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Non-canonical Transit Peptide for Import into the Chloroplast*[§]

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The large majority of plastid proteins are nuclear-encoded and, thus, must be imported within these organelles. Unlike most of the outer envelope proteins, targeting of proteins to all other plastid compartments (inner envelope membrane, stroma, and thylakoid) is strictly dependent on the presence of a cleavable transit sequence in the precursor N-terminal region. In this paper, we describe the identification of a new envelope protein component (ceQORH) and demonstrate that its subcellular localization is limited to the inner membrane of the chloroplast envelope. Immunopurification, microsequencing of the natural envelope protein and cloning of the corresponding full-length cDNA demonstrated that this protein is not processed in the N-terminal region during its targeting to the inner envelope membrane. Transient expression experiments in plant cells were performed with truncated forms of the ceQORH protein fused to the green fluorescent protein. These experiments suggest that neither the N-terminal nor the C-terminal are essential for chloroplastic localization of the ceQORH protein. These observations are discussed in the frame of the endosymbiotic theory of chloroplast evolution and suggest that a domain of the ceQORH bacterial ancestor may have evolved so as to exclude the general requirement of an N-terminal plastid transit sequence.

Higher plant plastids contain a genome with limited coding capacity. The large majority of plastid proteins are nuclear-encoded and thus, must be imported within these organelles. To this purpose, the two envelope membranes that surround chloroplasts contain a protein import apparatus constituted of the TOC and TIC complexes (translocon at the inner or outer membranes of the chloroplast envelope) (for recent reviews, see Refs. 1–4). The major element of this translocation complex is Toc75, which is the most abundant protein in the outer envelope membrane. Moreover, Toc75 seems to form the central pore of the outer envelope translocation channel (5, 6) and interacts specifically with the N-terminal transit peptide of precursor proteins during the import process toward chloroplast (7). This cleavable and N-terminal transit peptide was

shown to be necessary and sufficient for transport of precursors (i) across the two envelope membranes (8, 9) and (ii) across the thylakoid membrane if the transit sequence is bipartite (it contains additional targeting information for thylakoid lumen targeting) (9). The cleavage of the transit sequence is performed by two proteases, one in the stroma (10) and one in the thylakoid lumen for the bipartite transit sequences (11). Moreover it is interesting to note that all described inner membrane proteins and intermembrane space proteins possess an N-terminal cleavable transit peptide, while most outer envelope membrane proteins do not have any cleavable transit peptide. In the latter case, the targeting information is contained within the mature protein (12), and after their cytosolic synthesis, proteins are directly incorporated in the lipid bilayer through unknown interactions with the outer membrane lipids (13–15).

In this report, we describe the identification of a new component of the inner membrane of the chloroplast envelope, which exhibits similarities with a range of quinone oxidoreductase from various organisms (ceQORH for Chloroplast Envelope Quinone OxidoReductase Homologue). Immunolocalization, purification, and microsequencing of this protein and cloning of the corresponding full-length cDNA revealed the existence of a protein that does not require the presence of a cleavable N-terminal transit peptide to be targeted to the inner membrane of the chloroplast envelope. Transient expression of GFP¹-tagged and truncated forms of the ceQORH protein suggests that the localization of this protein into chloroplast is dependent on an essential but not sufficient internal domain located within the ceQORH amino acid sequence.

EXPERIMENTAL PROCEDURES

Purification of Chloroplast Envelope Membranes—All operations were carried out at 0–5 °C. Crude chloroplasts were obtained from 3–4 kg of spinach (*Spinacia oleracea* L.) leaves and purified by isopycnic centrifugation using Percoll gradients (16). At this step of purification, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM amino caproic acid) were added to prevent any protein degradation. Purified intact chloroplasts were lysed in hypotonic medium, and envelope membranes were purified from the lysate by centrifugation on sucrose gradients (16). Envelope subfractions respectively enriched in outer and inner membranes were obtained from purified intact spinach chloroplasts as previously described (17). To study polypeptides localized on the external face of the outer membrane, intact chloroplast were treated with thermolysin from *Bacillus thermoproteolyticus* as previously described (18). All types of envelope membranes preparations were stored in liquid nitrogen in 50 mM MOPS-NaOH, pH 7.8, in the presence of protease inhibitors (1 mM benzamidine and 0.5 mM amino caproic acid). Protein contents of mem-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains a figure and figure legend of the immunopurification of the natural spinach ceQORH protein.

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¹ The abbreviations used are: GFP, green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Q-TOF, quadrupole time-of-flight; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

brane fractions were estimated using the BIO-RAD protein assay reagent (19).

Solubilization of Membrane Proteins Using Treatment with Detergents—Solubilization of envelope membrane proteins with CHAPS or Triton X-100 were performed as follows. Envelope proteins (0.8 mg) were diluted in 1 ml of 50 mM MOPS, pH 7.8, containing 0.2% (v/v) Triton X-100 or 6 mM CHAPS. After incubation at 4 °C for 30 min, the mix was centrifuged (130,000 × *g*, 20 min, 4 °C) to separate (i) a supernatant containing membrane proteins solubilized by the treatment and (ii) a pellet containing the insoluble proteins.

SDS-PAGE and Western Blot Analyses of Envelope Proteins—SDS-PAGE analyses of chloroplast subfractions or chloroplast envelope subfractions were performed as described by Chua (20). For Western blotting experiments, gels were transferred electrophoretically to a nitrocellulose membrane (BA85, Schleicher & Schuell). The immunoblot assay was performed according to the protocol from BIO-RAD Laboratories except that 5% non-fat dry milk was used to saturate the membrane. The ceQORH protein was detected using the polyclonal antibodies raised against the recombinant *Arabidopsis* protein at a 1/5000 dilution using alkaline phosphatase detection.

Mass Spectrometry and Protein Identification—After SDS-PAGE, protein bands were excised from the Coomassie Blue-stained gel. Conditions for in-gel tryptic digestion and peptide elution were described previously (21). The peptide solution was introduced into a glass capillary (Protana) for nanoelectrospray ionization. Tandem mass spectrometry experiments were carried out on a Q-TOF (quadrupole orthogonal acceleration time-of-flight) hybrid mass spectrometer (Micromass) to obtain sequence information. Collision-induced dissociation of selected precursor ions was performed using argon as the collision gas and with collision energies of 40–60 eV. Tandem mass spectrometry sequence information were used for data base searching using the programs MS-Edman located at the University of California San Francisco (prospector.ucsf.edu) and BLAST located at the NCBI (www.ncbi.nlm.nih.gov/BLAST/). Prediction for membrane-spanning regions was achieved using the software programs TMPred (www.ch.embnet.org/software/TMPRED_form.html) (22).

Construction of Arabidopsis and Spinach cDNA Libraries for RACE-PCR Experiments—Purification of total RNAs from spinach or *Arabidopsis* leave were performed using the RNeasy Plant Mini Kit from Qiagen Laboratories. Purification of mRNA from the total RNA fraction was performed using the mRNA Direct Kit from Dynal Laboratories. Constructions of the spinach and *Arabidopsis* cDNA libraries were performed using the Marathon cDNA Amplification Kit from Clontech Laboratories. All RACE-PCR experiments were performed using the Advantage cDNA Polymerase Mix from Clontech Laboratories.

Cloning of the Arabidopsis ceQORH cDNA, Overexpression in Escherichia coli, and Purification of the Recombinant Protein to Obtain a Polyclonal Antibody—Cloning of the *Arabidopsis* cDNA coding the ceQORH from *Arabidopsis* was performed by PCR, on an *Arabidopsis* cDNA library using primers designed on the basis of the *Arabidopsis* genome sequence (AGI accession number AT4G13010) encoding the putative ceQORH protein (TrEMBL accession number Q9SV68). Two primers TCACATATGGCTGGAAAACCAATGCAC and ATGGATC-CAACGCTCTTATGGCTCGAC were designed respectively to introduce *NdeI* and *BamHI* recognition sites (*underlined residues*) at the 5' and 3' ends of the ceQORH cDNA. The amplified fragment was cloned in pBlueScript SK⁻. The insert was then digested with *NdeI* and *BamHI* and inserted into the expression vector pET-15b (Novagen). The resulting expression plasmid was used to express the N-terminal His-tagged ceQORH protein in *E. coli* strain BLR(DE3) (Novagen). The recombinant protein was purified by metal affinity chromatography (His-Bind resin, Novagen) and desalted (PD-10 column, Amersham Biosciences). This purified ceQORH from *Arabidopsis* was used (i) to raise a rabbit polyclonal antibodies against this protein (obtained from the *Eleveage Scientifique des Dombes*, F-01400 Châtillon sur Chalaronne, France) and (ii) as a tool in the immunopurification of the ceQORH from spinach chloroplast envelope membranes.

Immunopurification of the Spinach ceQORH—Envelope proteins (1 mg) were solubilized in 1 ml of 50 mM Tris/HCl, 150 mM NaCl, and 6 mM CHAPS and incubated 1 h at 4 °C with 33 μl of serum raised against the purified *Arabidopsis* recombinant ceQORH. Protein A-agarose (50 μg; Boehringer) was added, and the mix was incubated for 3 h at 4 °C. After three successive washings by centrifugation (Eppendorf 5415D, 16,000 × *g*, 20 min, 4 °C) of the protein A-agarose and pellet suspension in 1 ml of solubilization buffer (20 mM MOPS, pH 7.8, 150 mM NaCl, 6 mM CHAPS), 50 μg of His-tagged recombinant *Arabidopsis* protein, incubated in 200 μl of solubilization buffer, was added. The mix was incubated for 1 h at 4 °C and centrifuged for 20 min at 16,000 × *g*

(Eppendorf 5415D). Supernatant was incubated for 1 h with Ni-NTA resin (Qiagen), previously equilibrated in the solubilization buffer to remove the majority of the His-tagged recombinant *Arabidopsis* protein. After centrifugation (Eppendorf 5415D, 16,000 × *g*, 20 min, 4 °C) the supernatant was analyzed by SDS-PAGE.

Cloning of Spinach ceQORH cDNA—To isolate the spinach ceQORH cDNA, RACE-PCR were performed using degenerated primers synthesized on the basis of the spinach protein microsequences. Primers Rev (GCNCCYTCNGGNGTYTTRTARTC) and Fwd (GAYTAYAARACNCC-NGARGGNGC) were designed respectively for 5' or 3' RACE-PCR amplifications. Positive amplification was obtained from 5' RACE-PCR amplification performed with the primer Rev and the Adaptor Primer AP1 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGCA-GGT) provided with the Marathon cDNA Amplification Kit (Clontech Laboratories). Amplified fragments were inserted into the pBlueScript SK⁻ vector and sequenced from both ends with standard T3 and T7 primers as well as with insert-specific primers (the 5' sequence revealed the ceQORH translation start codon and a stop codon in frame with this ATG in the 5'-UTR). The complete cDNA sequence coding for the spinach ceQORH was obtained from 5' and 3' RACE-PCR performed with primers AP1 and non-degenerated primers D (CAGCAACATCA-GTTCCAGGTATAG) and A (ACACGCATGTAACAGCAACATGTG), respectively. Note that the existence of the stop codon localized within the 5'-UTR and in frame with the translation start codon was confirmed with this new 5' RACE-PCR using primers D and AP1.

Construction of GFP Reporter Plasmids for Transient Expression in Arabidopsis and Tobacco Cells—The GFP reporter plasmid 35Ω-sGFP(S65T) and the plasmid containing the transit peptide (TP) sequence from RBCS fused to GFP [35Ω-TP-sGFP(S65T)] were described previously (23). Construction of the plasmids for expression of truncated *Arabidopsis* ceQORH protein fused to GFP were performed as follows. The 35Ω-ceQORH-sGFP(S65T) plasmid corresponding to the coding region of *Arabidopsis* ceQORH was PCR-amplified using the two flanking primers *XhoI*-N-ter (CCTCTCGAGATGGCTGGAAAACCAATGCAC) and *NcoI*-C-ter (CAACCCATGGATGGCTCGACAATGATCTTC), and the PCR product was cloned into the pBlueScript SK⁻ Vector. The *XhoI*-*NcoI* fragment cleaved from this plasmid was inserted into the *SalI*-*NcoI* digested GFP reporter plasmid 35Ω-sGFP(S65T) to create the 35Ω-ceQORH-sGFP(S65T) vector. The protocol was similar for the other constructions. The 35Ω-Δ(1–31)ceQORH-sGFP(S65T) plasmid corresponding to ceQORH lacking the first 31 amino acids was PCR-amplified using the two flanking primers *SalI*-N-ter (CGGTTGTCGACATGAAGAGTAATGAGGTTTGCCTG) and *NcoI*-C-ter (CAACCCATGGATGGCTCGACAATGATCTTC). The 35Ω-Δ(1–59)ceQORH-sGFP(S65T) plasmid corresponding to ceQORH lacking the first 59 amino acids was PCR-amplified using the two flanking primers *SalI*-N-ter (GAATGGTTCGACATGTTTCTGCCCGCAAGTTC) and *NcoI*-C-ter (CAACCCATGGATGGCTCGACAATGATCTTC). The 35Ω-Δ(1–99)ceQORH-sGFP(S65T) plasmid corresponding to ceQORH lacking the first 99 amino acids was PCR-amplified using the two flanking primers *SalI*-N-ter (GGTTGTCGACATGCTAGGTGGAGGTGGACTTC) and *NcoI*-C-ter (CAACCCATGGATGGCTCGACAATGATCTTC). The 35Ω-(6–100)ceQORH-sGFP(S65T) plasmid containing the first six to 100 amino acids of ceQORH was PCR-amplified using the two flanking primers *XhoI*-N-ter (CCTCTCGAGATGGCTGGAAAACCAATGCAC) and *NcoI*-C-ter (ACCCATGGCTAGATGGCTAAGAACCCTAC). The 35Ω-(60–100)ceQORH-sGFP(S65T) plasmid containing the first 60–100 amino acids of ceQORH was PCR-amplified using the two flanking primers *SalI*-N-ter (GAATGGTTCGACATGTTTCTGCCCGCAAGTTC) and *NcoI*-C-ter (ACCCATGGCTAGATGGCTAAGAACCCTAC). The 35Ω-QORECOLI-sGFP(S65T) plasmid containing the QOR protein from *E. coli* (accession number P28304) was PCR-amplified using the two flanking primers *XhoI*-QORNter (GTTCTCGAGGACACATGGCAACAC) and *NcoI*-QORCter (CTATTCCATGGATGGAATCAG-CAGGCTGGAAC). Correct orientation and sequences of the inserted fragments were controlled. The plasmids used for tissue bombardment were prepared using the QIAfilter Plasmid Midi Kit (Qiagen Laboratories).

Arabidopsis and Tobacco Cells Bombardment and Fluorescence Microscopy—*Arabidopsis* cells were grown in light for 3 days in a Gamborg's B5 media (Sigma, pH 5.8) complemented with 1.5% sucrose and 1 μM naphthalene acetic acid. 15 ml of cell suspension (corresponding approximately to 0.5 g of fresh weight) were applied in Petri dishes containing the same growth media with 0.8% bacto-agar (Invitrogen) and were incubated for 18–36 h in light. BY2 tobacco cells were grown for 5 days at 27 °C in a Murashige and Skoog medium (Duchefa, pH 5.8) complemented with 3% sucrose, 0.2% KH₂PO₄, 0.2% myo-inositol, 1 μM 2,4-dichlorophenoxy acetic acid, and 3 μM thiamin. Cell suspension (2.5

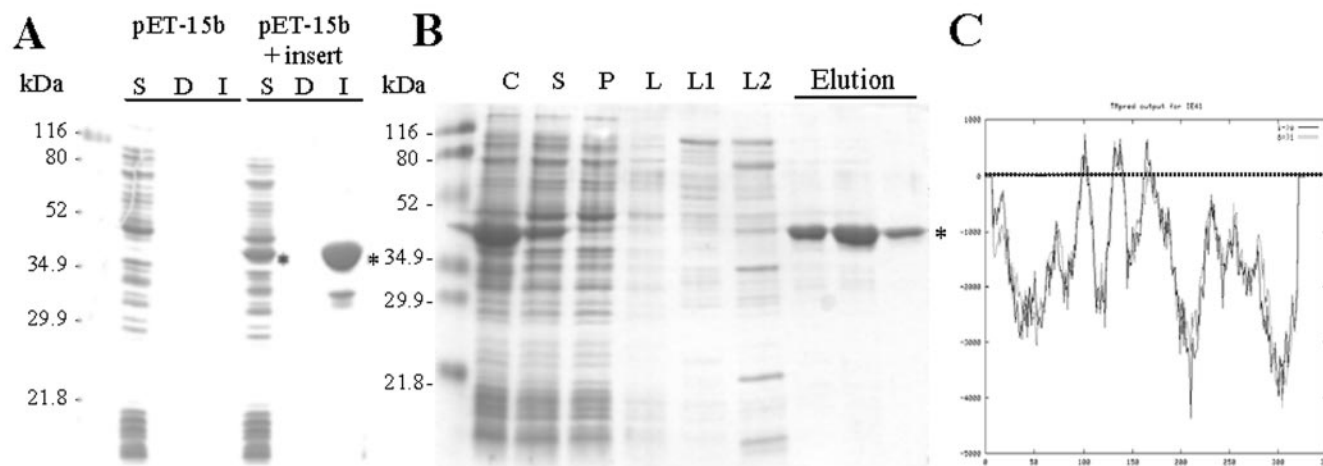


FIG. 1. The recombinant ceQORH protein behaves as a soluble protein. *A*, expression of the *Arabidopsis* ceQORH (*) protein in *E. coli*. After induction with isopropyl-1-thio- β -D-galactopyranoside and growth for 3 h, bacteria were concentrated by centrifugation, suspended in 1 ml of 20 mM Tris/HCl, pH 6.8, and disrupted by sonication. A first centrifugation (2 min, 16,000 \times g) released the soluble proteins (S). Insoluble proteins were suspended with 1 ml of detergent-containing buffer (20 mM Tris/HCl, pH 6.8, 0.5% Triton X-100). A second centrifugation allowed the separation of membrane proteins solubilized with Triton X-100 (D) from the non-solubilized proteins (I). These insoluble proteins were suspended in 1 ml of 20 mM Tris/HCl, pH 6.8, and analyzed. As a negative control, proteins from bacteria transformed with a non-recombinant plasmid (pET-15b) were analyzed in the same way. The samples were analyzed on a 12% SDS-PAGE. *B*, purification of the recombinant *Arabidopsis* ceQORH protein. The purification was performed by nickel affinity chromatography. *C*, crude bacterial extract previously diluted in disruption buffer. S, soluble bacterial proteins; P, unbound proteins; L, L1, and L2, proteins eluted in washings with disruption buffer containing 0, 35 and 60 mM imidazol, respectively. Elution, elution of the ceQORH protein in disruption buffer containing 250 mM imidazol. All fractions were analyzed on a 12% SDS-PAGE. *C*, hydropathy profile of the ceQORH protein from *Arabidopsis*. According to the TMpred program (34), no membrane-spanning segment could be deduced from the analysis of the ceQORH protein primary sequence.

ml, corresponding approximately to 0.3 g of fresh weight) were applied in Petri dishes containing the same growth media with 1% bacto-agar and stored at 27 °C during 18–24 h. Plasmids of appropriate constructions (1 μ g) were introduced into *Arabidopsis* and BY2 cells using a pneumatic particle gun (PDS-1000/He; Bio-Rad). The condition of bombardment was helium pressure of 1350 p.s.i., 1100 p.s.i. rupture disks (Bio-Rad), 10-cm target distance using 1 μ m of gold microcarriers (Bio-Rad). After bombardment, cells were incubated on the plates for 18–36 h (in light for the *Arabidopsis* cells). Cells were transferred to glass slides before fluorescence microscopy.

Localization of GFP and GFP fusions was analyzed in transformed cells by fluorescence microscopy using a Zeiss Axioplan2 fluorescence microscope, and the image was captured with a digital charge-coupled devices camera (Hamamatsu). The filter sets used were Zeiss filter set 13, 488013–0000 (exciter BP 470/20, beamsplitter FT 493, emitter BP 505–530) and Zeiss filter set 15, 488015–0000 (exciter BP 546/12, beamsplitter FT 580, emitter LP 590) for GFP and autofluorescence of chlorophylls, respectively. Cells were observed with a 400 \times magnification.

RESULTS

Identification of a Quinone Oxidoreductase Homologue (ceQORH) as a New Component of the Chloroplast Envelope—During the course of the identification of new chloroplast envelope protein components (21, 24) some peptide sequences were obtained, which shared homology with a putative *Arabidopsis* protein (AGI accession number At4g13010; TrEMBL accession number Q9SV68). Whereas most of the envelope proteins identified during these early publications were highly hydrophobic, this putative protein was structurally related to soluble bacterial, fungal, and animal proteins of known quinone oxidoreductase function. Because of its possible function and since this ceQORH protein could participate in the redox chains previously detected in the chloroplast envelope membranes (25), we decided to further investigate the subcellular localization of this ceQORH protein.

To obtain a polyclonal antibody raised against this protein the corresponding *Arabidopsis* cDNA was cloned and the full-length protein was overexpressed in *E. coli*. The recombinant ceQORH protein overexpressed in *E. coli* (Fig. 1A) was recovered in two distinct fractions: a soluble fraction in the *E. coli*

cytosol (Fig. 1A, lane S) and an insoluble fraction (Fig. 1A, lane I). As a high (0.5%) concentration of Triton X-100 did not release the insoluble fraction of the recombinant protein in the soluble phase, this fraction (Fig. 1A, lane I) is likely to correspond to aggregated protein (inclusion bodies). The soluble fraction of the N-terminal His-tagged recombinant protein was further purified by metal affinity chromatography. During this purification process, the ceQORH protein still behaved as a soluble protein (Fig. 1B) as expected for a member of this quinone oxidoreductase family (26).

The ceQORH Protein Is Localized in the Inner Membrane of the Chloroplast Envelope—Since the recombinant ceQORH protein appeared to be, at least in part, soluble in the *E. coli* cytosol (Fig. 1A), actual association of this protein with the chloroplast envelope remained to be demonstrated. Western blots performed on chloroplast subfractions demonstrated that this protein is only present within the chloroplast envelope (Fig. 2A, lane E) and not detected in other chloroplast subfractions (Fig. 2A, lanes S, T). From these data, it can be concluded that the ceQORH protein is associated exclusively with the envelope membrane and is not a soluble stroma protein contaminating the envelope preparations.

It is generally accepted that Percoll-purified intact chloroplasts are free from cytosolic contaminants (16). However, the ceQORH protein could be a soluble protein specifically interacting with the outer membrane of the chloroplast envelope thus, co-purifying with the purified chloroplast envelope membranes. To test this hypothesis, intact chloroplasts were treated with the thermolysin of *Bacillus thermoproteolyticus* as previously described (18). As presented in the Fig. 2B, the ceQORH protein remained unaffected by the thermolysin treatment performed on intact plastids. In contrast, complete degradation of the ceQORH protein was observed when the same proteolytic treatment was performed on solubilized envelope proteins, thus demonstrating the sensitivity of the ceQORH protein to thermolysin treatment (Fig. 2C). This result indicates that the ceQORH protein is not accessible to thermolysin from the cytosolic surface of the chloroplast, thus ruling out the hypothesis

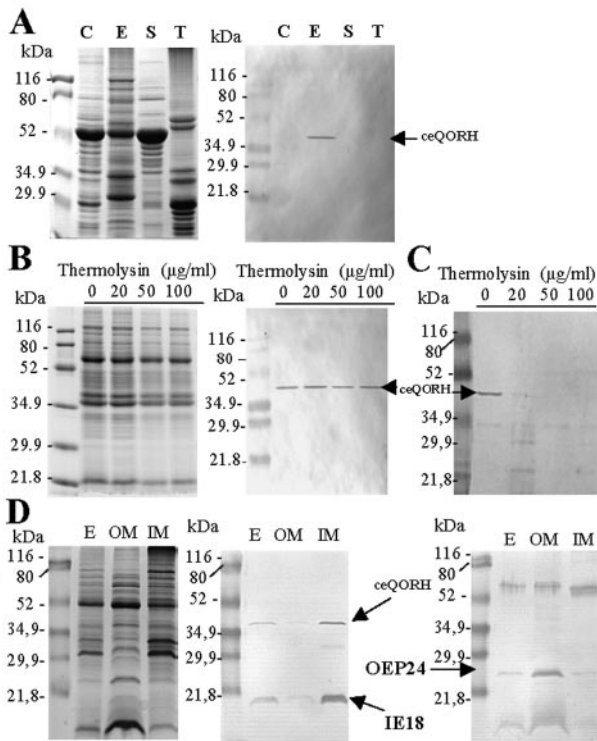


FIG. 2. Subcellular localization of the ceQORH protein. *A*, localization of the ceQORH protein in the chloroplast envelope. *Lane C*, crude chloroplast proteins; *lane E*, envelope membrane proteins; *lane S*, stromal proteins; *lane T*, thylakoid membrane proteins. Each fraction contained 15 μg of proteins. *B* and *C*, impact of the thermolysin treatment on the ceQORH protein. Envelope proteins (20 μg) derived from (*B*) intact chloroplasts treated with thermolysin (0, 20, 50, and 100 $\mu\text{g}/\text{ml}$) or, as a control, (*C*) solubilized envelope proteins (20 μg) treated with thermolysin (0, 20, 50, and 100 $\mu\text{g}/\text{ml}$). *D*, localization of the ceQORH protein in the inner membrane of the chloroplast envelope. *Lane E*, envelope membrane proteins; *lane IM*, inner membrane proteins; *lane OM*, outer membrane proteins. Each fraction contained 15 μg of proteins. All fractions were analyzed on a 12% SDS-PAGE. Western blots were performed with polyclonal antibodies raised against the recombinant ceQORH protein from *Arabidopsis*, the inner membrane protein IE18 (24), or the outer membrane protein OEP24 (27).

that the ceQORH protein could be a soluble cytosolic contaminant of the chloroplast envelope preparations or an envelope component associated with the outer face of the outer envelope membrane.

Finally, immunodetection of the ceQORH protein demonstrated that this protein is only present in the inner membrane of the chloroplast envelope (Fig. 2*D*, *lane IM*). The purity of the envelope subfractions was controlled using antibodies raised against IE18 and OEP24 proteins, which serve as marker molecules for inner and outer envelope membrane proteins respectively (24, 27). Thus, unlike the other members of this quinone oxidoreductase family (26), the ceQORH protein behaves as a genuine membrane protein.

The ceQORH Protein Interacts with the Inner Membrane of the Chloroplast Envelope through Electrostatic Interactions—At this stage, the apparent solubility of the recombinant ceQORH produced in *E. coli* and its exclusive localization in the inner membrane of the chloroplast envelope were contradictory. One hypothesis was that ceQORH could be a soluble protein localized in the intermembrane space between the inner and the outer membrane of the chloroplast envelope. This protein could be copurified with inner envelope preparations, sequestered in membrane vesicles. Some major soluble stroma proteins, which are sequestered in the envelope vesicles, are

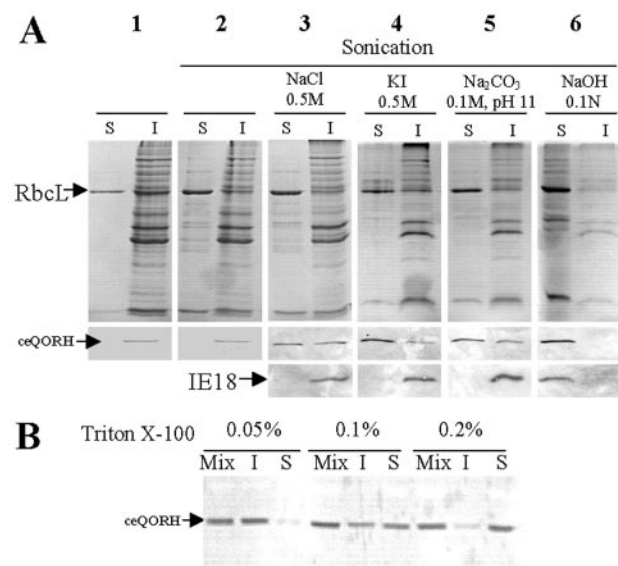


FIG. 3. The ceQORH protein is bound to the inner membrane of the chloroplast envelope through electrostatic interactions.

A, impact of salt or pH treatments. Envelope proteins (500 μg in 500 μl) were incubated for 30 min at 4 $^{\circ}\text{C}$ in 50 mM MOPS, pH 7.8, or in 10 mM MOPS, pH 7.8, containing 0.5 M NaCl or 0.5 M KI. For alkaline treatments, envelope proteins were directly incubated in 0.1 M Na_2CO_3 , pH 11 or 0.1 N NaOH. In some experiments (as indicated), sonication of envelope vesicles for 10 s and centrifugation (20 min at 72,000 $\times g$, Beckman L2 65B, rotor SW28) were performed to separate soluble (*S*) and insoluble membrane proteins (*I*). Insoluble proteins were then solubilized in 500 μl of the same buffers. *B*, impact of detergent treatment. Envelope proteins (100 μg in 100 μl) were incubated for 30 min at 4 $^{\circ}\text{C}$ in 50 mM Tris/HCl, pH 7.5, containing 0.05%, 0.1%, or 0.2% Triton X-100 (*Mix*) and centrifuged as above to recover soluble (*S*) and insoluble membrane proteins (*I*). Insoluble proteins were then solubilized in 100 μl of the same buffers. All resulting fractions were analyzed (20 μl) on 12% SDS-PAGE, and Western blots were performed with the polyclonal antibodies raised against the *Arabidopsis* ceQORH or IE18 proteins.

known to contaminate envelope fractions (e.g. large Rubisco subunit). These proteins can be released from envelope preparations by opening/closing of the membrane vesicles after sonication (Fig. 3*A*, *lane 2* and *RbcL*). Since the ceQORH protein was not solubilized after sonication of membrane vesicles (Fig. 3*A*, *lane 2*), it is likely to physically interact with the inner envelope membrane.

To test the nature of the association between ceQORH and the inner envelope membrane, membranes were washed by application of salt and alkaline treatments (28). One of the standard method to determine the interaction of a protein with a membrane is to examine the effects of an alkaline Na_2CO_3 solution, which strips peripheral membrane proteins leaving intrinsic proteins associated with the lipid bilayer. Alkaline extractions using Na_2CO_3 (0.1 M, pH 11) induced a release in the soluble phase of a major part of the protein, and all the protein was solubilized using NaOH (0.1 M) treatment (Fig. 3*A*, *lanes 5, 6*). These observations indicate that ceQORH is not a transmembrane protein, bound to membrane through strong hydrophobic interactions (in contrast with IE18, which is not solubilized using Na_2CO_3 treatment; Fig. 3*A*, *lane 5*). Indeed, these observations are consistent with the predicted hydrophobicity of the ceQORH protein, since no membrane-spanning domain could be predicted from its hydropathy profile (Fig. 1*C*). Moreover, salt treatments of envelope vesicles with NaCl (0.5 M) or KI (0.5 M) also induced a partial release of the ceQORH protein in the soluble phase (Fig. 3*A*, *lanes 3, 4*). Because ceQORH binding to the membrane is sensitive to high salt concentrations, this suggests that electrostatic interactions

play an important role in the ceQORH ability to bind to membranes.

Moreover, complete release of the ceQORH protein was achieved with a relatively low (0.2%) concentration of Triton X-100 (Fig. 3B), whereas high concentrations are required to release intrinsic membrane proteins (e.g. release of the intrinsic protein IE18 requires a detergent concentration of 2% Triton X-100, see Ref. 24).

Taken together, these results indicate that ceQORH is a genuine inner envelope membrane protein. It might be peripherally associated with the membrane via electrostatic interactions, with an intrinsic component of the inner envelope membrane or with the polar surface of this membrane. Moreover, since the recombinant ceQORH protein was, at least in part, soluble when produced in *E. coli*, this electrostatic interaction might occur via a plant specific posttranslational modification or via interaction with a specific envelope membrane component.

The ceQORH Protein Is Not Processed during Its Chloroplast Targeting—Surprisingly, while localized in the inner membrane of the chloroplast envelope, the ceQORH protein does not contain a classical N-terminal additional sequence when compared with bacterial or animal homologues (see later). This observation raised the possibility that, conversely to all other known inner envelope proteins, the ceQORH could be targeted to the inner envelope of the chloroplast without classical cleavage of an N-terminal transit sequence. To test this hypothesis, new peptide sequence informations were required in the N-terminal part of the envelope ceQORH protein. Because the purest chloroplast envelope preparations are obtained from spinach and due to requirement of large amounts of envelope protein to perform this experiment, the natural protein was purified from spinach envelope preparations.

Immunopurification of the native spinach ceQORH protein was performed (see supplemental data at <http://www.jbc.org>) using the polyclonal antibody raised against the *Arabidopsis* ceQORH and mass spectrometry analyses allowed obtaining several new peptide sequences from this purified protein. These peptide sequences were used to clone the spinach cDNA (1179 nucleotides) coding the ceQORH protein (329 amino acids). Identity of the clone was confirmed, since the nine different peptide sequences were identical to the primary sequence deduced from the spinach cDNA (Fig. 4). The spinach ceQORH protein presents 75.1% identity and 88.8% similarity with the *Arabidopsis* ceQORH protein. From screening all available databases, no other *Arabidopsis* protein was found that presents more than 35% identity with the ceQORH from *Arabidopsis* or spinach. This strongly suggests that orthologous *Arabidopsis* and spinach genes encode the two plant proteins. A stop codon, localized in the 5'-UTR of the cDNA and in frame with the spinach ceQORH translation start codon (Fig. 4), was identified by two independent 5' RACE-PCR amplifications performed with two independent primers, thus suggesting that the 5'-part of the cDNA was complete (Fig. 4). Finally, peptide sequences were obtained that correspond to the N-terminal region of the primary sequence deduced from the ceQORH spinach cDNA (Fig. 4). Surprisingly, these data strongly suggested that, in contrast with all other known inner envelope proteins, the spinach ceQORH protein is not processed during its targeting to the inner membrane of the chloroplast envelope.

However, these data could also be explained in another way. There are many reports on alternative splicing in higher plants (29), and alternative splicing may occur that would induce synthesis of multiple forms of ceQORH mRNA (with or without an extended N-terminal region coding for a chloroplast transit peptide). In this case, several 5' RACE-PCR experiments show-

gtaccattaccatctgaccgcaaatg	caaac	ctggg	taagata	ataca	22
gtaccattaccatctgaccgcaaatg	ctggg	ctgtaag	ctaatg	catgctg	82
				M A A K L M H A I Q Y S	12
ggctatggtggtggaactgatgctt	aaagc	atggtgga	agttgctg	tctctgatccaaag	142
G Y G G G T D A L K H V E V A V P D P R					32
tctgatgatttattgcttaaaattg	aggctg	caacctt	gaaccaat	tgattggaagatt	202
S D E L L L K I E A A T L N P I D W K I					52
cagaagggtgactctgcccctct	tacc	cccgca	agttcc	ctactatgac	262
Q K G V L R P L L P R R K F P T I A P G T D					72
gttgctggggaggtagctcaggc	tgatg	ctgctg	taaatg	tttaaacctggtgacaa	322
V A G E V V Q A G S A V N R F K T G D K					92
gtcgtggccgtgcttagtcatg	ctactg	ggggg	gcactag	ctgaatg	382
V V A V L S H A T G G A L A E Y A V A K					112
gagaacctgacagttgctagacc	cacaga	agtatc	agcagc	agaaggtgctg	442
E N L T V A R P P E V S A A E G A A L P					132
gttgctgcccctcagggctcac	caagct	caccag	cttgc	caacatcaagctg	502
V A A L T A H Q A L T Q F A N I K L D G					152
agtggtagaaggaacatattg	atgatc	acggct	gcacag	gggtggtggccactatg	562
S G E R K N I L I T A A S G G V G H Y A					172
gtccagctggcaagctcgggaa	cacgc	atgta	acagca	caactgtag	622
V Q L A K L G N T H V T A T C G A R N L					192
gatttctgtaaaggttgggtg	cggatg	agggtt	cttgact	acaacaac	682
D F V K G L G A D E V L D Y K T P E G A					212
tccttgacaagcccgtcagga	aaatg	atgact	agtagt	ccacgggtg	752
S L T S P S G R K Y D Y V V H G A S G I					232
ccttgctccactttagc	ccaatt	gagtag	aagc	aggtaatg	802
P W S T F E P N L S E A G K V I D L T F					252
ggcccaactgcaatgatgac	attgct	ggaaa	agctaac	attctccaaa	862
G P T A M M T F A W K K L T F S K Q L					272
gtgcctctgcttttgatacca	agaagat	cccac	cttga	aatatg	922
V P L L L I P K I P N F E Y V V N L V K					292
gaaaagaagcttaaacag	ctatag	actct	taaac	ctcccttg	982
E K K L K T V I D S K H P L S K G E D A					312
tgagtaggataatgggtg	ctgctg	acagga	agattata	atcagc	1042
W S R I M G G H A T G K I I E P *					330
aatattgatgcagaccctg	atata	attgct	gaa	ggttaca	1102
cttgagttatacttcttagt	gttaaac	ctcaag	tatttca	ataatg	1162
gtttcccca	aaaaaa				1179

FIG. 4. Nucleotide and deduced amino acid sequence of spinach ceQORH protein and positions of peptide sequences obtained from the purified spinach ceQORH protein. The translation start codon is indicated in bold character. The stop codon, localized in the 5'-UTR and in-frame with the translation start codon, is underlined. The shaded sequences correspond to the peptides obtained by mass spectrometry sequencing of the purified natural spinach ceQORH protein.

ing the presence of only one transcript may not be totally conclusive.

No Additional N-terminal Is Required for Plastid Localization of ceQORH in Vivo—Further evidence supporting the fact that no additional N-terminal sequence is required for the localization of ceQORH in chloroplast was obtained from *in vivo* expression of the ceQORH in plant cells. Transient expressions of GFP fusions were performed into both *Arabidopsis* and BY2 tobacco cells. As expected, the expressed GFP protein (negative control) was distributed uniformly in the cytosol and in the nucleus (see Fig. 6, GFP), whereas the plastid control (transit sequence of RbcS fused to GFP) was targeted to the chloroplast (Fig. 6, TP-GFP). Transient expressions of the complete ceQORH protein (Fig. 5) were then performed. As shown in Fig. 6, plastid localization was obtained with the ceQORH protein when GFP was fused to its C-terminus (Fig. 6, ceQORH). In conclusion, this result (together with previous experiments) indicates that the ceQORH does not require an additional N-terminal (a cleavable chloroplast transit peptide) for plastid localization.

Neither N-terminal nor C-terminal Parts of ceQORH Seem to Be Essential for Plastid Localization—Sequence comparison between the ceQORH proteins and other proteins from the same family, revealed that their 50 first residues in the N-terminal region are highly conserved from bacterial to plant and animal proteins (Fig. 5). This observation strongly suggests that the N-terminal region of the plant ceQORH proteins may not be directly required for the specific chloroplastic targeting but would have been most probably conserved through evolution due to selective pressure on a catalytic domain of the protein. For further examination of the domain required for plastid localization of the ceQORH protein, we generated several truncated versions of the ceQORH protein and transient expressions of corresponding GFP fusions were tested into Ara-

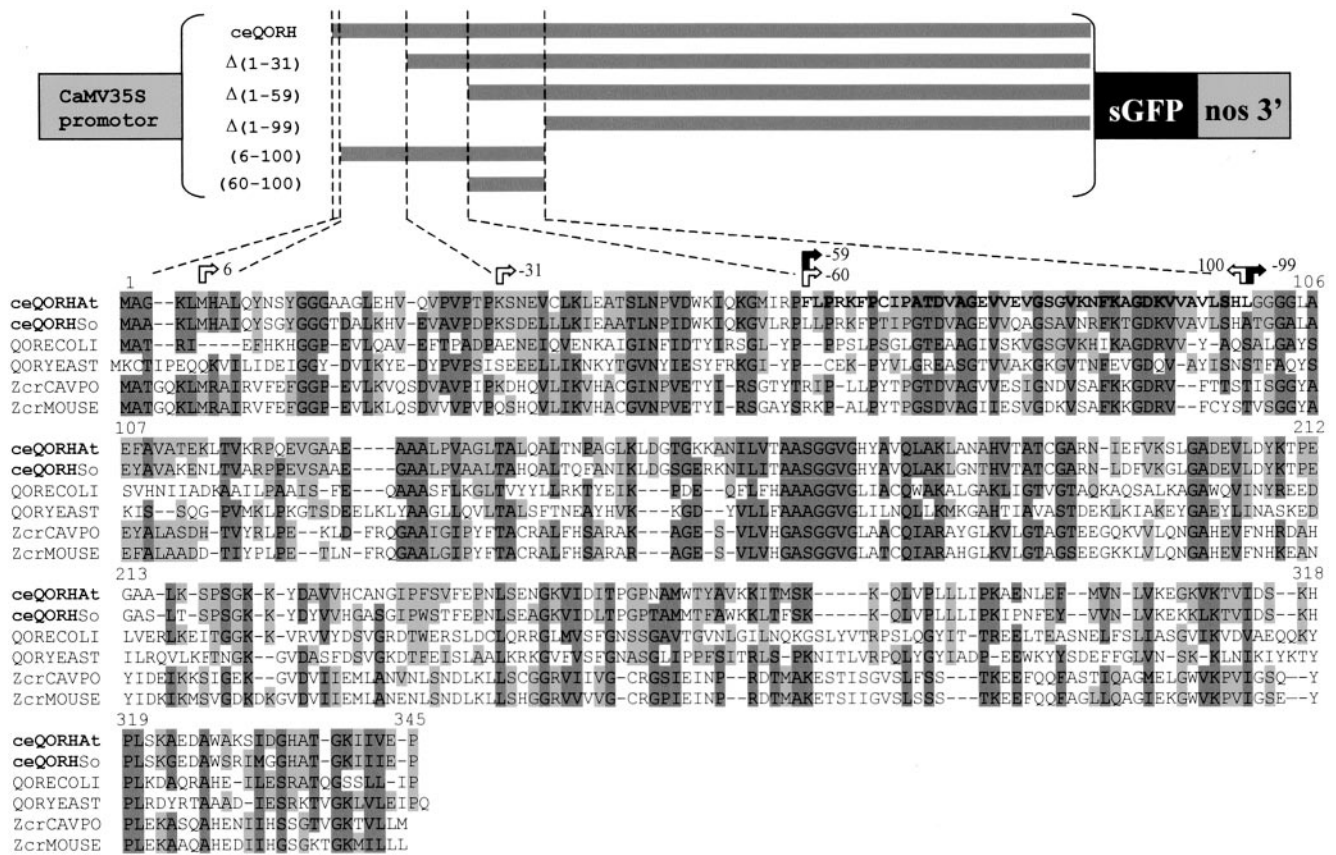


FIG. 5. Upper part, structure of the GFP-tagged and truncated forms of the *Arabidopsis* ceQORH protein used to localize the domain required for plastid localization. ceQORH, GFP fusion containing the complete ceQORH protein. $\Delta(1-31)$, GFP fusion containing the ceQORH protein lacking the first 31 amino acids. $\Delta(1-59)$, GFP fusion containing the ceQORH protein lacking the first 59 amino acids. $\Delta(1-99)$, GFP fusion containing the ceQORH protein lacking the first 99 amino acids. (6-100), GFP fusion containing the first six to 100 amino acids of the ceQORH protein. (60-100), GFP fusion containing the first 60-100 amino acids from the *Arabidopsis* ceQORH protein. Lower part, alignment of *Arabidopsis* and spinach ceQORH proteins with bacterial, yeast, and animal closest homologues. Residues conserved in the six proteins are dark gray colored. Similar residues (according to the following groupings: ASPTG, ILMV, KRH, NQ, DE, YWF and C) conserved in the primary sequence of ceQORH and in any other primary sequence are light gray colored. ceQORHSo (this work), ceQORHAT (Q9SV68), QORECOLI (P28304), QORYEAST (P38230), ZcrCAVPO (P11415), ZcrMOUSE (P47199).

biodopsis and BY2 tobacco cells.

Surprisingly, while lacking the N-terminal 31 or 59 amino acids (Fig. 5), truncated forms of the ceQORH protein could still drive the GFP to the plastids (Fig. 6, $\Delta(1-59)$ ceQORH). This demonstrated that the N-terminal part of ceQORH (59 first residues) is not essential for plastid localization. In contrast, a truncated form of the ceQORH protein lacking the first 99 amino acids and fused to the GFP (Fig. 5) was unable to drive the GFP to the plastids (Fig. 6, $\Delta(1-99)$ ceQORH). This suggests that this N-terminal part of ceQORH (first 100 residues) contains essential information to drive ceQORH to the plastids. To test whether this N-terminal part of ceQORH is sufficient to drive ceQORH to the plastid, the expression of the first six to 100 amino acids of the ceQORH protein fused to the GFP protein was tested (Fig. 5). These first six to 100 amino acids could drive GFP to plastids (Fig. 6 (6-100)ceQORH), thus suggesting that (i) this N-terminal sequence (100 residues) contains essential and sufficient information to drive ceQORH to the plastid and that (ii) the C-terminal sequence (229 residues) is not required for plastid localization. As the N-terminal sequence (100 residues) is sufficient to drive ceQORH to the plastid and since the first 60 residues are not required for plastid localization (Fig. 6, $\Delta(1-59)$ ceQORH), these strongly suggest that an internal region of 41 amino acids in the ceQORH protein (residues 59 to 100) is the only essential region required for plastid localization. Expression of this internal ceQORH domain (residues 59-100) fused to the GFP

protein was thus performed (Fig. 5). However, while essential for plastid localization of the protein, this domain appeared not to be sufficient to drive GFP to the plastids (Fig. 6, (60-100)ceQORH) thus suggesting that association of this internal ceQORH region with a downstream or upstream sequence is required for plastid localization.

DISCUSSION

This study describes the identification of a new protein localized in the inner membrane of the plastid envelope. Purification and microsequencing of the natural protein and cloning of the corresponding full-length cDNA suggested that this protein is not subjected to processing of an N-terminal transit sequence during its targeting to the chloroplast. Furthermore, *in vivo* expression of the protein in plant cells indicates that the ceQORH does not require an additional N-terminal (a cleavable chloroplast transit peptide) for plastid localization. To the best of our knowledge, this demonstrates that ceQORH is the first described protein to be targeted in the inner membrane of the plastid envelope without classical N-terminal cleavage of a plastid transit sequence. Finally, GFP fusion experiments suggest that neither the N- nor the C-terminal of this protein are essential for plastid localization. It is proposed that an internal region of the ceQORH protein (residues 59-100 of ceQORH protein) is essential but not sufficient for plastid localization since N- or C-terminal neighboring regions appear to be required.

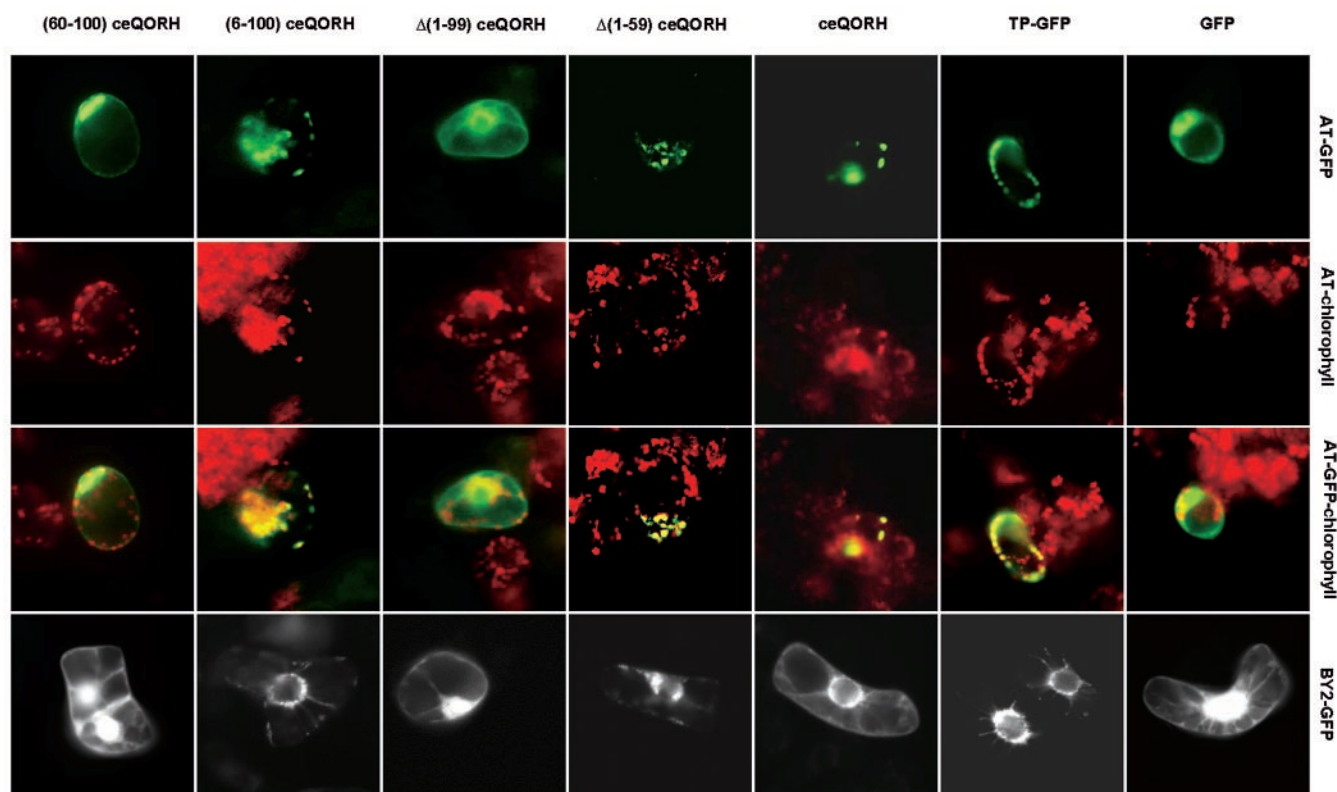


FIG. 6. Transient expression of GFP-tagged and truncated forms of the *Arabidopsis* ceQORH protein in *Arabidopsis* and BY2 tobacco cells. As subcellular localization controls, we used the 35 Ω -sGFP(S65T) plasmid (*GFP*) and the 35 Ω -TP-sGFP(S65T) plasmid (*TP-GFP*) containing the transit peptide (*TP*) sequence from RbcS fused to GFP. Detailed information concerning the structure of GFP-tagged and -truncated forms of the ceQORH protein is described in Fig. 5. As expected, colocalization of GFP and chlorophyll fluorescences was observed for both the plastidial control TP-GFP and the GFP-tagged ceQORH. Further colocalization of GFP and chlorophyll fluorescences observed with $\Delta(1-31)$ ceQORH (not shown), $\Delta(1-59)$ ceQORH and (6–100)ceQORH truncated forms of ceQORH suggest that the 60–100 region is the only essential domain for plastid localization of ceQORH.

It Is Generally Accepted That Most Proteins, which Are Targeted to the Outer Membrane of the Chloroplast Envelope, Do Not Contain Any Cleavable Targeting Sequence—Much progress has been achieved during the past few years on the protein import into chloroplast, and the basic framework for the protein import mechanism into this organelle is now in place (for recent reviews, see Refs. 1–4). Examples have been found for plastid targeting based on both N-terminal (SCE70; see Ref. 30) and C-terminal uncleaved targeting domains (OEP34; see Ref. 31) (Fig. 7). It seems that these proteins are directly incorporated after their cytosolic synthesis in the lipid bilayer by unknown interactions with the outer membrane lipids. To date, one exception has been identified, namely OEP75 (or Toc75), which requires a bipartite N-terminal cleavable transit peptide for productive targeting to the outer membrane of the chloroplast envelope (6, 32) (Fig. 7).

All Other Plastid Proteins Contain N-terminal Cleavable Transit Sequences—The only known envelope intermembrane space protein that has been cloned is Tic22 (Fig. 7). This protein is synthesized as a preprotein with a 50-residue N-terminal and cleavable presequence, thus suggesting that intermembrane proteins already require this type of targeting peptides (33, 34). Targeting of proteins to inner chloroplast envelope membrane, stroma, and thylakoid membrane or thylakoid lumen is strictly dependent on the presence of a cleavable transit sequence in the precursor N-terminal region (Fig. 7). In all cases, this transit sequence is cleaved by specific proteases after targeting, generally contains 40–100 residues, and is specifically recognized by the import apparatus.

Examples of exceptionally short chloroplast transit peptides have been described. A 13-amino acid transit peptide was de-

tected in the DHQase-SORase targeted to the stroma of tomato plastids (35), and a 16-amino acid transit peptide was detected in the PEND protein targeted to inner membrane of the chloroplast envelope (36). A stroma protein, BADH, was shown to have such a short transit peptide (8 residues or fewer) that the authors questioned whether it really had one (37). Experiments with *in vitro* translation of BADH mRNA originally showed that the primary translation product of BADH is only 1.2 kDa larger than the mature peptide (38). Since the mature peptide was not posttranslationally modified, the authors proposed that this result predicted a transit peptide of <10 amino acid residues (39). An economical interpretation of these data was that the transit peptide of BADH comprises the short fragment Met-Ala-Phe-Pro-Ile-Pro-Ala and that the N terminus of the processed stromal BADH monomer is a blocked arginine residue (39). Further experiments are required to validate the lack of a cleavable transit peptide sequence in plant BADH (37) or, if the protein is not processed, to identify the domain required for plastid localization of BADH.

Finally, an envelope component of the chloroplast protein import apparatus (Com44/Cim44) was more recently suspected to be targeted to both the outer and inner envelope membranes without further cleavage of an N-terminal transit peptide (40). However, this protein was finally demonstrated to be only targeted to the inner envelope and synthesized, in the cytosol, as a precursor with an N-terminal cleavable chloroplast targeting signal (41).

Examples of Mitochondria Proteins Lacking N-terminal Cleavable Transit Sequences Were Described—As for plastid proteins, most of the mitochondrial proteins are synthesized as precursors, contain a cleavable signal sequence in their N ter-

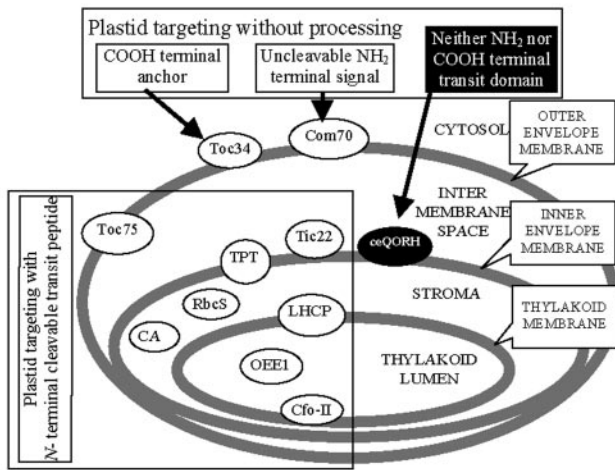


FIG. 7. Transit sequence requirements for protein targeting to various subplastidial compartments. Classical examples and original systems (referenced) are shown. An N-terminal and cleavable transit peptide is required for targeting to the intermembrane space of the chloroplast envelope (*Tic22*; see Refs. 33, 34), the inner membrane of the envelope (*TPT*; Triose-PiP_i translocator), the stroma (*RbcS*, small subunit of Rubisco; *CA*, carbonic anhydrase), the thylakoid membrane (*LHCP*, light harvesting complex; *Cfo-II*, ATPase subunit), and the thylakoid lumen (*OEEL*, oxygen evolving element 1). It is generally accepted that outer envelope proteins do not have any cleavable transit sequence. Examples have been found for plastid targeting based on both N-terminal (*Com70*; see Ref. 30) and C-terminal uncleaved targeting domains (*Toc34*; see Ref. 31). One exception has been identified that already requires a bipartite N-terminal cleavable transit peptide for productive targeting to the outer membrane of the chloroplast envelope (*Toc75*; see Refs. 6, 32). The ceQORH protein is the only known protein to be associated to the inner membrane of the plastid envelope while devoid of a cleavable N-terminal targeting peptide.

minus, and are posttranslationally targeted to the mitochondria (for review, see Ref. 42). However, some exceptions were described in the literature. It was demonstrated that a truncated precursor of the yeast mitochondrial inner membrane protein ADP/ATP carrier lacking the 103 amino acids from the N terminus (about one-third of the protein) present the same import characteristics of the authentic precursor. The signal sequence of this protein is localized in the C-terminal two-thirds of the protein (43). More recently, *in vitro* studies have demonstrated that another mitochondrial inner membrane protein, BCS1 (44), presents an internal signal sequence localized in its C-terminus part (45). This internal targeting signal contains a mitochondrial processing peptidase site, which is not cleaved when present in the context of an internal targeting sequence. Interestingly, the BCS1 import requires an obligatory sequence at the N-terminal side of the targeting signal and a facultative sequence in the C-terminal side of the targeting signal. As suggested for the plastid ceQORH protein, the internal targeting sequence of BCS1 is essential but not sufficient for directing BCS1 to the correct mitochondrial sublocation. Moreover, authors proposed that the translocation of BCS1 across the inner membrane uses the classical components of the MIM machinery (translocation Machinery of the Inner Membrane) and two new putative components of this import machinery (45).

Origin of the ceQORH Transit Domain—As described previously, the vast majority of chloroplastic proteins are targeted to the chloroplast as a precursor protein whose transport is dependent on an N-terminal and cleavable extension, known as transit peptide. These transit peptides are highly divergent in length, composition, and organization and were acquired by some unknown evolutionary process during endosymbiosis. An emerging notion indicates that during gene transfer to the

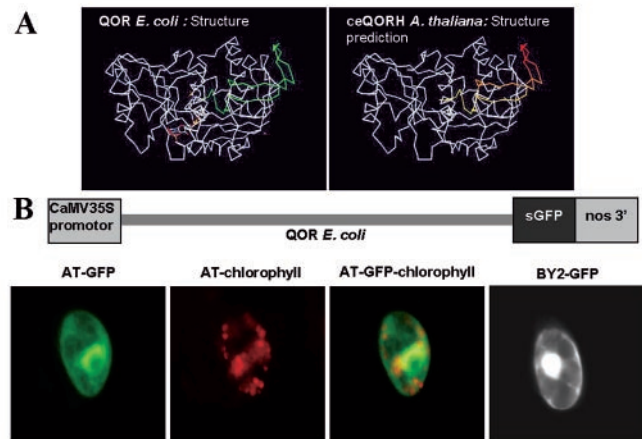


FIG. 8. QOR *E. coli* and *Arabidopsis* ceQORH structures comparison. A, structure of the QOR *E. coli* monomer (47) and predicted structure of the *Arabidopsis* ceQORH protein. Structures were obtained using the Swiss PDV viewer program (48). The 40 residues, which are essential for plastid localization of the ceQORH protein and the homologous domain in the *E. coli* QOR protein, are colored. B, transient expression in *Arabidopsis* and tobacco cells of the bacterial QOR protein fused to GFP.

nucleus, one or more sequences present within the cyanobacteria genome may have provided the source of coding information for modern transit peptides. These transit peptides should emerge by shuffling of existing cyanobacterial exons, which through selective pressure could yield a transit peptide capable of targeting and translocating the nuclear-encoded cyanobacteria proteins back into the plastid (for a review, see Ref. 46).

As suggested in this study, the ceQORH protein may be targeted to the internal membrane of the chloroplast envelope via an internal domain of 40 residues, which is essential but not sufficient for correct plastid localization. Surprisingly, sequence comparison between the plant ceQORH proteins and quinone oxidoreductases present in other organisms revealed that this internal domain is highly conserved from prokaryotic to eukaryotic proteins (Fig. 5). This information suggests that this domain was probably conserved through evolution due to selective pressure on a catalytic domain, essential for the quinone oxidoreductase activity. This observation suggests that the ancestral primary sequence of this protein may have evolved to provide sufficient information for correct targeting into the inner envelope membrane without addition of an N-terminal transit sequence. Interestingly, the 60–100 domain appears to be highly accessible in the structure of the *E. coli* QOR protein (47), and equivalent accessibility is suggested for the plant protein based on structure prediction (Fig. 8A). However, primary sequence evolution was required for correct targeting of the protein since the *E. coli* protein fused to GFP is not plastid-localized when expressed in *Arabidopsis* or BY2 tobacco cells (Fig. 8B). The observations presented in this article suggest that the ceQORH protein possess a new type of chloroplastic targeting signal, specific for the inner membrane of the chloroplast envelope. For this reason, the mechanism involved in the import of the ceQORH protein may require specific elements. The understanding of the ceQORH import pathway could allow the identification of new proteins, either additional to the classical import system or constitutive of an original system, to integrate the chloroplast.

Finally, further experiments are required to understand the role of the ceQORH protein in the inner membrane of the chloroplast envelope. Jager-Vottero *et al.* (25) presented spectroscopic evidence for the presence of electron carriers (redox chains) in chloroplast envelope membranes. However, to date,

no protein components of these redox chains were identified. During this earlier work, an NADPH quinone oxidoreductase activity was detected in the chloroplast envelope presumably associated to these redox chains (25). This activity may result from the presence of the ceQORH protein since this protein is structurally related to bacterial, fungal, and animal proteins of known quinone oxidoreductase function. As previously discussed (25), the physiological significance of these redox chains may be related to the presence of desaturase activities associated to the envelope membranes and/or to the regulation of the stromal pH during photosynthesis.

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