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Plastid Import of a Transit Sequence-Less Precursor Protein in *Arabidopsis thaliana*

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Abstract

Chloroplasts make use of translocon complexes in the outer and inner plastid envelope membranes called the TOC and TIC machineries for importing the major part of their proteins from the cytosol. Using the chloroplast envelope quinone oxidoreductase homologue ceQORH as model, we recently showed that import of this transit sequence-less precursor into *Arabidopsis* chloroplasts occurs through a unique pathway. Import of ceQORH is mediated by three distinct chloroplast members of the preprotein and amino acid transporter (PRAT) family, designated HP20, HP30 and HP30-2. This family comprises 17 evolutionarily conserved preprotein and amino acid transporters that obviously operate both in mitochondria and chloroplasts and mediate the import of certain subsets of cytosolic precursor proteins including those lacking cleavable transit sequences. Here we further dissected the PRAT-dependent pathway and identified three classes of additional components: (i) proteins of the TOC GTPase superfamily of receptors (TOC120, TOC90 and TOC34), (ii) redox-sensors (TIC55), and (iii) molecular chaperones of the HSP93-V and HSC70 families. We propose a unique modular arrangement of PRAT, TOC and TIC components for import of ceQORH into the inner plastid envelope membrane of chloroplasts.

Keywords Chloroplast biogenesis; Envelope biochemistry; Protein translocation

Introduction

Plastids comprise a family of partially interconvertible forms with highly specialized functions and different protein complements [29]. Chloroplasts are the predominant plastid type of green plants. As semiautonomous cell organelles which contain only limited coding information in their own DNA, chloroplasts must import roughly 3000 different proteins from the cytosol [46]. Dahlin and Cline [15] demonstrated that chloroplast protein import is determined by age and developmental state of the plant.

Due to their endosymbiotic origin, chloroplasts are surrounded by an outer and an inner envelope membrane that need to be crossed when cytoplasmic precursors enter the plastids. Translocons at the outer and inner chloroplast envelope membranes, called the TOC and TIC machineries, have been identified that mediate precursor protein import [5,20,22].

It was for a long time believed that all of the different cytosolic precursors would enter the chloroplast through the same, jointly acting TOC and TIC machineries. Recent evidence, however, suggests the operation of multiple, regulated import pathways in *Arabidopsis thaliana* [3,25,32,51]. According to recent studies, different combinations of TOC and TIC proteins establish different import sites. For example, Jarvis et al. [25] isolated twin components of the regulatory GTPase AtTOC33/34 that are differentially expressed in dark-grown and light-adapted plants. Bauer et al. [3] identified three receptor components in addition to the main precursor receptor AtTOC159. Smith et al. [51] demonstrated that the presequence receptors AtTOC159, AtTOC130 and AtTOC120 have different precursor protein specificities. While AtTOC159 participates in import of photosynthetic proteins, AtTOC130 and AtTOC120 bound non-photosynthetic precursors in pull-down assays [51]. In addition, mutants that are defective in AtTOC159 were shown to be seedling lethal, whereas AtTOC130 and AtTOC120 knock-out mutants were not [32].

Another, interesting case is made by proteins destined to the outer or inner plastid envelope membranes. Most of the outer and at least some of the inner plastid envelope membrane proteins lack predictable NH_2-terminal transit peptides for import [36,37,39]. For example, inner envelope proteins such as TIC32 and the chloroplast envelope quinone oxidoreductase homologue ceQORH contain internal targeting information that governs their import and membrane insertion [36,37,39]. TIC32 and ceQORH do not interact with the standard protein import machinery containing the receptor TOC159 and translocation channel TOC75 [36,37,39]. Chemical cross-linking identified three new components not previously implicated in protein import that represent chloroplast members of the family of preprotein and amino acid transporters PRAT [43,47,48].

In the present work, we identified additional components operating in chloroplast import of ceQORH in *A. thaliana*. We report the purification of at least three classes of envelope proteins, comprising previously identified TOC receptors (AtTOC120, AtTOC90 and AtTOC34), TIC components (TIC55), as well as molecular chaperones such as AtHSP93-V/TIC40 and stromal HSC70, both known to act as ATP-powered import motors during protein translocation. Our data resolve previous findings on the receptor and energy requirements of ceQORH import and support a central role of the PRAT proteins in the plastid envelope for conductance of ceQORH.

Materials and Methods

Plant growth

Arabidopsis thaliana of the indicated ecotypes was grown at 25°C under continuous white light illumination provided by fluorescent bulbs (30 W/m^2) on soil for appropriate periods. *Attoc159 (ppi2)* seedlings [3] were grown on half-strength Murashige and Skoog medium supplemented with 100 mM sucrose [7].

Plastid isolation, manipulation, and protein import

Plastids were isolated from seedling or leaf homogenates by density gradient centrifugation on Percoll (Pharmacia LKB Biotechnol. AB, Sweden) [45]. Re-isolated, intact plastids were resuspended in import buffer lacking ATP [45], energy depleted [45] and added to the radiolabeled precursors given in the text. Either bacterially expressed precursors or *in vitro*-synthesized precursors were used. *In vitro*-synthesis of ^{35}S-containing precursors was carried out in a wheat germ system by coupled transcription/translation of respective cDNA clones. All precursors were denatured in 8 M urea prior to use and diluted to 0.2 M final urea concentration before being added to the import reactions [44]. Final 50 µL import assays consisted of 25 µL of the doubly-concentrated import buffer, 10 µL of the plastid suspension containing, if not stated otherwise, 5·10^7 plastids, 5 µL of the different urea-denatured, radiolabeled precursors, and Mg-ATP and Mg-GTP as indicated in the text. If needed, doubly-distilled water was added to adjust the final reaction volume. Import reactions were conducted at 23°C for 15 min. in darkness. Plastids then were re-isolated on Percoll [45]. Post-import protease treatment of plastids with thermolysin and extraction of membranes with sodium carbonate, pH 11, or 1 M NaCl were made as described [13]. Plastid sub-fractionation into envelopes, stroma and thylakoids was carried out according to Li et al. [34].

Production of import intermediates

^{35}S-(60-100)-ceQORH-GFP and ^{35}S-ceQORH-GFP were expressed in *Escherichia coli* strain SG13009 (Qiagen) in the presence of ^{35}S-methionine and recovered from inclusion bodies by dissolution in 8 M urea containing 20 mM imidazole-HCl, pH 8.0. Protein was passed over a G-25 column which yielded approximately 90% pure precursor fractions. These in turn were incubated with isolated, energy-depleted *Arabidopsis* plastids in the presence of 0.1 mM Mg-ATP and 0.1mM Mg-GTP for 15 min. After the reaction, the plastids were re-isolated, lysed under hypertonic conditions, and total membranes were sub-fractionated by flotation into linear 20-38% sucrose gradients [49]. Individual gradient fractions were harvested and their proteins precipitated with trichloroacetic acid. After washing with acetone, ethanol and ether, protein was separated by SDS-PAGE, transferred onto nitrocelluose filters and detected with the antisera described in the text. ^{35}S-(60-100)-ceQORH-GFP and ^{35}S-ceQORH-GFP were detected by autoradiography. Alternatively, import intermediate-associated proteins interacting with ^{35}S-(60-100)-ceQORH-GFP and ^{35}S-ceQORH-GFP were solubilized from the OM-IM fraction obtained after sucrose gradient centrifugation in a buffer containing 2% Triton X100, 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole-HCl, pH 8.0, and 1mM phenylmethylsulfonyl fluoride [49,56]. After a step of centrifugation at 100,000g for 15 min, 10 mL portions of the supernatant were incubated for 1 h at 4°C with 0.25 mL Ni^{2+}-NTA-agarose beads in solubilization buffer [56]. The beads were washed twice, and the bound protein was eluted with 2% SDS, 100 mM EDTA, 50 mM PIPES-NaOH, pH 7.4, precipitated by methanol/chloroform, and suspended in SDS-sample buffer [33]. Protein was separated by either 1D-SDS-PAGE on 10-20% polyacrylamide gels or 2D-SDS-PAGE including isoelectric focusing in the first dimension and SDS-PAGE gradient gel electrophoresis in the second dimension [41]. Protein was stained with Coomassie Brilliant Blue G25 and subjected to sequencing according to Chang et al. [9].

Other Biochemical Procedures

Immunoprecipitation was performed according to Wiedmann et al. [58], using the antisera described in the text. Western blotting was done according to Towbin et al. [57], using either an enhanced chemiluminescence (ECL) detection system or anti-rabbit, anti-goat, alkaline phosphatase detection system with 4-nitroblue-tetrazoliumchloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Bio-computational Methods and Sequence Data Sources

Multiple sequence alignments were performed using Clustal W method with Gonnet 250 residue weights within the Megalign® program (DNAStar, Madison, WI), as described in the SI section. This web site also contains information on software and internet databases used to predict the subcellular localizations and topologies of AtHP20 and AtHP30.

Arabidopsis Mutants

The *Attoc159*, *Attoc132*, *Attoc120*, *Attoc90* as well as *Attoc33* and *Attoc34* mutants of *A. thaliana* have been described [3,14,21,23,25,32]. The *Atcphsc70-1* (Salk_140810) and *cphsc70-2* (Salk_095715) were identified as described previously by Su and Li [53,54]. The *Athsp93-v-1* and *Athsp93-v-2* mutants (Salk_014058) were identical to those described previously [31,50]. The *Attic40* mutant used is the *tic40-2* null allele previously reported [12].

Results and Discussion

Isolation of components interacting with ceQORH during its import into chloroplasts of *A. thaliana*

Kouranov and Schnell [30] have shown that binding of transit sequence-containing precursors to the plastids is initially is reversible and occurs in an equilibrium reaction unless nucleoside triphosphates are present. Low (<0.1 mM) ATP concentrations favor partial integration of the receptor-bound precursors into the import machinery [30]. This step, which originally has been referred to as binding, is stimulated by GTP [30,35,42]. The precursors then insert across the outer envelope membrane and also interact with components of the inner envelope [30]. As a result, early import intermediates are formed [49]. Similar to these results Miras et al. [37] found that also binding of ceQORH used as a model of a transit sequence-less precursor was initially reversible and that this step was stimulated by low (<0.1 mM) Mg-ATP concentrations. ceQORH then inserted across the outer envelope membrane and established strong interactions with HP20 [47], a member of the PRAT protein family in chloroplasts [43,48]. ceQORH also established contact sites with components of the inner envelope membrane; the latter step was favored when the assays were supplemented with low (<0.1 mM)

concentrations of Mg-GTP [37,47]. ceQORH then tightly interacted with two other components, named HP30 and HP30-2 [47], that are additional members of the PRAT protein family of chloroplasts [43,48]. Pretreatment of chloroplasts with thermolysin abolished any step of ceQORH import [37,47] and suggested an essential role of protease-sensitive receptors in import of transit sequence-less precursors. Indeed, chemical cross-linking using isolated chloroplasts from respective knock-out mutants revealed that TOC120 and TOC90 are likely candidates for these protease-sensitive import receptors [47].

To pinpoint these and other, yet to be characterized interactions and partners, a fusion protein was used consisting of ceQORH, the green fluorescence protein (GFP), and a COOH-terminal hexa-histidine [$(His)_6$] tag (ceQORH-GFP-$[His]_6$) [37]. The protein was radio-labeled (^{35}S-Met) and expressed in *Escherichia coli*, purified on Ni-NTA agarose, and added to isolated, energy-depleted *Arabidopsis* chloroplasts. Incubations were performed in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. These ATP and GTP concentrations were used to permit the isolation of proteins interacting with ceQORH in the outer and inner plastid envelope membranes, henceforth collectively referred to as import intermediate-associated proteins (IAPs) [28,45]. As control to the full-length ^{35}S-ceCORH-GFP-$[His]_6$, a truncated version of ^{35}S-ceQORH was employed that lacked the NH_2- and COOH-terminal parts of ceQORH and contained only the central piece of amino acids between positions 60 and 100, fused to GFP, and the $(His)_6$ tag [^{35}S-(60-100)-ceQORH-GFP-$(His)_6$]. This truncated version of ceQORH has been shown to be imported via the main import pore, TOC75 [37].

We first incubated ^{35}S-ceQORH-GFP-$(His)_6$ and ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ separately with isolated chloroplasts. After a step of re-isolation on Percoll, intact plastids were rapidly disrupted and processed to yield a crude membrane fraction [28] which was loaded onto a linear 20-40% (w/v) sucrose gradient and separated by flotation into a light outer envelope membrane fraction (OM), a heavy inner envelope membrane (IM) fraction, and an intermediate density (OM-IM) fraction [28] (Figure 1A and B). Autoradiography and Western blotting revealed that ^{35}S-ceQORH-GFP-$(His)_6$ was enriched in the OM-IM fraction obtained after centrifugation, indicating the formation of IAP complexes (Figure 1A and B). By contrast, most of the outer envelope membrane marker protein OEP37 was present in the light outer membrane fraction, and most of the inner envelope membrane protein IEP36 was present in the inner membrane fraction (Figure 1A). Similar results were obtained when ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ was used as import substrate, demonstrating that also this precursor formed IAP complexes (Figure 1A and B). When ^{35}S-ceQORH-GFP-$(His)_6$ and ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ were incubated together, they co-purified in the OM-IM fraction of chloroplasts (Figure 1B). This finding demonstrated that they did not compete for the same import site. As shown previously by competition and antibody blocking experiments [37], full-length ceQORH enters the plastids in a TOC159- and TOC75-independent manner. By contrast, ^{35}S-(60-100)-ceQORH-GFP is imported into the plastids in a TOC75-dependent way [37], explaining why (60-100)-ceQORH-GFP and ceQORH-GFP formed IAPs independently from each other.

To purify envelope proteins bound to ^{35}S-ceQORH-GFP-$(His)_6$, the IAP fractions (cf. Figure 1) were pooled and subjected to mild detergent solubilization [28,45]. The resulting higher molecular weight protein complexes were purified by Ni-NTA-agarose chromatography [28,45] and analyzed further by 1D- and 2D-SDS polyacrylamide gel electrophoresis (PAGE) and Coomassie staining. The eluted proteins consisted of the ^{35}S-ceQORH-GFP-$(His)_6$ and at least 10 main polypeptide bands (Figure 2) that were characterized further by protein sequencing.

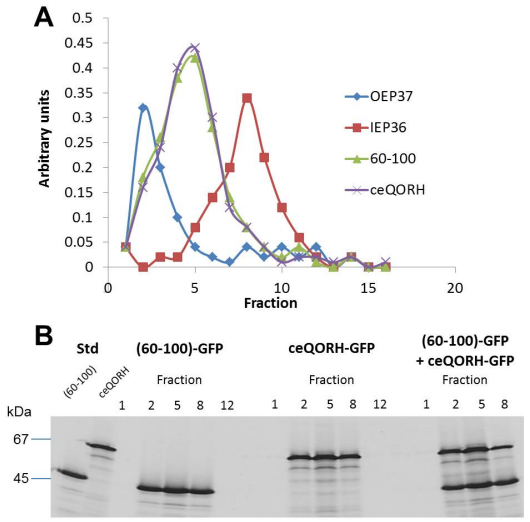

Figure 1: Production of import intermediates during the transport of bacterially expressed, urea-denatured ^{35}S-ceQORH-GFP-$(His)_6$ and ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ into isolated and energy-depleted *A. thaliana* chloroplasts. A, Distribution of OEP37 and IEP36 as well as ^{35}S-ceQORH-GFP-$(His)_6$ and ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ in outer membranes (OM, fractions 1 and 2), OM-IM junction complexes (fractions 3-7) and inner membranes (IM, fractions 8-10), obtained after sucrose density gradient centrifugation. Arbitrary units correspond to signal intensities on Western blots after detection with NBT-BCIP and to the amount of radioactivity of the ceQORH precursors quantified by liquid scintillation counting. B, SDS-PAGE analysis and autoradiography of ^{35}S-ceQORH-GFP-$(His)_6$ and/or ^{35}S-(60-100)-ceQORH-GFP-$(His)_6$ in the indicated sucrose density gradient fractions. Each lane contained 20 µg proteins. Std defines import standards.

Table S1 shows that the proteins interacting with ceQORH fall into four major classes: (i) members of the TOC GTPase superfamily such as AtTOC120, AtTOC90 and AtTOC34, (ii) redox-regulated proteins such as TIC55, (iii) molecular chaperones such as HSP93-V/TIC40 and chloroplast (cp) HSC70, acting as import motors and providing the driving force for the translocation of cytosolic precursors across the outer and inner envelope membranes, and (iv) AtHP20, AtHP30 and AtHP30-2 that had previously been shown to provide cross-link partners of ceQORH. Figure S1 and Table S2 underscore that (60-100)-ceQORH-GFP interacted with a different set of plastid envelope proteins, among them being AtTOC159, AtTOC75, AtTOC33 and AtTIC110.

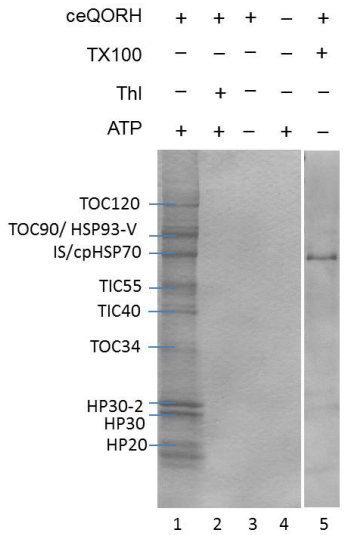

Figure 2: Purification of envelope proteins bound to ^{35}S-ceQORH-GFP-$(His)_6$ in chloroplasts of *A. thaliana*. Coomassie stain of envelope proteins that interact with ^{35}S-ceQORH-GFP-$(His)_6$ used as import substrate (IS) at different conditions (in the presence or absence of either Triton X-100 (TX100), thermolysin (Thl) or ATP as indicated, lanes 1-5).

The following control experiments proved that ^{35}S-ceQORH-GFP-$(His)_6$ interacted with the identified envelope proteins specifically and in an import-dependent manner. First, chloroplasts that had been pretreated with thermolysin and re-isolated were unable to import ^{35}S-ceQORH-GFP-$(His)_6$ and therefore they did not produce the co-purifying envelope protein bands (Figure 2, lane 2). This result is consistent with previous data [37,47] that ceQORH import requires protease-sensitive components (AtTOC120 and AtTOC90; see below), on the outer plastid surface. Second, omission of Mg-ATP during incubation yielded neither 35S-ceQORH-GFP-$(His)_6$ nor the co-purifying polypeptide bands (Figure 2, lane 3), a result consistent with the published requirement of ceQORH import for Mg-ATP [37,47] and the presence of HSP93-V and HSC70, acting as ATP-powered import motors, in the IAP fraction. Third, an import reaction in the absence of import substrate did not yield envelope polypeptides that bound to Ni-NTA agarose (Figure 2, lane 4). This control excluded the possibility that envelope polypeptides present in residual OM-IM junction complexes that were formed in the absence of precursor bound non-specifically to the affinity column. Fourth, no envelope polypeptides co-purified with import substrate when it was added during the solubilization of the OM-IM envelope fraction (Figure 2, lane 5). This result underscored that ^{35}S-ceQORH-GFP-$(His)_6$ interacted with the identified envelope polypeptides in an import-dependent reaction.

Three TOC GTPase receptors operate in ceQORH import into chloroplasts

Interestingly, three of the identified proteins interacting with ceQORH belong to the superfamily of TOC GTPases. This family comprises TOC159 [30,35,42,49] and TOC34 [19,26,55] that mediate preprotein recognition and initiate membrane transport [4,20,22,24]. In *A. thaliana*, both TOC-GTPases are encoded by small gene families [3,20-25]. *Arabidopsis* has four TOC159 homologs, named AtTOC159, AtTOC132, AtTOC120 and AtTOC90, and two TOC34 homologs, named AtTOC33 and AtTOC34 [3,20-25]. All four TOC159 proteins represent receptors and exhibit a characteristic tripartite structure, consisting of an NH_2-terminal acidic domain, a central GTP binding domain, and a COOH-terminal membrane-anchor domain, although the A-domain is greatly reduced in AtTOC90 [3,10,20,21].

Several lines of evidence suggest that there is a functional specialization amongst the AtTOC159 family members and that they display distinct precursor specificities. For example, an AtTOC159 knockout mutant, termed *ppi2*, was isolated which was conditionally seedling lethal [3]. *ppi2* seedlings are defective in the expression and import of light-induced proteins associated with chloroplasts (mostly photosynthetic proteins) but accumulate normal levels of house-keeping proteins [3,59]. The latter proteins are imported via AtTOC132 and AtTOC120, operating redundantly [3,24]. Whereas single *Attoc132* and *Attoc120* mutants displayed no visible phenotypes, respective double mutants were embryo-lethal [3,59]. Similar results were obtained for the *ppi1* and *ppi3* mutants that lack AtTOC33 and AtTOC34, respectively, which had only weak phenotypes in the single mutant states but were embryo-lethal when present in a double mutant background [14,25]. Biochemical and genetic studies suggest that AtTOC159 interacts with AtTOC33, whereas AtTOC132 and AtTOC120 interact with AtTOC34, to establish different import machineries [32].

If both AtTOC120 and AtTOC90 would be operative in the import of ceQORH into chloroplasts, their lack in respective *Attoc120* and *Attoc90* single mutants and particularly in *Attoc120*::*Attoc90* double mutants should have severe effects. In order to test this hypothesis, we used the published single *Attoc120* and *Attoc90* mutants as well as *Attoc120*::*Attoc90* double mutants [3,21,32] and carried out (i) Western blot analyses on total leaf proteins as well as IAPs formed by ceQORH, and (ii) *in vitro* protein import studies using ^{35}S-ceQORH. As control, the *ppi1* mutant (lacking AtTOC33) [25], *ppi2* mutant (lacking *Attoc159*) (3) and *ppi3* mutant (lacking AtTOC34) [14] were used.

Figure 3A shows that ceQORH protein levels were similar in wild-type as well as *Attoc159* and *Attoc132* plants. By contrast, ceQORH protein levels were drastically reduced, in most cases below the limit of detection, in *Attoc120* and *Attoc90* single mutant as well as *Attoc120*::*Attoc90* double mutant seedlings. *In vitro* import experiments (Figure 3B) confirmed that the diminished ceQORH protein levels are most likely due to an import defect rather than a reduction in expression of the *ceQORH* gene.

When the pattern of total IAPs was compared, insights were obtained on how AtTOC120 and AtTOC90 may function in *ceQORH* import (Figure 3C). While *Attoc120* mutant chloroplasts were unable to establish any IAPs with *ceQORH*, the two *Attoc90* mutants analyzed still formed early IAPs. However, no late IAPs containing HP20 and HP30 were found. These results demonstrated that

AtTOC120 and AtTOC90 act sequentially. Whereas *Attoc120* is indispensable for ceQORH binding to chloroplasts, AtTOC90 most likely plays a role in transferring the plastid-bound ceQORH into the HP20 and HP30 import channels in the outer and inner plastid envelope membranes, respectively.

When the amount of ceQORH was examined for *ppi1* and *ppi3* plants, only the latter lacking AtTOC34 showed reduced protein levels (Figure 3D, panel a). These results corroborated the data reported in Figure 2, showing co-fractionation of ceQORH and AtTOC34 in IAPs in wild-type chloroplasts. Protein import experiments underscored the role of AtTOC34 in ceQORH import (Figure 3D, panel b).

A Rieske, non-heme iron-sulfur protein is part of the import machinery for ceQORH

TIC55 belongs to a family of non-heme oxygenase proteins sharing conserved Rieske and mononuclear iron binding domains in plants and bacteria [8,17,18]. In addition to TIC55, this family comprise pheophorbide *a* oxygenasee (PAO), chlorophyllide *a* oxygenase (CAO), and a 52 kDa protein (PTC52) associated with the precursor NADPH: protochlorophyllide (Pchlide) oxidoreductase (pPOR) A translocon [45] (Figure 4A). Some of these chloroplast proteins have documented roles in chlorophyll biosynthesis (CAO) and degradation (PAO), and biochemical evidence indicates that PTC52 encodes a Pchlide *a* oxygenase activity [45]. A biochemical function for TIC55 has not yet been determined so its exact role in the TIC complex remains unknown. In addition to the highly conserved Rieske ($CxHx_{16-17}Cx_2H$) and mononuclear iron binding ($Nx_2Dx_{3-4}Hx_4H$) motifs, PTC52, PAO and TIC55 proteins from different plant species share the presence of a conserved, CxxC motif reminiscent of thiolreductases at approximately 73 amino acids from the carboxy terminus which is also found in homologs from *Nostoc* and other cyanobacteria (Figure 4B). Via this CxxC motif, TIC55, PTC52 and PAO are prone to regulation by the thioredoxin system and also respond to oxidative stress [2]. On the basis of these results we propose a role of TIC55 as redox-sensor in the ceQORH import pathway while retaining the possibility that it acts upon a substrate that is co-imported with an enzymatic protein.

Both HSP93-V and stromal HSC70 function in ceQORH import into chloroplasts

The third group of ceQORH interacting proteins is constituted by molecular chaperones of the heat shock protein (HSP) and heat shock cognate protein (HSC) families and comprises HSP93 (also known as caseinolytic protein C-class chaperone, ClpC) and HSC70. Stromal HSP93 belongs to the HSP100 subfamily of triple A (AAA+) ATPases that are associated with various cellular activities. Stromal HSP93 is a soluble protein that interacts with other translocon components and with importing precursors [1,40]. *Arabidopsis* has two genes encoding chloroplast HSP93: HSP93-III (ClpC2) and HSP93-V (ClpC1). HSP93 requires ATP and the co-chaperone TIC40 for activity [6,12,52]. The second class of chaperones implicated in chloroplast protein import is represented by the chloroplast (cp) hsc70s [11,53,54]. Studies with single *cphsc70-1* and *cphsc70-2* knock-out mutants defined a developmental role of cpHSC70s in chloroplast protein import [11,53,54]. Double mutant analyses revealed that the cpHSC70 and HSP93/TIC40 systems function in parallel *in planta* [11,53,54].

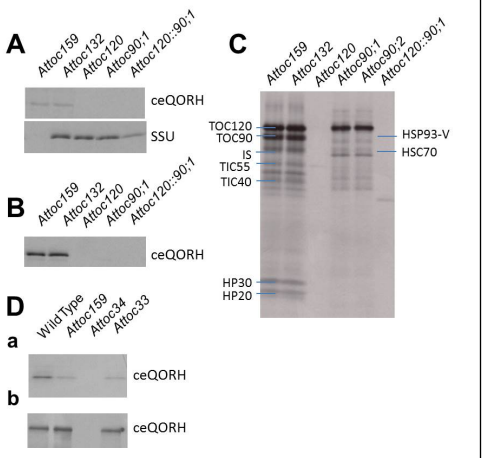

Figure 3: Lack of import of ceQORH-GFP into chloroplasts of the *Attoc120* and *Attoc90* single and double mutants. A, Western blot analysis of ceQORH in chloroplasts isolated from the indicated *Attoc120* and *Attoc90* single and double mutants as well as *Attoc159* and *Attoc132* mutants. As control, the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU) was used. B, *In vitro* chloroplast import of ^{35}S-ceQORH into the same plastids as those used in A. C, Isolation of IAPs from chloroplasts of the *Attoc159*, *Attoc132*, *Attoc120* and *Attoc90* mutants after incubation with ceQORH-GFP-$(His)_6$. Note the formation of IAPs in the *Attoc159* and *Attoc132* mutants, the absence of any IAPs in the *Attoc120* mutant, and the lack of late IAPs containing HP20 and HP30 in the *Attoc90* mutants. D, Reduced accumulation of ceQORH in *Attoc34*, but not *Attoc33*, chloroplasts *in vivo* (a) and after an *in vitro* import reaction using the radiolabelled protein (b).

Basically the same *cphsc70-1* and *cphsc70-2*, *hsp93-v-2* and *tic40-2* mutants as those described by Su and Li [54] were used for testing the role of cpHSP70 and HSP93/TIC40 in ceQORH import. Figure 5 depicts results of respective Western blot analyses carried out for ceQORH on total leaf extracts and IAPs. Apparently, very weak if any effect was seen for the *cphsc70-1* and *cphsc70-2* single and double mutants, indicating that both HSC70 chaperones have a limited role in import of ceQORH *in planta*. By contrast, more severe effects were seen for the *Athsp93-v* and *Attic40* mutants that were unable to import ceQORH (Figure 5A). *Athsp93-v-2* and *Attic40-2* formed no late IAPs containing HP20 and HP30, but still formed early IAPs containing TOC120 (Figure 5C). In the *Athsp93-v-2::Attic40-2* double mutant, IAP formation was abolished, indicating that the HSP93-V/TIC40 system is indispensable for ceQORH import. This conclusion is corroborated by results of *in vitro* import experiments and protein gel blot analyses which demonstrated a complete lack of ceQORH import in the *Athsp93-v::Attic40* double mutant (Figure 5B) and *Attoc34::Attic40* double mutants (Figure S2).

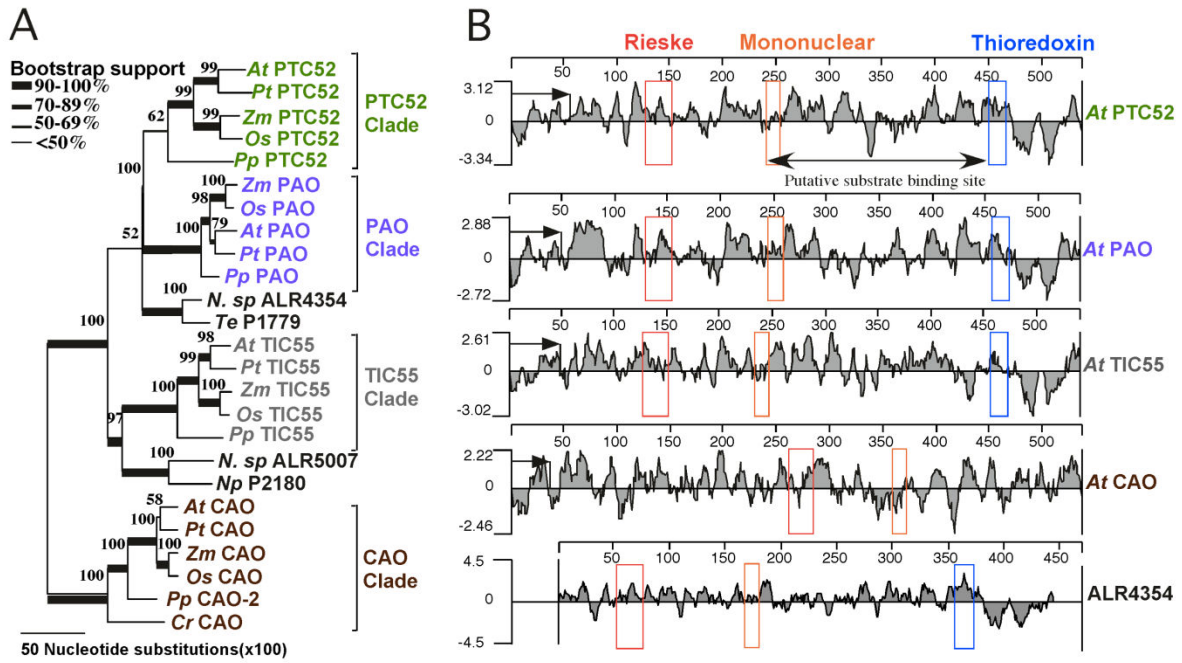

Figure 4: Phylogenetic tree and evolution of TIC55 and related non-heme Rieske iron-sulfur proteins. A, Phylogenetic tree of 25 non-heme oxygenases estimated using maximal parsimony. Proteins are named according to their abbreviated species name followed by the gene name *(Arabidopsis thaliana, At; Chlamydomonas reinhardtii, Cr; Nostoc punctiforme, Np; Nostoc species PCC2170, PCC; Physcomitrella patens, Pp; Populus trichocarpa, Pt; Trichodesmium erythraeum, Te; Oryza sativa, Os; Zea mays, Zm).* A multiple sequence alignment of PTC52-related proteins was determined using Clustal W (default parameters). Branch lengths are proportional to the expected number of nucleotide substitutions. The reliability of each bifurcation was estimated using bootstrap analysis (percentage values over 50% are shown next to nodes), and the support for each of the branches is indicated by line thickness. The tree is drawn using the chlorophyll oxygenase CAO clade as a monophyletic ingroup. B. Comparison of hydropathicity plots between *A. thaliana* PTC52-related non-heme oxygenases and *Nostoc* species PCC7120 ALR4354. Hydropathy plots were determined using an experiment-based Wimley-White whole-residue hydrophobicity octanol-interface scale and the Membrane Protein Explorer (MPEx) tool (**http://blanco.biomol.uci.edu/mpex/**). The hydropathy profile is shown in black with a superimposed version in green. Red bars indicate possible trans-membrane or membrane-associated regions. Aromatic residues (F, W, and Y) are highlighted in purple and H residues in yellow.

HP20, HP30 and HP30-2 are Preprotein and Amino acid Transporter (PRAT) family members with intriguing functions in plastid import of ceQORH

The fourth group of ceQORH interacting proteins comprises the Preprotein and Amino acid Transporter (PRAT) family members HP20, HP30 and HP30-2 (Figure 6). HP20 was originally identified by proteomics studies as an outer plastid envelope membrane protein of unknown function in *A. thaliana*, annotated as Q9SZ09 (encoded by At4g26670) [16]. The closest relative of HP20 is HP22 (encoded by At5g55510) that shares 79% amino acid sequence identity [16,38]. The second PRAT protein interacting with ceQORH is HP30 (encoded by At3g49560) [Figure 6] and the third is HP30-2 (encoded by At5g24650) [16,38]. HP30 and HP30-2 are closely related members of the PRAT family and exhibit 83% amino acid sequences identity [16,38]. In phylogenetic studies, HP20 and HP22 as well as HP30 and HP30-2 establish different clades suggestive of their functional specialization [38]. Computer-assisted topology modelling carried out using different algorithms (see supporting information) predicted the presence of 3 or 4 trans-membrane spans for HP20 and HP30 (Figure S3).

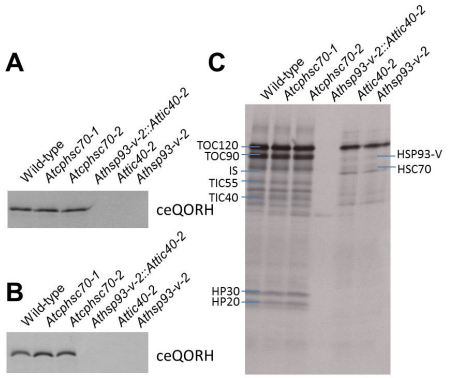

Figure 5: Lack of import of ceQORH-GFP into chloroplasts of the *Athsp93-v* and *Attic40* single and double mutants. A, Western blot analysis of ceQORH in chloroplasts isolated from the indicated *Athsp93-v* and *Attic40* single and double mutants as well as the *Atcphsc70;1* and *2* mutants used as control. B, *In vitro* chloroplast import of ^{35}S-ceQORH into the same plastids as those used in A. C, Isolation of IAPs from chloroplasts of the *Athsp93-v* and *Attic40* versus *Atcphsc70* mutants after incubation with ceQORH-GFP-$(His)_6$. Note the formation of IAPs in the *Atcphsc70* mutants, complete absence of IAPs in the *Athsp93-v::Attic40* double mutant, and the lack of late IAPs containing HP20 and HP30 in the *Athsp93-v* and *Attic40* single mutants.

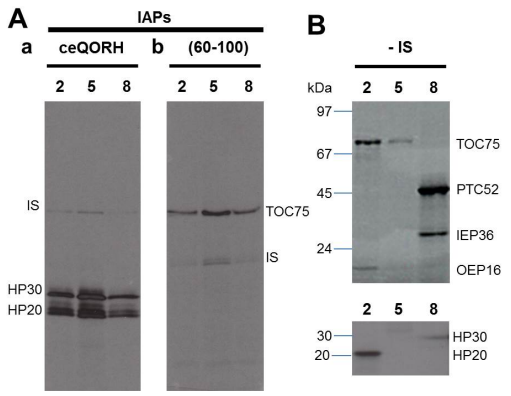

Figure 6: IAP formation in the presence and absence of ceQORH-GFP-$(His)_6$. A, Western blots to detect HP20 and HP30 in IAPs formed with ceQORH-GFP-$(His)_6$ (a) and TOC75 in IAPs formed with (60-100)-ceQORH-GFP-$(His)_6$ (b) in chloroplasts. ceQORH-GFP-$(His)_6$ and (60-100)-ceQORH-GFP-$(His)_6$ were affinity-purified via their His tags from detergent-solubilized gradient fractions 2, 5 and 8 that had been obtained as described in Figure 1 and subjected to co-precipitation with antibodies against HP20 and HP30 (a) or TOC75 (b). Then, the precipitated proteins were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and the blots were probed with anti-TOC75, anti-HP20 and anti-HP30 immune sera. B, Identification of TOC75, PTC52, IEP36 and OEP16-1 in fractions 2, 5 and 8 of a gradients separating OM, OM-IM and IM proteins from chloroplasts that had been incubated in the absence of added import substrate. The upper panel shows a Western blot probed with a mixed antiserum against the indicated plastid envelope proteins. The lower panel shows a replicate filter probed with only the HP20 and HP30 antisera.

We used previously characterized antibodies [47] to confirm the presence of HP20 and HP30 in IAPs with ceQORH. Figure 6A demonstrated that both HP20 and HP30 co-purified in IAPs formed with ceQORH-GFP, but not in IAPs established with (60-100)-ceQORH-GFP interacting with TOC75. When IAP formation was analyzed in the absence of either ceQORH-GFP or (60-100)-ceQORH-GFP, HP20 was present in the OM fraction, whereas HP30 was present in the IM fraction (Figure 6B). This suggested that both types of HPs were actively recruited into IAPs comprising ceQORH and that these IAPs spanned both the outer and inner chloroplast envelope membranes.

Conclusions

In the present study, proteins were identified and characterized that interact with ceQORH during its import into chloroplasts of *A. thaliana*. We show that ceQORH interacts with at least two receptors, AtTOC120 and AtTOC90. Both receptors play distinctive roles in ceQORH import. Mutant studies revealed that AtTOC120 mediates the initial binding of the transit sequence-less protein to the outer plastid envelope. AtTOC90, by contrast, has a subsequent role and is needed for the establishment of late IAPs of ceQORH with HP20 and HP30. AtTOC120 and AtTOC90 both contain protease-sensitive regions protruding into the cytosol [20,21], explaining why thermolysin pretreatment abolished IAP formation and import of ceQORH.

Formation of late IAPs critically depends not only on the presence of AtTOC90 but also on the presence of HSP93-V/TIC40. Mutant studies suggest that the HSP93-V/TIC40 system plays a by far more important role than the cpHSC70 system. In the absence of HSP93-V, only early IAPs containing AtTOC120 were produced, but no late IAPs containing HP20 and HP30 were detectable. In the *Athsp93-v::Attic40* double mutant, surprisingly no IAPs were detectable at all. This observation suggests a thus far unprecedented role of TIC40 in the assembly of membrane complexes in the inner plastid envelope, comprising the one engaged by ceQORH. Indeed, this hypothesis was vindicated by our mutant studies with the *Athsp34::Attic40* double mutant which failed to accumulate ceQORH *in vitro* and *in planta*. The presence of HSP93-V and TIC40 operating as ATP-powered import motors explains why no IAPs were formed when ATP was omitted from the assays.

The fact that TOC75 and TIC110 are dispensable for plastid import of ceQORH suggests that other components must establish a hydrophilic environment for membrane passage across the outer and inner plastid envelopes. Likely candidates are HP20 and HP30/HP30-2 that were co-isolated along with ceQORH in IAPs (this study) and also

identified by chemical cross-linking [47]. Both types of HPs are predicted to be trans-membrane proteins and belong to the PRAT family of preprotein and amino acid transporters operative in mitochondria and chloroplasts [43,47,48]. Recent genetic, cell biological and biochemical evidence is supportive of the notion of HP20 and HP30 as representing crucial components in the import pathway of ceQORH and other, transit sequence-less chloroplast proteins [47,48]. Together, our study shows that chloroplasts make use of a unique combination of PRAT, TOC and TIC components to drive the import of proteins lacking cleavable transit sequences into the inner plastid envelope membrane.

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