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Toc159- and Toc75-independent Import of a Transit Sequence-less Precursor into the Inner Envelope of Chloroplasts^{*[5]}

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Chloroplast envelope quinone oxidoreductase (ceQORH) is an inner plastid envelope protein that is synthesized without cleavable chloroplast transit sequence for import. In the present work, we studied the *in vitro*-import characteristics of *Arabidopsis* ceQORH. We demonstrate that ceQORH import requires ATP and is dependent on proteinaceous receptor components exposed at the outer plastid surface. Competition experiments using small subunit precursor of ribulose-bisphosphate carboxylase/oxygenase and precursor of ferredoxin, as well as antibody blocking experiments, revealed that ceQORH import does not involve the main receptor and translocation channel proteins Toc159 and Toc75, respectively, which operate in import of proteins into the chloroplast. Molecular dissection of the ceQORH amino acid sequence by site-directed mutagenesis and subsequent import experiments *in planta* and *in vitro* highlighted that ceQORH consists of different domains that act concertedly in regulating import. Collectively, our results provide unprecedented evidence for the existence of a specific import pathway for transit sequence-less inner plastid envelope membrane proteins into chloroplasts.

Plastids conduct vital biosynthetic functions, and many essential reactions are located exclusively within these organelles. However, plastids contain only limited coding information in their own DNA. Endosymbiotic evolution has resulted in the transfer to the nuclear genome of genes encoding the vast majority of plastid proteins. As a consequence of this displacement of genetic material, plastids had to evolve mechanisms to reimport cytoplasmically synthesized precursor proteins from the cytosol (1–6). In most cases, nucleus-en-

coded plastid proteins are synthesized in the cytosol as higher molecular weight precursors, with a cleavable N-terminal sequence called transit peptide (7). Transit peptides contain all the information that is necessary and sufficient for import. During the actual import process, transit peptides are proteolytically removed by a stromal processing peptidase. Transit peptides can be simple, as found for stroma proteins, or bipartite, as found for proteins destined to thylakoids. In the latter case, the N-terminal part directs the precursor to the stroma, whereas the non-cleaved C-terminal part directs the partially processed precursors to their final intraorganellar destination; *i.e.* the thylakoid membranes and the thylakoid lumen. Ultimate precursor maturation occurs by virtue of the thylakoid-processing peptidase (8).

During chloroplast import, the transit peptide first recognizes the chloroplast surface in a process involving membrane lipids and the translocon at the outer chloroplast envelope (TOC)³ (9). The TOC complex consists of three distinct core subunits: the GTP-dependent Toc34 and Toc159 receptors and the translocation channel protein Toc75 (10, 11). Translocation across the inner envelope is mediated by another multiprotein complex, the translocon at the inner chloroplast envelope (TIC) and requires ATP in the stroma, most likely providing energy for the activity of chaperones (12). In the inner envelope, the integral membrane component Tic110 is supposed to form at least part of the translocation channel, whereas the integral membrane protein Tic20 is presumably involved in the formation of the protein translocation pore. In addition to these components of the presumed basic TIC complex, several auxiliary subunits have been identified, including the regulatory redox components Tic55, Tic32, and Tic62, the intermembrane space protein Tic22, and the chaperone coordinating factor Tic40 (10). All proteins targeted to the five different intraplastidic subcompartments (the inter-membrane space, the inner enve-

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, Figs. S1–S4, and references.

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³ The abbreviations used are: TOC, translocon at the outer envelope membrane of chloroplasts; TIC, translocon at the inner envelope membrane of chloroplasts; ceQORH, chloroplast envelope quinone oxidoreductase homologue; GFP, green fluorescent protein; pSSU, precursor to the small subunit of Rubisco; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PORA, -B, NADPH:protochlorophyllide oxidoreductases A and B; pFD, precursor of ferredoxin.

lope, the stroma, the thylakoid membranes, and the lumen) contain cleavable N-terminal transit peptides that are quite variable in length and actual amino acid composition. In contrast, almost all proteins located at the outer envelope membrane do not bear such cleavable extensions, and their targeting signals reside within the mature part of the proteins (with the only known exception of Toc75 (13)). Insertion of the overwhelming part of the outer envelope membrane proteins does not require either surface-exposed receptors or energy and has generally been assumed to be accomplished by a spontaneous mechanism or through interaction with the lipid components of the outer membrane (14, 15). Although early work suggested otherwise, the best studied outer membrane proteins are now known to use both proteins within the chloroplast and NTPs for insertion (5, 16–18).

With the completion of the *Arabidopsis* genome-sequencing project, it was possible to identify multiple isoforms of many TOC and TIC proteins, including Toc159 (19), Toc34 (20, 21, 22), Toc75 (23), and Tic22 and Tic20 (24). An emerging concept suggests that multiple types of import complexes could be present within the same cell, each having a unique affinity for different plastid precursor proteins, depending upon the mix of TIC/TOC isoforms it contains. In a recent report, evidence was provided for the existence of distinct plastid import pathways for NADPH:protochlorophyllide oxidoreductases A and B (PORA and PORB) (25). Studies of Ivanova *et al.* (26) and Kubis *et al.* (27) revealed that different TOC subcomplexes are capable of harboring different precursors. Last but not least, Nada and Soll (28) described unusual plastid import characteristics for the inner envelope protein Tic32. This protein is imported into plastids thanks to an uncleavable pre-sequence, and the targeting information is restricted to the N-terminal extremity of this protein. Interestingly, Tic32 import is not dependent on protease-sensitive receptors at the chloroplast surface and does not use the classic TOC machinery to be translated across the outer envelope membrane (28). Tic32 was also demonstrated to be an essential component in chloroplast biogenesis (29), and, more recently, calcium regulation of chloroplast protein translocation was demonstrated to be mediated by calmodulin binding to Tic32 (30).

In a previous study (31), we identified an inner chloroplast envelope quinone-oxidoreductase homologue (ceQORH), which lacks a canonical cleavable N-terminal transit peptide and is, to date, the only known example of an inner envelope protein that contains internal targeting information for import. Here we demonstrate that ceQORH does not embark the trimeric TOC159/75/34 complex during import but uses a novel, proteinaceous, and evolutionarily conserved site. Moreover, molecular dissection of ceQORH has allowed us to identify domains in the polypeptide chain of ceQORH that act concertedly in directing the protein to this hitherto unknown import pathway.

EXPERIMENTAL PROCEDURES

Purification of Chloroplast and Chloroplast Subfractions from *Arabidopsis*—All operations were carried out at 0–5 °C. Percoll-purified chloroplasts were obtained from 200–300 g of *Arabidopsis thaliana* leaves. Crude cell extracts and chloroplast

subfractions were purified and stored as previously described (32). Protein content of the fractions was estimated using the Bio-Rad protein assay reagent (33).

SDS-PAGE and Western Blot Analyses—SDS-PAGE analyses were performed as described by Chua (34). For Western blot analyses, gels were transferred to a nitrocellulose membrane (BA85, Schleicher & Schuell). ceQORH and ceQORH fusions were detected using a previously described α ceQORH rabbit polyclonal antiserum raised against the recombinant *Arabidopsis* ceQORH (31) at a 1/5000 dilution. Green fluorescent protein (GFP) and GFP fusions were detected using a commercially available α GFP mouse monoclonal antibody (GFP-2A5, Euromedex, 67458 Mundolsheim, France) at a 1/4000 dilution.

Construction of Vectors for Stable Expression in *Arabidopsis* and *In Vitro* Targeting—A detailed description of the strategies developed to construct the vectors used for stable expression in *Arabidopsis* and for *in vitro* import experiments are provided as supplemental test (supplemental data 1 and 2, respectively).

Plant Material and Growth Conditions—*Arabidopsis* plants, Wassilevskija background, were germinated in Petri dishes containing solidified medium (Murashige and Skoog, 1% (w/v) sucrose, and 1% (w/v) agarose) for 2 weeks before transfer to soil. Plants were then grown in growth rooms at 23 °C (12-h light cycle) with a light intensity of 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The *Arabidopsis* *ceqorh* mutant (line DRM40) was identified by screening the FLAGdb/FST data base (35) from the INRA (Versailles, France) collection of T-DNA insertion mutants. These lines, produced by *Agrobacterium*-mediated transformation (36), were transformed with a T-DNA construct (pGKB5 binary vector) that carries both *bar* and *nptII* selection markers (37). Primary transformants were self-pollinated to obtain plants homozygous for the insertion.

***Arabidopsis* Transformation**—Wild-type and transgenic *Arabidopsis* plants ecotype Wassilevskija background were transformed by dipping the floral buds of 4-week-old plants into an *Agrobacterium tumefaciens* (C58 strain) solution containing a surfactant (Silwett L-77) according to Clough and Bent (38). Primary transformants were selected on Murashige and Skoog medium containing 100 $\text{mg}\cdot\text{liter}^{-1}$ kanamycin. Only lines segregating 3:1 for the resistance to kanamycin and expressing the recombinant protein were selected for further analyses. Primary transformants were then self-pollinated to obtain plants homozygous for the insertion. Fluorescence microscopy was performed with a confocal laser-scanning microscope (TCS-SP2, Leica, Deerfield, IL).

***In Vitro* Import Experiments**— $[^{35}\text{S}]$ Methionine-radiolabeled molecules were synthesized by *in vitro* transcription/translation (TNT Coupled Wheat Germ Extract Systems, Promega, Madison, WI) of the cDNA clones (as described in supplemental data 2) and added to import mixture (50 mM HEPES-KOH, pH 7.5, 330 mM Sorbitol, 2.5 mM MgCl_2 , and 8.5 mM L-Met) in the presence of Mg-ATP and Mg-GTP at the desired mM concentrations. Seeds of barley (*Hordeum vulgare* cv. Carina) were germinated on moist vermiculite at 25 °C and grown under continuous white-light illumination (30 $\text{watts}\cdot\text{m}^{-2}$) for 8 days. Chloroplasts were prepared by differential centrifugation and Percoll density gradient centrifugation and were then depleted of energy sources (39). Purified plastids (5×10^7) were then

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added to the import mixture, and import reactions were performed at various incubation times at 23 °C in the dark. After incubation, plastids were sedimented by centrifugation and treated (when specified) with thermolysin (40). Proteins were precipitated with trichloroacetic acid (5% (w/v) final concentration) and separated by denaturing SDS-PAGE on 11–20% (w/v) polyacrylamide gradients (41), and finally radiolabeled molecules were detected by autoradiography. Competitive receptor binding and translocation studies were performed with the indicated radiolabeled precursors. The import mixture was complemented, as indicated, either by 0.25 μM , 2.5 μM , or 25 μM of bacterially expressed unlabeled pSSU, pFd, or Tic32, by 500-fold excesses of pFd or by 150-fold excesses of pPORB and pPORA (42). In this latter case, import experiments were performed using chloroplasts that had been incubated or not with 5-aminolevulinic acid as previously described (39). Plastids were then sedimented by centrifugation and used for import experiments as described above.

Quantitative Receptor Binding Studies—The methodology used was that described in Friedman and Keegstra (43). Chloroplasts were isolated, and depleted of ATP, as described (44). Chloroplasts were then incubated at 4 °C with 0.1 mM Mg-ATP, and increasing concentrations of the radiolabeled ceQORH::GFP and (60–100)-ceQORH::GFP. After a 15-min incubation in the dark and a subsequent step of centrifugation, the numbers of plastid-bound ceQORH::GFP and (60–100)-ceQORH::GFP molecules recovered in the sediment fractions and of nonbound precursor molecules present in the supernatant fractions were determined (43) and plotted as a function of the total precursor concentrations in the assays (45).

Cross-linking Experiments—Cross-linking with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (40, 46) was performed by activating the cysteine residues in ceQORH::GFP and (60–100)-ceQORH::GFP and incubating the derivatized proteins with isolated plastids in the presence of Mg-ATP and Mg-GTP, as given in the text. Protein was recovered from the different samples by precipitation with trichloroacetic acid (5% (w/v) final concentration), resolved by SDS-PAGE on 10–20% (w/v) polyacrylamide gradients under reducing or nonreducing conditions (46), and detected by autoradiography.

RESULTS

In Vitro Import of ceQORH into Isolated Chloroplasts—*In vitro* import experiments were performed with ceQORH, which had been synthesized by coupled *in vitro*-transcription/translation of a respective cDNA clone. The ^{35}S -labeled protein was incubated with isolated barley chloroplasts. As control, the small subunit precursor of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) was used. Although ^{35}S -pSSU was rapidly imported and processed to mature size (Fig. 1A, lanes 1–4), ^{35}S -ceQORH did not undergo a proteolytic maturation during incubation (Fig. 1B, lanes 1–5). At first glance this result suggested that ceQORH was not imported. When chloroplasts were re-isolated after import and treated with thermolysin, however, ceQORH was easily detectable in a protease-resistant, plastid-bound form (Fig. 1B, lanes 6–10). Moreover, in the absence of chloroplasts, ^{35}S -ceQORH was degraded by the pro-

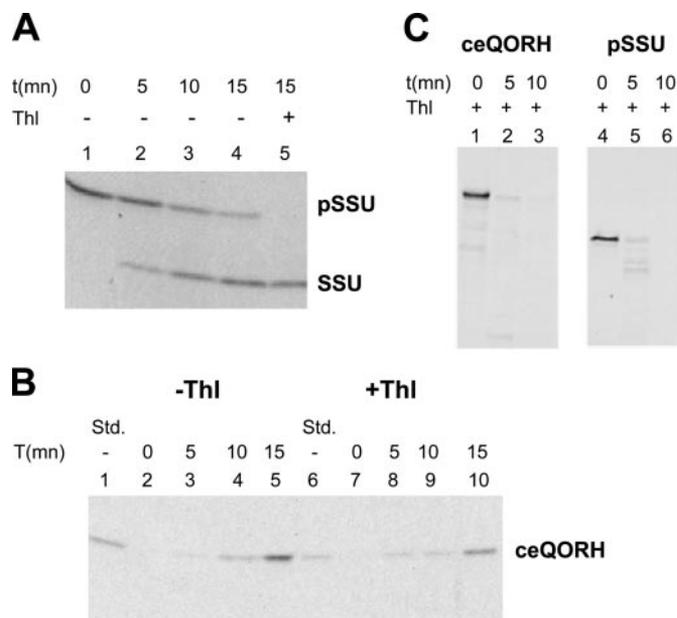


FIGURE 1. ceQORH is imported *in vitro* into isolated barley chloroplasts and is not cleaved during its import process. Barley chloroplasts were isolated and energy-depleted. *A*, as a control, import of the small subunit ^{35}S -precursor of Rubisco (pSSU) was performed. Barley chloroplasts were incubated with the small subunit ^{35}S -precursor of Rubisco (pSSU) in the presence of 2 mM Mg-ATP and 0.1 mM Mg-GTP. In this panel, total assays are shown. The mature form was annotated SSU. *B*, import of the ^{35}S -ceQORH (ceQORH) was performed. Barley chloroplasts were incubated with ^{35}S -ceQORH in the presence of 2 mM Mg-ATP and 0.1 mM Mg-GTP. At the indicated time intervals, the plastids were sedimented by centrifugation. Plastids found in the pellet fraction were treated with (+Thl) or without (-Thl) thermolysin as specified. Std., input standard. *C*, time course of degradation of ^{35}S -ceQORH and ^{35}S -pSSU incubated with thermolysin in the absence of chloroplasts.

tease validating its protease-sensitivity (Fig. 1C, ceQORH, lanes 1–3). These observations revealed that ceQORH had been imported but without detectable proteolytic cleavage, a result that is consistent with our previous work (31).

ceQORH Uses a Novel Pathway to Cross the Outer Envelope—We next tested whether the trimeric TOC159/75/34 complex was involved in chloroplast import of ceQORH. In a first set of experiments, *in vitro*-import experiments were carried out in the presence or absence of chemical amounts (corresponding to large, 150-fold excesses of bacterially expressed and purified, unlabeled pSSU or pFd). Both precursors have previously been demonstrated to use the TOC159/75/34 import machinery for uptake (47, 48).

As internal control, import of ^{35}S -pSSU was tested in the presence or absence of unlabeled competitor. In fact, this control verified that no import of ^{35}S -pSSU was detectable in the presence of excess pSSU and pFd. This is evident from (i) the lack of protease-protected precursor after incubation and (ii) the lack of processed, mature SSU (Fig. 2A, lanes 2–3 and 7–8). By contrast, import of ^{35}S -ceQORH in chloroplasts (presented in Fig. 1B) occurred in the presence of excess pSSU and pFd competitors (Fig. 2B, panels a and b, lane 9). As a control, the same competition experiment was reproduced in the presence of excess pFd competitor using “minus competitor” and “plus competitor” conditions in the same experiment (Fig. 3).

In a second set of experiments, isolated barley chloroplasts were preincubated with antibodies directed against the TOC

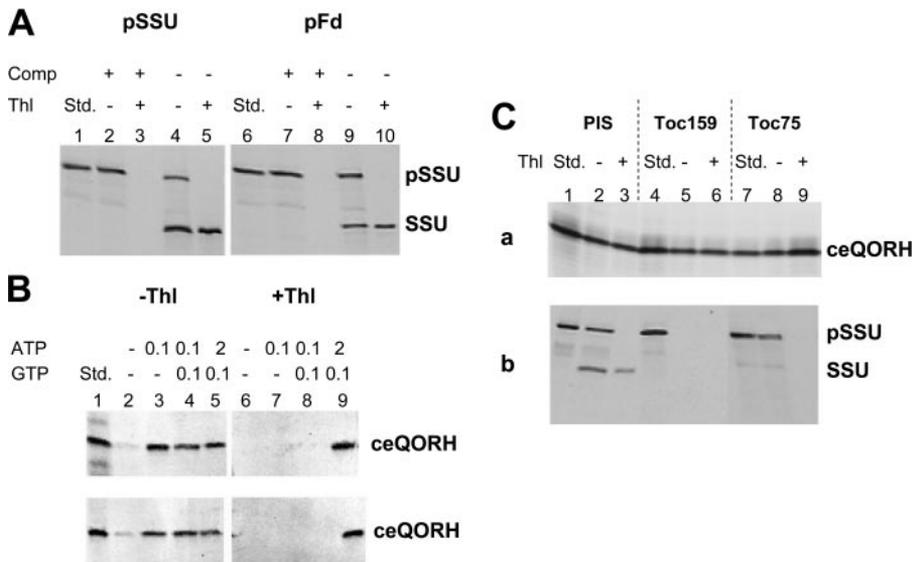


FIGURE 2. ceQORH import does not involve the main receptor and translocation channel proteins Toc159 and Toc75. Barley chloroplasts were isolated and energy-depleted. Import reactions were performed for 15 min in the dark and the plastids were then sedimented by centrifugation. Plastids found in the pellet fraction were treated (+Thl) or left untreated (-Thl) as indicated. Std, input standard. A, as control, import of ^{35}S -pSSU was performed in the presence of 2.5 μM of bacterially expressed unlabeled pSSU (lanes 1–5) or pFd (lanes 6–10). Import reactions were performed with 2 mM Mg-ATP and 0.1 mM Mg-GTP. The mature form was annotated SSU. B, ^{35}S -ceQORH import was tested in the presence of 2.5 μM of bacterially expressed unlabeled pSSU (a) or pFd (b), in the presence of Mg-ATP and Mg-GTP at the indicated mM concentrations. C, barley chloroplasts were preincubated with pre-immune serum (Toc159 pre-immune serum: PIS, Toc75 pre-immune serum not shown) and with antibodies directed against Toc159 or Toc75 before ^{35}S -ceQORH import reactions (a) and ^{35}S -pSSU import reactions (b). Import reactions were performed in the presence of 2 mM Mg-ATP and 0.1 mM Mg-GTP. Std, input standard.

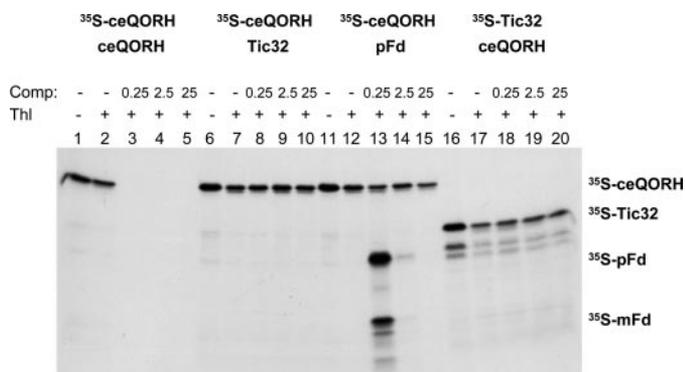


FIGURE 3. ceQORH and Tic32 do not use the same import site into chloroplasts. ^{35}S -ceQORH and ^{35}S -Tic32 were synthesized *in vitro* and added to incubation mixtures containing 2 mM Mg-ATP and the indicated 0.25 μM , 2.5 μM , and 25 μM concentrations of bacterially expressed unlabeled ceQORH, Tic32, or pFd, used as competitors. The concentration of 0.25 μM ferredoxin in the incubation mixture was obtained using ^{35}S -pFd (lane 13). Mock incubations lacked competitor. At time zero (lanes 1, 6, 11, and 16) and after 15 min (lanes 2–5, 7–10, 12–15, and 17–20) the reactions were stopped on ice. Plastids were repurified on Percoll and supplemented with thermolysin (Thl) to degrade non-imported protein, as indicated. Proteins were detected by autoradiography following SDS-PAGE.

components Toc75 (the translocation channel of the outer envelope) or Toc159 (the major receptor at the outer envelope) (49) and were then used for new import experiments (Fig. 2C). The preincubation of plastids with antibodies raised against Toc159 strongly affects pSSU binding to plastid surface (Fig. 2C, panel b, lane 5) and leads to the inhibition of the pSSU import into plastids (Fig. 2C, panel b, lane 6). Similarly,

although the preincubation of plastids with antibodies raised against Toc75 did not largely affect pSSU binding to plastid surface (Fig. 2C, panel b, lane 8), these antibodies obstructed the translocation channel of the outer envelope, and therefore, strongly inhibited the pSSU import into plastids (Fig. 2C, panel b, lane 9). Finally, although preincubation of plastids with antibodies raised against Toc159 and Toc75 strongly affected the import of pSSU, the same pretreatment did not abolish the ceQORH import into plastids (Fig. 2C, panel a, lanes 6 and 9). As control, preincubation of plastids with pre-immune serum did not affect both the pSSU and the ceQORH import into plastids (Fig. 2C, panels a and b, lane 3). The lack of competition using excess of pSSU and pFd, as well as the lack of antibody blocking indicated that ceQORH did not utilize Toc159 and Toc75 for import.

Evidence for the operation of distinct import sites was previously reported for the chloroplast import

of PORA and PORB (42, 25, 50). Results, shown in supplemental Fig. S1, established that ^{35}S -ceQORH import into chloroplasts is not affected in the presence of excess pPORA and hence did not involve the recently discovered protochlorophyllide-dependent translocon (25). Furthermore, in a recent report, Nada and Soll (28) reported on import of another transit-sequenceless inner envelope protein, Tic32, into isolated chloroplasts. Competition experiments were used to address the question of whether ceQORH and Tic32 would share the same import pathway or not (Fig. 3). In a first set of experiments, bacterially expressed and purified Tic32 of *Arabidopsis thaliana* was added to import reactions containing ^{35}S -ceQORH. Three different competitor concentrations were used: 0.25 μM , 2.5 μM , and 25 μM . In all three cases no competition occurred (Fig. 3, lanes 6–10). *Vice versa*, import of ^{35}S -Tic32 was insensitive to an excess of bacterially expressed ceQORH (Fig. 3, lanes 16–20). Controls proved that either radiolabeled precursor was sensitive to an excess of the respective unlabeled protein (Fig. 3, lanes 1–5; and data not shown). Because import of ^{35}S -ceQORH also occurred in the presence of excess ferredoxin (which is well known to utilize the standard protein import machinery containing Toc159 (51, 52)), we concluded that ceQORH import does not involve either the Tic32 or Toc import pathway (Fig. 3, lanes 11–15).

We next asked whether the ceQORH import site may be (a) of proteinaceous nature and (b) universally conserved in monocotyledonous and dicotyledonous plants species. We therefore performed import experiments with purified plastids isolated from wheat, spinach, and *A. thaliana* and pretreated or not with

ceQORH Import Into Chloroplast

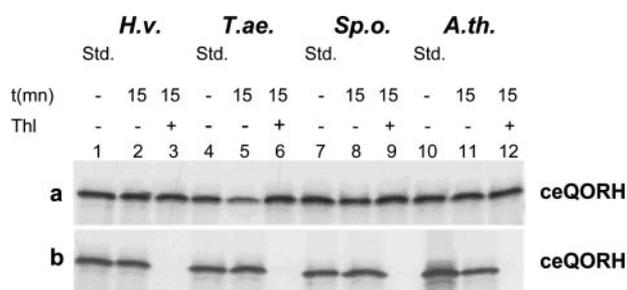


FIGURE 4. ceQORH import is dependent on proteinaceous receptor components exposed at the outer plastid surface. Plastids from barley (*H. vulgare*, *H.v.*), wheat (*T. aestivum*, *T.ae.*), spinach (*S. oleracea*, *Sp.o.*), and *A. thaliana* (*A. th.*) were isolated, depleted of energy sources, and pretreated without (a) or with (b) thermolysin. Plastids were then re-purified and incubated with ^{35}S -ceQORH in the presence of 2 mM Mg-ATP and 0.1 mM Mg-GTP. After 15-min incubations in the dark, plastids were sedimented by centrifugation. Plastids found in the pellet fraction were supplemented, or not, with thermolysin and incubated for 30 min on ice. Lanes 1, 4, 7, and 10 show input standards.

thermolysin, a protease which degrades surface-exposed outer envelope proteins, including receptors facing the cytosol (53, 40, 54). We observed that ceQORH was imported into chloroplasts from all tested plant species (Fig. 4-a, lanes 3, 6, 9, and 12). Moreover, the results uncovered the proteinaceous nature of the ceQORH import site, because pretreatment of chloroplasts with thermolysin led to a lack of import (Fig. 4, panel b, lanes 3, 6, 9, and 12, panels b versus a). Interestingly, the amount of ceQORH bound to plastids seems not to be drastically affected (Fig. 4, lanes 2, 5, 8, and 11, panels b versus a). This could indicate that ceQORH binding to plastids is, at least for a part, mediated either by proteinaceous components insensitive to thermolysin treatment or by non-proteinaceous components. As a matter of fact, several studies described the implication of lipid components in the targeting of outer membrane proteins (14, 15). This can also be the case for ceQORH binding to plastids.

In Planta Evidence for the Novel ceQORH Import Pathway—In a previous report (31), we analyzed the chloroplast targeting of ceQORH using transient expression in *Arabidopsis* cells. To investigate the chloroplast targeting of ceQORH in the different plant tissues, we constructed *Arabidopsis* plants stably expressing ceQORH fused to GFP. We also analyzed, as controls, plants expressing fusions of the transit peptide of pSSU and GFP (TP-pSSU::GFP), or GFP alone without a plastid signal attached to it.

The intracellular fluorescence of the various reporter proteins was first examined for leaf mesophyll cells (Fig. 5, mesophyll cells). Whereas GFP alone (negative control) was distributed in the cytosol and in the nucleus (Fig. 5, GFP), TP-pSSU::GFP was detectable in chloroplasts, as evidenced by the co-localization of GFP fluorescence and chlorophyll autofluorescence (Fig. 5, TP-pSSU::GFP). The same figure was also obtained for ceQORH::GFP (Fig. 5, ceQORH::GFP).

In contrast to TP-pSSU::GFP (Fig. 5, guard cells, TP-pSSU::GFP), ceQORH::GFP was not targeted to chloroplasts in guard cells and was found in small structures (Fig. 5, guard cells, ceQORH::GFP). Controls experiments excluded that these structures correspond to mitochondria (supplemental Fig. S2), and their identity still remains to be determined. Obviously

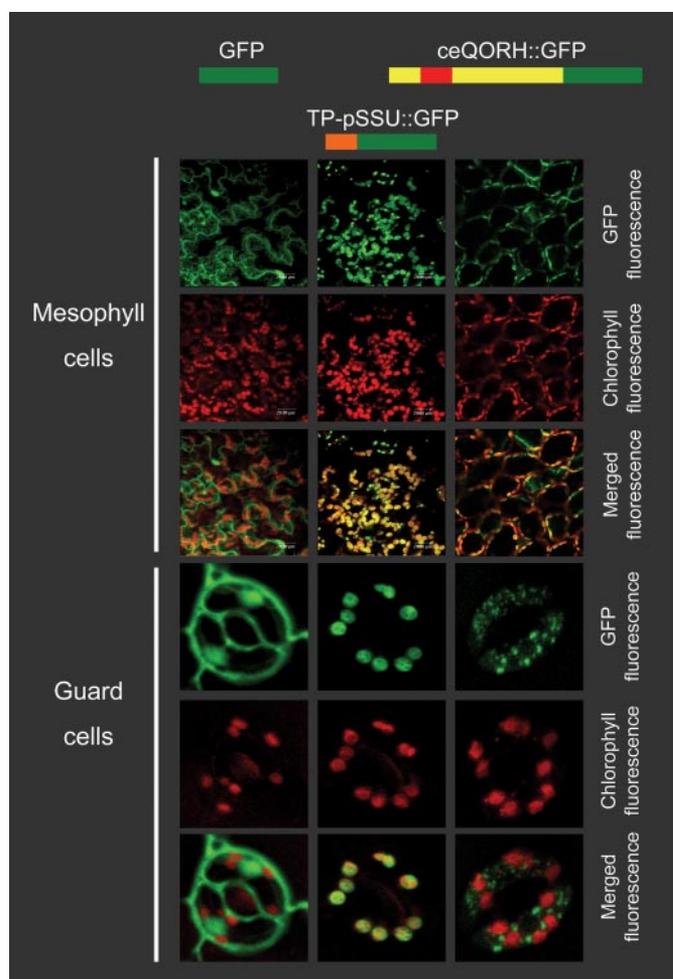


FIGURE 5. Distinct and tissue specific plastid targeting of ceQORH. Localization of GFP (GFP), of the transit peptide of the Rubisco small subunit fused to GFP (TP::GFP), and of the ceQORH protein fused to GFP (ceQORH::GFP) was analyzed in transgenic *Arabidopsis* plants by fluorescence microscopy. The intracellular fluorescence of the various reporter proteins was observed in mesophyll cells and in guard cells. Cells were observed with 400 \times magnification.

import of pSSU and ceQORH into leaf mesophyll and guard cell chloroplasts *in planta* was differentially regulated. Similar differences between plastid import into mesophyll cells and guard cells were previously reported for the PORA and PORB fused to GFP (55).

The C Terminus Part of ceQORH Is Implicated in Chloroplast Targeting and Is Essential for Specific Targeting of the Protein to the Chloroplast Envelope—Using transient expression experiments, we have previously demonstrated that an internal region of the ceQORH protein (residues 60–100) was essential for plastid targeting (31). Nevertheless, *in vivo*, this internal region was not sufficient to provide sufficient targeting information for plastid import. Additional domains both in the N-terminal part (residues 6–60) and the C-terminal part (residues 100–349) were identified to play important roles in governing import of ceQORH (31). To gain further insights into the respective functions of these ceQORH domains, we combined two complementary approaches, including *in planta* tests and *in vitro*-import experiments. Several truncated versions of ceQORH fused to GFP were constructed (Fig. 6). In a first set of

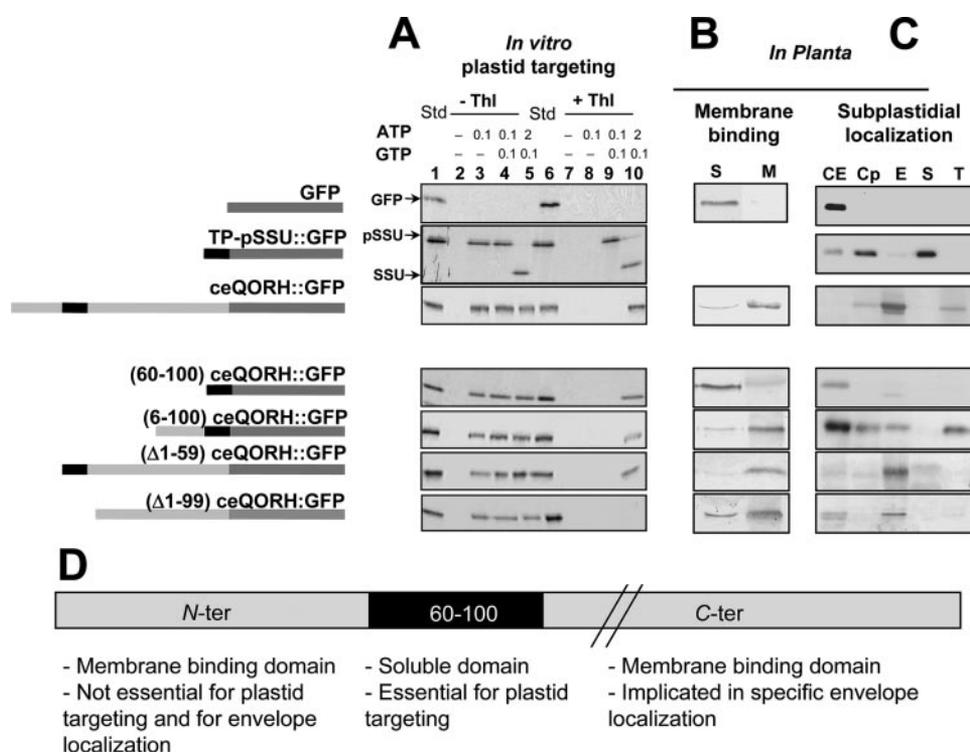


FIGURE 6. *In planta* and *in vitro* analysis of the ceQORH domains function in targeting and import into chloroplasts. *A*, barley chloroplasts were isolated, energy-depleted, and incubated with ^{35}S -ceQORH-GFP fusions in the presence of Mg-ATP and Mg-GTP at the indicated millimolar concentrations. Import reactions were performed for 15 min in the dark, and the plastids were then sedimented by centrifugation. Plastids found in the pellet fraction were treated (+Thl) or left untreated (–Thl) as indicated. Std, input standard. As control, import of the ^{35}S -GFP and the small subunit ^{35}S -precursor of Rubisco (pSSU) was performed. The mature form of the small subunit of Rubisco was annotated SSU. *B*, distribution of GFP and ceQORH::GFP fusions in crude soluble (S) or membrane (M) fractions of transgenic *Arabidopsis* plants. Western blot analyses were performed with the αGFP antibody. Each lane contains 20 μg proteins. *C*, distribution of GFP, TP-pSSU::GFP, and ceQORH::GFP fusions in transgenic *Arabidopsis* plants. CE, crude leaf extract; Cp, chloroplast; E, envelope; S, stroma; T, thylakoid. Each lane contains 20 μg of proteins. Western blot analyses were performed using the αceQORH antibody (for the WT ceQORH), or using the αGFP antibody (for GFP, TP-pSSU::GFP, and ceQORH fusions). *D*, representative model of ceQORH domains functions.

experiments, these ceQORH fusion proteins, as well GFP and pSSU used as control proteins were synthesized *in vitro* and were used to perform *in vitro*-import experiments (Fig. 6A). To dissect the different stages of the import process, we manipulated the concentrations of Mg-GTP and Mg-ATP according to previously published procedures. The different precursors were incubated with isolated, energy-depleted barley chloroplasts either in the absence of added nucleoside triphosphates (-NTPs), in the presence of 0.1 mM Mg-ATP, in the presence of 0.1 mM Mg-ATP plus 0.1 mM Mg-GTP or in the presence of 2 mM Mg-ATP plus 0.1 mM Mg-GTP. Taking into account the previous findings of Kouranov and Schnell (9) these different nucleoside triphosphate combinations and concentrations were used to distinguish energy-independent binding (-NTPs) and energy-dependent binding (0.1 mM Mg-ATP) of the precursors to the plastids from their insertion (2 mM Mg-ATP plus 0.1 mM Mg-GTP) across the respective import machineries. Fig. 6A shows the results of a representative *in vitro*-import experiment. In the presence of 0.1 mM ATP, pSSU interacted with the plastids to reach a still protease-sensitive conformation, indicating that the protein was bound to but not imported into the plastids (Fig. 6A, pSSU, lanes 3 and 8). pSSU import and maturation required raising the Mg-ATP

concentration up to 2 mM (Fig. 6A, pSSU, lanes 5 and 10). Similarly, ceQORH::GFP was readily imported in the presence of exogenous added GTP and ATP (Fig. 6A, ceQORH::GFP). By contrast, GFP alone, without a transit peptide attached to it, was unable to interact with the chloroplast and was not imported (Fig. 6A, GFP, lanes 3 and 8).

In a second set of experiments, these truncated versions of ceQORH fused to GFP, as well as the respective controls (GFP and TP-pSSU::GFP), were stably expressed in *Arabidopsis* plants to analyze their respective subcellular and subplastidial localization. The expression of these fusion proteins in *Arabidopsis* was firstly validated by immunodetection (supplemental Fig. S3). We then analyzed the distribution of these truncated ceQORH::GFP forms (*i*) between crude soluble and membrane fractions derived from these transgenic plants (Fig. 6B) and (*ii*) between crude cellular extract and purified chloroplast fractions (envelope, stroma, and thylakoids, respectively). Purified thylakoid fractions were analyzed to demonstrate their low contamination by envelope proteins, thus validating the specificity of association of trun-

cated ceQORH with thylakoid membranes (supplemental Fig. S4). These experiments confirmed the cytosolic and plastid localizations of GFP and TP-pSSU::GFP, respectively (Fig. 6, B and C). Moreover, the *in vitro* and *in planta* experiments both proved the plastid import of ceQORH::GFP (Fig. 6, B and C).

When we performed the same type of experiments with the truncated ceQORH forms fused to GFP, some quite unexpected results were obtained. When the internal domain of ceQORH (amino acids 60–100) was fused to GFP, the corresponding fusion protein was imported into chloroplasts *in vitro* (Fig. 6A, (60–100)-ceQORH::GFP) but not *in planta* (Fig. 6C). (60–100)-ceQORH-GFP is likely to be localized in the cytosol *in planta* and hence was easily detectable in crude-soluble leaf extracts, but not in the respective membrane fraction (Fig. 6B). One proposed explanation is that the complexity of the cellular milieu is not fully reproduced *in vitro*. Indeed, in *in vitro* conditions, the (60–100)-ceQORH polypeptide is in close contact to purified plastids, and this vicinity may favor its recognition by the plastid import machinery. However, *in planta*, a membrane binding domain could be required to efficiently target the (60–100)-ceQORH polypeptide to plastids. These results strongly suggest that, *in planta*, either the C- or the N-terminal

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parts of ceQORH operate as additional targeting signals and/or membrane anchors.

Indeed, when the N-terminal portion (amino acids 6–100) of ceQORH was fused to GFP, the corresponding reporter protein was imported into the plastids both *in vitro* and *in planta* (Fig. 6A and B, (6–100)-ceQORH::GFP), and it faithfully associated with membranes (Fig. 6B). Interestingly, (6–100)-ceQORH::GFP appeared to be evenly distributed between envelope and thylakoid fractions *in planta* (Fig. 6C). These results indicated that the C terminus part of ceQORH, which had been eliminated during the construction of the chimeric protein, was required for the proper and exclusive localization of ceQORH in the inner envelope membranes.

Next, ceQORH was truncated from its N-terminal end, leaving the remainder of the polypeptide chain and especially region 60–100 unaffected and fused to GFP. The resulting fusion protein was imported into the plastids both *in vitro* and *in planta* and localized to the inner envelope membrane fraction (Fig. 6A–C, (Δ 1–59)-ceQORH::GFP). This finding is consistent with a role of the C-terminal part of ceQORH in correct membrane targeting/binding. Finally, the C terminus part alone was fused to GFP. Lacking the amino acid 60–100 internal ceQORH region, the resulting fusion protein had been shown to bind to the plastids *in vitro* but remained sensitive to protease treatment; therefore, it was not imported (Fig. 6A, (Δ 1–99)-ceQORH::GFP). Nevertheless (Δ 1–99)-ceQORH::GFP was detected in the envelope fraction *in planta*, suggesting that the N-terminal part of ceQORH alone provided some targeting information. However, because the protein was not imported into plastids *in vitro*, (Δ 1–99)-ceQORH::GFP most probably interacted with the outer surface of the chloroplast (Fig. 6C).

In summary, the *in vitro* and *in planta* results provoke the view that the 60–100 internal domain of ceQORH is neighbored by two membrane-interacting domains (Fig. 6D). The N-terminal part of ceQORH may mediate some nonspecific interactions with cell membranes (Fig. 6D). On the contrary, the C-terminal part of ceQORH could mediate a specific interaction with the envelope membranes and thereby would be essential for the specific and unique localization of the protein within the inner chloroplast envelope (Fig. 6D). These interactions may involve membrane lipids and proteins.

In Vitro and In Planta Experiments Indicate That the C Terminus Part of ceQORH Confers Specificity on the Import Process and Directs the Polypeptide to a Novel Import Site—The results presented thus far implied that ceQORH uses an unprecedented import site to be targeted to plastids and that several domains of ceQORH provided distinct functions for plastid targeting and subplastidial localization of the protein. To dissect the role of the identified domains of ceQORH in the regulation of import and membrane binding, *in vitro*-import experiments were performed with the same truncated ceQORH forms as before, and with isolated barley chloroplasts that had been preincubated, or not, with antibodies against Toc75, the translocation channel protein of the TOC complex (Fig. 7A). Consistent with the results shown before, the presence of α TOC75 antibodies did not abolish the ceQORH-GFP import (Fig. 7A, ceQORH-GFP, lanes 8 and 10). Nevertheless, it seems that the ceQORH-GFP signal observed in lane 10 would be slightly less

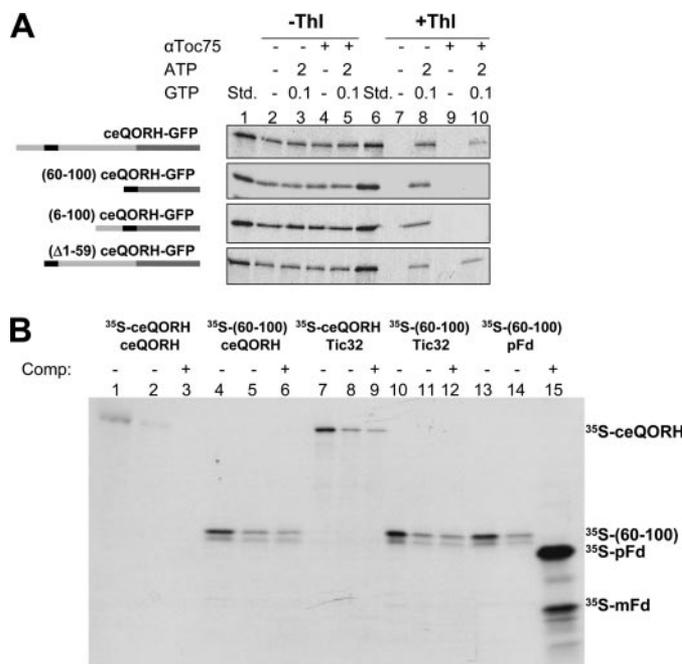


FIGURE 7. The C terminus part of ceQORH is essential *in vitro* to drive polypeptides to the original import site. A, barley chloroplasts were isolated as described, energy-depleted, and preincubated or not with the α Toc75 antiserum (\pm α Toc75). Chloroplasts were then incubated with 35 S-ceQORH::GFP fusions in the presence of Mg-ATP and Mg-GTP at the indicated millimolar concentrations. Import reactions were performed for 15 min in the dark, and the plastids were then sedimented by centrifugation. Plastids found in the pellet fraction were treated (+Thl) or untreated (–Thl) with thermolysin as indicated. Std, input standard. B, import reactions for 35 S-ceQORH::GFP and 35 S-(60–100)-ceQORH::GFP fusion proteins was performed in a mixture containing 2 mM Mg-ATP and in absence (lanes 2, 5, 8, 11, and 14) or presence (lanes 3, 6, 9, 12, and 15) of bacterially expressed unlabeled ceQORH (25 μ M, lanes 1–6) or TIC32 (25 μ M, lanes 7–12) or 35 S-pFd (500-fold excess, lanes 13–15), relative to time point zero (lanes 1, 4, 7, 10, and 13). After 15 min the reactions were stopped on ice. Plastids were repurified on Percoll and supplemented with thermolysin (Thl) to degrade non-imported protein. Proteins were detected by autoradiography following SDS-PAGE.

than in lane 8 (in presence or not of α TOC75 antibodies, respectively). Even if this slight decrease of ceQORH-GFP import was not present in the blocking experiments presented in Fig. 2C, we cannot exclude that α TOC75 antibodies lead to a slight inhibition of ceQORH import.

When both N- and C-termini parts of ceQORH were deleted, the resulting (60–100)-ceQORH::GFP fusion protein was still imported into chloroplasts but in a TOC75-mediated manner (Fig. 7A, (60–100)-ceQORH::GFP, lanes 8 and 10). This unexpected finding was confirmed using the fusion protein (6–100)-ceQORH::GFP, which was also routed to the classic, TOC75-containing import site (Fig. 7A, (6–100)-ceQORH::GFP, lanes 8 and 10). By contrast, the exclusively N-terminally truncated ceQORH derivative ((Δ 1–59)-ceQORH::GFP) was targeted to the novel, TOC75-independent import site (Fig. 7A, lanes 8 and 10). These results suggest that the C-terminal part of ceQORH is necessary and sufficient to select for the ceQORH original import site.

Competition experiments were then carried out with the ceQORH-GFP and (60–100)-ceQORH::GFP fusion proteins (Fig. 7B). We have demonstrated that ceQORH import was insensitive to chemical amounts of pFd (Fig. 2b, lane 9). By

contrast, (60–100)-ceQORH protein was no longer imported into chloroplasts in the presence of excess ferredoxin (Fig. 7B, lanes 13–15). This result confirmed and extended that deletion of the C-terminal part of ceQORH abolished the correct targeting of ceQORH and led to its mistargeting to the Toc complex. Because the proteins including or not the C-terminal part of ceQORH were routed to a different import site, they were expected to interact with different receptors at the chloroplast surface.

A way to demonstrate this would be to determine the number of receptor sites and respective K_D values. Kouranov and Schnell (9) have shown that binding of precursors to the plastids initially is reversible and occurs in the absence of nucleoside triphosphates. Low (<0.1 mM) ATP concentrations, however, favor partial integration of the receptor-bound precursors into the import machinery (9). This step, which previously has been referred to as binding (57, 58) is stimulated by GTP. The precursors then insert across the outer envelope membrane and also interact with components of the inner envelope (9). As a result, early import intermediates are formed (59). The precursor concentrations that are necessary to saturate the sites for establishing early import intermediates were found to be nearly identical to those seen for energy-independent binding. This allows extrapolating the number of energy-independent preprotein-binding sites and suggests that they may limit the number of preproteins that associate with the outer envelope membrane (9). If high (>2.5 mM) ATP concentrations are present, the precursors ultimately translocate across the inner envelope (57, 58).

Taking into account these findings, chloroplasts were isolated on Percoll cushion and subsequently depleted of ATP (44). Then, various amounts of the radiolabeled ceQORH::GFP and (60–100)-ceQORH::GFP were added to incubation mixtures containing 0.1 mM Mg-ATP. All assays were supplemented with wheat germ extract, to obtain identical reaction mixtures. After a 15-min incubation at 4 °C in the dark, the plastids were sedimented by centrifugation and repurified using Percoll cushion, and the amounts of bound ceQORH::GFP and (60–100)-ceQORH::GFP were determined. ceQORH::GFP and (60–100)-ceQORH::GFP molecules that were not bound to the chloroplasts were recovered from the supernatant obtained after sedimentation of the plastids by precipitation with trichloroacetic acid and quantified as described (43). At saturating precursor concentration, each chloroplast bound ~ 850 ceQORH::GFP and 2200 (60–100)-ceQORH::GFP molecules (Fig. 8, A and B, respectively). Scatchard analysis of the binding data indicates that K_d values are quite similar for ceQORH::GFP and (60–100)-ceQORH::GFP (8.3 and 8, respectively) (Fig. 8, A and B). These experiments showed that binding of ceQORH::GFP and (60–100)-ceQORH::GFP most likely involved different receptors. The number of receptors for (60–100)-ceQORH::GFP binding well matched those obtained with pSSU (43) and was clearly different for ceQORH.

Cross-link assays were then performed using the ceQORH::GFP and (60–100)-ceQORH::GFP proteins (Fig. 9). Tokatlidis *et al.* (46) have shown that precursors in transit through the outer and inner plastid envelope membranes are in such close physical proximity to components of the import machinery that allows the formation of mixed disulfide bonds. If a thiol group of the precursor is activated with

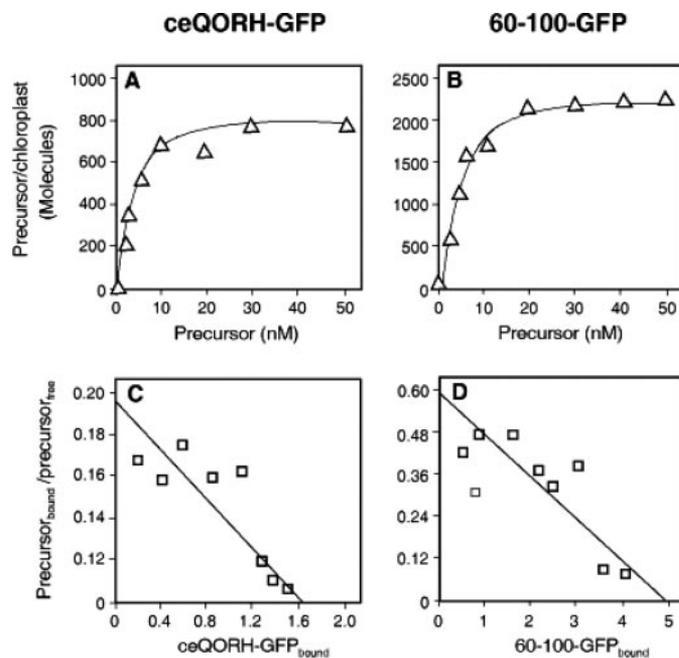


FIGURE 8. Quantitative receptor binding assays reveal different numbers of receptor sites for ceQORH::GFP and (60–100)-ceQORH::GFP. *Arabidopsis* chloroplasts were isolated, energy-depleted, and incubated at 4 °C in the presence of 0.1 mM Mg-ATP with different amounts of ^{35}S -ceQORH::GFP and ^{35}S -(60–100)-ceQORH::GFP. The assay mixtures were then centrifuged, and the amount of radiolabeled precursors left in the supernatants or bound to the plastids, respectively, were quantified by liquid scintillation counting (A and B). To determine the number of receptor sites as well as K_D values, Scatchard analyses were used (C and D).

DTNB, it can react with a second thiol group to give rise to a covalent cross-link product (60). ceQORH::GFP and (60–100)-ceQORH::GFP were synthesized from corresponding cDNA clones, activated with DTNB and incubated with barley chloroplasts in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. These experiments gave rise to a ~ 100 kDa cross-link product for the ceQORH::GFP protein and a ~ 250 kDa cross-link product for the (60–100)-ceQORH::GFP protein (Fig. 9A). By subtracting the molecular mass of ceQORH::GFP or (60–100)-ceQORH::GFP, the molecular mass of the protein(s) linked to ceQORH was ~ 30 kDa, whereas the protein linked to (60–100)-ceQORH was ~ 215 kDa. Toc159 has previously been detected at ~ 215 kDa on Laemmli SDS-PAGE gels instead of 159 kDa (the calculated molecular mass of Toc159) (61, 62). The abnormal mobility of Toc159 is presumably due to its highly acidic N-terminal region (54, 62). This suggests that the protein of 215 kDa linked to (60–100)-ceQORH may well correspond to Toc159 and corroborate our previous results demonstrating that (60–100)-ceQORH uses the classic TOC machinery to be translated across the outer envelope membrane. Taken together, these results clearly demonstrate that ceQORH::GFP and (60–100)-ceQORH proteins are tightly bound to different envelope receptors.

To validate the physiological significance of the *in vitro* results, *in planta* experiments were performed with the same type of ceQORH derivatives fused to GFP as used before (Fig. 10). Whereas ceQORH::GFP and ($\Delta 1$ –59)-ceQORH::GFP as well as (6–100)-ceQORH::GFP were efficiently targeted to leaf mesophyll cell plastids (Fig. 10, ceQORH::GFP, ($\Delta 1$ –59)-

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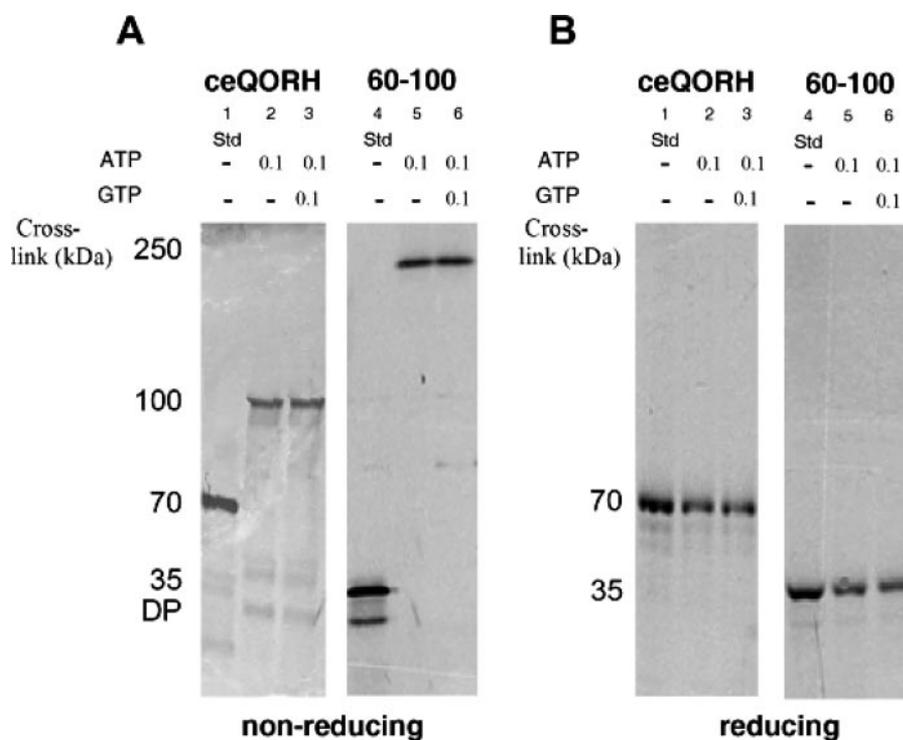


FIGURE 9. ceQORH::GFP and (60–100)-ceQORH interact with different receptors at the chloroplast surface. ^{35}S -ceQORH::GFP and ^{35}S -(60–100)-ceQORH::GFP were synthesized *in vitro* and activated with DTNB. Precursors in turn were incubated with isolated, energy-depleted *Arabidopsis* chloroplasts in the presence of either 0.1 mM Mg-ATP or 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. After 15 min in darkness, mixed envelope membranes were isolated on a sucrose gradient, solubilized with SDS, and separated by reducing (A) or non-reducing (B) SDS-PAGE. The autoradiograms show the levels of ^{35}S -ceQORH::GFP and ^{35}S -(60–100)-ceQORH::GFP, and their respective cross-link products, marked by their M_r , and after incubation (15 min). *Std* defines input standards. *DP* indicates a degradation product of ^{35}S -(60–100)-ceQORH::GFP.

ceQORH::GFP, and (6–100)-ceQORH::GFP), the full-length ceQORH::GFP protein was not imported into guard cell chloroplasts (Fig. 10, ceQORH::GFP). This result suggested that the ceQORH import site was not expressed in guard cells, whereas the TOC machinery comprising Toc75 may be ubiquitously expressed in different cell and tissue types and associated plastid forms. In good agreement with *in vitro* data, (6–100)-ceQORH::GFP was imported into guard cell chloroplasts (Fig. 10 (6–100)-ceQORH::GFP). An exception to the rule seems to be (Δ 1–59)-ceQORH::GFP that also entered guard cell plastids *in planta* (Fig. 10, (Δ 1–59)-ceQORH::GFP). This difference between the *in vitro* and *in vivo* approaches could be explained by the simplification of the cellular context in the *in vitro* import assays. We hypothesized that some cytosolic factors (absent in the *in vitro* import assays) could play a role in the (Δ 1–59)-ceQORH::GFP plastid targeting *in planta*. In this way, similar differences between *in vitro* experiments and *in planta* observations were also described by other authors and indicate that *in vitro* import assays may not fully match the more complex *in vivo* situation suggesting that some cytosolic components provide back-up systems to assure import of certain precursors under different environmental conditions (64, 65).

DISCUSSION

ceQORH was previously demonstrated to be the first identified chloroplast protein to be targeted to the inner envelope

membrane while lacking a cleavable N-terminal transit peptide (31). Using both *in planta* and *in vitro* approaches, we have shown that ceQORH does not use the classic TOC complex comprising Toc75 to cross the outer envelope but uses a different, yet to be identified import site. These results demonstrate that ceQORH import is due to the operation of a novel and hitherto unknown import site.

This import site is of proteinaceous nature and universally conserved in mono-cotyledonous and dicotyledonous plant species. Cross-link experiments as well as competition experiments, determined receptor numbers and K_D values, underscore its unique properties. Moreover, the energy requirement of ceQORH import seems a distinguishing feature from that known for other nucleus-encoded plastid proteins. Generally, the energy requirement of import of nucleus-encoded plastid proteins is closely linked to the final subplastidial localization of the import substrate. Indeed, (i) the insertion of most integral outer envelope pro-

teins does not require an energy source (15), (ii) transit across the outer envelope and access to the intermembrane space compartment requires only low ATP concentration (<2 mM) (66), and finally (iii) access to the stromal compartment requires high ATP concentration (2 mM) (44). Interestingly, import of ceQORH is only observed at high ATP concentration in the uptake medium (2 mM ATP, Fig. 6A). This energy requirement is higher than the one observed (<50 μM) for import of Tic22 into the intermembrane space (67) and for Tic32, which is proposed to be targeted in the intermembrane space prior its integration in the inner envelope (28). The larger energy requirement of 2 mM ATP is reminiscent of that of pSSU import into the chloroplast stroma (Fig. 6A). However, whether or not the translocation across the inner envelope membrane would require some of the previously identified TIC components operating in translocation across the inner envelope membrane remains to be determined.

For its proper targeting, ceQORH has evolved distinct subdomains. The ceQORH amino acid sequence can be divided into three subdomains, which accomplish distinct but also complementary roles. A soluble and internal domain (residues 60–100) dictates its proper targeting to the chloroplast. This internal domain is flanked by two membrane interacting domains in the N- and the C-terminal parts of ceQORH. The N-terminal domain (residues 1–100) has a general and nonspecific membrane-interacting function, whereas the C-terminal domain (residues 100–329) plays a role in (i) mediating the

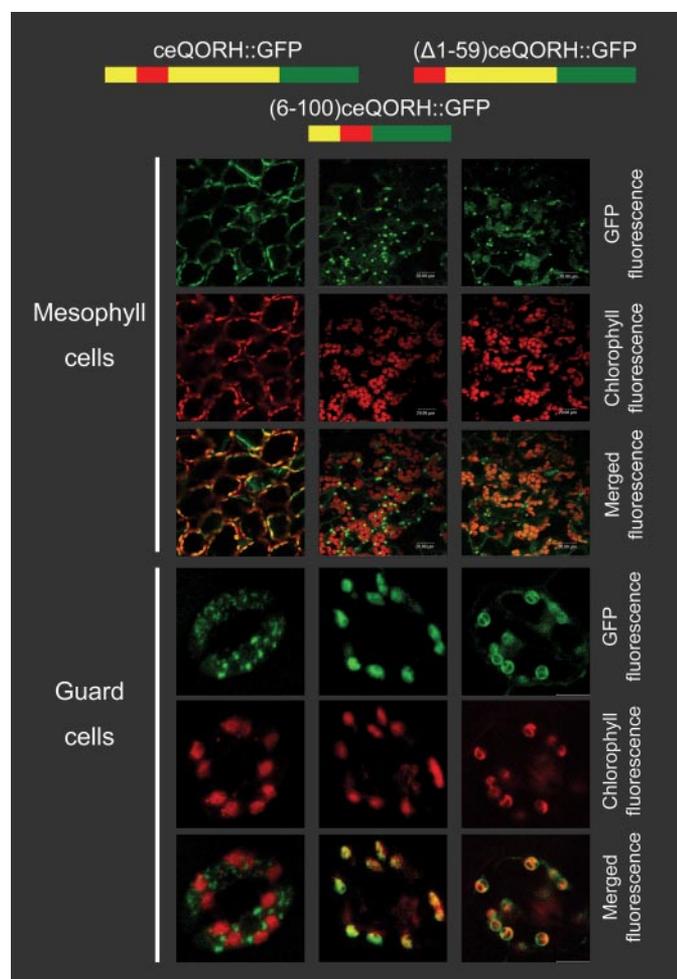


FIGURE 10. ceQORH domains act concertedly in directing the precursor to distinct and tissue-specific plastid targeting pathways. Localization of truncated ceQORH forms fused to GFP was analyzed in transgenic *Arabidopsis* plants by fluorescence microscopy. Detailed information concerning the structure of GFP-tagged and -truncated forms of the ceQORH protein is described in Fig. 6. The localization of ceQORH described in Fig. 6 is shown (ceQORH::GFP). Cells were observed with 400 \times magnification.

specific interaction with the outer envelope import machinery and, (ii) directing its targeting to the inner envelope membrane.

Classic cleavable N-terminal transit peptides contain all the information that is necessary and sufficient for import, including receptor recognition and membrane translocation. Despite their great primary sequence divergence, distinct “homology blocks” were identified in plastid transit peptides (68). An emerging concept suggests that transit peptides contain multiple domains that provide distinct and complementary information as to how to recognize the components of the outer and inner envelope import machineries. Generally, transit peptides seem to consist of (i) two membrane-interacting domains, generally localized in the N- and C-terminal extremities, which are implicated in lipid-mediated binding (51, 69, 70), and (ii) a central region that could be involved in the recognition of the respective import machinery (70). At first glance, the functional organization of the entire amino acid sequence of ceQORH well matches that of a classic transit peptide, with the only exception that it is uncleavable and rather long. Indeed, our study identified two lipid-interacting domains in the N- and C-termini of

ceQORH. These domains are separated by a soluble domain that is essential for faithful plastid targeting. Rather than having acquired a cleavable N-terminal transit peptide during evolution, ceQORH could have evolved in its total amino acid sequence to acquire plastid targeting information.

Multiple isoforms of many TOC/TIC proteins have been identified in the *Arabidopsis* genome (20–24). Furthermore, subcellular proteomics allowed the identification of (i) new envelope proteins presenting homology with known TOC and TIC components as well as mitochondrial TOM and TIM proteins, and (ii) new plastid proteins that are not predicted to contain classic plastid transit peptides (32, 52, 56, 63). A rising concept is that multiple translocon complexes may exist in the plastid envelope that harbor the few thousand nucleus-encoded plastid proteins during import and assure a cell-, tissue-, and organ-specific protein complement that may vary at different stages of plant development.

In line with such a proposal, we provide strong evidence that ceQORH is imported into plastids through an unprecedented proteinaceous import site and that several domains of this protein act concertedly in directing the precursor to this hitherto unknown import pathway. Future challenges in understanding the various chloroplast protein import machineries will be to identify alternative translocon complexes and to define their respective substrate specificities. In fact, ceQORH seems an ideal bait for identifying components involved in import of transit less precursors, and work is in progress to do so by biochemical and genetic approaches.

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