

Calcium regulation in endosymbiotic organelles of plants

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Key words: mitochondria, chloroplasts, calcium, calmodulin, EF-hand proteins

In plant cells calcium-dependent signaling pathways are involved in a large array of biological processes in response to hormones, biotic/abiotic stress signals and a variety of developmental cues. This is generally achieved through binding of calcium to diverse calcium-sensing proteins, which subsequently control downstream events by activating or inhibiting biochemical reactions. Regulation by calcium is considered as a eukaryotic trait and has not been described for prokaryotes. Nevertheless, there is increasing evidence indicating that organelles of prokaryotic origin, such as chloroplasts and mitochondria, are integrated into the calcium-signaling network of the cell. An important transducer of calcium in these organelles appears to be calmodulin. In this review we want to give an overview over present data showing that endosymbiotic organelles harbour calcium-dependent biological processes with a focus on calmodulin-regulation.

Introduction

Calcium has long been acknowledged as one of the most important signaling components in plants and is crucial for the activation of environmental stress responses as well as for the regulation of developmental processes.¹⁻⁵ Many abiotic and biotic signals are transduced into a cellular response by temporary and spatial changes in calcium concentration.⁶⁻¹⁰ These changes in calcium concentration serve as triggers for various calcium sensors, i.e., calcium-dependent protein kinases, calmodulin and calcineurin, usually by binding to a domain called the EF-hand.³ Despite the importance of calcium signaling in plants many aspects of this ubiquitous signaling pathway remain elusive.

A large variety of metabolic processes essential for plant viability take place in mitochondria and chloroplasts and thus their biogenesis and function has to be carefully balanced in accordance with the developmental stage and metabolic requirements. Therefore, both organelles are tightly integrated into diverse regulatory networks of the cell. Regulation by calcium is considered as a eukaryotic trait and has not been described for prokaryotes. Nevertheless, experimental evidence indicates the existence of calcium regulation in chloroplasts and mitochondria. However, the impact of calcium on mitochondrial and chloroplast processes

is not well understood. In this manuscript we will present an overview on the current knowledge about calcium regulation in chloroplasts and mitochondria with a special focus on calcium/calmodulin.

Calmodulin and Calmodulin-Binding Proteins

Calmodulins are important transducers of calcium signals and the Arabidopsis genome contains more than 50 genes coding for potential calmodulins and calmodulin-like proteins. Calmodulin is a relatively small and acidic protein and is found ubiquitously in eukaryotes.¹¹⁻¹⁵ It has a dumbbell-shaped structure and typically contains four EF-hands, which are located in pairs at the two globular ends of the folded protein.¹⁶ Calmodulins can interact with a wide range of calmodulin-binding proteins. Upon binding of calcium, the calmodulin molecule undergoes a conformational change that alters its affinity to the downstream target proteins. Most experimental data on calcium regulation in chloroplasts and mitochondria are due to the identification of calmodulin-binding proteins and the analysis of calmodulin-regulated processes.

Calcium/calmodulin regulation in chloroplasts. Several processes in chloroplasts have been shown to be affected by calcium/calmodulin. One of the most detailed studied examples of calcium regulation is the import of nuclear encoded chloroplast proteins.^{17,18} The majority of chloroplast proteins are synthesized in the cytosol and are post-translationally transported into the organelle by means of two translocation machineries, the TOC (translocon at the outer envelope of chloroplasts) and TIC (translocon at the inner envelope of chloroplasts) complex utilizing an N-terminal cleavable transit peptide (reviewed in refs. 19 and 20). The protein import via TOC and TIC complexes is a highly regulated and energy-dependent process which requires ATP and GTP. The translocation at the TOC complex is regulated by GTP/GDP-binding and by phosphorylation. For the TIC complex a redox regulation was proposed on the basis of the properties of its proteinaceous components.

Inhibition analysis of the import into chloroplasts revealed a new regulation at the TIC complex through calcium.¹⁸ The nature of the inhibitors used in that study suggested that the mediator of this regulation is the calcium-sensor calmodulin. Subsequently, Tic32, a component of the TIC translocon, was identified as a calmodulin-binding protein.¹⁷ Binding between calmodulin and Tic32 was shown to occur in a calcium-dependent manner and the calmodulin-binding domain could be identified. These results indicated that calcium-regulation of chloroplast protein import takes place in the inside of the organelle.

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Submitted: 06/06/09; Accepted: 06/08/09

Previously published online:

www.landesbioscience.com/journals/psb/article/9234

Interestingly, Tic32 has also been proposed as a site for redox-regulation since it is a bona fide NADPH-dependent dehydrogenase.^{17,21} NADPH binding to Tic32 affects the assembly of the TIC complex, which subsequently would result in chloroplast import inhibition. Since binding of calmodulin and NADPH to Tic32 was shown to be mutually exclusive, the calcium-dependent binding of calmodulin to Tic32 would in contrast promote the interaction of Tic32 with the TIC complex and thereby provide the base of the positive effect of calmodulin on the protein import into the chloroplast.¹⁷

Such bipartite regulation was also shown in a recent study for Tic110, the pore-forming protein of the TIC complex.²² Electrophysiological characterisation of Tic110 revealed properties of a cation-selective channel sensitive to chloroplast transit peptides.²³ Balsera and co-workers demonstrated that addition of calcium drastically affected the channel activity of Tic110 in vitro thereby changing the gating properties of the channel.²² Additionally, the authors proposed Tic110 to be a site for redox-regulation since it harbours a redox-active disulfide bridge which may influence the gating behavior of the pore and therefore protein import. Thus, the physiological involvement of calcium in the protein import process seems to be a result of a dual effect on Tic32 and Tic110. Furthermore, the findings support the idea that Tic32 and Tic110 could serve as a link between two different signaling pathways, calcium and redox state, to differentially regulate the protein import process in chloroplasts.

While protein import is the most extensive studied example for calcium regulation in chloroplasts, there is evidence for other plastidal processes that are regulated in this fashion. It has been shown that vesicular transport events in the cytosol are affected by inhibitors of calmodulin as well as by calcium antagonists at the stage of membrane fusion.²⁴ A similar sensitivity was shown for the vesicle transport system inside chloroplasts. Application of calmodulin inhibitors as well as calcium depletion leads to accumulation of vesicle-like structures in the stroma of the chloroplasts.^{25,26} While very little is known so far about the cargo and function of this transport system inside chloroplasts, a role in the biogenesis of the thylakoid membrane was proposed.²⁵⁻²⁹

Calcium regulation of thylakoid formation ties in very well with a further chloroplast process which seems to be regulated by calcium: oxygenic photosynthesis. Using inhibitors of calmodulin as well as calcium-chelators Jarrett et al.³⁰ demonstrated that NAD kinase is activated by calcium/calmodulin. NAD kinase regulates the light-induced conversion of NAD to NADP. This is important for photosynthesis since NADP is the terminal electron acceptor of photosystem I. A further study to test the phosphorylation of thylakoid membrane proteins suggested a regulation of thylakoid kinase(s) by calcium and calmodulin.³¹ Reversible phosphorylation of thylakoid membrane proteins has been shown to be mainly influenced by light and temperature and is implicated in various adaptive and regulatory responses of the photosynthetic machinery. In plants, phosphorylation and calcium-regulation are often directly tied together by the action of calcium-dependent protein kinases^{32,33} but so far there is no experimental evidence that members of this protein family are present in chloroplasts.

Several more potential targets for calcium regulation in chloroplast were identified by a screen for calmodulin-binding proteins in *Arabidopsis thaliana*.³⁴ Chloroplast proteins identified in this screen include PsaN, a component of photosystem I, the chaperonin Cpn10, a AAA-ATPase and the ATPase ACA1. Cpn10 had previously been shown to bind calmodulin in a calcium-dependent manner corroborating the results of the screen.³⁵ Chloroplast Cpn10 is a homolog of the bacterial GroES and seems to be involved, together with the chaperonin Cpn60, in the assembly of the ribulose-1,5-bisphosphate carboxylase/oxygenase complex, the key enzyme in photosynthetic CO₂-fixation. ACA1 is a Ca²⁺-ATPase found in the chloroplast envelope and appears to have a function as a Ca²⁺-transporter.³⁶ In contrast to these proteins, the specific function of the AAA-ATPase remains so far elusive.

A new study recently discovered a AAA⁺-ATPase (AFG1L1) as a calmodulin target protein in *A. thaliana*.³⁷ AAA⁺-ATPases (ATPases associated with a variety of cellular activities) are ubiquitously found in all kingdoms and play an essential role in protein degradation, DNA replication and homo- and heterotypic membrane fusion.^{38,39} Interaction of AFG1L1 with calmodulin occurs in a calcium-dependent manner and the calmodulin-binding domain of AFG1L1 is localized within the N-terminal region of the catalytic AAA-domain, which is necessary for ATP binding and hydrolysis. While homologues of AFG1L1 can be found in all kingdoms of life, the calmodulin-binding domain is present exclusively in plants indicating that the ability of AFG1L1 to bind calmodulin is an evolutionary novel, plant-specific trait. The study on AFG1L1 furthermore revealed that the protein is present in both endosymbiotic organelles, chloroplast and mitochondria. Dual localization of AFG1L1 as calmodulin-binding protein thereby provides a means to regulate a specific function in both organelles in a concerted fashion via incorporation into the calcium-signaling network of the cell. It also provides evidence for the presence of calcium/calmodulin regulation in plant mitochondria (see below).

Calcium/Calmodulin Regulation in Mitochondria

Compared to chloroplasts, the examples for calmodulin-mediated calcium regulation in mitochondria are less abundant. A recent study showed that the import of nuclear encoded proteins into plant mitochondria, similar to chloroplasts, is influenced by calcium and calmodulin.⁴⁰ As their chloroplast counterparts, most mitochondrial proteins are encoded in the nucleus and are post-translationally translocated into the organelle and sorted into the respective sub-compartment. Proteins residing in the mitochondrial matrix and the inner membrane are translocated by the TIM23 and/or TIM22 complex (reviewed in refs. 41 and 42). Kuhn and co-workers showed that calmodulin inhibitor ophiobolin A as well as the calcium ionophores A23187 and ionomycin inhibited translocation across both translocons.⁴⁰ This effect could be counteracted by addition of external calmodulin or calcium, respectively. Interestingly, the authors exclusively observed this inhibition of protein translocation for plants but not for protein translocation into mitochondria of yeast. Therefore, calcium/calmodulin regulation of protein import into mitochondria

seems to be a plant-specific trait. The mediator of this regulation so far remains elusive.

Inhibitor studies using calmodulin antagonists showed that the mitochondria pyruvate dehydrogenase complex in *P. sativum* is activated by calmodulin.⁴³ The authors observed the influence of calmodulin either using the partially purified pyruvate dehydrogenase complex or isolated intact mitochondria. The complex assists in the conversion of pyruvate into acetyl-CoA. This component is used in the citric acid cycle, which provides reduction equivalents for the mitochondrial respiratory chain.

The studies on protein import indicate certain similarity in calcium regulation in plant chloroplasts and mitochondria. This is further supported by studies in *Z. mays* and *A. sativa* revealing that a calcium/calmodulin-regulated NAD kinase isoform is located in mitochondrial membranes. NAD kinase catalyzes the synthesis of NADP by phosphorylation of NAD and controls cellular metabolism through the intracellular concentration of NADP.^{44,45} A study by Dieter and Marmé showed that the regulatory calmodulin-binding domain of NAD kinase, which is located in the mitochondrial outer membrane, faces the cytosol. According to the authors, this provides a means to change the activity of the NAD kinase by its ability to sense changes in the cytosolic free calcium concentration. Two other studies however, support the localization of a calcium/calmodulin-regulated NAD kinase within the inner membrane of mitochondria.^{46,47} This agrees with the fact that NADP is of physiological importance within the mitochondrial matrix. Moreover, this localization is also supported by the isolation of a calmodulin from *A. sativa* mitochondria, which is localized in the intermembrane space of mitochondria.⁴⁸

Calcium-Binding and EF-Hand Proteins

Most proteins that function as sensors of calcium signals contain a common structural motif, termed “EF-hand”, which is a helix-loop-helix structure that binds a single Ca²⁺ ion. A bioinformatic approach analysing the entire genome of *Arabidopsis thaliana* revealed that over 250 proteins contain such EF-hand motifs,⁴⁹ including proteins involved in transcription and translation, protein- and nucleic-acid-binding proteins and a large number of unknown proteins. These proteins likely modulate the activity of cellular and developmental processes via calcium binding. Among these putative calcium binding proteins, several are predicted to be targeted to the chloroplasts as well as to mitochondria. For most of these proteins, neither the cellular localization nor the calcium binding has yet been verified experimentally. In case of a mitochondrial-localized glutamate dehydrogenase, the presence of potential EF-hands had been annotated before⁵⁰ and the localization in mitochondria has been corroborated by MS/MS,⁵¹ indicating that some of the proteins identified by the

bioinformatic approach represent good candidates for organelle-localized calcium-binding proteins.

In addition to these in silico data, experimental evidence for chloroplast localized EF-hand proteins has been obtained.⁵² Tozawa and co-workers showed the presence of a calcium-activated (p)ppGpp synthetase in chloroplasts. The protein contains two EF-hand motifs in its C-terminus and represents an interesting connection between calcium and (p)ppGpp mediated signaling in the organelle. Furthermore, type II NAD(P)H:quinone oxidoreductases of *A. thaliana*, proteins shown to be attached to the inner and outer surface of the inner membrane of mitochondria, also contain EF-hand motifs⁵³ indicating that oxidation of cytosolic NADH and NADPH within plant mitochondria might be regulated by calcium.

Not all proteins bind calcium via EF-hand motifs and several such proteins have been identified in both mitochondria and chloroplasts by their ability to bind calcium. An example for such different calcium regulation is CaS, a protein originally identified as a calcium sensing protein of the plasma membrane.⁵⁴ In vitro analysis showed the ability for calcium binding probably through a low-affinity calcium-binding site at its N-terminus. Later on it was shown that CaS resides in the chloroplast and is an extrinsic protein of the thylakoid membrane, where it is subjected to light-dependent phosphorylation.⁵⁵ Analysis of T-DNA mutant lines suggested that CaS plays a role in signaling cascades that coordinate growth and the response to environmental cues. Alternative studies suggested a role in calcium regulated stomatal responses.⁵⁶ While the exact role of the protein remains to be elucidated it provides further evidence for the inclusion of the chloroplast into the calcium signaling pathway via direct calcium binding of chloroplast proteins.

Outlook

All in all, recent data and previous results show that direct calcium regulation takes place in both endosymbiotic organelles. Future studies will have to show which environmental or developmental changes are transduced into the organelles via the calcium signaling pathway. Moreover, the calmodulins that mediate calcium regulation have to be identified. Biro and co-workers⁴⁸ have purified a protein with features characteristic of calmodulin from *A. sativa* mitochondria and similarly, Jarrett and co-workers⁵⁰ enriched a potential calmodulin from chloroplasts of *P. sativum*. In neither case was the protein clearly identified or further characterized.⁴⁸ Nevertheless, several of the over 50 calmodulin and calmodulin-like proteins encoded in the Arabidopsis genome contain potential targeting sequences for mitochondria and chloroplasts but their exact intracellular localization remains to be elucidated.

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