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Arabidopsis calcium-binding mitochondrial carrier proteins as potential facilitators of mitochondrial ATP-import and plastid SAM-import

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ABSTRACT

Chloroplasts and mitochondria are central to crucial cellular processes in plants and contribute to a whole range of metabolic pathways. The use of calcium ions as a secondary messenger in and around organelles is increasingly appreciated as an important mediator of plant cell signaling, enabling plants to develop or to acclimatize to changing environmental conditions. Here, we have studied the four calcium-dependent mitochondrial carriers that are encoded in the *Arabidopsis* genome. An unknown substrate carrier, which was previously found to localize to chloroplasts, is proposed to present a calcium-dependent *S*-adenosyl methionine carrier. For three predicted ATP/phosphate carriers, we present experimental evidence that they can function as mitochondrial ATP-importers.

1. Introduction

Plants can react rapidly to a changing environment or stress conditions by employing immediate signaling pathways, such as calcium signaling. This involves the generation of specific information in transient or oscillating spikes of free calcium ions that are decoded by calcium binding proteins, ultimately leading to a physiological change of the plant (recently reviewed in [1–3]). Canonical calcium binding proteins contain EF-hands with a high affinity for calcium ions (Ca^{2+}) [4,5]. Fluxes of free Ca^{2+} have been reported to occur upon a myriad of stresses and developmental cues and take place mainly in the cytoplasm [6]. However, Ca^{2+} fluxes and Ca^{2+}-dependent signaling pathways in and around plant organelles, such as mitochondria and chloroplasts have also been reported [7]. In chloroplasts, Ca^{2+} fluxes have been measured upon the transition from light to dark [8] and in mitochondria, they were elicited by a range of stimuli, including application of H_2O_2, touch stimulation and anoxia [9,10]. An evolutionary conserved enzyme of the bacterial stringent response, which contains two EF-hands (CRSH) and a non-canonical calcium binding protein that influences the cytosolic calcium fluxes observed during stomatal closure (CaS) localize to chloroplasts [11–15]. In addition, the mitochondrial type II NAD(P)H:quinone oxidoreductases contain EF-hands and are Ca^{2+}-regulated [16]. Despite these initial reports, only a few more Ca^{2+}-binding proteins have been found in these organelles and even less data exist on the roles that Ca^{2+} signaling in mitochondria or chloroplasts play in adjusting the physiology of the plant to its changing environment.

The mitochondrial carrier family (MCF) contains transmembrane transporter proteins that transport a diverse set of substrates such as ATP/ADP [17], citrate [18] and glutamate [19] (for a complete overview see [20]). Originally they were found to localize exclusively to mitochondria [21], however, in plants certain prominent members are also located in other organelles. For example, the *S*-adenosylmethionine (SAM) transporter SAMT1 localizes to chloroplasts [22] and the peroxisomal ATP/ADP translocases PNC1 and PNC2 localize to peroxisomes [23]. MCF proteins have three tandemly repeated homologous domains [24], each

Abbreviations: APC, ATP/phosphate carrier; BKA, bongkrekic acid; MCF, mitochondrial carrier family; SAM, *S*-adenosyl methionine; SAMTL, SAM transporter-like; TM, transmembrane domain; YFP, yellow fluorescent protein

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containing two transmembrane helices that form a pore lined with specific amino acids that determine the substrate specificity [25]. This protein family is conserved in eukaryotes and contains 35 putative members in *Saccharomyces cerevisiae*, about 50 in *Homo sapiens* and 58 in *Arabidopsis thaliana*. The rare examples found in pathogenic prokaryote genomes appear to be pseudogenes resulting from horizontal gene transfer. The high conservation of functional residues between species allows the predictive assignment of substrate specificity based on protein homology [20]. Interestingly, the aspartate/glutamate and ATP-Mg/P_i carrier subfamilies, which have been described in yeast and human have EF-hands containing N-terminal protein extensions and their transport activities are Ca^{2+}-dependent [26–29]. Out of our interest for calcium signaling in organelles we set out to describe and characterize the four EF-hand containing MCF proteins that are present in the Arabidopsis genome.

2. Materials and methods

2.1. Plant material and yeast strains

Tobacco plants (*Nicotiana tabacum* cv. Petite Havana SR1) were grown in soil for approximately 6–7 weeks under short day conditions (8 h light) in a climate chamber with a light strength of 150 μM photons/m^2 s. Yeast strains were W303 (MATα leu2-3.112 trp1-1 can1-100 ura3-1 ade2-1 his3-11.15) and Δsal1 (as W303, but, AAC1–3, Δsal1::kanMX4). *Agrobacterium tumefaciens* strain was AGL1 (recA::bla pTiBo542ΔT Mop+ CbR; Lazo et al, 1991).

2.2. Computational procedures

Sequences of plant orthologs from SAMTL (At2g35800) and APC1 (At5g61810.1), APC2 (At5g51050), and APC3 (At5g07320) were retrieved from the Phytozome database (http://www.phytozome.net/). Algae sequences and the sequences from *S. cerevisiae* and *H. sapiens* were obtained from a BLAST search of the NCBI nr protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [30]. Sequences were screened with ScanProsite (http://www.expasy.org/tools/scanprosite/) [31] and only those containing EF-hands were considered for further evaluation. Transmembrane domains were predicted by Aramemnon (http://aramemnon.botanik.uni-koeln.de/) [32]. Sequences (NCBI protein accession numbers listed in Supplementary Table 1) were aligned with the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [33] and visualized as phylogenetic tree. For substrate prediction of SAMTL, all plant and algae orthologs were aligned and the relative positions of the functional residues were compared to the sequence of the bovine ATP/ADP carrier. Residues that line the carrier cavity were identified, in particular the contact points of the predicted substrate binding site and the salt bridge networks. These were compared to known transporters to predict the nature of the substrate and the translocation mechanism [25,34].

2.3. Subcellular localization analysis of YFP-fusion proteins

Full-length coding sequences of APC1, 2 and 3 were cloned in the plant binary expression vector pBIN19 containing a C-terminal YFP-tag, transformed into AGL1 and infiltrated in tobacco leaves as described previously [35]. Tobacco protoplasts were prepared from infiltrated leaves two days after transfection according to [36] and stained with MitoTracker Red CMXRos (Molecular Probes; final concentration of 100 nM). Images were taken on a confocal laser scanning microscope Zeiss 510 model, with a Plan-Neofluar 40x/1.3 oil DIC objective.

2.4. Radiolabeled calcium overlay assays

Assays were performed as described earlier [37] with minor modifications using recombinant proteins expressed in *Escherichia coli*. The N-terminal parts of SAMTL (amino acids 1–424), APC1 (1–187), APC2 (1–200) and APC3 (1–188) were expressed and purified with the IMPACT™ System (New England Biolabs) that allows the purification of untagged proteins. After purification 2.5 and 0.25 μg of protein were spotted on PVDF membranes. As controls, the recombinant EF-hand protein aequorin and commercially available bovine serum albumin (BSA, New England Biolabs) were used. Membranes were incubated three times for 20 min at room temperature with buffer containing 60 mM KCl, 5 mM $MgCl_2$, 60 mM imidazole-HCl (pH 6.8), before incubation in the same buffer containing 0.1 μM $^{45}CaCl_2$ (13.90 mCi/mg; Perkin Elmer) and 0.1 mM 'cold' $CaCl_2$ for 10 min at room temperature. Membranes were subsequently washed for 5 min with 50% ethanol. Autoradiographs were visualized on a FUJI FLA-3000 (FUJIFILM). Membranes were subsequently stained with amido black.

2.5. Yeast functional complementation assay

Full-length coding sequences of APC1, 2 and 3 were cloned in the yeast expression vector YEp351 behind the constitutive methionine-repressible promoter (pMet25) and were transformed into W303 and Δsal1 yeast strains. An empty vector served as a negative control. Cultures were grown aerobically to an OD600 of 1.0 and were subsequently diluted to an OD600 of 0.1, 0.01, and 0.001. 5 μl of each dilution were plated on SD-Leu plates (pH 4.0) containing 0.0, 0.5 or 1 μM bongkrekic acid (BKA, Enzo Life Sciences) and the plates were incubated for 2 days at 30 °C according to [38].

3. Results

3.1. The calcium-binding MCF proteins form two phylogenetically distinct groups in plants

In a proteomic approach aiming at the identification of novel Ca^{2+}-binding proteins in the chloroplast, we identified recently a member of the MCF with unknown function containing one EF-hand, to be targeted to the chloroplast envelope and called it SUC (substrate carrier) [39]. To avoid confusion with the sucrose-H^+ symporters, named SUC1-9 [40], we will refer to this protein from now on as SAMTL, for SAM transporter-like. From the 58 other members of this family in Arabidopsis, three more MCF proteins contain EF-hands: APC1, 2 and 3 (ATP/Phosphate Carrier 1, 2 and 3) as referred to by Palmieri et al. [20], due to their high homology to the mitochondrial ATP-Mg/P_i carriers in yeast and human. These carriers have not been described experimentally before in Arabidopsis.

A phylogenetic analysis of the aforementioned Arabidopsis proteins with their orthologs in dicots, monocots, mosses, green algae, fungi and humans is shown in Fig. 1. Based on phylogenetic distance as well as on differences in their primary protein structure and functional residues in the predicted binding site, SAMTL and APC1, 2 and 3 are most likely not functionally related. SAMTL seems to be specific for higher plants, mosses and algae. No homologues proteins containing EF-hands could be found in the genomes of yeast and humans. The closest homolog found, using profile hidden Markov models of conserved portions of SAMTL, is the SAM transporter of plants (Arabidopsis SAMT1), animals and fungi. However, the SAM transporters do not contain EF-hands. *S. cerevisiae* has a single ortholog of the APC1, 2 and 3 proteins, named Suppressor of Aac2 Lethality 1 (SAL1; [29]), and humans have three orthologs, called Short Calcium-binding Mitochondrial