FEBS 29889 FEBS Letters 579 (2005) 4991–4996

Localization of a putative ClC chloride channel in spinach chloroplasts

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Received 19 July 2005; accepted 2 August 2005

Available online 15 August 2005

Edited by Julian Schroeder

Abstract **Seven genes seem to encode for putative ClC chloride channels (AtClC-a to AtClC-g) in *Arabidopsis thaliana*. Their function and localization is still largely unknown. AtClC-f shares considerable sequence similarity with putative ClC channel proteins from *Synechocystis*, considered to represent the precursor of chloroplasts. We show by biochemical and mass spectrometry analysis that ClC-f is located in the outer envelope membrane of spinach chloroplasts. Consistent with the plastidial localization of ClC-f, *p*-chlorophenoxy-acetic acid (CPA) reduces photosynthetic activity and the protein is expressed in etioplasts and chloroplasts but not in root tissue. These findings may represent a step toward the molecular identification of ion channel activities in chloroplast membranes.**

Keywords: Chloroplast; Anion channel; ClC family; Envelope membranes; Photosynthesis

1. Introduction

ClC channels are widely expressed in varying organisms, including bacteria, yeast, plants and mammals. In mammals, ClCs function as chloride selective channels. Nine ClC family members have been identified, five of them having intracellular location.

An important step forward in our understanding of ClC channels is resolution of the X-ray structure of StClC from *Salmonella typhimurium* and EcClC from *Escherichia coli* [1]. In *E. coli*, the product of gene *eriC* has been shown to function as a proton-chloride exchange transporter [2].

The genes encoding for the anion channels, recorded by electrophysiology in plant cells, are mostly still undefined. Among the candidates are those of the ClC family. The first ClC channel has been cloned from *Nicotiana tabaccum* [3]. However, a clear-cut demonstration that plant ClC proteins may indeed function as chloride selective channels is still lacking [4]. AtClC-c and AtClC-d from *Arabidopsis* [5] functionally complemented *Saccharomyces cerevisiae* mutant lacking the single ClC protein Gef1, which is associated with chloride channel activity [6].

The location of ClC proteins in *Arabidopsis* is not known. All seven AtClC proteins are encoded by genes in the nuclear genome and genes for putative ion channels are missing from plastidial genome. Bioinformatic analysis, based on nature of N-terminal aminoacid sequence (see Aramemnon http://aramemnon.botanik.uni-koeln.de [7]), does not predict a clear location for AtClC-f. However, many organelle proteins have an internal targeting peptide.

The present paper provides biochemical evidence that ClC-f protein is present in the outer envelope membrane fraction of spinach chloroplasts and this location is not due to contamination by other membranes. This work thus defines for the first time the intracellular location of ClC-f in plants, and also represents a step forward in the molecular identification of non-abundant chloroplast channels.

2. Materials and methods

2.1. Protein concentration

Protein concentrations were determined by BCA kit.

2.2. SDS–PAGE and Western blotting

Proteins were separated in 7.5% SDS–PAGE or 7.5–15% acrylamide gradient gel (with 6 M urea). Percoll-purified chloroplasts from *Arabidopsis thaliana* or spinach leaves were prepared according to [8]. Anti-ClC3- antibody (Sigma) was used at a 1:300 dilution for 3 h. Differing lots always recognized the 82 kDa band in spinach, whereas cross-reaction with other bands varied among lots. Anti-OEP21 and anti-ferredoxin NADP$^+$ oxidoreductase, anti-P45, anti-H^+-ATP-ase of the plasmamembrane, anti-BiP, anti-calreticulin and anti-AtClC-e antibodies were kind gifts of Profs. J. Soll, N. Rolland, I. DeMichelis, A. Vitale, B. Baldan and H. Barbier-Brygoo, and were used at 1:500, 1:1000, 1:5000, 1:5000, 1:10 000, 1:2000 and 1:250 dilutions, respectively. Horse-radish peroxidase coupled anti-rabbit IgG was used as a secondary antibody and blots were developed using the ECL system.

2.3. Purification of outer, inner and thylakoid membrane vesicles

Vesicles were prepared following the procedures in [9]. Briefly, Percoll-purified chloroplasts were incubated in 0.7 M sucrose, broken by 50 strokes and diluted to 0.3 M sucrose. Membranes were separated on a discontinuos sucrose gradient at 120 000 g for 3 h. PMSF was added throughout the procedure and all steps were performed at 4 °C.

2.4. Proteolysis experiments

Leaves were homogenized with 340 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA, 2 mM HEPES, pH 7.8, filtered twice, and centrifuged for 10 s at 3500 × *g* [10]. The same medium was used to obtain total extracts. Pellets were resuspended in 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES, pH 7.8, and contained intact chloroplasts. Chlorophyll concentration was measured, 0.5, 2.5 and 5 μg proteinase K per 10 μg chlorophyll was added and mixture was incubated for 20 min on ice. Following addition of PMSF

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.005

(5 mM) and washing twice, chloroplasts were loaded on 40% Percoll and centrifuged for 10 min at 6000 × *g*. Intact chloroplasts were recovered in pellet, washed twice and loaded on SDS–PAGE.

2.5. Bidimensional electrophoresis

IPGphor (Pharmacia) was used for isoelectric focusing with Immobilin strips (linear pH gradient 4–7 or 3–10). 2% ASB-14 was added to the rehydration buffer (2% Chaps, 9 M urea, 0.5% IPG buffer, and 18 mM DTT). 2D gels, obtained in parallel, were either transblotted to PVDF membrane and decorated with anti-ClC antibodies, or stained with Colloidal Coomassie solution. Spots were excised and analyzed by mass spectrometry (MS). All experiments were repeated at least three times and yielded similar results.

2.6. Mass spectrometry

Protein digestion was performed according to Shevchenko et al. [11], with minor modifications. The dried tryptic digest samples were reconstituted in 10 μl of 0.5% TFA in water and were purified with a ZipTip$_{C18}$ (Millipore). For electrospray MS analysis, the peptides were eluted in 50% acetonitrile containing 0.2% formic acid. Data were collected on a Micromass Q-Tof Micro mass spectrometer (Manchester, UK) (capillary voltage: 3000–3200 V; cone voltage: 45 V; scan time: 1 s; interscan: 0.1 s). Spectra were analyzed using Micromass MassLynx. The MASCOT program (www.matrix-science.com) was used to search for all MS/MS spectra against the Swiss-Prot database. The parameters were set to allow parent ion mass tolerance to be 1 Da and fragment mass tolerance to be 0.8 Da, and up to two missed trypsin cuts were allowed.

2.7. Oxygen evolution measurements

Leaves were homogenized with 340 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA, 2 mM HEPES, pH 7.8, filtered twice, and centrifuged for 10 s at 3500 × *g* [10]. Pellets were resuspended in the assay medium. Purity and intactness was checked by inverted microscope. Experiments were performed using a Clark electrode (Hansatech) as described in [12]. Briefly, the assay medium for intact chloroplasts contained 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES–KOH (pH 7.6), 0.2% bovine serum albumin and 10 mM D,L-glyceraldehyde (included to inhibit CO_2 fixation). The intactness of chloroplasts was assayed by the Hill reaction [12]. Electron acceptor $K_3Fe(CN)_6$ (2.7 mM) was added. Thylakoids were obtained by osmotic shock of intact chloroplasts (corresponding to 78 μg Chlorophyll, diluted in 620 μl sterile water) and then assayed in the same final medium as above. Chorophyll concentration was equal to 60 μg/ml in all experiments and oxygen evolution was measured at 25 °C in the dark, followed by application of light with an intensity of 2000 μE m^{-2} s^{-1}. Oxygen evolution in control condition in intact chloroplasts was 15.11 ± 0.99 μmol O_2/(mg Chl h) ($n = 20$) while in osmotically shocked chloroplasts (thylakoids) it was 49.6 ± 2.8 μmol O_2/(mg Chl h) ($n = 20$). When applied, chloroplasts or thylakoids were incubated with CPA for 10 min in the dark on the ice prior to measurement. Controls were performed to exclude any effect of the solvent. Oxygen evolution was determined by using the FIP program and expressed as μmol O_2/(mg Chl h). The absolute values measured under control conditions varied slightly among preparations depending on spinach sample, and oxygen evolution values in the presence of the inhibitor were normalized accordingly. Mean ± S.D. values are reported in the graphic. Independent unpaired *t*-tests were performed.

2.8. Chlorophyll fluorescence

Analyses were performed with a PAM101 fluorometer (Waltz), 2000 μE m^{-2} s^{-1} intensity light was applied.

3. Results

Proteins with transport function of chloroplast membranes may derive from the endosymbiontic cyanobacterium, as indeed is the case for some constituents of the protein import machinery [13]. The results shown in Table 1 indicate the cyanobacterial origin of AtClC-f and AtClC-e (see also Aramemnon). If these two channel proteins had a predicted chloroplast transit peptide, they would be good candidates as plastidial channels. TargetP algorithm (www.cbs.dtu.dk/services) predicts plastidial location with a probability of 0.413 for AtClC-e and 0.19 for AtClC-f. However, some heterospe-

Table 1
AtClC-f, AtClC-e and cyanobacterial putative ClC channels share sequence similarity

	AtClC-e (710) (At4g35440)	AtClC-f (781) (At1g55620)
Synechocystis sp. *PCC 6803*	2×10^{-12}	3×10^{-15}
sll1864 (899) (GI: 16331172)	20% (579)	21% (624)
Synechocystis sp. *PCC 6803*	2×10^{-6}	2×10^{-10}
sll0855 (451) (GI: 16330386)	19% (316)	19% (433)
Synechococcus sp. *PCC 7942*	2×10^{-15}	2×10^{-16}
EriC (463) (GI: 22002515)	21% (401)	21% (422)
Synechocystis sp. *WH 8501*	10^{-11}	10^{-14}
EriC (822) (GI: 46119460)	20% (580)	22% (450)
Thermococcus elongatus sp. (628)	10^{-58}	6×10^{-60}
(GI: 22293866)	31% (583)	30% (616)
Nostoc sp. *PCC 7120*	10^{-19}	9×10^{-21}
alr4891 (871) (GI: 17134028)	22% (619)	22% (513)
Nostoc sp. *PCC 7120*	10^{-63}	10^{-57}
alr2079 (586) (GI: 17131170)	30% (590)	29% (619)
Gloebacter violaceus PCC 7421	3×10^{-20}	10^{-24}
(858) (GI: 37523751)	22% (566)	22% (627)

Aminoacid sequences of ClC putative chloride channels (GI: Accession Nos. in NCBI data bank; numbers beside names: protein length) were compared pairwise by BLASTP algorithm (www.ncbi.nih.gov). *E* values: number of hits expected to be found by chance. % of identity is shown and length of aligned sequence reported in brackets. Similarity is considered to be of higher degree when *E* value is lower and percentage of identity is higher over a longer region.

Table 2
Rice and tomato homologs of AtClC-e and AtClC-f may be located in chloroplasts

	AtClC-e	AtClC-f	TargetP	Predicted MW (kDa)
Oryza sativa (718) (GI: 34910358)	10^{-154} 47% (642)	2×10^{-91} 35% (614)	0.922	76.36
Oryza sativa (750) (GI: 50947293)	10^{-84} 33% (615)	0 59% (669)	0.975	80.23
Lycopersicon esculentum (750) (GI: 13620228)	10^{-101} 37% (622)	0 58% (780)	0.874	79.98

Sequence similarity was evaluated as described in Table 1. Predicted MW was calculated by Compute MW/pI tool (www.us.expasy.org).

cific homologs of both AtClC-e and AtClC-f display predicted chloroplast location with high probability (Table 2).

Western blotting was performed on isolated *Arabidopsis* chloroplasts using an antibody against rat ClC-3 (aa 592–661) (GI: 1705905). This antibody may recognize a priori both AtClC-e and AtClC-f, given the presence of highly conserved aminoacid stretches in the epitope region. The similarity of this region with other AtClC proteins, except for AtClC-d, is less pronounced. Fig. 1A shows that anti-ClC3 antibody did recognize in *Arabidopsis* chloroplasts a single band of 75.0 kDa apparent molecular weight (MW) (note: the predicted MW of AtClC-e is 75.5 kDa). In order to define the exact location of ClC in chloroplast membranes, both outer and inner envelopes and thylakoid membranes had to be purified. A method for such purification in *Arabidopsis* has not been reported up to now, therefore spinach was used. The antibody in isolated Percoll-purified chloroplasts from spinach clearly recognized a band of 82 kDa apparent MW (close to the predicted 83.5 kDa MW of AtClC-f) while the 75 kDa band was not revealed (Fig. 1A and B). However, when assaying purified outer envelope vesciles (OE), the 75 kDa band, which was recognized also by a specific anti-AtClC-e antibody (not shown), became evident (Fig. 1B), suggesting either the presence of small amount of ClC-e in spinach chloroplasts, or a decreased affinity of the antibody for spinach ClC-e with respect to AtClC-e. Concerning the 82 kDa band, presumed to correspond to ClC-f, a 2.9-fold enrichment in OE with respect to chloroplasts was observed (based on densitometry, $n = 5$) (Fig. 1B). The OE location of the 82 kDa band was further examined by proteolytic treatment of isolated, intact chloroplasts with different quantities of Proteinase K. The intensity of the 82 kDa band greatly diminished by increasing the quantity of the protease (Fig. 1C) – as expected, if the protein was accessible from the cytosolic side of OE. OEP21, another OE protein is also accessible to proteinase K, while P45 of the inner membrane as well as the ferredoxin NAD(P)$^+$ oxidoreductase (FNR), which interacts on the stromal side with the IE protein Tic62, are not digested by proteinase K (Fig. 1C). This latter data, as well as the fact that control and protease treated isolated chloroplasts displayed basically identical oxygen evolution in the presence of ferricyanide (Hill reaction) (12.12 ± 0.44 and 12.81 ± 0.78 μmol O_2/(mg Chl h), respectively; $P = 0.46$, $n = 5$), indicate the intactness of the inner membrane.

The purity of our membrane preparations was tested using specific antibodies against proteins of OE, IE and thylakoid membrane (THY). The outer membrane protein OEP21 [14], a 45 kDa inner envelope protein [15] and the photosystem II

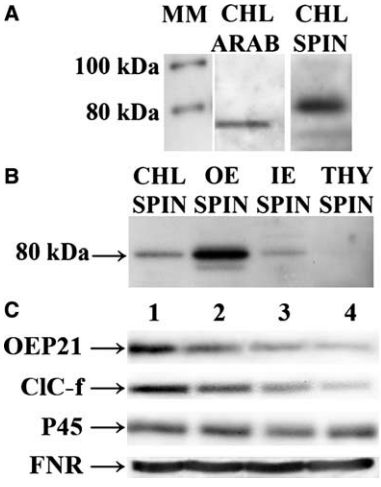

Fig. 1. Location of ClC-f in outer membrane of spinach chloroplasts. (A) Chloroplasts from *A. thaliana*, *S. oleracea* (50 μg protein each) assayed by anti-ClC3 antibody. (B) *S. oleracea* chloroplasts, OE, IE and THY (30 μg each). Lower band in OE: 75 kDa protein. (C) Isolated chloroplasts left untreated (lane 1) or treated with Proteinase K (lanes 2, 3,and 4 correspond to treatment with 0.5, 2.5 and 5 μg proteinase K per 10 μg chlorophyll, respectively); developed with antibodies, as indicated.

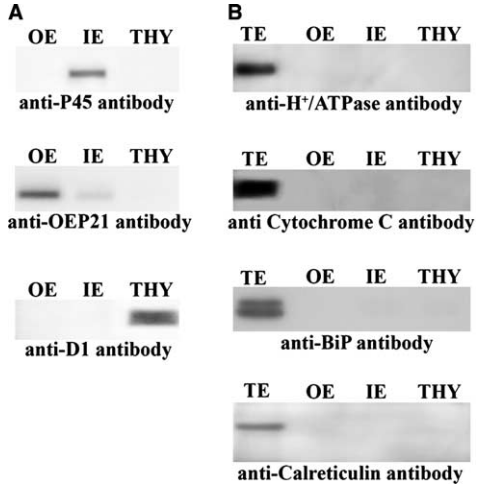

Fig. 2. Presence of ClC-f and ClC-e in outer membrane is not due to contamination. (A) OE, IE and THY (5 μg each) from same preparation; developed with antibodies, as indicated. (B) 20 μg of total extract (TE), OE, IE and THY assayed as indicated. Apparent MWs of 98, 13, 78 and 52 kDa for PM H^+ATP-ase, cytochrome *c*, BiP and calreticulin fit those reported in the literature.

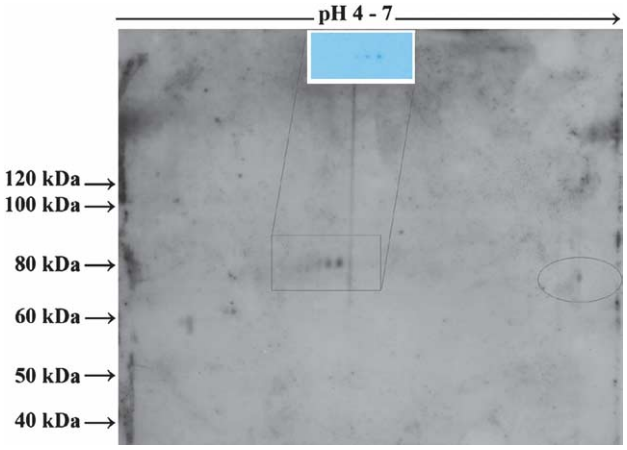

Fig. 3. Bidimensional electrophoresis and Western blot of outer membrane vesicles. 2D PAGE was performed in parallel with two aliquots of OE (80 μg each). One gel was transblotted and assayed with anti-ClC3 and one was stained with Colloidal Coomassie. Rectangle: 81 kDa; circle: 76 kDa protein. Inset: 81 kDa spots (in the same position of spots recognized by anti-ClC3 on Western blot).

component D1 protein in the thylakoid were used as marker proteins. OE and THY were pure membranes, whereas IE was slightly contaminated by OE (Fig. 2A). Densitometric analysis indicated that the 82 kDa band in IE (Fig. 1B) was due mainly to contamination by OE and/or to the presence of contact sites.

The vesicle preparations were assayed also using antibodies against marker proteins of various membraneous compartments. Antibodies against plasmamembrane H^+-ATP-ase [16], BiP of ER [17], mitochondrial cytochrome *c* and calreticulin were used (Fig. 2B). The latter antibody, when used for immunogold labeling, located on both ER and Golgi membranes [18]. These data indicate that the presence of ClC-f in the OE is not due to extra-plastidial contamination.

Further proof of the identification of the 82 kDa band as ClC-f was obtained by 2D electrophoresis followed by mass spectrometry (MS) analysis. Fig. 3 shows the Western blot of an exemplary 2D-PAGE of OE vesicles, developed with anti-ClC3. The antibody clearly recognized two series of spots (81 kDa and 5.3 p*I* (isoelectric point); 76 kDa and 6.8 p*I*). The 76 kDa protein, which was recognized also by anti-AtClC-e antibody, was not present in sufficient quantity for MS analysis. Instead, the results of MS/MS analysis of the 81 kDa spot is shown in Fig. 4A. Exactly the spot which was recognized by the used antibody was cut out from the 2D-SDS–PAGE obtained in parallel and subjected to tryptic digestion and analyzed. MS analysis identified three long peptides with aminoacid sequences identical to those of AtClC-f (Fig. 4B). The aminoacid sequences of heterospecific homologs of AtClC-f are very similar, especially in the regions identified by MS analysis, but are significantly different from the se-

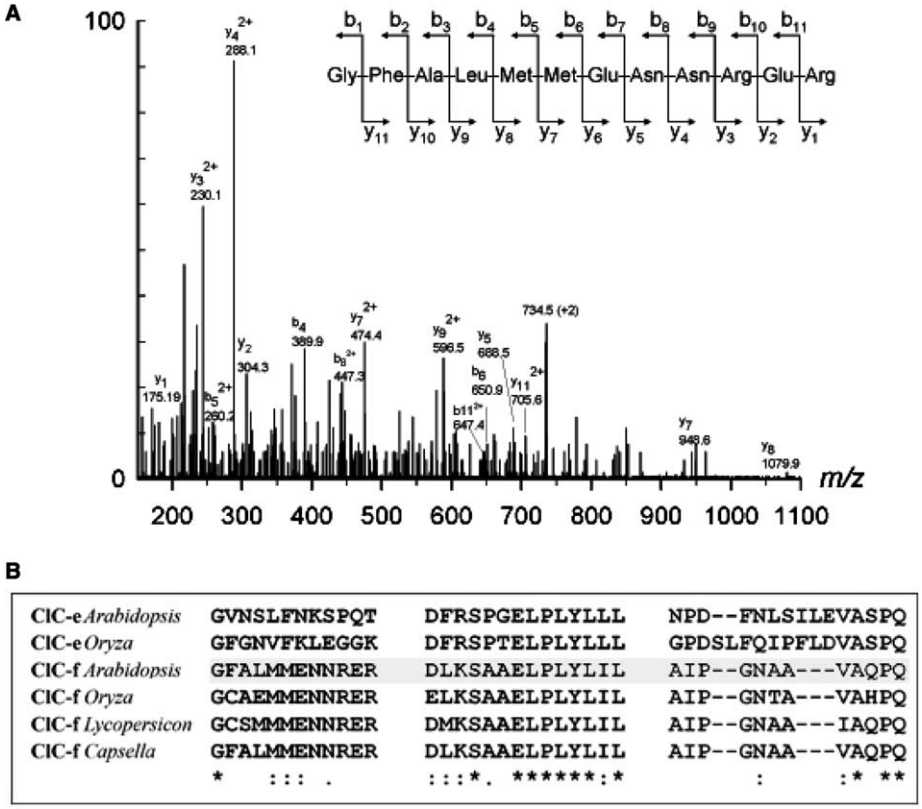

Fig. 4. Mass spectrometry analysis confirms presence of ClC-f in the outer membrane. (A) MS/MS spectrum of precursor ion with *m*/*z* 734.5 detected in ESI-MS spectrum of tryptic digest of 81 kDa spot from experiment shown in Fig. 3. One of the peptides is indicated in spectrum together with location of y and b ions. (B) Sequence alignments (T-Coffee algorithm) of selected regions of ClC-e and ClC-f heterospecific homologs. Highlighted sequences correspond to those identified by MS/MS analysis in our experiments. "*": residues which are identical in all sequences in alignment; ":": conserved, ".": semi-conserved substitutions.

quence of other putative ClC channel proteins (Fig. 4B). OEP75 (identified sequences: GYNMGEIGAAR; the same peptide with oxidized methionine and SAEVSTEWSIVPGR) and IEP110 (KQAEVLLADGQLTK) were also detected by MS (not shown) in OE and IE vesicles loaded on two separate 2D-PAGE, respectively.

Chloride ion fluxes have been proposed to regulate photosynthesis [19]. Oxygen evolution was measured in the presence of *p*-chlorophenoxy-acetic acid (CPA), an inhibitor of ClC-0 and ClC-1, which was mapped in the ClC-1 channel to a binding pocket that partially overlaps with the central chloride ion binding site revealed in the crystal structure of EriC [20]. Given that CPA interacts with highly conserved residues which are present also in plant ClC channels and especially with residues that are important for gating it is probable that CPA blocks plant ClCs as well, although direct proof is missing. Other chloride channel inhibitors were excluded because of interaction with porins or photosystem II proteins [21]. Oxygen evolution was measured in intact chloroplasts and thylakoids and expressed as μmol O_2/(mg Chl h). The absolute values measured under control conditions varied slightly among preparations obtained from different spinach samples, therefore oxygen evolution values in the presence of CPA were normalized accordingly, with respect to control (100%). Chloroplasts were preincubated with 100 μM CPA for 10 min before measurement, as CPA is only slowly membrane-permeable [20]. Oxygen evolution was significantly diminished by CPA, by 33 ± 6% ($n = 13$, $P < 0.05$). CPA diminished oxygen evolution only when added to intact chloroplasts, but not when added to thylakoids (5 ± 10% inhibition, $n = 13$, $P > 0.05$), indicating lack of direct interaction with photosystem II (Fig. 5A). Intact chloroplasts, isolated from the same spinach leaves soaked with 100 μM CPA or left with water without CPA for 3 h, showed significant difference in oxygen evolution (30.35 ± 2.7 for control and 22.45 ± 0.5 μmol O_2/(mg Chl h) for CPA-treated; $P = 0.023$, $n = 4$), indicating the ability of CPA to cross plant membranes. An effect of CPA on photosynthetic efficiency was found also in intact spinach plants, grown first in the absence of CPA, and then watered with either a solution containing 100 μM CPA for 3 days or with water (control). Chlorophyll fluorescence measurements performed on intact leaves showed a significant difference in the ratio of variable fluorescence and maximal fluorescence (F_v/F_m) between control and CPA-treated plants (0.761 ± 0.006 and 0.6621 ± 0.017, respectively; $P = 0.0007$, $n = 6$).

In accordance with the above observations, which suggest a role for ClC channels in the regulation of photosynthesis, the 82 kDa ClC-f protein was found to be expressed in stem and leaf but not in root extract (Fig. 5B). Furthermore, leaf tissue extract from etiolated plants, which contain plastids committed to become chloroplasts, displayed ClC-f, and this expression was maintained upon shift to light (Fig. 5C).

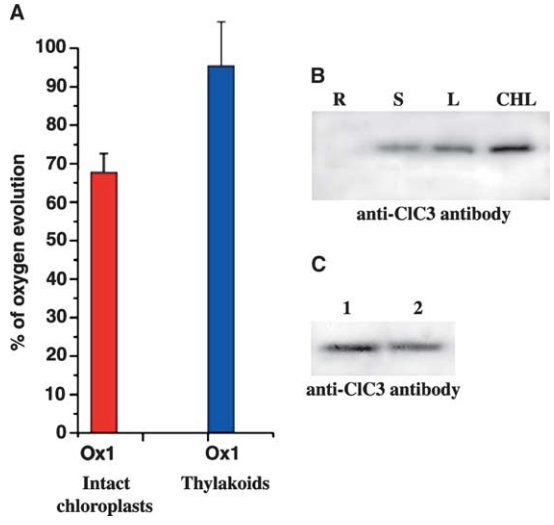

Fig. 5. Oxygen evolution in the presence of CPA and expression of ClC-f protein. (A) CPA decreases oxygen evolution in intact chloroplasts but not when directly added to thylakoids (see text for description). (B) Expression of ClC-f in root (R), stem (S), leaves (L) and chloroplasts (CHL). In all lanes equal quantities of proteins were loaded (40 μg). (C) Expression of ClC-f in total leaf extracts of dark-grown etiolated (lane 1) and etiolated shifted to light (lane 2) spinach plants. In both lanes 60 μg of total proteins was loaded (please note that in etiolated plants the relative amount of ClC-f with respect to total proteins is higher than in non-etiolated ones, due to the lack of expression of, e.g., Rubisco, LHC (light harvesting complex), etc. in the former plants).

4. Discussion

The present paper identifies a homolog of *Arabidopsis* ClC-f in spinach, as non-abundant component of the chloroplast outer envelope and suggests functional expression. To our knowledge, the intracellular location of ClC-f has not been published until now. This work also reports for the first time the identification of a plant ClC protein by mass spectrometry.

Chloroplast membrane proteins have been intensively studied by proteomics over the past few years [22], but ClC proteins have not been found. The MS data presented in this work leave little doubt about the presence of a ClC-f homolog in the OE of spinach. Biochemical data suggest also the presence of a ClC-e homolog in the OE. However, our data do not exclude the simultaneous presence of ClC-f and/or ClC-e in other subcellular compartments. AtClC-f has a short form (At1g55620.1, predicted MW of 62.5 kDa), due to alternative splicing (www.tigr.org). Alternative splicing may well account for the differential subcellular location of ion channels (e.g. [23]). OE membrane proteins generally do not require an N-terminal transit peptide for chloroplast targeting. The transit peptides in the heterospecific homologs of AtClC-f are very short (4–7 aminoacids, Tplen algorithm). Therefore, we cannot determine whether spinach ClC-f is present in the full-length form or not in OE.

Several chloroplast outer envelope ion channels have been identified, purified and studied over the past decade [24,25]. These studies indicate that the permeability of OE is regulated by, e.g., metabolites and solutes acting on porins [24]. In addition to these pores, several distinct anion-selective channels have been recorded by the patch clamp technique directly in chloroplasts [26] and by incorporating envelope vesicles into the planar lipid bilayer [19]. It is difficult to predict whether any of these activities can be ascribed to ClC, in view of the limited pharmacological characterization, and also the lack of knowledge concerning plant ClC activity. A further difficulty is the low single-channel conductance of ClC channels, which, if were also true for plant ClC channels, would largely

prevent detection of ClC activity directly in chloroplast OE or purified vesicles.

The fact that a ClC blocker decreases photosynthetic efficiency suggests that ClC proteins are functionally active in chloroplasts. It is well known that the presence of chloride ions is necessary for the oxygen evolving complex to be able to work at maximal rate [27]. To reconcile the presence of ClC in the OE with the regulation of photosynthesis, two possibilities may be envisioned. A part of ClC putative channels may operate at contact sites where they may sense voltage and effectively regulate chloride flux into the stroma. Another possibility is that ClC channels/transporters (see below) as well as other pores in the outer envelope membrane are regulated [24]. In any case, determination of the exact way in which ClC proteins contribute to the regulation of photosynthesis will be possible only when the capability of plant ClC channels to form anion-selective channels is demonstrated and their selectivity and pharmacology will be clarified. This is of utmost importance, given that mutant plants may not be useful in this context because of ClC redundancy. It is also possible, a priori, that ClC-f and ClC-e function as proton/chloride antiporters, like EriC [2], ClC-4 and ClC-5 [28,29]. Hopefully, future work with recombinant and/or heterologously expressed ClC-f and ClC-e will clarify these points.

Acknowledgments: The authors are grateful to Drs. P. Scannapieco and A. Cestaro for help with bioinformatic analysis. We also thank Drs. M. Zoratti, E. Schleiff, G. Ephritikhine, H. Barbier-Brygoo and P. Polverino de Laureto for useful discussions. We also thank all laboratories who provided us with various antibodies. I.S. is grateful to EMBO Young Investigator Program and I.S. and G.M.G. to the University of Padova (Progetto Ateneo) for financial support. This work was partially supported also by a PRIN grant from the Italian Ministry of the University and Research (MIUR).

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