PNAS BRIEF REPORT | PLANT BIOLOGY OPEN ACCESS

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

Sanghun Baek[a], Sousuke Imamura[b,c], Takeshi Higa[a,1], Yumi Nakai[d], Kan Tanaka[b], and Masato Nakai[a,2]

Edited by Philip Benfey, Duke University, Durham, NC; received May 18, 2022; accepted July 14, 2022

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 $Tic20_{pt}$ localized to the chloroplast envelope and was transiently associated with preproteins during import, suggesting its conserved function as a TIC constituent. Besides plastome-encoded $FtsH_{pt}$ 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.

chloroplast protein import | organelle biogenesis | algal evolution | intracellular protein traffic | Rhodophyta

All extant photosynthetic eukaryotes are thought to have originated from a unique ancestral endosymbiotic relationship between a host eukaryotic cell and a cyanobacterium-like endosymbiont (1). Following the establishment of the protein transport mechanism for cytosolically synthesized chloroplast-destined proteins through translocons at the outer and inner envelope membranes, the endosymbiont evolved into chloroplasts by massive transfer of the endosymbiont genes to the nucleus. Subsequently, the Glaucophyta and Rhodophyta diverged from the Chlorophyta, the ancestor of land plants. All Archaeplastida are believed to share common chloroplast protein transport mechanisms involving conserved TOC and TIC machineries (2). Mechanisms of chloroplast protein import have been extensively studied in plants; TOC consists of preprotein receptor GTPases Toc34/Toc159 and the Toc75 channel, whereas TIC consists of the central Tic20 channel and other indispensable constituents (2, 3). The conservation of their functional orthologs in Chlorophyta was experimentally demonstrated (4). However, Rhodophyta appear to lack some counterparts of these constituents, including Toc75 (2, 5). This study aims to obtain clues about chloroplast protein import mechanisms in Rhodophyta.

Results and Discussion

$Tic20_{pt}$ Localizes to the Chloroplast Envelope and Interacts with $FtsH_{pt}$. 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 ($Tic20_{pt}$) would be part of a TIC. Anti-$Tic20_{pt}$ 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 $FtsH_{pt}$ 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*C*). A fraction of the digitonin-solubilized $FtsH_{pt}$ associated with $Tic20_{pt}$ (Fig. 1*D*), suggesting their functional cooperativity, like the TIC-Ycf2/FtsHi motor supercomplex in green lineages (8).

$Tic20_{pt}$ Associates with Translocating Preproteins during Import In Vivo. We adopted an in vivo approach to analyze preprotein-interacting proteins during import using *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

Author affiliations: [a]Institute for Protein Research, Osaka University, Suita 565-0871, Japan; [b]Institute of Innovative Research, Tokyo Institute of Technology, Yokohama 226-8503, Japan; [c]NTT Space Environment and Energy Laboratories, Nippon Telegraph and Telephone Corporation, Tokyo 180-8585, Japan; and [d]Faculty of Medicine, Osaka Medical and Pharmaceutical University, Takatsuki 569-8686, Japan

Author contributions: M.N. designed research; S.B., S.I., T.H., Y.N., K.T., and M.N. performed research; S.B. and M.N. analyzed data; S.I. and K.T. generated algal transformants; and S.B. and M.N. wrote the paper.

The authors declare no competing interest.

Copyright © 2022 the Author(s). Published by PNAS. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

[1]Present address: Graduate School of Arts and Sciences, The University of Tokyo, Tokyo 153-8902, Japan.

[2]To whom correspondence may be addressed. Email: nakai@protein.osaka-u.ac.jp.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2208277119/-/DCSupplemental.

Published August 15, 2022.

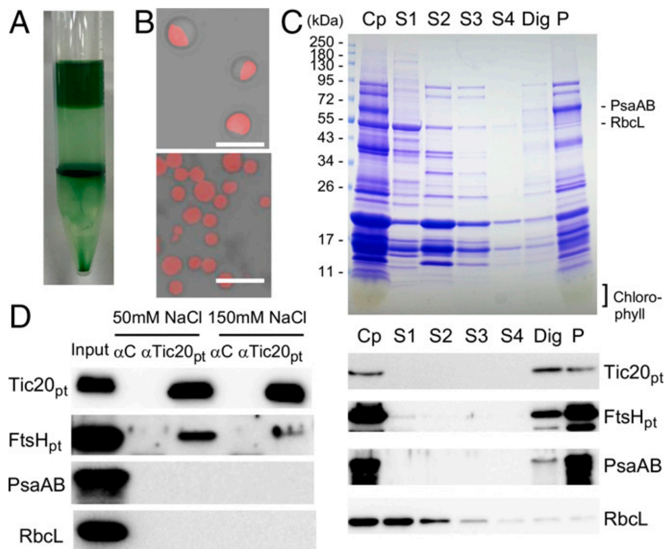

Fig. 1. (*A*) Intact chloroplasts (interface) isolated from *C. merolae*. (*B*) Microscopy images (chlorophyll autofluorescence is in red in the bright-field image) of unbroken cells (*Upper*) and chloroplasts (*Lower*). (Scale bars: 5 µm.) (*C*) Suborganellar distribution of $Tic20_{pt}$ and $FtsH_{pt}$ (*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 $Tic20_{pt}$ and $FtsH_{pt}$ shown by immunoprecipitation using anti-$Tic20_{pt}$ ($\alpha Tic20_{pt}$) or control (αC) Immunoglobulin Gs.

protein) with the 3xFLAG-tag) (Fig. 2*A*). 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. 2*B*). At each time point, the membrane-bound proteins were solubilized with digitonin and immunoprecipitated with anti–3xFLAG-tag antibody. Both $Tic20_{pt}$ and $FtsH_{pt}$ were coimmunoprecipitated upon transient accumulation of the TpGFP-3xFLAG precursor, demonstrating their participation in chloroplast protein import (Fig. 2 *B* and *C*). By mass spectrometry (MS) analysis (Fig. 2*D*), other than $FtsH_{pt}$, cytosolic and chloroplast stromal chaperones $Hsp70_{cyt}$, $Hsp70_{pt}$, and $Cpn60_{pt}$ were detected, suggesting that they maintain preproteins in an unfolded state during import (Fig. 2*E*).

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. 2*C*), distinct from preprotein receptor Toc34/Toc159 GTPase in green lineages (2), was transiently bound to preproteins. Notably, a 67-kDa protein was observed with relatively high abundance (Fig. 2*C*), which was confirmed as CMI311C (PTF1) by MS. PTF1, PTF2, and PTF3 contain a typical GTP-binding motif near the amino terminus, and their amino-terminal 400 to 500 residues show sequence similarity with those of eukaryotic Sey1/RHD3 (Fig. 3*A*), a member of the dynamin superfamily required for homotypic endoplasmic reticulum (ER) membrane fusion (9). CMN141C, the Sey1/RHD3 ortholog in *C. merolae*, was absent in the preprotein-associated protein fraction. While Sey1/RHD3 possesses two transmembrane segments near the C terminus for ER association, PTF1, PTF2, and PTF3 do not. Instead, their C-terminal ~200 residues contain conserved glutamines (Q) and are even Q rich (notably for PTF1), and they show sequence similarity with those of PPP1, the *Toxoplasma gondii* (Apicomplexa) peripheral plastid protein required for apicoplast protein import (10) (Fig. 3 *A* and *B*). While PTF1 and PTF3 mainly localized in the cytosol (Fig. 3*C*), both associated with preproteins purified from the membrane fraction (Fig. 2*C*), suggesting their involvement at the membrane boundary, such as at the chloroplast surface. Like Sey1, PTF1 to -3 proteins may form homo- or heterodimers in vivo and interact with $Hsp70_{cyt}$ (Fig. 3*D*).

Purified GST(glutathione-*S*-transferase)-PTF1 and GST-PTF3 fusion proteins, but not GST alone, pulled down a model preprotein pApcC, and this interaction depended on the pApcC Tp (Fig. 3 *E*–*G*). We tested another preprotein pNDH (CMM178C) (Fig. 3*F*). Some of the purified pNDH preprotein was amino-terminally degraded. GST-PTF1 and GST-PTF3 pulled down only the full-length protein, indicating that this interaction requires intact Tp.

The chloroplast protein import mechanisms in Rhodophyta were assumed to be similar to those in green lineages (2), but this possibility should be reevaluated. Chloroplast Tps from Rhodophyta differ from those of green lineages (5). A true Toc75 ortholog appears absent in Rhodophyta (5). In *C. merolae*, *CMP284C*

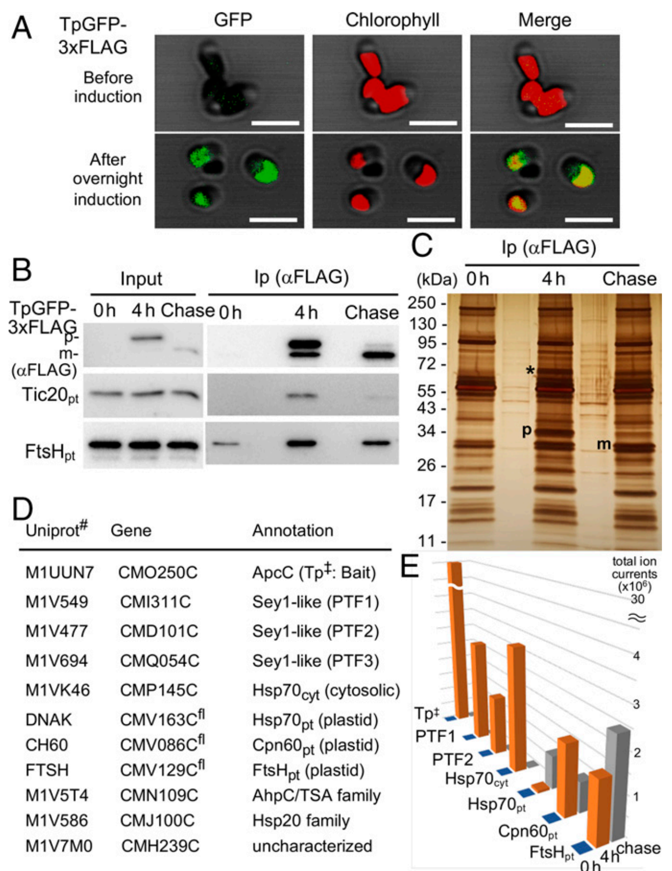

Fig. 2. (*A*) Chloroplast localization of TpGFP-3xFLAG after overnight induction. (Scale bars: 5 µm.) (*B*) Induced accumulation of preproteins (p) of TpGFP-3xFLAG (*Left*) and their transient association with $Tic20_{pt}$ and $FtsH_{pt}$ analyzed by immunoprecipitation (Ip) using αFLAG antibody (*Right*) from digitonin-solubilized membrane fractions (Input) before induction (0 h), after induction (4 h), and after a further 3-h incubation in the dark (chase). (*C*) Silver staining of the immunoprecipitated proteins prepared as in *A* (67-kDa protein [asterisk], precursor [p], and processed mature [m] forms of TpGFP-3xFLAG). (*D*) Preprotein-interacting candidates detected by MS. Photosynthetic and other abundant proteins similarly observed in 0-h samples were excluded. Fl, plastid genes. #_CYAM1 was omitted; ‡for ApcC, peptides derived from Tp of TpGFP-3xFLAG were detected. (*E*) Sum of total ion currents of peptides for each time point detected by MS (refer to *SI Appendix*). ‡For ApcC, peptides derived from Tp of TpGFP-3xFLAG were detected.

 https://doi.org/10.1073/pnas.2208277119

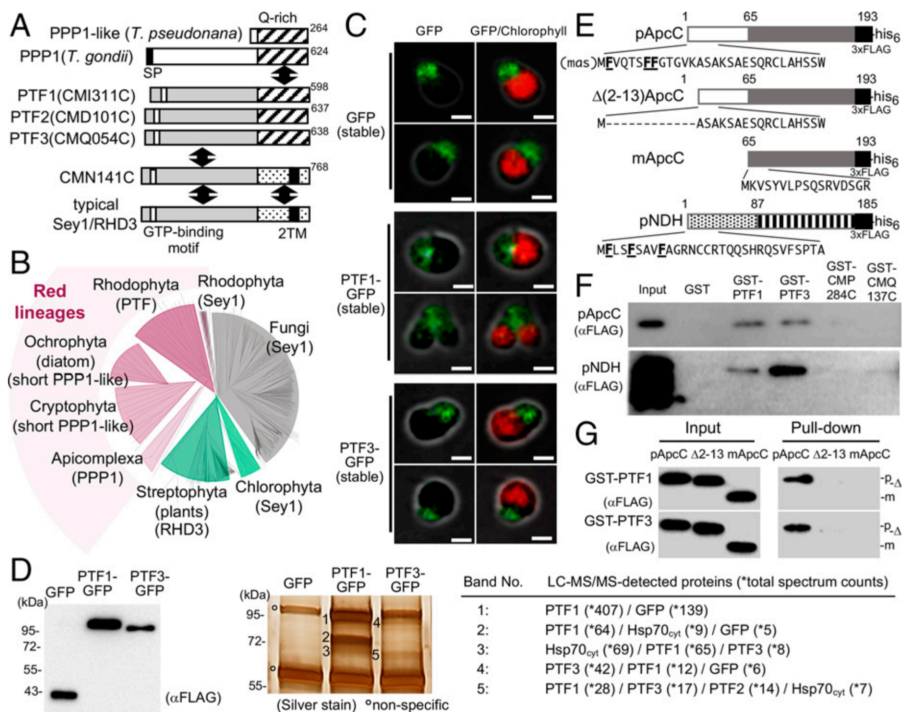

Fig. 3. (*A*) Structures of PTF1- to -3 and CMN141C from *C. merolae* and PPP1 from *T. gondii* and *Thalassiosira pseudonana* in relation to typical Sey1/RHD3. Double-headed arrows indicate sequence homology. SP, signal peptide; TM, transmembrane segment. (*B*) Phylogeny of PTF, PPP1, Sey1/RHD3, and related proteins. (*C*) Localization of GFP, PTF1-GFP, and PTF3-GFP in *C. merolae* cells. GFP (*Left*) and merged GFP/chlorophyll autofluorescence (*Right*) with phase-contrast images. (Scale bars: 1 μm.) (*D*) Purification of PTF1-GFP and PTF3-GFP from *C. merolae* cells (*Left* and *Center*) and MS-detected copurified proteins (*Right*). GFP, GFP-expressing cells. LC-MS/MS, liquid chromatography-tandem mass spectrometry. (*E*) Preproteins and deletion mutants for binding experiments. Phenylalanine residues, characteristic of Rhodophyta Tps, are underlined. (*F*) Binding ability of GST-PTF1 and GST-PTF3 to model preproteins (input) pApcC and pNDH, examined by a pull-down assay using glutathione-Sepharose. GST-CMP284C and GST-CMQ137C were also analyzed with GST as a control. Preproteins were detected using αFLAG antibody. (*G*) The same as in *F* but pApcC, Δ(2-13)ApcC, and mApcC were analyzed.

and *CMQ137C* were tentatively annotated as Toc34 homologs. MS analysis of preprotein-associated proteins failed to detect them (Fig. 2*D*). GST-CMP284C and GST-CMQ137C showed much weaker binding abilities to preproteins (Fig. 3*F*). Hence, 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 N-terminal GTP-binding domain is conserved among Rhodophyta but is not present in diatoms, and it is replaced by unrelated sequences in Apicomplexa (Fig. 3 *A* and *B*). In Rhodophyta, preproteins are targeted to the outer envelope after synthesis on cytosolic ribosomes, while in Apicomplexa or diatoms, this targeting only occurs after passing through symbiont-specific ERAD (endoplasmic reticulum-associated degradation)-like machinery located at the ER membranes surrounding the apicoplast/chloroplast (11), 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 $Tic20_{pt}$-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. merolae 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.

ACKNOWLEDGMENTS. We thank Gaku Fujii and Yukari Asakura for assistance with the initial stage of this study. This work was supported by Japan Ministry of Education, Culture, Sports, Science, and Technology Grant 19H03183 and by the Cooperative Research Program of the "Network Joint Research Center for Materials and Devices."

1. J. M. Archibald, Endosymbiosis and eukaryotic cell evolution. *Curr. Biol.* **25**, R911–R921 (2015).
2. M. Nakai, New perspectives on chloroplast protein import. *Plant Cell Physiol.* **59**, 1111–1119 (2018).
3. S. Kikuchi *et al.*, Uncovering the protein translocon at the chloroplast inner envelope membrane. *Science* **339**, 571–574 (2013).
4. S. Ramundo *et al.*, Coexpressed subunits of dual genetic origin define a conserved supercomplex mediating essential protein import into chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 32739–32749 (2020).
5. M. Knopp, S. G. Garg, M. Handrich, S. B. Gould, Major changes in plastid protein import and the origin of the chloroplastida. *iScience* **23**, 100896 (2020).
6. A. R. Kasmati, M. Töpel, R. Patel, G. Murtaza, P. Jarvis, Molecular and genetic analyses of Tic20 homologues in *Arabidopsis thaliana* chloroplasts. *Plant J.* **66**, 877–889 (2011).
7. G. G. van Dooren, C. Tomova, S. Agrawal, B. M. Humbel, B. Striepen, *Toxoplasma gondii* Tic20 is essential for apicoplast protein import. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13574–13579 (2008).
8. S. Kikuchi *et al.*, A Ycf2-FtsHi heteromeric AAA-ATPase complex is required for chloroplast protein import. *Plant Cell* **30**, 2677–2703 (2018).
9. J. Hu *et al.*, A class of dynamin-like GTPases involved in the generation of the tubular ER network. *Cell* **138**, 549–561 (2009).
10. L. Sheiner *et al.*, A systematic screen to discover and analyze apicoplast proteins identifies a conserved and essential protein import factor. *PLoS Pathog.* **7**, e1002392 (2011).
11. U. G. Maier, S. Zauner, F. Hempel, Protein import into complex plastids: Cellular organization of higher complexity. *Eur. J. Cell Biol.* **94**, 340–348 (2015).
12. D. Köhler *et al.*, Identification of protein N-termini in *Cyanophora paradoxa* cyanelles: Transit peptide composition and sequence determinants for precursor maturation. *Front Plant Sci* **6**, 559 (2015).