Chloroplasts were further treated with 200- $\mu\text{g}/\text{ml}$ thermolysin after import. Intact chloroplasts were reisolated and analyzed by SDS-PAGE. An equal amount of protein was loaded in each lane of the same gel, except for the Tr lanes. The gels were stained with Coomassie blue and dried for fluorography. The region around endogenous CAB is shown below the fluorograph of the same gel. Imported mature protein was quantified using a phosphorimager and further corrected for loading by quantifying the Coomassie-stained gels by ImageJ. The amount of mature protein imported in the absence of recombinant protein was set as 100%. Values represent mean \pm SD of three independent experiments

3.3 | Import of prTic22 into *mar1* and *tic236* mutant chloroplasts

To explore if import of prTic22 is facilitated by the translocon components Toc75 and Tic236, we assayed prTic22 import into chloroplasts isolated from Arabidopsis lines with knockdown mutations in *TOC75* and *TIC236*. In initial experiments, we found that the import efficiency of prTic22 into isolated Arabidopsis chloroplasts was lower than that of import into pea chloroplasts. Furthermore, mature Tic22 ran to the same gel position as the abundant chloroplast *a/b*-binding protein (CAB), which often obscured the signal of mature Tic22 when gels had to be exposed for a long period of time. Therefore, we constructed a C-terminus-truncated prTic22 by removing the most C-terminal 26 amino acids so that the imported mature protein could be separated from CAB upon SDS-PAGE. To increase signal intensity further, we added two methionine residues at the C-terminus, resulting in a prTic22(1-242)MM construct (full-length prTic22 is 268 amino acids long). Truncation of the C-terminus should not affect prTic22 import because previous studies have shown that the transit peptide of prTic22 is sufficient to target a passenger protein to the IMS (Kouranov et al., 1999). Furthermore, when the prTic22 transit

peptide was fused to a passenger protein, import of the fusion protein also showed the uncoupled translocation-processing characteristic of prTic22 (Kouranov et al., 1999), suggesting that the prTic22 transit peptide determines the targeting, translocation, and processing characteristics of prTic22.

The *mar1* (*modifier of arg1*) mutant of Arabidopsis contains a missense mutation in Toc75 (At3g46740), resulting in mutation of the glycine at residue 658 to arginine (Stanga et al., 2009). Although *mar1* is the first known hypomorphic allele of Toc75, to our knowledge, no direct import experiment has been reported using chloroplasts isolated from *mar1*. Therefore, we assayed import of the preprotein of stromal Hsp93 (prHsp93) as a control. We selected prHsp93, rather than the most commonly used prRBCS, as a control because the size of prRBCS is similar to that of prTic22(1-242)MM, whereas prHsp93 and imported mature Hsp93 are 100 and 93 kD, respectively, so they are well separated from Tic22(1-242)MM in SDS-PAGE. We coimported prHsp93 and prTic22(1-242)MM into chloroplasts isolated from *mar1* and wild-type control (*WS2* ecotype) plants. As shown in Figure 3, we observed reduced amounts of mature Hsp93 and Tic22(1-242)MM after import into *mar1* chloroplasts, illustrating that Toc75 facilitates the import of prHsp93 and prTic22.

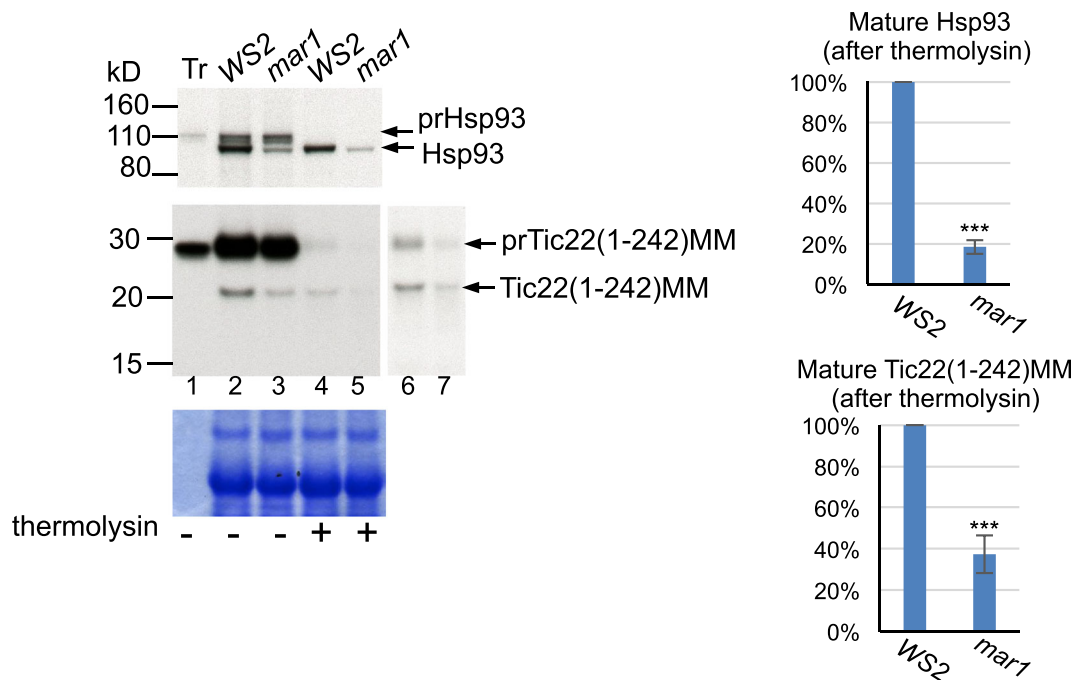


FIGURE 3 Import of prTic22(1-242)MM into chloroplasts of *mar1* and the corresponding wild type. [³⁵S]Met-prHsp93 and [³⁵S]Met-prTic22(1-242)MM produced by in vitro translation (Tr) were imported into chloroplasts isolated from *mar1* and the corresponding wild-type (WS2) plants in 5-mM ATP at room temperature for 30 min. Half of the chloroplasts were further treated with thermolysin. Reisolated intact chloroplasts were analyzed by SDS-PAGE, and the gels were stained with Coomassie blue and dried for fluorography. The region of the gel around CAB is shown below the fluorograph. An equal amount of protein was loaded in each lane, except for the Tr lane. In the prTic22(1-242)MM panel, Lanes 6 and 7 are a longer exposure of Lanes 4 and 5. Quantifications show the amounts of mature Hsp93 and Tic22(1-242)MM from thermolysin-treated chloroplasts using a phosphorimager, further corrected for loading by quantifying the Coomassie-stained gels by ImageJ. The amount of mature protein imported into the wild-type chloroplasts of the same experiment was set as 100%. Values represent mean \pm SD of three independent experiments. Statistical analysis by two-tailed *t* test, ****p* < .001

Next, we tested import of prTic22(1-242)MM into chloroplasts isolated from *tic236* mutants. We used two mutant alleles, *tic236-2* (*Col* ecotype) and *tic236-3* (*No-0* ecotype) in our experiments. As shown in Figure 4, before thermolysin treatment, amounts of prTic22(1-242)MM were higher in the mutant lines than in their respective wild types. Upon thermolysin treatment, amounts of prTic22(1-242)MM in mutant chloroplasts were lower than detected in wild types. Moreover, amounts of mature Tic22(1-242)MM in the mutant lines were lower relative to controls, both before and after thermolysin treatment. Therefore, it is likely that prTic22(1-242)MM crosses the outer membrane using Toc75 that is assembled with Tic236, with amounts of this latter protein being reduced in the *tic236* mutants (Chen et al., 2018). Accordingly, nontranslocated prTic22 accumulated on the chloroplast surface of the *tic236* mutant, whereas levels of fully translocated prTic22 and mature Tic22 were diminished due to reduced amounts of Toc75-Tic236 complex in the mutant lines.

4 | DISCUSSION

In previous studies, prTic22 from pea (AF095284, Kouranov et al., 1999) and Arabidopsis (At4g33350, Vojta et al., 2007) were used to study prTic22 import. The import efficiencies reported in both

studies seemed low, rendering quantifications difficult. By using the SP6 promoter to direct transcription and a C-terminally truncated clone to which methionine residues had been added, we obtained very clear import signals. We found that addition of excess amounts of prRBCS competed with prTic22 for import. Moreover, prTic22 import was impaired in knockdown mutants of Toc75 and Tic236. Together, these results indicate that, like most chloroplast preproteins with cleavable transit peptides, prTic22 uses the TOC-TIC translocon for import into chloroplasts.

We have shown previously that Tic236 mutant lines display reduced import of preproteins destined for the inner envelope membrane, the stroma, and the thylakoid, suggesting that preproteins targeted to these subcompartments all use TOC-TIC supercomplexes (Chen et al., 2018). Only outer-membrane proteins such as OEP14 and Toc34 can insert into the outer membrane via Toc75 not assembled with the TIC complex (Chen et al., 2018; Tu et al., 2004). Our results presented herein imply that preproteins of the IMS also use the TOC-TIC supercomplexes for import. This is an interesting finding because these preproteins should only need to cross the outer membrane and not the inner membrane. It is possible that Toc75 not assembled with the TOC-TIC supercomplexes only allows lateral diffusion of inserted proteins into the outer membrane and that it cannot support complete translocation across the outer membrane.

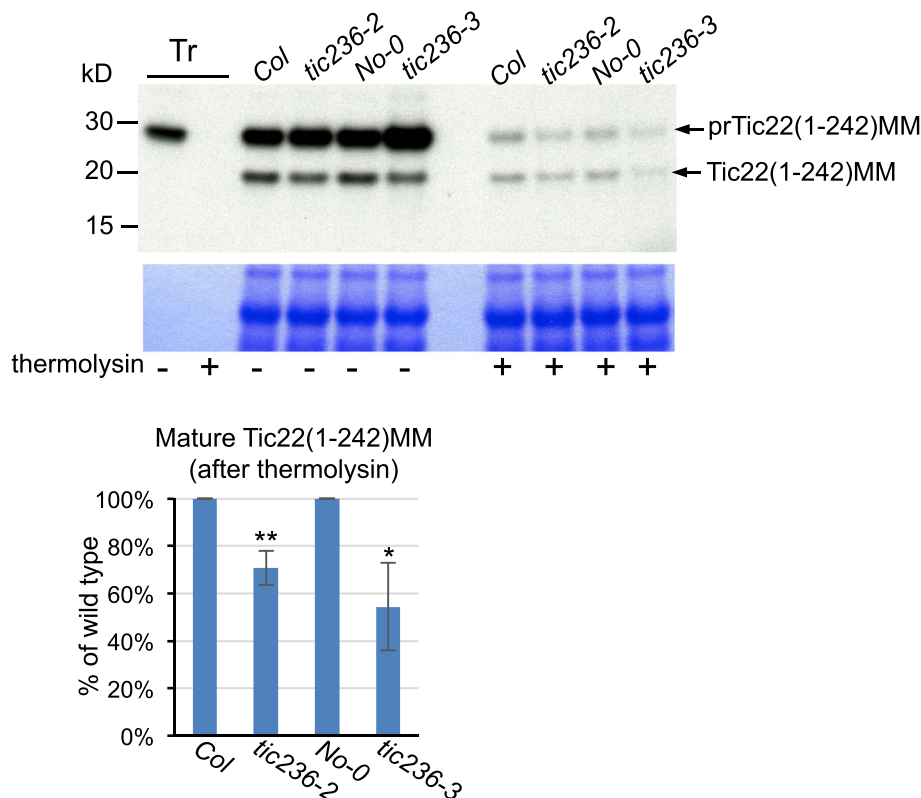


FIGURE 4 Import of prTic22(1-242)MM into chloroplasts of *tic236-2* and *tic236-3* mutants, as well as their corresponding wild types. [³⁵S] Met-prTic22(1-242)MM produced by in vitro translation (Tr) was imported into chloroplasts isolated from the *tic236-2* and *tic236-3* mutant lines, as well as their corresponding wild-type (*Col* and *No-0*) plants, in 5-mM ATP at room temperature for 30 min. Half of the chloroplasts were further treated with thermolysin. Reisolated intact chloroplasts were analyzed by SDS-PAGE, and the gels were stained with Coomassie blue and dried for fluorography. The region of the gel around CAB is shown below the fluorograph. An equal amount of protein was loaded in each lane, except for the Tr lanes. Quantifications show the amount of mature Tic22(1-242)MM from thermolysin-treated chloroplasts using a phosphorimager, further corrected for loading by quantifying the Coomassie-stained gel by ImageJ. The amount of mature Tic22(1-242)MM imported into the corresponding wild-type chloroplasts of the same experiment was set as 100%. Values represent mean \pm SD of three independent experiments. Statistical analysis by two-tailed *t* test, **p* < .05 and ***p* < .01

Accordingly, prTic22 would still need Toc75 assembled with Tic236 to fully transit the outer membrane. Notably, the peptidase responsible for cleaving the prTic22 transit peptide is still unknown. It remains possible that the peptidase is located in the stroma, so that prTic22 must be partially translocated across the inner membrane for processing, necessitating TOC-TIC supercomplex-mediated transport. Future investigations on the import dynamics and processing of other IMS proteins are required to gain further understanding of the mechanism underlying protein import into the chloroplast IMS.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the publication of this manuscript.

AUTHOR CONTRIBUTIONS

H.-m. L. conceived the study, designed the experiments, and wrote the manuscript. M.-R. C. and L.-J. C. designed and performed the experiments and analyzed the data.

DATA AVAILABILITY STATEMENT

All relevant data are included in the manuscript. Access to raw data is available on request.

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