Minireview

Calmodulin-like proteins, CMLs: New players in plant defense regulation?

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Introduction

In natural ecosystems plants continuously face the presence of other living organisms such as pathogens and pests (Figure 1A) and this interaction is complex (Mithöfer et al. 2009). Among others, plants have developed a sophisticated system to deal with challenges coming along with herbivores. Plants perceive herbivore-derived physical (mechanical wounding) and chemical cues, such as elicitors in insects' oral secretions and in oviposition fluid, which are collectively referred to as herbivore-associated molecular patterns (HAMPs) (Mithöfer et al. 2008). In order to defend themselves against insect herbivores, plants dramatically reshape their transcriptomes, proteomes, and metabolomes (Wu et al. 2010). Phytohormones, anti-herbivore secondary metabolites like alkaloids and proteins with anti-nutritional properties are produced by the plant to deal with diverse environmental stresses (Mithöfer and Boland 2012). Those defensive compounds from the plant can act as toxins, target physiological processes in the insect and thus affect insect survival. One of the most popular examples is that of the alkaloid nicotine, which is produced in tobacco plants. Nicotine acts as an acetylcholine receptor agonist and is highly toxic to herbivores (Steppuhn et al. 2004; Mithöfer and Boland 2012).

Plant defense response against herbivory is tightly coordinated by a network of interacting phytohormones, in particular jasmonic acid (JA) dependent signaling pathways. Jasmonates represent a family of jasmonic acid derivatives that regulate plant response to biotic stress as well as growth and development. Jasmonic acid is synthesized from an unsaturated fatty acid, linolenic acid. Synthesis of JA is initiated with the three step conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA). OPDA then undergoes a reduction and three rounds of β-oxidation to form (+)-7-*iso*-JA. JA is further conjugated to the amino acid, isoleucine (Ile) to form the bioactive form JA-Ile, (+)-7-*iso*-Jasmonoyl-*L*-isoleucine. Bioactive JA-Ile further binds to jasmonate receptor COI1. This F-box protein, coronatine insensitive 1 (COI1), is a component of the SCF E3 ubiquitin ligase complex and mediates jasmonate signaling by degradation of JA transcriptional repressor JAZ proteins. JAZ degradation relieves the repression of transcription factor MYC2 which in turn activates JA-responsive genes (Fonseca et al. 2009). Although much is known about downstream JA pathways, the initial recognition process in plant-herbivore interactions and the mediating signal transduction pathways connecting it to downstream defense induction are less understood.

Calcium signaling

Calcium ions (Ca²⁺) play a crucial role in almost every aspect of life. Ca^{2+} is a key second messenger and important component of signal transduction machinery in many cellular processes. In plants, Ca^{2+} plays a vital role during various developmental processes and in response to environmental stimuli (Clapham 2007). Calcium ion concentration in the cell is tightly regulated as high concentration of Ca^{2+} can form insoluble complexes with proteins, membranes and organic acids and is thus toxic to cells. Ca^{2+} concentration in the cytosol is very low, in the range of nM, with higher levels of Ca^{2+} (mM) in the apoplast and organelles like ER and vacuole where they are stored (Clarkson et al. 1988; Bush et al. 1989). Upon perception of a stimulus by receptors, Ca^{2+} channels open to release Ca^{2+} from different stores into the cytosol and these elevations in cytosolic Ca^{2+} activate signaling pathways leading to specific responses. Calcium ions are involved in many kind of abiotic and biotic stress responses in plant (Sanders et al. 1999). Change in cytosolic free Ca^{2+} occur in response to various biotic and abiotic signals (Sanders et al. 2002). Biotic signals like phytohormones, e.g. abscisic acid (ABA), jasmonates (Walter et al. 2007) and gibberellins (Gilroy et al. 1992), pathogens (Mithöfer et al. 1999; Blume et al. 2000) and the growth promoting endophytic fungus *Piriformospora indica* (Vadassery et al. 2009) act on Ca^{2+} concentrations in the cell. Abiotic stress factors like touch and light can change the Ca^{2+} concentration (Sai et al. 2002). Changes in cytosolic free Ca^{2+} concentration was also detectable in response to drought stress (Knight et al. 1997). Many more stimuli that evoke rapid change in cytosolic calcium concentration are reviewed (Knight et al. 2001; Rudd et al. 2001). In general, Ca^{2+} acts as a second messenger in plant cells and at the same time links many input signals to many divers and specific responses.

Early events in plant-insect interaction include damage-induced ion imbalances, causing variations in membrane potentials, Ca^{2+}-signaling, and production of reactive oxygen species, leading to phytohormone elevation and activation of defense (Maffei et al. 2007). Feeding of lepidopteran *Spodoptera littoralis* larvae on lima bean (*Phaseolus lunatus*) leaves cause increase of intracellular Ca^{2+} concentration (Maffei et al. 2004). Ca^{2+} elevation is also measured further downstream in herbivore-induced signaling cascades and it is reported that jasmonates and synthetic jasmonate analogues induce Ca^{2+} elevations in tobacco BY-

2 cell culture either in the cytosol or in the nucleus or in both compartments (Walter et al. 2007; Mazars et al. 2009). Moreover grasshopper herbivore *Schistocerca gregaria*- and *Spodoptera littoralis* -derived oral secretion treatment resulted in increased cytosolic Ca^{2+} concentration within a few seconds and JA accumulation in *Arabidopsis thaliana* (Schaefer et al. 2011; Vadassery et al. 2012a).

Calcium sensor proteins

Various stimuli can cause changes in cellular Ca^{2+} concentration. The activation time, amplitude, frequency and localization varies according to the particular stimulus, and this is supposed to be a unique Ca^{2+} signature (Sanders et al. 2002; Batistič et al. 2012). Subsequently, such a change of Ca^{2+} intracellular concentrations can be recognized via calcium sensing proteins, which evoke cellular responses and determine further specificity (Figure 1B). Calcium sensors can be divided in two types, sensor relays and sensor responders. Sensor relays like calmodulin (CaM) and calmodulin-like proteins (CMLs) possess no enzymatic activity. They undergo Ca^{2+}-induced conformational change and relay the information to an additional interacting partner. This information then can be further transmitted through enzymatic activity or conformational change of the

particular interacting partner. The second type of calcium sensors, sensor responders, undergoes Ca^{2+}-induced conformational change and this causes a change in its own enzymatic activity and results in specific responses. Ca^{2+}-dependent protein kinases belong to the class of sensor responders. Sensor relays function through bimolecular interactions, sensor responders however function by intermolecular interactions (Sanders et al. 2002).

250 EF-hand- containing proteins have been identified in Arabidopsis. Those proteins include protein binding proteins, proteins in the processes of transcription and translation (Day et al. 2002). EF-hand is a prevalent Ca^{2+} binding motif in Ca^{2+} binding proteins. An EF-hand is composed of typical helix-loop-helix structure. A specific 12-amino acids Ca^{2+}-binding loop bridges two α-helices (Gifford et al. 2007). It is currently hypothesized that EF-hand containing proteins undergo Ca^{2+}-induced conformational change that makes it possible to regulate their target proteins and in this way co-ordinate many signaling pathways. The three main classes of this EF-hand calcium sensor family of calcium binding proteins are calmodulins (CaMs) including CML proteins, calcium dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs) (DeFalco et al. 2010).
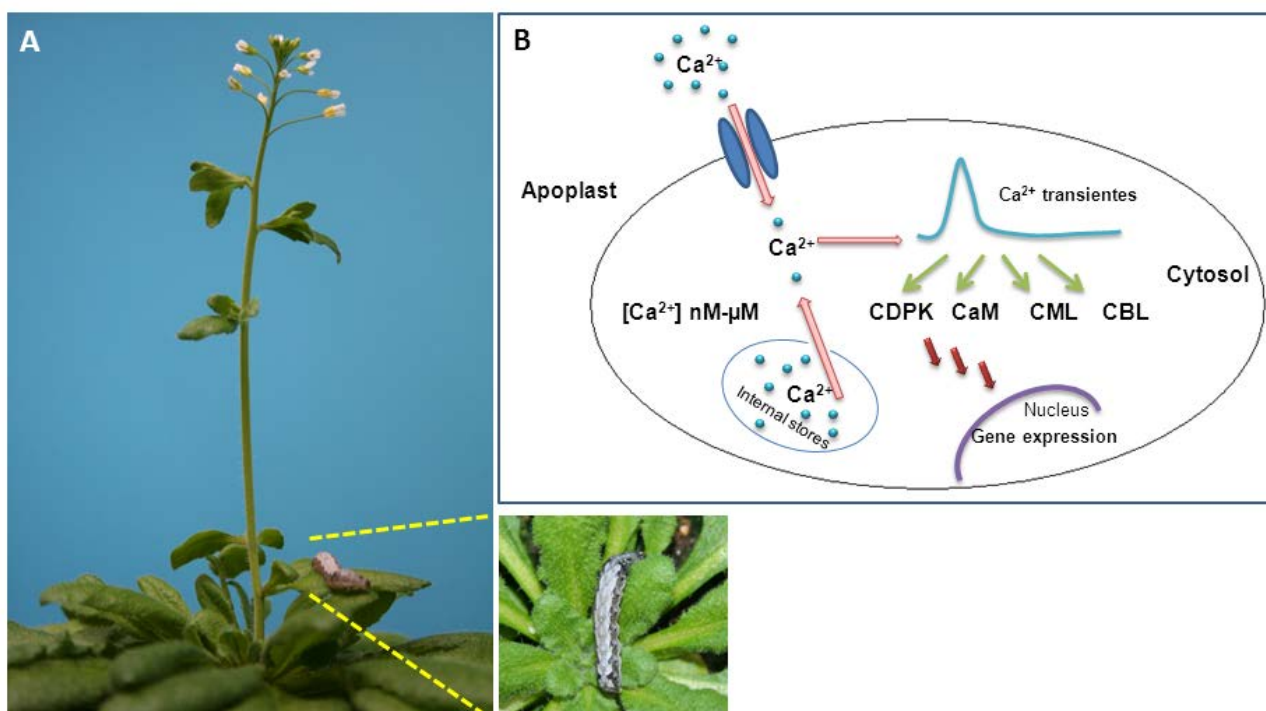

Figure 1: (A) *Spodoptera littoralis* larva feeding on *Arabidopsis thaliana*. (B) Simplified model of calcium signaling in plant cells induced by external stimuli. Upon an external stimulus, cytosolic Ca^{2+} level increase due to influx from apoplasm and internal stores, thereby generating signal-specific Ca^{2+} transients, which can modulate the activities of downstream Ca^{2+} sensor proteins and finally regulate gene activation. CaM: calmodulin; CML: calmodulin-like proteins; CDPK: calcium dependent protein kinase; CBL: calcineurin B-like proteins.

Calmodulin-like proteins, CMLs

A family of 50 genes was identified in Arabidopsis, which encode for CMLs. CMLs belong to the sensor relay family of calcium sensor proteins. Those proteins have no identifiable functional domains and share at least 16% amino acid identity with CaM and have 2-6 identifiable EF-hand motifs (McCormack et al. 2003). CMLs differ from CaMs in following ways: target specificity, subcellular localization and affinity for calcium (Luan et al. 2002; Zielinski 2002). Dif-

ferent CML proteins have different role in stress perception and plant development (McCormack et al. 2005). It was reported that biotic and abiotic stress, phytohormone and chemical treatment regulate transcripts of *CML37*, *CML38*, and *CML39* (Vanderbeld et al. 2007). CML24 seems to be involved in pathogen-induced innate immune responses (Ma et al. 2008). Moreover, CML24 has a function in responses to ABA, day length and ion stress (Delk et al. 2005). This is similar to CML9, which is induced by abiotic stress

and ABA in young Arabidopsis seedlings; using *cml9* knock-out mutants it was demonstrated that CML9 has a function in modulating responses to salt stress and ABA (Magnan et al. 2008). CML43 and, again, CML9 are involved in plant defense by modulating responses to bacterial strains of *Pseudomonas syringae* (Chiasson et al. 2005; Leba et al. 2012). In addition, expression of CML9 is rapidly induced by phytopathogenic bacteria, flagellin and salicylic acid (Leba et al. 2012).

Spodoptera littoralis feeding on Arabidopsis leaves (Figure 1A) or mimicking herbivory with oral secretion treatment results in cytosolic calcium ion elevation as well as in induction of a set of different CML-genes: *CML9, 11, 12, 16, 17, 23, 42*. These genes belong to two groups that respond with different kinetics to the treatment with oral secretion, early and transiently expressed CMLs- (*CML11, 12, 16* and *42*) and late and sustained expressed CMLs-(*CML9, 17* and *23*) (Vadassery et al. 2012b).

CML42 was the first member of the CML- family that was identified as herbivory-related (Vadassery et al. 2012a). CML42 shares ~ 35% sequence identity with CaM. Three molecules of Ca^{2+} can bind to this 191-amino acid protein, which displays a classical α-helical secondary structure and has Ca^{2+}-binding affinities ranging from 30 to 430 nM (Dobney et al. 2009). Functional analysis revealed that CML42 acts as a negative regulator of plant defense against *S. littoralis,* and plant defense is increased in *cml42* knock out lines. The mechanism of negative defense regulation is coordinated via increased COI1 mediated JA perception. Upon *S. littoralis* feeding, JA-responsive genes *VSP2* and *Thi1.2* are highly up-regulated in *cml42* mutants, and constitutive glucosinolate levels also increase. Apart from its role in insect herbivory it also functions in many abiotic stress response pathways. ABA accumulation upon drought stress is higher in *cml42* lines then in WT. On the other hand, flavonol accumulation is lower in *cml42* lines and they have decreased UV-B tolerance compared to WT plants (Vadassery et al. 2012a). In addition, CML42 is involved in the regulation of trichome branching in Arabidopsis, because knock out mutants show increased number of branches (Dobney et al. 2009). CML42, thus, has multiple functions in plant cells upon perception of different stimuli, but how a specific response is determined remains unknown. Different signal transduction pathways could play crucial role in the various physiological responses. Because sensor relay proteins such as CML42 as well as other CMLs have no enzymatic function and their ability to be involved in multiple biotic and abiotic stress responses is through interaction with different target proteins, those proteins have to be identified in future studies to understand the role and function of CMLs in plant defenses. In addition, both biochemical and physiological characterization of these calcium sensor proteins would unravel specificity within the CML family.

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