A distinct class of GTP-binding proteins mediates chloroplast protein import in Rhodophyta

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Chloroplast protein import is mediated by translocons named TOC and TIC on the outer and inner envelope membranes, respectively. Translocon constituents are conserved among green lineages, including plants and green algae. However, it remains unclear whether Rhodophyta (red algae) share common chloroplast protein import mechanisms with the green lineages. We show that in the rhodophyte Cyanidioschyzon merolae, plastome-encoded Tic20pt localized to the chloroplast envelope and was transiently associated with preproteins during import, suggesting its conserved function as a Tic constituent. Besides plastome-encoded FtsHpt and several chaperones, a class of GTP (guanosine 5′-triphosphate)-binding proteins distinct from the Toc34/159 GTPase family associated transiently with preproteins. This class of proteins resides mainly in the cytosol and shows sequence similarities with Sey1/RHD3, required for endoplasmic reticulum membrane fusion, and with the periplastid-localized import factor PPP1, previously identified in the Apicomplexa and diatoms. These GTP-binding proteins, named plastid targeting factor for protein import 1 (PTF1) to PTF3, may act as plastid targeting factors in Rhodophyta.

Results and Discussion

**Tic20pt Localizes to the Chloroplast Envelope and Interacts with FtsHpt.** In *Cyanidioschyzon merolae*, two Tic20 homologs are encoded by the nuclear gene CMS050C (group 1) and the conserved red algal chloroplast gene CMV078C (ycf60, http://czon.jp; group 2) (6). Since all known protein import–related Tic20 are classified into group 2 (7), we hypothesized that the plastome-encoded CMV078C (Tic20p) would be part of a Tic. Anti-Tic20pt antibody recognized a *C. merolae* chloroplast 20-kDa protein found in the digitonin-solubilized membrane fraction, which enriched the envelope proteins (Fig. 1 A–C, Lower). Plastome-encoded FtsHpt was also found in this fraction to a significant extent as compared with thylakoidal PsaAB, suggesting its dual localization to the envelope and the thylakoids (Fig. 1 O). A fraction of the digitonin-solubilized FtsHpt associated with Tic20pt (Fig. 1 D), suggesting their functional cooperativity, like the Tic-Ycf2/FtsHi motor supercomplex in green lineages (8).

**Tic20pt Associates with Translocating Preproteins during Import In Vivo.** We adopted an in vivo approach to analyze preprotein-interacting proteins during import with *C. merolae* transformants with inducible expression of TpGFP-3xFLAG (consisting of a transit peptide [Tp] of the chloroplast protein ApcC [CMO250C] and GFP (green fluorescent protein import | organelle biogenesis | algal evolution | intracellular protein traffic | Rhodophyta

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The authors declare no competing interest.

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protein) with the 3xFLAG-tag (Fig. 2A). Four hours after induction, the TpGFP-3xFLAG precursor had accumulated, and after a subsequent 3-h chase incubation, the protein had been processed to its mature size, indicating completion of chloroplast import (Fig. 2B). At each time point, the membrane-bound proteins were solubilized with digitonin and immunoprecipitated with anti-Tic20pt (αTic20pt) or control (αC) immunoglobulin Gs.

**A Distinct Class of GTP-Binding Proteins May Act as a Chloroplast Targeting Factor in Rhodophyta.** A class of GTP-binding proteins (CMI311C, CMD101C, and CMQ054C; hereafter named plastid targeting factor for protein import 1 [PTF1], PTF2, and PTF3, respectively) (Fig. 2C), distinct from preprotein receptor Tic34/Toc159 GTPase in green lineages (2), was transiently bound to preproteins. Notably, a 67-kDa protein was observed (asterisk), precursor (p), and processed mature (m) forms of TpGFP-3xFLAG were detected. (Scale bars: 5 μm.) (C) Subcellular distribution of Tic20pt and FtsHpt (Lower). Chloroplasts (Cp) were ruptured hypotonically (S1), successively washed with high (S2 and S3) and low salt (S4), and solubilized by digitonin to obtain an envelope-rich fraction (Dig) and membrane pellets (P) containing thylakoids. (Upper) Coomassie Brilliant Blue staining. (Lower) Immunoblotting. Chlorophylls, thylakoid; PsaAB, thylakoid; RbcL, stroma. (D) Interaction between Tic20pt and FtsHpt shown by immunoprecipitation using anti-Tic20pt (αTic20pt) or control (αC) immunoglobulin Gs.

For ApcC, peptides derived from Tp of TpGFP-3xFLAG were detected. For ApCp, peptides derived from Tp of TpGFP-3xFLAG were detected.
and CMQ137C were tentatively annotated as Toc34 homologs. MS analysis of preprotein-associated proteins failed to detect their identities as TOC constituents must be carefully examined. A wide range of lineages with red algal–derived plastids, including Apicomplexa and diatoms, commonly lacks clear Toc75, Toc34, or Toc159 orthologs.

Our data suggest that the distinct GTP-binding protein family participates in preprotein targeting to chloroplasts by recognizing Tps. Their C-terminal Q-rich domains are conserved among Rhodophyta and other red lineages, indicating that this domain may be crucial for targeting to the outer envelope or for binding Tps. Their C-terminal Q-rich domains are conserved among Rho-phyta and other red lineages, indicating that this domain may be crucial for targeting to the outer envelope or for binding Tps. Their C-terminal Q-rich domains are conserved among Rho-

which may reflect the difference in their N-terminal domains. Our data suggest that Rhodophyta utilize conserved mechanisms for protein translocation across the inner envelope involving Tic20pc centered TIC but retain a distinct preprotein targeting mechanism, which has been conserved among the red lineages and possibly, among another non-Chloroplastida, Glaucophyta (5, 12).

Materials and Methods

C. mero1e 10D was used. MS was performed on Q-Exactive (ThermoFisher). Protein sequences were obtained from GenBank. Experimental procedures are in SI Appendix.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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