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

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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.

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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].