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No plastidial calmodulin-like proteins detected by two targeted mass-spectrometry approaches and GFP fusion proteins

Elisa Dell'Aglio[a,b,c,d,1], Daniel Salvi[a,b,c,d], Alexandra Kraut[a,f,g], Mathieu Baudet[a,f,g], David Macherel[e], Martine Neveu[e], Myriam Ferro[a,f,g], Gilles Curien[a,b,c,d,*], Norbert Rolland[a,b,c,d]

[a] Univ. Grenoble Alpes, F-38000 Grenoble, France
[b] Commissariat à l'Energie Atomique et aux Energies Alternatives, Direction des Sciences du Vivant, Institut de Recherches en Technologies et Sciences pour le Vivant, F-38054 Grenoble, France
[c] INRA, USC1359, 17 rue des Martyrs, F-38054 Grenoble, France
[d] CNRS, Laboratoire de Physiologie Cellulaire & Végétale, UMR 5168, 17 rue des Martyrs, F-38054 Grenoble, France
[e] Université d'Angers, UMR 1345 Institut de Recherche en Horticulture et Semences, SFR 4207 QUASAV, Angers F-49045, France
[f] CEA, iRTSV, Laboratoire Biologie à Grande Echelle, U880, F-38054 Grenoble, France
[g] INSERM, U1038, F-38054 Grenoble, France

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ABSTRACT

Background: CaM-like proteins (CMLs) are localized in the cytosol and others in organelles such as the mitochondria, the peroxisomes and the vacuole. To date, although several plastidial proteins were identified as CaM/CML interactors, no CMLs were assigned to the chloroplast. Absence of clues about the genetic identity of plastidial CMLs prevents investigating their regulatory role.
Results: To improve our understanding of plastidial Ca^{2+} regulation, we attempted to identify plastidial CMLs with two large scale, CaM-specific proteomic approaches, and GFP-fusions.
Conclusions: Despite the use of several different approaches no plastidial CML could be identified. GFP fusion of CML 35 CML36 and CML41 indicate a cytosolic localization.

1. Introduction

Calmodulin (CaM) is an eukaryotic calcium (Ca^{2+}) sensor protein containing two pairs of EF-hand Ca^{2+}-binding sites connected by a central α-helix [1]. CaM is known to modulate several cellular processes, like muscle contraction and enzyme activation, by binding to protein partners in response to dynamic changes in Ca^{2+} concentration [2].

In yeast, CaM is cytosolic and coded by an essential single gene [3]. In human, a multigene family of three divergent cytosolic members is present, all of them coding for the same protein sequence [4,5], plus one CaM-like – a protein constituted only by EF-hand domains with 85% amino-acid sequence identity compared to human CaM [6]. By contrast, in plants a wide variety of CaM and CaM-like proteins (CMLs, with at least 15% amino acid identity with CaM and a variable number of Ca^{2+}-binding sites) exists, with about 50 different members depending on the plant species [7], as well as many other Ca^{2+} sensors which include CPKs (Ca^{2+}-dependent Protein Kinases) and CRKs (CPK-related kinases) [8].

Such a high number of plant Ca^{2+}-binding proteins is thought to allow a precise and localized control of cell responses to developmental and environmental stimuli [9–14]. Indeed, CMLs differ in their expression profile [15] and bind specific target proteins [16].

Unlike classic CaMs which are cytosolic or nuclear [17–20], CMLs are present in various subcellular compartments, thanks to target sequences at their N-terminal or C-terminal. Indeed, some of them were found in the nucleus (AtCML19/AtCEN2, At4g37010, [21]), the plasma membrane (petunia CaM53[19], AtCML4-5 [22]), the mitochondria (AtCML30, At3g29000, [23]), the peroxisomes (AtCML3, At3g07490 [23]), and the extracellular space [24], whereas AtCML18 (previously called AtCaM15) was shown to

Abbreviations: CaM, calmodulin; CML, calmodulin-like protein; NADK, NAD^+ kinase.

* Corresponding author at: CNRS, Laboratoire de Physiologie Cellulaire & Végétale, 17 rue des Martyrs UMR 5168, Grenoble 38054, France.
E-mail address: gilles.curien@cea.fr (G. Curien).
[1] Present address: Department of Botany and Plant Biology, University of Geneva, 1211 Geneva, Switzerland.

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interact with the C-terminal region of AtNHX1, a vacuolar Na^+/H^+ antiporter [25].

With 15 mM of total Ca^{2+} concentration, the chloroplast is considered one of the main reservoirs of Ca^{2+} in the plant cell [26]. Only 150 nM free stromal Ca^{2+} is however present, the rest of it being bound to the thylakoid membrane [27]. Increasing evidence suggests also a signalling role for Ca^{2+} in the chloroplast. In particular, stromal Ca^{2+} fluxes were shown to take place at light/dark transition and to be proportional to the duration of previous light exposure [28]. These findings suggested a role for Ca^{2+} in light responses and circadian clock regulation. Ca^{2+} fluxes in chloroplasts have also been reported under conditions mimicking pathogen attacks [29] and Ca^{2+}-dependent phosphorylation of plastidial proteins (in particular CAS, Var1 and PsaN) was observed [30]. Current knowledge on plastidial proteins involved in regulating Ca^{2+} signalling and Ca^{2+}-dependent responses is however still limited.

Some plastidial proteins were shown to interact *in vitro* with CaMs and/or CMLs [31–34]. In particular, CaM was reported to interact *in vitro* with NADK2 – a NAD kinase isoform localized into the chloroplast [33,35], suggesting a role of Ca^{2+} in the regulation of photosynthesis. *In vitro* CaM was also shown to bind Tic32, a putative component of the protein import machinery [36–38]. The interaction of CaM with Tic32 was reported to prevent Tic32 binding to NADPH, thus suggesting that Ca^{2+} regulation of chloroplast protein import could be mediated by Tic32-CaM [32]. We also recently identified around 200 new putative plastidial CaM/CML interactors, thanks to a proteomic approach [33]. These findings indirectly supported the theory of a role of CaM-related proteins in orchestrating the plastidial response to physiological and environmental stimuli [39–41].

The physiological relevance of all the interactions mentioned above is however still unclear, especially because the presence of CMLs in the chloroplast has never been proven, neither by GFP-fusions nor by proteomics of plastidial subcompartments [42,43]. Some CMLs contain N-terminal sequences that might act as plastidial transit peptides, but absence of clues about the genetic identity of plastidial CMLs prevents investigating their regulatory role by CML mutants/overexpressors, or *in vivo* co-localization studies.

In this work, in order to improve current knowledge of plastidial Ca^{2+} signalling, we attempted to identify plastidial CMLs using subcellular localization of GFP-fusions in protoplasts and two protein purification strategies coupled to LC–MS/MS analyses. Our results call into question the role of this protein family in the chloroplast, as well as the previously identified interactions.

2. Material and methods

2.1. CML-GFP fusion construction

The full-length predicted coding sequence of AtCML35 (AT2G41410), AtCML36 (AT3G10190) and AtCML41 (At3g50770) were amplified from a cDNA library previously described [44] with primers containing the restriction sites SacI (forward primer) and NcoI (reverse primer). After 5 min of denaturation at 95°, PCR was conducted with 35 cycles of a denaturation step (95°, 1 min) a primer annealing step at 55° (1 min) and an amplification step (72°, 1 min). Phusion DNA polymerase (New England Biolabs, Inc) was used for all cloning procedures. PCR products were cloned into a pUC vector [45] in a frame with a C-terminal GFP sequence by classic restriction enzyme digestion and subsequent ligation with T4 ligase.

Primers sequences were as follows:

CML35GFP forward: TTCGTCGACATGAAGCTCGCCGCTAGCCT

CML35GFP reverse: CAACCATGGAATGATGATGATCATTCATCGC

CML36GFP forward: AAGTCGACACTATGAAACTCGCCAAACTAATTCC

CML36GFP reverse: AAACCATGGAACGCTGGAGATCCATCATTCGTGAG

CML41GFP forward: TATAAAGTCGACGATATGGCAACTCAAAAAGAGAAACC

CML41GFP reverse: ATTTAATCCATGGAAACCGTCATCATTTGACGAAACTC

2.2. Protoplast transformation

Arabidopsis protoplasts transformation and confocal microscopy were carried out according to [46].

2.3. Plant material and preparation of chloroplast protein extracts

Arabidopsis plants, Wassilewskija background (Ws), were grown in culture chambers at 23 °C (12-h light cycle) with a light intensity of 150 $\mu mol.m^{-2}\,s^{-1}$ in standard conditions [47]. Purification of chloroplasts, stroma, and thylakoids from Arabidopsis leaves were carried out according to [42].

2.4. CML41 cloning and production in E. coli

The CML41 predicted mature protein (*i.e.* without the predicted *N*-terminal transit peptide comprising aminoacids 1–46) was amplified with primers CML41-forward (CAACCTTAAACTCTCCCATGGGCAACAGTGATGAC) and CML41-reverse (GGTAATTACGTAAAAGCTCGAGTTAATTACACTAAAC), containing a NcoI and a XhoI restriction site, respectively, and cloned into pET-30(a+), without any tag. The purified plasmid was introduced into Rosetta™-2(DE3) *E.coli* bacteria (Novagen, Darmstadt, Germany). Liquid cultures supplemented with antibiotics (kanamycin and chloramphenicol) reaching 0.8 OD were induced with 0.4 mM IPTG overnight at 20 °C. Bacteria were harvested, washed once with 50 mM Tris-HCl pH 8.0, then re-suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM DTT, 0.5 M NaCl, 5% (v/v) glycerol, 5 mM ε-aminocaproic acid and 1 mM benzamidine), and sonicated for 5 min at 4 °C on a Benson sonifier. Streptomycin sulphate 0.1% (w/v) was added to precipitate DNA. The sonicated bacteria were centrifuged for 20 min at 30,000*g* at 4 °C.

AtCML41 expressing bacterial soluble extract (30 mg, 3 mg/ml) was adjusted to a Ca^{2+} concentration of 5 mM, heat-shocked for 5 min at 95 °C, immediately cooled on ice for 2 min and centrifuged 20 min at 16 °C. The supernatant was loaded twice on a 4-mL Phenyl Sepharose column, equilibrated with Ca^{2+} buffer (50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$). The column was washed 10 times with Ca^{2+} buffer (40 mL), then with the same buffer supplemented with 200 mM NaCl, for a more stringent washing (20 mL). Then, bound proteins were eluted in EGTA buffer (50 mM Tris-HCl pH 7.5, 2 mM EGTA). The eluted fractions containing the protein were pooled and concentrated on a 3 K Amicon filter unit (Merck).

An identical protocol was followed for the *Arabidopsis* stroma and thylakoid highly purified subfractions [42] (1 mg/ml for the stroma and 1.5 mg/ml for the thylakoids, volume 2 mL) in order to purify putative plastidial CMLs. In this last case, the eluted fractions were precipitated with an equal volume of 20% (v/v) TCA, vortexed and incubated for 1 h at −20 °C, then centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was removed and the precipitate was resuspended in 100 μL of SDS loading buffer.

2.5. Protein purification

ceQORH (At4g13010) was purified according to [48]; Threonine synthase 2 (AT1G72810) was purified according to Supplementary data of [49]. DAHPS3 (AT1G22410) was purified according to [33].

2.6. Coupling of putative CaM binding proteins to CNBr sepharose beads for purification of native CMLs from chloroplast fractions

CNBr-activated Sepharose 4 B (500 μL GE Healthcare, ref 17-0430-01) coupled with 3.5 mL of the protein of choice (ceQORH, Threonine synthase 2 or DAHPS3) was used for the experiment.

Stroma purified fraction (1 mg/mL, 2.5 mg in total) were diluted in buffer IPP150 CBB 2X (Tris–HCl 20 mM, pH 8, NaCl 300 mM, 2 mM $Mg(CH_3COO)_2$, 2 mM imidazole, 4 mM $CaCl_2$, 2% NP40). Protein extracts were loaded on the CNBr Sepharose column equilibrated in the same buffer and the flow through was discarded. The column was then washed three times with buffer IPP150 CBB (Tris–HCl 10 mM, pH 8, NaCl 150 mM, 1 mM Mg $(CH_3COO)_2$, 1 mM imidazole, 2 mM $CaCl_2$, 1% NP40). One aliquot of this washing for each protein was kept for mass spectrometry analysis.

Ca^{2+}-specific bound proteins were finally eluted in buffer CEB (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM $Mg(CH_3COO)_2$, 1 mM Imidazole, 2 mM EGTA, 1% NP40) and collected in 17 fractions of 0.5 mL. To detach all possible remaining proteins, a final elution was performed with 1 M NaCl.

Eluted fractions were loaded on SDS PAGE and were subjected to LC MS/MS analyses as previously described [33].

3. Results

3.1. GFP fusion experiments showed that AtCML36, 37 and 41 are not localized in the chloroplast of arabidopsis protoplasts

In order to identify candidates for plastidial localization among Arabidopsis CML proteins which were listed by [7], available information about their localization was compiled, and protein sequences were analyzed by the SUBA subcellular localization tool. Table 1 lists Arabidopsis CMLs, the number of predicted EF-hand sites (column III), and the occurrence of putative N-terminal extension which could correspond to a target peptide (column IV).

In silico subcellular localization predictions reported in SUBA [50] are displayed in Column V. Columns VI – VIII contain all available experimental data about CMLs localization, as well as the techniques used to assess the localization. Finally, column IX states the presence, in the protein sequence, of peptides that can be detected by mass spectrometry after trypsin digestion, according to the MASCP database ([51] http://gator.masc-proteomics.org/).

Among all Arabidopsis CML proteins, only AtCML41 is predicted to harbor a N-terminal putative transit peptide and to localize in the chloroplast, and thus appeared as the best candidate for plastidial localization. The mitochondrial-predicted AtCML35 and AtCML36, whose localization has never been reported to our knowledge, were also retained as potential candidates for plastidial localization, as prediction software are limited in their ability to differentiate mitochondrial and plastidial transit peptides [52,53].

The expression profile of *AtCML35*, *−36* and *−41* was then analyzed using Genevestigator [54] (see **Fig. S1**). The expression profile of *AtCML36* did not reveal special features, with low or medium expression levels in most organs/tissues, the highest level being found in hypocotyl. Both *AtCML35* and *AtCML41* appeared to be mostly expressed in photosynthetic organs such as adult and senescent leaves. The enrichment in transcripts appeared higher for *AtCML41*, whose expression in other tissues – with the exception of the sepals – appears very low. Considering the major role and metabolic activity of chloroplasts in photosynthetic tissues, the expression profiles of *AtCML35* and *−41* could fit with a plastidial localization of the corresponding proteins.

The whole cDNA sequences corresponding to AtCML35, AtCML36 and AtCML41 were cloned as C-terminal GFP fusions, under control of the CaMV 35S promoter. These constructs were used to transiently transform Arabidopsis leaf protoplasts. As shown in Fig. 1 the three AtCML constructs displayed a pattern similar to LEA1G, a cytoplasmic protein GFP-fusion rather than LEA23G (plastidial GFP fusion) [55]. For none of our construct GFP fluorescence could be detected in chloroplast.

3.2. Establishment of a protocol for partial purification of CMLs

Previous mass spectrometry analysis of plastid proteome did not allow the identification of any plastidial CaM-related proteins, but this could result from their low abundance or difficulties to detect their proteolytic peptides. In order to improve detection of such proteins, we applied a CaM/CML purification protocol to plastidial subfractions to enrich these proteins in our samples before MS analysis. In this protocol, which was previously used for the purification of several CaM isoforms [56], CaM proteins are bound to a phenyl-sepharose resin in the presence of an excess of $CaCl_2$, and after washing, the proteins are eluted with an excess of EGTA.

To evaluate whether such an approach would be suitable for CML enrichment, a recombinant protein corresponding to the mature AtCML41 (without its predicted N-terminal targeting sequence) was produced in *E.coli*. Fig. 2A illustrates the production of the recombinant protein and its detection in crude extracts of bacterial proteins corresponding to total or soluble protein extracts.

The soluble bacterial extract containing the recombinant protein was then submitted to heat shock followed by centrifugation to remove all precipitated proteins. The supernatant contained a protein with the predicted size of the recombinant mature AtCML41, suggesting that this CML, as true CaMs, was heat resistant. This fraction was then loaded on a Phenyl Sepharose resin equilibrated with a $CaCl_2$-containing buffer. Unbound proteins were washed from the column using the same buffer. Then, proteins still bound to the matrix were eluted with an excess of EGTA. As *E. coli* does not produce any endogenous CaM/CML protein, a single band corresponding to AtCML41 was expected after this purification step. Indeed, as shown in Fig. 2B (last lane) the eluted fraction contained a nearly homogeneous protein which migrated at the expected molecular weight for AtCML41.

A mobility-shift assay was performed on the purified AtCML41 (Fig. 2C) and revealed a slightly faster migration of the protein in the presence of $CaCl_2$ versus EGTA, as previously reported for true CaMs or CMLs [57,58].

Together these results show that such an enrichment method is well suited for the purification of CaM-related proteins from a complex protein extract.

3.3. Application of the CaM purification protocol to Arabidopsis chloroplast fractions combined with mass spectrometry did not reveal any plastidial CML

The CML enrichment method successfully used for the recombinant AtCML41 was applied to stroma and thylakoids protein samples of *Arabidopsis* chloroplasts, purified as previously described [42]. The proteins specifically retained on the Phenyl Sepharose column in the presence of $CaCl_2$ were eluted with EGTA, TCA precipitated and visualized on SDS-Page by silver staining. The gel showed enrichment of specific bands that correspond to the usual size of CaM/CMLs, *i.e.* between 15 and 25 kDa (Fig. 3).

In order to assess the presence of CMLs in the chloroplast, two samples of EGTA-eluted proteins for both stroma and thylakoids were subjected to LC–MS/MS. The first sample corresponded to a

fraction of the total elution, while the second sample was generated by cutting the low molecular weight part from an identical, not stained SDS-Page. We also ran on SDS-PAGE the protein content of the supernatant of the heat shocked stroma and thylakoids, before the loading on the Phenyl Sepharose column. The low molecular part of these runs was cut from the gel and analyzed by LC–MS/MS.

A total of 54 proteins were detected in the 6 samples analyzed by LC–MS/MS (heat shock band, Phenyl Sepharose total sample or gel bands, for both the stroma and the thylakoids).

Most of the identified proteins had a molecular weight lower than 30 kDa (Table S1). Many of them belonged to ribosomes, the ATP synthase or photosystems – namely highly abundant protein complexes of the chloroplasts, but none of them belonged to the CML family.

Table 1
Arabidopsis CML features. *Arabidopsis* CMLs listed according to [7] and their subcellular localization predicted using SUBA (column V), as well as the putative length of the predicted transit peptide (column IV), when present. The table also shows the putative number of Ca^{2+}-binding sites predicted by UniProt (column III) and experimental data about their subcellular localization, from either GFP-fusion constructs or LC–MS identification (VI–VIII). The last column (IX) reports the presence/absence of detectable peptides in mass spectrometry after trypsic digestion, according to MASCP [51,68–76].

I	II	III	IV	V	VI	VII	VIII	IX
CML	AGI	EF hands	N-terminal extension	SUBAcon	Verified localization	References	Technique	MASCP
1	At3g59450	4	no	nucleus	–			no
2.	At4g12860	4	no	cytosol	cytoplasm	[67]	GFP-tag	yes
3	At3g07490	4	no	peroxisome	peroxysome	[23]	GFP-tag	no
4	At3g59440	4	yes – 20 aa	extracellular	–			yes
5	At2g43290	4	yes – 25 aa	plasma membrane	–			yes
6	At4g03290	4	no	extracellular	cytoplasm; nucleus	[67,17]	GFP-tag; proteomics	yes
7	At1g05990	4	no	cytosol	–			no
8	At4g14640	4	no	cytosol	–			no
9	At3g61920	4	no	cytosol	–			no
10	At2g41090	4	no	cytosol	–			yes
11	At3g22930	4	yes – 54 aa	cytosol	–			yes
12	At2g41100	6	no	cytosol	plasmodesmata; vacuole	[68,69]	proteomics; proteomics	no
13	At1g12310	3	no	cytosol	cytoplasm; nucleus; cytosol; plasma membrane; plasma membrane	[67,17,70,71,72]	GFP-tag; proteomics; proteomics; proteomics; proteomics	yes
14	At1g62820	3	no	cytosol	nucleus; cytosol	[17,70]	proteomics; proteomics	yes
15	At1g18530	4	no	cytosol	–			no
16	At3g25600	4	no	cytosol	–			yes
17	At1g32250	4	no	cytosol	–			yes
18	At3g03000	4	no	vacuole	vacuole	[25]	GFP-tag	yes
19	At4g37010	4	yes – 70	vacuole,plasma membrane				yes
20	At3g50360	4	yes – 70	cytosol,nucleus	plasma membrane; plasma membrane	[71,72]	proteomics	yes
21	At4g26470	3	no	plasma membrane	–	[67]	GFP-tag	yes
22	At3g24110	4	no	cytosol	–			no
23	At1g66400	4	no	nucleus	–			yes
24	At5g37770	4	no	plasma membrane	–			yes
25	At1g24620	4	no	cytosol	cytosol; nucleus	[67,17]	GFP-tag; proteomics	yes
26	At1g73630	4	no	cytosol	–	[67]	GFP-tag	yes
27	At1g18210	4	no	nucleus	vacuole	[73]	proteomics	yes
28	At3g03430	2	no	cytosol	cytoplasm	[67]	GFP-tag	yes
29	At5g17480	2	no	cytosol	cytoplasm; cytoplasm	[67,74]	GFP-tag; immunolocalization	yes
30	At2g15680	4	yes – 57	mitochondrion	mitochondria	[23]	GFP-tag	yes
31	At2g36180	4	no	cytosol	–			no
32	At5g17470	4	no	cytosol	–			no
33	At3g03400	4	no	cytosol	–			no
34	At3g03410	4	no	cytosol	–			no
35	At2g41410	4	yes – 73	mitochondrion	–			yes
36	At3g10190	4	yes – 19	mitochondrion	–			yes
37	At5g42380	4	yes – 18	cytosol,nucleus	cytosol, nucleus	[75]	GFP-tag	no
38	At1g76650	4	yes – 22	nucleus	–			yes
39	At1g76640	4	no	nucleus	–	[67]	GFP-tag	no
40	At3g01830	2	no	nucleus	–			yes
41	At3g50770	4	yes – 46	plastid	–			no
42	At4g20780	3	no	cytosol,nucleus	–			yes
43	At5g44460	3	yes – 21	cytosol	–			yes
44	At1g21550	2	no	cytosol	–			no
45	At3g29000	3	yes – 29	endoplasmic reticulum	–			no
46	At5g39670	3	no	plasma membrane	–			no
47	At3g47480	2	yes – 34	plasma membrane	–			no
48	At2g27480	2	no	nucleus	–			no
49	At3g10300	2	no	nucleus	–			yes
50	At5g04170	2	no	nucleus	–			yes

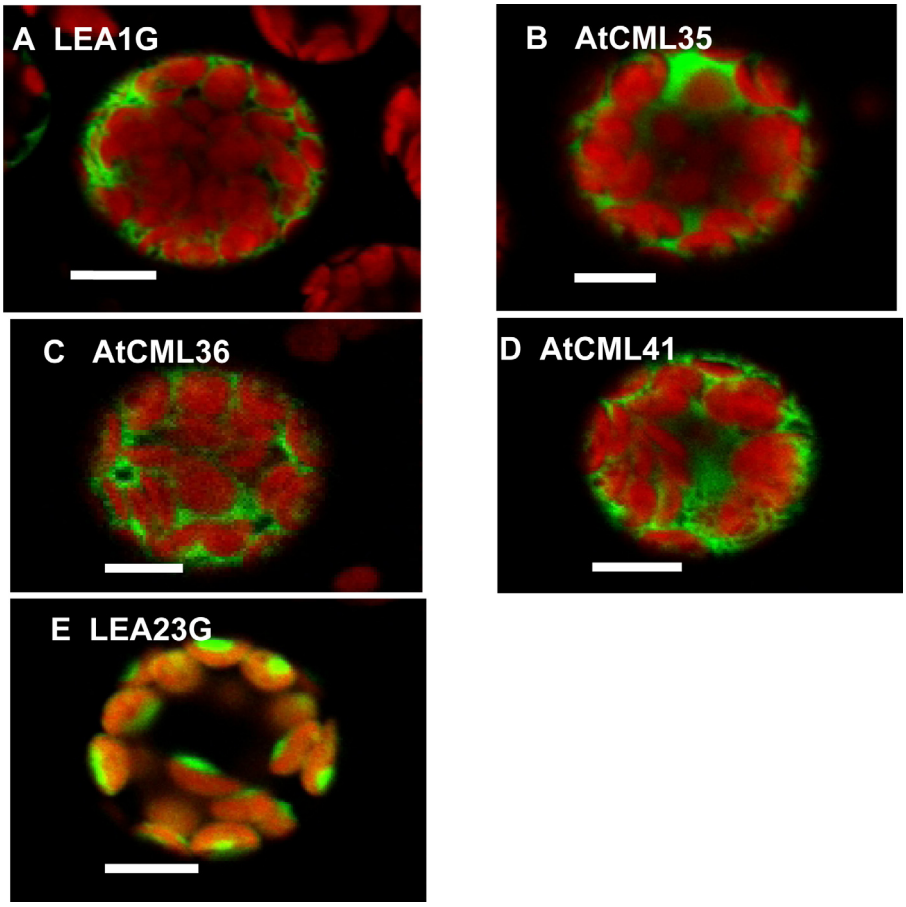

Fig. 1. Subcellular localization of AtCML35, AtCML36 and AtCML41 GFP fusion proteins in transformed Arabidopsis protoplast. (A): cytoplasmic control (LEA1G); (B): CML35-GFP; C: CML36-GFP; D: CML41-GFP, E: plastidial control (LEA23G). In blue, chlorophyll autofluorescence; in green: GFP fluorescence. White bars represent 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Affinity chromatography coupled with mass spectrometry failed to detect CMLs in the stroma

The purification method described above was optimized for AtCML41, but might not be suitable for other AtCMLs. We therefore attempted to use affinity chromatography to trap plastidial CMLs using putative plastidial CaM partners as baits. Three proteins which were shown to bind AtCaM1 in a previous study [33]: ceQORH (At4G13010 [45,48,59]), TS2 (Threonine synthase 2, At1g72810), and DAHPS3 (3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase, At1g22410).

An *in vitro* CaM binding test (Fig. 4) revealed that ceQORH has a high, Ca^{2+} dependent affinity for CaM. In contrast, as shown before [33], the affinity of DAHPS3 and TS2 for AtCaM1 was weaker and not Ca^{2+}-dependent in the case of TS2. These proteins were nevertheless employed as well to trap putative plastidial CMLs, as the specificity and affinity of AtCaM1 can be different from that of other CaMs or CMLs [16,35].

Bait proteins were cross-linked to CNBr Sepharose beads for affinity chromatography of the stromal extract as described in the Material and Methods section. LC–MS/MS analyses of the eluted fractions from ceQORH, TS2 and DAHPS3-bound columns allowed identifying 69, 188, and 168 proteins, respectively (Table S2) but none of them belonged to the CML group.

4. Discussion

The aim of this work was to identify putative CMLs which may mediate Ca^{2+} signalling in the plastids. To achieve this goal, we expressed GFP-fusions of CML candidates in Arabidopsis protoplasts and conducted mass spectrometry analyses of plastidial subfractions that were subjected to two purification protocols – one specific for CMLs and a second one based on an affinity chromatography strategy. None of these approaches was successful.

In the chloroplast, Ca^{2+} is suspected to have a role in orchestrating light/dark transition [28], metabolic adjustments [35], responses to elicitors [29], and plastidial protein import [32]. Many CMLs were previously detected in almost all cell compartments, such as the nucleus [21], the vacuole [25], the plasma membrane [19] the mitochondria and the peroxisomes [23], and several putative plastidial CaM/CML-binding proteins were previously identified [31,33,60,61]. For these two reasons, failing in finding plastidial CMLs was a rather unexpected result.

CaMs and CMLs likely play a role in plant responses to stress conditions, therefore their expression is expected to be finely tuned ([15], see also Fig. S**1** for an expression profile of the three CMLs investigated). As a starting material for our mass spectrometry studies we chose chloroplasts purified from 3 week old Arabidopsis seedlings. Thus, some CMLs expressed at a later or previous stage, as well as CMLs expressed in chloroplasts of a particular cell type (such as the stomata) or in response to stress could have been missed in this study. To assess this possibility, it will be necessary to generate CML GFP fusions under the control of native promoters and stable plant transformants. In addition, CMLs interacting with other plastidial proteins might have been partially or totally precipitated with their partners during the heat shock treatment used for the first mass spectrometry experiment. Finally,

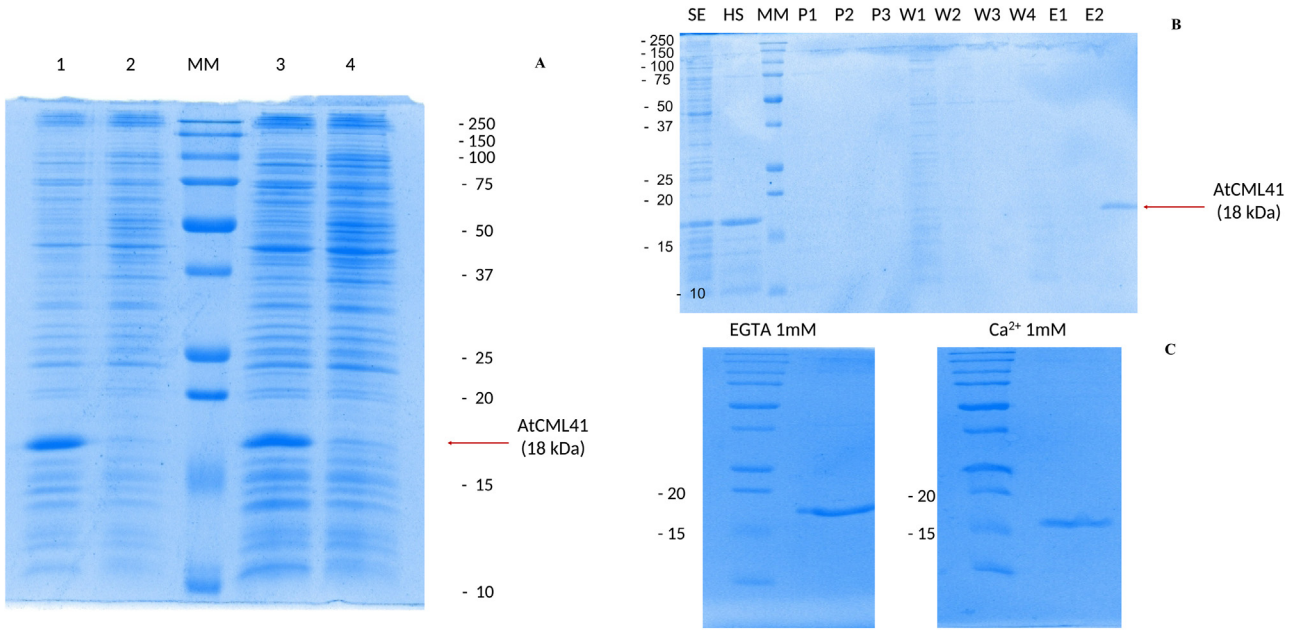

Fig. 2. Production and purification of recombinant AtCML41 without its putative plastidial transit peptide. (A): expression of AtCML41 in the total (1) and soluble (3) bacterial extracts of a culture induced with 0.4 mM IPTG. The overproduced protein is detected at the expected size (18 kDa, arrow). The corresponding band is not detected in non-induced total (2) and soluble (4) bacterial extracts. MM: molecular weight marker. (B): SDS-PAGE profile of the purification of recombinant AtCML41. 20 μg of each protein extract were loaded. MM: molecular weight markers. SE: soluble bacterial protein extract corresponding to a culture of bacteria in which protein expression was induced with IPTG (0.4 mM) overnight HS: Soluble protein fraction following the heat shock (95 °C, 2 min). P1-3: Pass-through of the Phenyl Sepharose column charged with the heat shock extract after 20 min of centrifugation at 4 °C and dilution in 1 mM Ca^{2+} buffer. W1-4: washes in the presence of 1 mM Ca^{2+}. E1-2: protein fractions eluted from the Phenyl Sepharose column after the EGTA treatment. c: mobility shift assay of 10 μg of AtCML41 in the presence of 5 mM EGTA (left) or 1 mM $CaCl_2$ (right). The predicted molecular weight of recombinant AtCML41 (minus the predicted transit peptide) is 18 kDa.

according to Table 1, column IX, some CMLs with N-terminal extensions are inaccessible to peptide fractionation, and therefore difficult to detect by LC–MS/MS. Future attempts may include a control plant sample spiked with a candidate protein purified in vitro (such as AtCML41 or another good candidate) to ensure the protein is detectable by LC–MS/MS.

CaMs and CMLs represent a huge family of plant Ca^{2+} sensors but increasing evidence suggests that other proteins are present in the chloroplast to fulfill this role. An intriguing Ca^{2+}-binding protein located in the thylakoid membrane, CAS, and its Ca^{2+} affinity was shown to be in the mM range. Recently, CAS role in chloroplast physiology was linked to photoacclimation and retrograde signalling [62,63]. Other Ca^{2+}-binding proteins that might play a major role in the chloroplast include serine-threonine phosphatases [64], the two EF-hand containing protein CRSH [65], the one EF-hand containing protein AtSAMTL (AT2G35800, [66]), and the CP12 protein [67]. It is noteworthy that the EF-hand of the SAML protein is more likely localized in the intermembrane space rather than the stroma [66]. The role of all these Ca^{2+} interactors will require further studies. In addition, a large number of plastidial proteins is still not associated to any function and many of them might contain known and/or new Ca^{2+}-binding motifs.

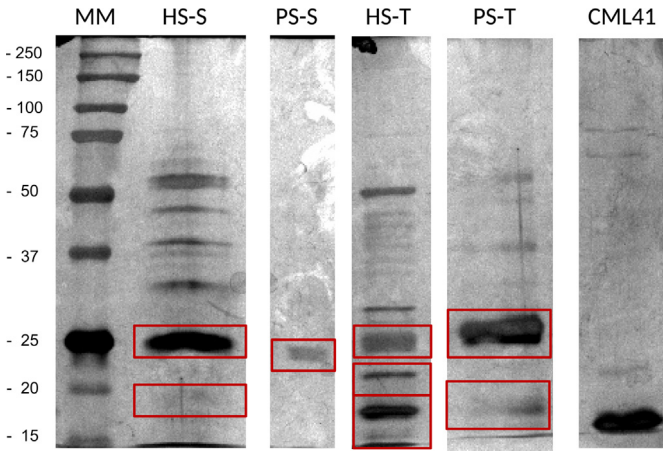

Fig. 3. Silver staining gel of the SDS-PAGE of the proteins present in the heat shock and phenyl sepharose elutions from Arabidopsis plastidial subfractions. For heat-shock extracts, 20 μg of protein were loaded. For Phenyl Sepharose purifications, 10 μL of each TCA precipitation were loaded. MM: molecular weight marker; HS-S: stroma supernatant after heat-shock; PS-S: stroma Phenyl Sepharose elution; HS-T: thylakoids supernatant after heat-shock; PS-T: thylakoids Phenyl Sepharose elution; CML41: pure CML41 (10 μg). Squares indicate the regions that were excised and submitted to mass spectrometry analysis.

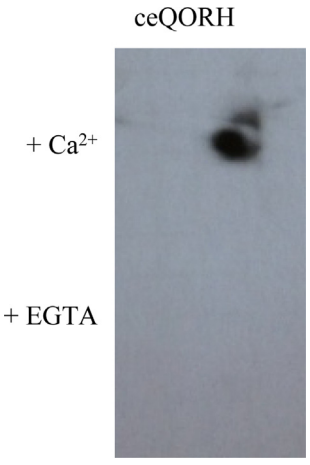

Fig. 4. Overlay assay of ceQORH The *in vitro* binding test was conducted in the presence of $CaCl_2$ (1 mM) or EGTA (5 mM) as described in [33].

In conclusion, according to this study AtCML35, 36 and 41– three putative plastidial CMLs – are targeted to the cytoplasm rather than the chloroplast. Our two mass spectrometry-based analyses failed to identify any CML protein in this cell compartment, suggesting that the main Ca^{2+} regulators in the chloroplast likely belong to other protein families. As Ca^{2+} signalling inside the chloroplast is currently receiving great attention [39–41], the identification of plastidial Ca^{2+}-binding proteins is a main goal of plant physiology that will require more efforts in the characterization of the role and localization of each CMLs by multiple approaches as well as of plastidial proteins of unknown function.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neps.2016.08.001.

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