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Review

Experimental and computational approaches for the study of calmodulin interactions

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ABSTRACT

Ca^{2+}, a universal messenger in eukaryotes, plays a major role in signaling pathways that control many growth and developmental processes in plants as well as their responses to various biotic and abiotic stresses. Cellular changes in Ca^{2+} in response to diverse signals are recognized by protein sensors that either have their activity modulated or that interact with other proteins and modulate their activity. Calmodulins (CaMs) and CaM-like proteins (CMLs) are Ca^{2+} sensors that have no enzymatic activity of their own but upon binding Ca^{2+} interact and modulate the activity of other proteins involved in a large number of plant processes. Protein–protein interactions play a key role in Ca^{2+}/CaM-mediated in signaling pathways. In this review, using CaM as an example, we discuss various experimental approaches and computational tools to identify protein–protein interactions. During the last two decades hundreds of CaM-binding proteins in plants have been identified using a variety of approaches ranging from simple screening of expression libraries with labeled CaM to high-throughput screens using protein chips. However, the high-throughput methods have not been applied to the entire proteome of any plant system. Nevertheless, the data provided by these screens allows the development of computational tools to predict CaM-interacting proteins. Using all known binding sites of CaM, we developed a computational method that predicted over 700 high confidence CaM interactors in the Arabidopsis proteome. Most (>600) of these are not known to bind calmodulin, suggesting that there are likely many more CaM targets than previously known. Functional analyses of some of the experimentally identified Ca^{2+} sensor target proteins have uncovered their precise role in Ca^{2+}-mediated processes. Further studies on identifying novel targets of CaM and CMLs and generating their interaction network – "calcium sensor interactome" – will help us in understanding how Ca^{2+} regulates a myriad of cellular and physiological processes.

Contents

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1. Introduction

Ca^{2+} is an important messenger for relaying signals and regulating many aspects of plant growth and development and plant responses to stresses. A change in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is implicated in the regulation of diverse physiological processes and plant defense and stress responses (Hepler, 2005; Reddy, 2001; White and Broadley, 2003). The cell signaling process via Ca^{2+} has three phases: generation of a Ca^{2+} signature, sensing the signature and transduction of the signal (Reddy and Reddy, 2004). The Ca^{2+} concentration in the cytoplasm is maintained at a low concentration (100–200 nM), while in the cell wall and vacuole, the concentration can be millimolar (Hirschi, 2004; Reddy and Reddy, 2004; Rudd and Franklin-Tong, 2001; McAinsh and Pittman, 2009 #2771). The generation of a cellular Ca^{2+} signature in response to signals is orchestrated by Ca^{2+} channels that allow Ca^{2+} to flow from the stores to the cytoplasm and then by Ca^{2+} pumps restoring the resting $[Ca^{2+}]_{cyt}$ (McAinsh and Pittman, 2009). The change in $[Ca^{2+}]_{cyt}$ is sensed by Ca^{2+}-binding proteins, the Ca^{2+} sensors (Day et al., 2002; Dodd et al., 2010; Reddy and Reddy, 2004). The sensors themselves may become active and transduce the signal by their activity or may bind to other proteins affecting their activity and, thereby, transduce the signal through their interacting proteins.

A majority of Ca^{2+} sensors have one or more EF-hand motifs (InterPro IPR002048) that are responsible for binding Ca^{2+}. Each EF hand is composed of two helices (E and F) flanking a Ca^{2+}-binding loop capable of binding one Ca^{2+} ion. Two other types of proteins have been shown to bind Ca^{2+}, annexins and C2-domain proteins, and a few other proteins with novel sequences have also been shown to bind Ca^{2+} (Reddy and Reddy, 2004).

Bioinformatics analysis of EF hand-containing proteins in Arabidopsis and rice has revealed a large number of putative Ca^{2+} sensors (Boonburapong and Buaboocha, 2007; Day et al., 2002). A phylogenetic analysis of the Arabidopsis EF-hand proteins revealed the presence of six major groups, which were further classified into several subfamilies of proteins (Boonburapong and Buaboocha, 2007; Day et al., 2002). A major group of EF-hand proteins includes calmodulin (CaM) and CaM-like proteins (CMLs). CaM is an acidic EF-hand protein with no catalytic activity of its own. The prototype CaM found in all eukaryotes is 148 amino acids long with two globular domains, each containing two EF hands connected by a long flexible helix (Chin and Means, 2000). CML proteins are defined as proteins composed of EF hands and no other known or identifiable functional domains, and that share at least 16% amino acid identity with CaM (McCormack et al., 2005). There are seven *CaM* and 50 *CML* genes in Arabidopsis (Supplementary Table 1). Upon binding Ca^{2+}, CaM and CMLs can bind to target proteins involved in many cellular processes modulating their activity (Bouche et al., 2005). McCormack et al. (2005) reviewed expression analysis of the *CaM* and *CML* genes and found that while most *CaMs* are expressed somewhat similarly, the *CMLs* have a greater difference in expression at different developmental stages and under different conditions.

The CaM-binding domain of target proteins is composed of 12–30 contiguous amino acids with positively charged amphiphilic characteristics that tend to form an alpha-helix upon binding to CaM (Bouche et al., 2005; Reddy and Reddy, 2004). However, the amino acid sequence in different CaM-binding proteins (CBPs) is variable and some CBPs do not have typical motifs, and so the identification of unique CBPs does not lend itself to database searches. The proteomic challenge is to identify all CBPs and CML binding proteins (CMLBPs) and study the specificity of their interacting partners. This will provide the information to construct a "calcium sensor interactome" and further our understanding of CaM/CBL-regulated processes.

2. Calmodulin interacting proteins

CaM, one of the best characterized Ca^{2+} sensors, has been studied extensively in animals and plants. Many proteins that are the targets of CaM have been identified in numerous expression screens with labeled CaM. The targets have been found to be involved in regulation of transcription, metabolism, ion transport, protein folding, cytoskeleton-associated functions, protein phosphorylation and dephosphorylation and phospholipid metabolism (Bouche et al., 2005; Reddy and Reddy, 2004; Yang and Poovaiah, 2003). Some CBPs are common in plants and animals while others are specific to either animals or to plants (Reddy et al., 2002; Yang and Poovaiah, 2003). Animal studies have shown three types of CaM-activation mechanisms for target proteins (Hoeflich and Ikura, 2002). One is relief of autoinhibition with the CaM binding site adjacent to or within the autoinhibitory domain of the target. The second is active site remodeling and the third is CaM-induced dimerization.

There are many experimental approaches to study protein–protein interactions (PPIs) (Reddy and Reddy, 2004; Shoemaker and Panchenko, 2007). However, only a few of these (see below) have been used extensively to identify CaM interactors. Since the interaction of CaM with its targets is dependent on Ca^{2+}, the yeast two-hybrid (Y2H) method, a widely used approach to identify direct protein–protein interactions, is not an ideal system because of the problems associated with cellular Ca^{2+} manipulation during the screening process. CaM-binding proteins in the human proteome and *Caenorhabditis elegans* were identified using the mRNA display technique (Shen et al., 2008, 2005). Although this is a powerful method for discovering the targets of not only CaM, but also other Ca^{2+} sensors, it has not been used in plants. TAP, a mass spectrometry-based approach for identifying interacting proteins that form complexes (Rohila et al., 2004, 2006) has the potential to uncover proteins that complex with CaM/CML binding proteins. So far, this approach has not been applied to CaMs/CMLs.

2.1. Isolation by screening expression libraries

Historically the method used to identify CaM-binding proteins was to screen an expression library with ^{125}I or ^{35}S labeled CaM

(Fromm and Chua, 1992; Reddy et al., 1993, 1996b; Sikela and Hahn, 1987). Biotinylated or horseradish peroxidase (HRP)-labeled CaM was also used for identifying CBPs (Fordham-Skelton et al., 1994; Lee et al., 1999; Safadi, 2000). The labeled CaM methods use expression libraries grown on plates and induced to express protein. The plaques are overlaid with filters containing IPTG to induce expression. The filters are lifted and probed with labeled CaM. Positives are picked and rescreened through additional rounds and then the positive clones are rescued and sequenced (See Fig. 1). Verification of CaM binding in a Ca^{2+}-dependent manner has been done in a variety of ways including CaM overlay assays, gel shift assays, CaM immunoprecipitation or using CaM Sepharose columns (O'Day, 2003; Reddy and Reddy, 2004). Fig. 2 shows examples of the CaM overlay, gel shift, and CaM Sepharose column assays.

Early screens of Arabidopsis expression libraries identified interacting proteins such as GAD (Arazi et al., 1995; Bouche et al., 2004), KCBP (Reddy et al., 1996a,b) and EICBP also called CAMTA3 or SR1 (Reddy et al., 2000; Yang and Poovaiah, 2002a) and many others (Bouche et al., 2005; Yang and Poovaiah, 2003). A more global effort at identifying CBPs in Arabidopsis was done using the labeled CaM method to identify new CBPs by screening a number of expression libraries prepared from different tissues/plant parts that were exposed to hormones and other signals, and then searching the Arabidopsis genome with sequences of the newly identified proteins and known plant and animal CBPs (Reddy et al., 2002). Several expression libraries from flower meristem, seedlings or tissues treated with hormones, an elicitor, or a pathogen were used. Of the twenty identified CBPs in the search, 14 were previously unknown. Searching the genome revealed a total of 27 CBPs in Arabidopsis. Sixteen of the CBPs are represented by families with 2–20 members while 11 were single gene-encoded CBPs, totaling about 100 proteins. Thirteen of the 27 protein types were plant specific and 29 animal CBPs were not found in the Arabidopsis genome reflecting the differences in plant and animal Ca^{2+}/CaM signaling. Although this screen and database search revealed many new CBPs, it was not exhaustive and more CBPs have been found in CaM labeled screens using a protein microarray.

2.2. Identification of CBPs using a protein chip

Given that there are 7 CaMs and 50 CMLs in Arabidopsis, screening libraries is too laborious to provide global identification of the Ca^{2+} sensor interactome. Protein chip technologies are providing a platform for large-scale analysis of proteins in plants. Popescu et al. (2007) developed a protein microarray to screen with CaM and CML proteins. A key element of the protein microarray was that the proteins were expressed in plants rather than bacteria or yeast. Using a *Nicotiana benthamiana* transient expression system they expressed 1133 Arabidopsis ORFs and isolated and purified the proteins for use on the microarray chip. The chips containing the 1133 Arabidopsis proteins, mostly transcription factors and signaling proteins, were then probed in the presence of Ca^{2+} with CaM1, CaM6, CaM7, CML8, CML9, CML10, or CML12. A total of 173 targets were identified, 122 interacted with CaM1, 99 with CaM6, 117 with CaM7, 77 with CM8, 113 with CML9, 102 with CML10, and 86 with CML12. Six of nine known Arabidopsis homologs of CaM targets including SAUR (At5g20810), phosphofructokinase (At5g47810), diacylglycerol kinase (At5g63770), Hsp70-1 (At5g02500), TGA3 (At1g22070) and WRKY21 (At2g30590) were identified as CaM/CML binding but three (At4g30360-CNGC-like.m, At5g60390-EF-1a.a, and At3g094490-CNGC4.d) were not detected. The identified targets included 70 putative intracellular and receptor protein kinases, 60 transcription factors, and 43 other classes of proteins. To validate their data, coimmunoprecipitation assays were performed for 20 target proteins with extracts from plant cells coexpressing a Ca^{2+} sensor and its target. They were able to coimmunoprecipitate 17 of the targets with at least one of the CaM/CML proteins. Several previously unknown targets were verified including CBL-interacting protein kinase (CIPK) 6 and CIPK24, which is interesting in that CBLs are Ca^{2+}-binding proteins (Kolukisaoglu et al., 2004). Also the binding of CaM/CML to two Ca^{2+}-dependent protein kinases (CDPK6 and 30) was verified (Popescu et al., 2007). Results with the protein chip show that novel CaM-binding proteins can be identified using this high throughput method. Extension of this approach to the whole proteome is likely to result in many more targets of CaMs and CML proteins. Using the above approaches about 200 proteins

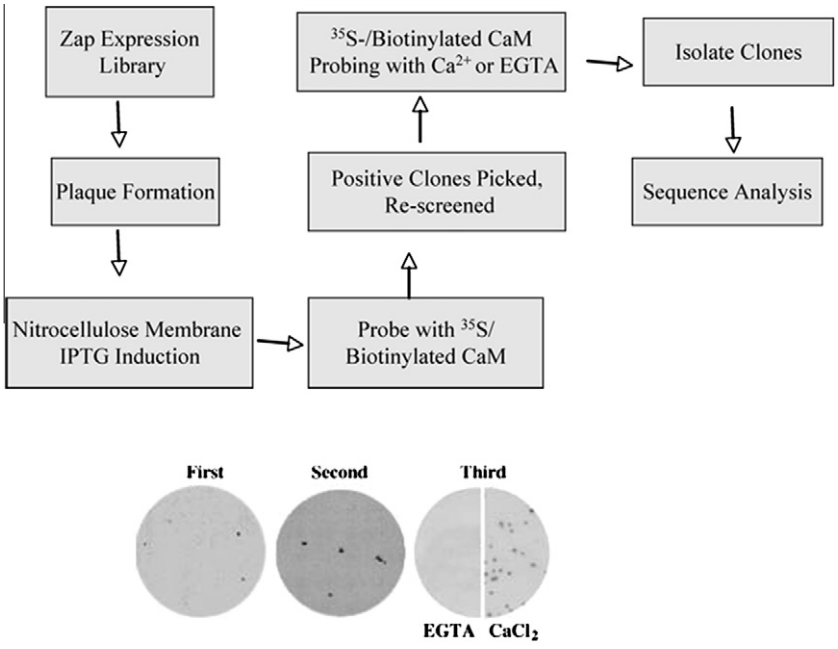

Fig. 1. Screening of expression libraries for CaM targets. A flow sheet describes the labeled CaM-screening method. The three filters below show the first and second screen filters probed with CaM and the third shows one side of the final screen probed with CaM in the absence of Ca^{2+} (EGTA) or the presence of Ca^{2+} ($CaCl_2$).

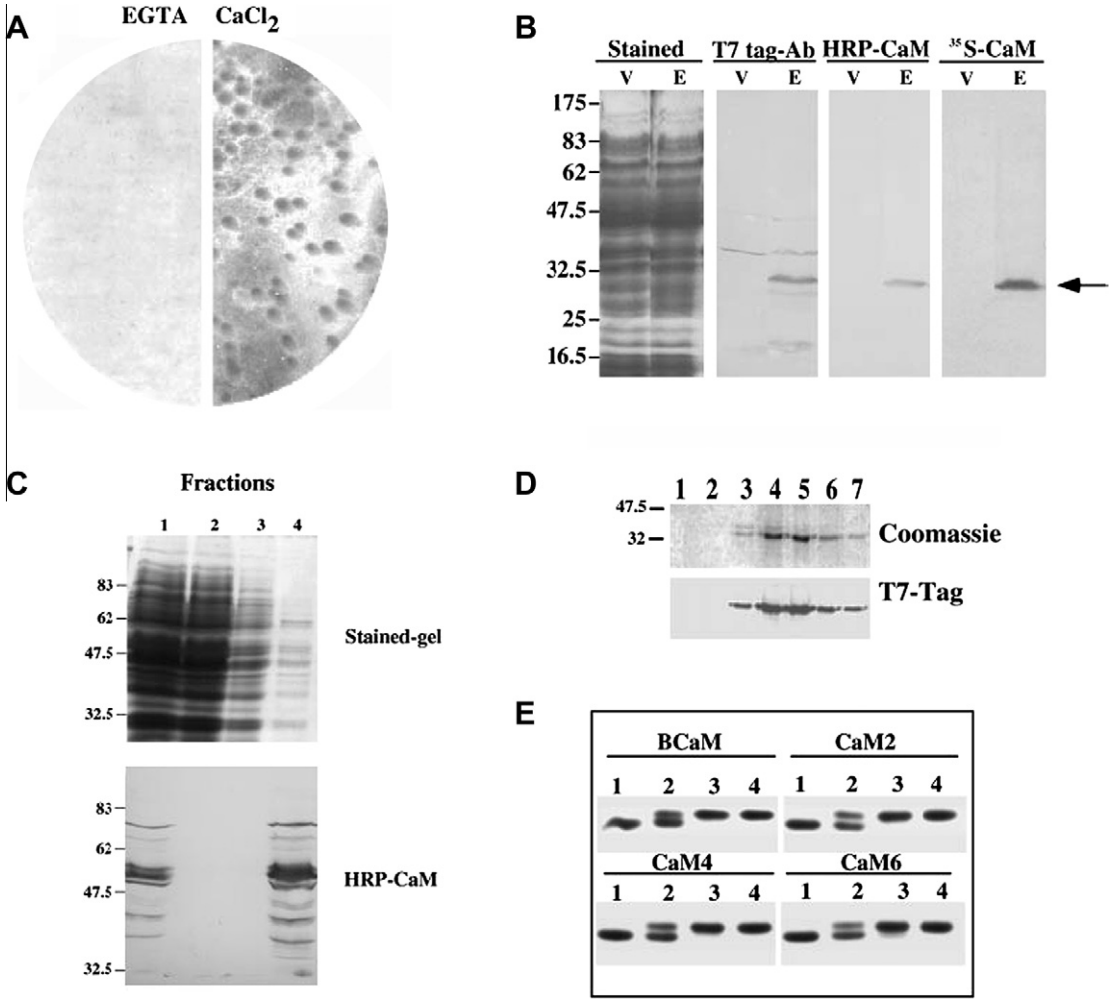

Fig. 2. (A) Overlay assay to verify CaM-binding protein from an expression library. Autoradiogram shows Ca^{2+}-dependent binding of ^{35}S-labeled CaM to CAMTA3. (B) Overlay assay showing detection of CaM-binding protein using either radioactive or non-radioactive probes. Bacterially expressed truncated EICBP (CAMTA3) (aa 770–1042) and its binding to CaM. Soluble proteins from induced cultures containing vector alone (V) or the truncated form of EICBP (E) were separated on four gels. One gel was stained with Coomassie blue (stained), and the other three were blotted and probed with either T7 tag-antibody (T7 tag-Ab), HRP-conjugated CaM (HRP-CaM) or ^{35}S-labeled CaM (^{35}S-CaM). The location of EICBP is indicated by an arrow. (C) CaM-binding proteins eluted from a CaM-Sepharose column. Soluble protein from pollen was passed through CaM-Sepharose and bound proteins were eluted with EGTA-containing elution buffer. Soluble protein (1), flow-through (2), wash (3), and elution fractions (4) were separated on SDS polyacrylamide gels and were either stained with Coomassie Blue (stained gel), or blotted and probed with HRP-CaM (HRP-CaM). (D) Purification by CaM-Sepharose column. A fusion protein of maize pollen CaM-binding protein (MPCBP) was purified by passing crude protein through a CaM-Sepharose column. Lanes 1–7 are elution fractions either stained with Coomassie Blue or probed with a tag to the fusion protein T7. (E) Gel-shift assay. CaMs 2, 4 or 6 or bovine CaM (166 pmol) were run on a gel alone (lane 1) or with 166, 332, 664 pmol of synthetic peptide corresponding to the CaM-binding domain of MPCBP. From Reddy et al. (2000) and Safadi et al. (2000).

were identified as CaM-binding proteins, suggesting that it is one of the few proteins in plants that interacts with numerous other proteins (Lee et al., 2010).

3. Computational approaches for prediction of protein–protein interactions (PPIs)

The identification of hundreds of targets for CaM and CMLs permits development of computational tools to predict targets of calcium sensors and PPIs in general. In discussing computational approaches we consider two related problems – the prediction of the whole interactome of a given organism and prediction of interactions of a given protein, using CaM as an example.

In general, large scale high-throughput screens for PPIs suffer from a high rate of false positives and false negatives (up to 50% for the false positive rate) (von Mering et al., 2002). Computational prediction and filtering of PPIs is therefore extremely useful even in model organisms that have been studied using high-throughput experimental methods, and more so in plants, where such efforts have been limited so far (see Section 4). As the number of experimentally determined interactions increases, computational models will become more and more accurate in their predictions. The high false-positive rate of high-throughput experimental methods has led to the development of computational methods that assign confidence scores to the results of high-throughput experimental methods (Suthram et al., 2006). Being able to predict interactions *de novo* or verify a given set of interactions are related problems – both of them rely on data that serve to lend credibility to a putative interaction. These data are surveyed next.

3.1. Sources of data for interactome prediction

There are many sources of information indicative of an interaction between two proteins. Each of these can be used to predict interactions by itself, but better accuracy can be obtained by integrating several sources of data and building a model to predict interactions on the basis of a collection of features of a pair of proteins. These integrative models typically use classification methods

from the field of machine learning (Tarca et al., 2007). In what follows we present various sources of data that are predictive of protein interactions (summarized in Table 2) and then discuss methods for integrating them.

3.1.1. Interlogs

Perhaps the simplest method for predicting PPIs is by orthology. A pair of proteins are called "interlogs" if there is a pair of orthologs in another species that are known to interact (Matthews et al., 2001; Walhout et al., 2000). This was the first method to be used in Arabidopsis for creating a draft interactome (Geisler-Lee et al., 2007). To improve the accuracy of the resulting interactions, De Bodt et al. (2009) predicted interlogs in Arabidopsis and filtered the resulting interactions by their co-expression and similarity in functional annotations. Chen et al. (2009) provide a web-server that can be used to search for interlogs in a large number of species.

3.1.2. Domain/motif methods

Domain-based methods operate under the assumption that PPIs are mediated by domains, and that proteins with the same domain will exhibit similar interactions (Ta and Holm, 2009). The first of these methods was proposed in Sprinzak and Margalit (2001); they score each pair of domains according to their tendency to co-occur in PPIs more frequently than chance. A pair of proteins is then scored according to the maximum scoring pair of domains they contain. This method has been extended using more sophisticated maximum likelihood approaches that aim at explaining a set of interactions as domain–domain interactions (Wang et al., 2007). Perhaps surprisingly, even a simple approach based on amino acid composition is able to perform on-par with some domain composition methods (Roy et al., 2009).

3.1.3. Comparative genomics methods

Comparative genomics methods that consider the genomic context of genes are providing information that is complementary to orthology and domain methods. Table 1 lists several such methods – gene neighborhood methods (Dandekar et al., 1998), the gene-fusion method (Enright et al., 1999; Marcotte et al., 1999), phylogenetic methods that quantify the co-occurrence of a pair of proteins in a set of genomes (Pazos and Valencia, 2001), and co-evolution methods that quantify the likelihood of an interaction by the similarity of the phylogenetic trees of the families of proteins that two proteins belong to (Goh et al., 2000; Pazos and Valencia, 2001). These methods have been applied mainly in prokaryotic genomes and yeast, and a large compendium of such predictions is available via the STRING database (Jensen et al., 2009).

Table 1
CaMs and CMLs in the Arabidopsis genome (Day et al., 2002; McCormack et al., 2005).

ATG No.	Gene name	ATG No.	Gene name
AT5G37780	CaM1	AT1G66400	CML23
AT2G41110	CaM2	AT5G37770	CML24
AT3G56800	CaM3	AT1G24620	CML25
AT1G66410	CaM4	AT1G73630	CML26
AT2G27030	CaM5	AT1G18210	CML27
AT3G43810	CaM7	AT3G03430	CML28
AT3G59450	CNL1	AT5G17480	CML29
AT4G12860	CML2	AT2G15680	CML30
AT3G07490	CML3	AT2G36180	CML31
AT3G59440	CML4	AT5G17470	CML32
AT2G43290	CML5	AT3G03400	CML33
AT4G03290	CML6	AT3G03410	CML34
AT1G05990	CML7	AT2G41410	CML35
AT4G14640	CML8	AT3G10190	CML36
AT3G51920	CML9	AT5G42380	CML37
AT2G41090	CML10	AT1G76650	CML38
AT3G22930	CML11	AT1G76640	CML39
AT2G41100	CML12	AT3G01830	CML40
AT1G12310	CML13	AT3G50770	CML41
AT1G62820	CML14	AT4G20780	CML42
AT1G18530	CML15	AT5G44460	CML43
AT3G25600	CML16	AT1G21550	CML44
AT1G32250	CML17	AT3g29000	CML45
AT3G03000	CML18	AT5G39670	CML46
AT4G37010	CML19	AT3G47480	CML47
AT3G50360	CML20	AT2G27480	CML48
AT4G26470	CML21	AT3G10300	CML49
AT3G24110	CML22	AT5G04170	CML50

3.1.4. Co-expression and co-regulation

Proteins that interact need to be present in the cell at the same time and in similar concentrations under the conditions that re-

Table 2
Methods for predicting PPIs and the rationale behind them.

Method	Biological rationale
Orthology to known interactions	A pair of proteins is suspected to interact if there is a pair of interacting orthologs in another organism; such putative interactors are called "interlogs" (Walhout et al., 2000; Matthews et al., 2001)
Domain composition	PPIs are mediated by domains, and proteins with the same domain will exhibit similar interactions (Ta and Holm, 2009)
Co-expression	Proteins that interact need to be present in the cell at the same time and in similar concentrations; co-expression has been used for both scoring the reliability of PPIs (Deane et al., 2002) and predicting PPIs (Soong et al., 2008)
Co-regulation	The co-expression required for an interaction can be achieved by co-regulation of interacting proteins
Network topology	Pairs of interacting proteins have similar interaction neighborhoods, i.e. interact with similar sets of proteins. This has led to the development of scores that capture local topological features of the network that are indicative of an interaction (Goldberg and Roth, 2003)
Similarity in functional annotations	Proteins that interact tend to participate in similar biological processes and have similar cellular localization
Phylogenetic profiles	Given that the homologs of interacting proteins are also likely to interact, the existence of homologs for a pair of interacting proteins will tend to be coordinated – either both or none of them will have homologs in other organisms. The pattern of homology of a protein across organisms is captured by what's called a phylogenetic profile, and interacting proteins tend to have similar phylogenetic profiles (Pazos and Valencia, 2001)
Gene neighbors	Proteins that are encoded by genes that are on the same bacterial operon tend to be functionally related, and often physically interact (Dandekar et al., 1998)
Gene fusion (Rosetta Stone method)	Some interacting proteins have homologs in other genomes that are fused into a single protein - the so-called Rosetta Stone protein (Marcotte et al., 1999). Existence of a Rosetta Stone protein is an indication of a functional linkage or protein interaction (Enright et al., 1999; Marcotte et al., 1999)
Sequence co-evolution	Proteins that interact need to co-evolve so as to maintain their interaction. This will show itself as similarity between phylogenetic trees for the corresponding families of the two proteins
Docking	Proteins interact because they have structures that support the interaction
Literature mining	Automatic extraction of interaction data from the literature is complementary to the work of database curators that perform this task manually

quire their interaction; co-expression, as measured using DNA microarrays has been used for both scoring the reliability of PPIs (Deane et al., 2002) and predicting PPIs (Soong et al., 2008). In most cases, it has been used in conjunction with other signals for *de novo* prediction of PPIs, as expression is not a very specific signal for PPIs. The co-expression of two genes can occur if the genes are regulated by the same transcription-factors. Since transcription factor binding can be quantified in a high-throughput manner, co-regulation has proven to be a useful source of information for prediction of PPIs (Lu et al., 2005).

3.1.5. Network topology methods

In organisms with well-characterized interaction networks it has been observed that pairs of interacting proteins have similar interaction neighborhoods, i.e. interact with similar sets of proteins. This has led to the development of scores that capture local topological features of the network that are indicative of an interaction (Chen et al., 2008; Goldberg and Roth, 2003).

3.1.6. Shared functions

Proteins that interact tend to participate in similar biological processes and have similar cellular localization. Researchers have therefore used the similarity of Gene Ontology (GO) terms associated with a pair of proteins as a source of data for predicting PPIs (Lu et al., 2005; Ben-Hur and Noble, 2005). In fact, shared GO biological processes are one of the most predictive sources of data (Lu et al., 2005). However, shared process annotations are not useful in the case of proteins with unknown function that are often characterized with the help of PPIs.

3.1.7. Using structure

The three-dimensional structure of a protein is what determines its biochemical activity and ability to interact with proteins and other biological molecules. Docking methods that use protein structure in order to predict a complex of two or more proteins at atomic-scale resolution can be used to infer whether a pair of proteins interact. It has been noted that docking methods are "not sufficiently accurate to predict whether or not two proteins actually interact" (Russell et al., 2004). Despite the significant improvements in docking algorithms in recent years as evidenced by their performance in Capri evaluations this is still likely to be the case (Ritchie, 2008). There are other ways of using structure to infer interactions without resorting to docking, which is computationally intensive (Hue et al., 2010).

3.1.8. Automatic extraction of interactions from the biomedical literature

The curators of PPI databases can't keep up with the large number of interactions that are reported in the literature (Baumgartner et al., 2007). Since there are many ways of expressing interactions in natural language, extraction of biological knowledge from the biomedical literature is a difficult task. It turns out that even detecting which genes are being referred to is difficult (known as the problem of gene normalization). An overview of the state-of-the-art of literature mining is provided in Krallinger et al. (2008). A large number of interactions that are automatically extracted are found in the STRING database (Jensen et al., 2009).

3.2. Integrative methods

Each of the methods described above constitutes noisy evidence for a PPI. Furthermore, individual sources of data have limited coverage, and cannot provide support for every interaction (Lu et al., 2005). Integration of several of these can lead to more accurate predictions, increasing both the number of predicted interactions and the confidence with which they are predicted (Ben-Hur and Noble, 2005; Lu et al., 2005; Qi et al., 2006). This is crucial when performing predictions on a genome-wide scale since even a modest error-rate can lead to millions of false-positives when applied to all pairs of proteins. Integration is carried out using statistical and machine learning techniques (Tarca et al., 2007). This involves the training of a model for distinguishing between interacting and non-interacting pairs of proteins. The training process requires a set of known PPIs (positive examples), and a set of pairs of proteins that are not known to interact (negative examples). In view of the high rate of false positives in high-throughput datasets, assessing the performance of the resulting models is usually performed on trusted interactions – interactions that have been found using multiple methods or by reliable small-scale methods.

The simplest and most widely used integration method is the Naïve Bayes classifier, which was employed in several publications (Jansen et al., 2003; Jensen et al., 2009; Lu et al., 2005). These studies use a small number of features, making this a viable approach. When incorporating all possible combinations of domains for example, more sophisticated machine learning approaches that can handle high dimensional data are required. Support-vector machines are one such approach (Ben-Hur et al., 2008), and this has been applied successfully for prediction of PPIs using such extremely high dimensional data (Ben-Hur and Noble, 2005). The random forests classification method has done well in a comparison of several machine learning approaches (Qi et al., 2006).

Most of the work on integrative methods has been carried out in yeast, where there is a wealth of genomic data. A comprehensive integration of all the possible signals supporting PPIs is a challenging bioinformatics task; the most comprehensive integration to-date has been carried out by the designers of the STRING database, that combines information from orthology, sequence evolution methods, co-expression, and literature mining, providing over 50 million predictions in 630 organisms (Jensen et al., 2009).

3.3. Prediction of CaM-binding proteins in Arabidopsis

An alternative approach to the construction of a global interactome prediction model is to train models for the prediction of PPIs for individual proteins. Bleakley et al. (2007) trained models for each protein in the yeast genome that has known interaction partners using a collection of genomic data that included expression, localization information, and phylogenetic profiles. This approach has the advantage over a global model in that it is able to take into account specific propensities of individual proteins. Their results show the advantage of this approach over global modeling of the interactome. An issue with this approach is that for many proteins very few interaction partners are known, so extensions of their method were proposed to address this point (Yip and Gerstein, 2009).

Focusing on prediction of interactions of specific proteins allows the design of a classifier that is specifically tailored towards the properties of the protein and the modes in which it interacts with other proteins. Whereas all the models used in Bleakley et al. (2007) employ the same data in the same way for all proteins, recent work on prediction of interactions of CaM uses the sequence characteristics of the interaction partners of CaM to design accurate classifiers that can predict both interaction sites and interaction partners of CaM (Radivojac et al., 2006) (Hamilton et al., 2010). Taking advantage of the fact that CaM is highly conserved across eukaryotes these methods model CaM binding sites using data from a variety of organisms collected in the CaM Target Database (Yap et al., 2000). Hamilton et al. (2010) used the support-vector machine classifier, which has shown its usefulness in a large variety of bioinformatics domains (Ben-Hur et al., 2008); a support-vector machine finds a boundary between known binding sites and non-binding sites such that the margin between these

two classes is maximized. This concept is illustrated in Ben-Hur et al. (2008). Hamilton et al. (2010) considered several representations of CaM binding sites and found that using the amino acid composition of a binding site yields results that are almost as accurate as more sophisticated methods that use evolutionary information and the presence of short motifs. The success using amino acid composition is related to the fact that CaM binding sites tend to be disordered, a property driven by amino-acid composition (Radivojac et al., 2006). When run on the whole Arabidopsis proteome their method produces over 700 high-confidence predictions with an estimated false positive rate less than 0.05. This adds to the 200 or so CaM binders identified by Popescu et al. (2007), again highlighting how computational approaches complement high-throughput approaches in saturating interaction networks.

4. Databases of protein–protein interactions

There are many databases that store both experimental and predicted PPIs. Table 3 provides a list of interaction databases – both generic, and plant-specific. This table highlights the fact that high-throughput interaction data in plants is not as widely available as in yeast and animal model organisms. The IntAct database (Aranda et al., 2010), which contains the largest number of experimentally-derived interactions in plants (most of them in Arabidopsis), contains 8000 interactions in Arabidopsis, compared to nearly 70,000 in yeast, over 40,000 for human, close to 30,000 for fly, over 10,000 for *C. elegans*, and close to 10,000 for mouse. This relative paucity is likely to change soon, as large-scale projects are under way to interrogate the interactomes of several plants (see Table 3).

Because of the small number of experimentally determined interactions in plants, computationally predicted interactions have been the main source of PPIs. The first database of predicted interactions in Arabidopsis was published in 2007, and consisted mostly of interlogs, with confidence scores assigned by their level of co-expression (Geisler-Lee et al., 2007). Additional resources soon followed. AtPID integrates interlogs, information from co-expression, shared Gene-Ontology biological processes, domain composition, gene fusion events, and phylogenetic profile similarity scores using a simple Naïve-Bayes method (Cui et al., 2008). The AtPIN database pools interactions from several databases and its latest release (#10) contains 96,827 interactions (Brandao et al., 2009). A similar number of interactions are available from the STRING database that contains 89,009 Arabidopsis interactions when considering their most stringent threshold for prediction confidence (Lars Jensen, personal communication). VisANT (Hu et al., 2008) is another useful resource that provides both a database back end and tools for analyzing interaction networks. Many of these databases have features for visualization of interaction networks, where AtPIN, for example, allows visualization of query results using Cytoscape, which is one of the standard tools in network analysis (Cline et al., 2007). The CaM Target Database (Yap et al., 2000) is a collection of around 200 proteins that interact with CaM where the binding site location is known. This database has not been updated in recent years, and additional data is likely available.

5. Specificity of interaction with CaM and CML proteins

There is little sequence difference in the seven CaMs in Arabidopsis. One isoform is encoded by *CaM1* and *CaM4*, while *CaM2*, *CaM3*, and *CaM5* encode a second isoform. The CaM1/CaM4 isoform differs by four amino acids from CaM7 while the CaM2/CaM3/CaM5 isoform and CaM6 differ from CaM7 by only one amino acid (McCormack et al., 2005; Reddy et al., 1999). The CMLs are more highly divergent having as little as 16% amino acid identity with one to six EF hands. CaMs have been shown to have many targets and some targets have been shown to bind more than one CaM isoform, raising the question of specificity of CaM/CBP interactions. KCBP is a kinesin-like protein that binds CaM in a Ca^{2+}-dependent manner and the binding of CaM blocks the motor interaction with microtubules (Reddy et al., 1996a). Crystallization studies suggest that Ca^{2+}/CaM inhibits the binding of KCBP to microtubules by blocking the microtubule binding sites (Vinogradova et al., 2008, 2004). In a yeast two-hybrid assay, a

Table 3
A lists of protein–protein interaction databases. We list both generic PPI databases that contain interactions for all species, and plant specific databases. For the generic databases we list the total number of interactions in the database across all species, and the plant interactions (most of them are in Arabidopsis). The plant specific databases typically collect interactions from other databases (we list the number of such interactions as "collected"); two of the databases contain predicted interactions.

Database	Number of interactions	URL/publication
BioGRID	241,173 (total) 3304 (plant)	http://thebiogrid.org/ (Breitkreutz et al., 2008)
DIP	69,463 (total) 54 (plant)	http://dip.doe-mbi.ucla.edu/dip/ (Xenarios et al., 2002)
IntAct	154,639 (total) 8000 (plant)	http://www.ebi.ac.uk/intact/ (Aranda et al., 2010)
Mint	83,571 (total)	http://mint.bio.uniroma2.it/mint/ (Ceol et al., 2010)
VisANT	918,312 total (300,297 predicted) 147,204 in Arabdiopsis	http://visant.bu.edu/Contains interactions collected from other databases and predicted interactions (Hu et al., 2008)
STRING	Over 60 million predicted interactions in 630 organisms 89,009 (predicted high confidence in Arabidopsis)	http://string.embl.de/Contains interactions collected from several databases and predictions based on evolutionary methods and text mining (Jensen et al., 2009).
Plant specific		
TAIR (Arabidopsis)	2656 (Arabidopsis)	http://www.arabidopsis.org/
Arabidopsis predicted interactome	4300 (collected) 70,944 (predicted)	ftp://ftp.arabidopsis.org/home/tair/Proteins/Interactome2.0http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgiMostly interlogs (Geisler-Lee et al., 2007)
Arabidopsis thaliana protein interactome database (AtPID)	4666 (collected) 23,396 (predicted)	http://atpid.biosino.org/Predicted from orthology, co-expression, shared annotations, evolutionary information, and domain pairs (Cui et al., 2008)
Arabidopsis thaliana protein interaction network (AtPIN)	96,827(collected)	http://bioinfo.esalq.usp.br/atpin/atpin.plCollected from all the above databases (Brandao et al., 2009)
Rice kinase database	378	http://rkd.ucdavis.edu/Interactions of rice kinases (Dardick et al., 2007)
Plant interactome database	Coming soon	http://interactome.dfci.harvard.edu/A_thaliana/A Y2H and protein chip map of the Arabidopsis interactome

second protein (KIC, KCBP Interacting Ca^{2+} sensor) was found to bind KCBP and also inhibit microtubule binding (Reddy et al., 2004). It is also a Ca^{2+}-binding protein with one EF hand. Although both Ca^{2+}-KIC and Ca^{2+}-CaM are able to interact with KCBP and inhibit its microtubule binding and ATPases activity, the concentration of Ca^{2+} required to inhibit the microtubule-stimulated ATPase activity of KCBP by KIC is threefold less than that required for CaM. At the same time, two KIC-related Ca^{2+}-binding proteins and CML19 from Arabidopsis, which contain one and four EF-hand motifs, respectively, bound Ca^{2+} but did not affect microtubule binding and microtubule-stimulated ATPase activities of KCBP. Probing of thirteen purified Ca^{2+}-binding proteins on a slot blot with KCBP showed interaction with three CaMs (1, 4 and 6) and KIC in the presence, but not absence, of Ca^{2+}. KCBP did not bind to the tested CBL, CPK or to KRP1, a KIC-related protein that binds Ca^{2+} but does not regulate KCBP/tubulin binding and other tested CMLs. These findings indicate that there are specific CaMs/CMLs that bind targets but multiple targets for a given CaM/CML are also possible. These findings were supported by the Arabidopsis protein microarray probed with three CaMs and four CMLs (Popescu et al., 2007). Each of the CaMs/CMLs interacted with 86–122 of the target proteins, suggesting that large numbers of targets can interact with individual CaMs/CMLs. Looking at the specificity of the targets, approximately 25% of the proteins interacted with all the CaMs/CMLs, 50% interacted with two or more and 25% interacted with only one CaM/CML (Popescu et al., 2007). The interaction networks that they constructed showed four hubs containing different sets of CaMs/CMLs. One hub contained CaM1, CaM6, CaM7, CML9 and CML10, which share more common targets than any other hub. Another hub containing CML8 and CML12 interacted with a larger number of unique targets. In fact, CaM4 and CaM7 represent the top two of three hubs in Arabidopsis (Lee et al., 2010). These data indicate that there is a range of specificity in targets from one to multiple interacting CaM/CML proteins.

6. Functions of CaM and CML targets

Interaction screens and predictions have resulted in identification of numerous targets of CaMs and CMLs in plants. Functions of only a few of these have been elucidated. Here we discuss briefly the functions of CaM/CML targets in plant growth and development and stress responses.

6.1. Calmodulin-binding proteins in plant growth and development

Several of the identified CBPs that are transcription factors, enzymes and motors have functions in plant growth and development. Plant hormones regulate many aspects of plant growth and development and some CBPs are involved in response to hormonal changes. A group of proteins are upregulated in response to auxin, the small auxin up RNA (SAUR) proteins. Yang and Poovaiah (2000) isolated a *Zea mays* SAUR using labeled CaM; an Arabidopsis homolog to a bean SAUR was also identified as a CBP (Reddy et al., 2002) and SAUR.B was found to interact with CaM in the protein chip assay (Popescu et al., 2007). Two other transcription factors involved in auxin signaling were also found to bind CaM/CMLs, GRAS and AUX/IAA (IAA31 was verified by immunoprecipitation), which play a role in root development and gibberellin signaling (GRAS) and in many developmental processes (AUX/IAA) (Popescu et al., 2007b).

The CAMTA family of transcription factors is comprised of ethylene and stress induced CaM-binding proteins (Bouche et al., 2002; Reddy et al., 2000; Yang and Poovaiah, 2002a). A mutant CAMTA3 line is smaller than WT Arabidopsis (Du et al., 2009; Galon et al., 2008) indicating a role for CAMTA3 in growth and development. DWARF1 (DWF1), another CBP in Arabidopsis, is responsible for an early step in brassinosteroid biosynthesis (Du and Poovaiah, 2005). Brassinosteroids have a role in coupling environmental factors with plant growth and development. Ca^{2+}/CaM binding was shown necessary for DWF1 function using site-directed and deletion mutants (Du and Poovaiah, 2005).

Some CBPs are involved in pollen germination and the directed growth of pollen tubes. The Arabidopsis homolog of maize pollen CaM-binding protein (MCPCBP), NPG1 (no pollen germination1), is essential for pollen germination (Golovkin and Reddy, 2003; Safadi et al., 2000). Pollen forms normally in *npg1* mutants but germination of pollen grains containing the *npg1* allele was affected (Golovkin and Reddy, 2003). ACA9 is a Ca^{2+}/CaM activated Ca^{2+}-ATPase that has been shown to be important for normal pollen tube growth (Schiott et al., 2004). Three mutant alleles of *aca9* showed poor growth of the pollen tube and low frequency of fertilized ovules. Another possible Ca^{2+}/CaM regulated protein that may be involved in pollen growth is apyrase. There are two apyrases in Arabidopsis but only one of them has a CaM binding domain (Steinebrunner et al., 2003). When both apyrases were knocked out, pollen failed to germinate and growth of hypocotyls was inhibited (Steinebrunner et al., 2003; Wu et al., 2007).

KCBP, a kinesin regulated by Ca^{2+}/CaM (see above) was isolated as a protein involved in trichome morphogenesis (Oppenheimer et al., 1997). *ZWITCHEL* mutants had aberrant trichomes with only one or two branches instead of the normal three (Oppenheimer et al., 1997). KCBP has also been localized to the phragmoplast during cell division and has a role in cytokinesis (Bowser and Reddy, 1997; Voss et al., 2000). Other cell cycle proteins were identified in the protein chip assay as possible CaM/CML-binding proteins including CDKB2 (verified by immunoprecipitation) and two other CDK-like proteins (Popescu et al., 2007).

GAD, an enzyme that catalyzes the conversion of glutamate to GABA, has been shown to be regulated by Ca^{2+}/CaM and has been identified in many plant species (reviewed in Bouche et al., 2005). Using a GAD deletion mutant with no CaM-binding domain resulted in constitutive GAD activity, increased amounts of GABA and abnormal plant development (Baum et al., 1996).

Recently the Arabidopsis transcription factor Telomerase Activator1 (TAC1) was found to bind the promoter of AtBT2, a CBP known to be involved in transcription regulation suggesting a role for AtBT2 in telomerase regulation (Du and Poovaiah, 2004; Ren et al., 2007). A functional analysis of the BT family in Arabidopsis showed that there is functional redundancy among the family members, and that the expression of specific BT genes is up- or down-regulated when null mutations occur in other BT genes (Robert et al., 2009). It was shown that BT proteins play an essential role during gametogenesis, and probably throughout plant development in plants containing multiple null mutations in the different BT genes.

Membrane bound proteins have also been shown to bind CaM. A protein involved in translocation of proteins across the chloroplast membrane was isolated using a CaM-Agarose column (Chigri et al., 2006). Calmodulin-binding assays corroborated the finding and domain analysis located a CaM-binding domain between Leu-296 and Leu-314 close to the C-proximal end of the pea Tic32. Tic32 is a dehydrogenase and its dehydrogenase activity is affected by CaM (binding of NADPH and CaM to Tic32 appear to be mutually exclusive). A homolog in Arabidopsis, At4g23430, has a similar binding domain (Chigri et al., 2006).

A 50 kDa protein encoded by AT4G30490 (AFG1L1) with similarity to the ATPase family gene 1 protein from yeast was identified using affinity chromatography on CaM-agarose with chloroplast proteins (Bussemer et al., 2009). The protein has a single AAA-domain characteristic for members of the AAA+-ATPase and *in vitro* pull-down as well as cross-linking assays corroborate Ca^{2+}-dependent binding of the protein to CaM. The CaM-binding domain was

located in a region of 20 amino acids within the AAA-domain. The protein localizes to both mitochondria and chloroplasts.

An S-locus RLK (receptor-like kinase) CaM-binding kinase (CBRLK1, AT1G11350) identified in a CaM screen was localized to the plasma membrane (Kim et al., 2009a). Calmodulin bound specifically to a Ca^{2+}-dependent CaM-binding domain in the C-terminus of CBRLK1. The bacterially expressed CBRLK1 kinase domain could autophosphorylate and phosphorylates general kinase substrates (Kim et al., 2009a).

6.2. Calmodulin-binding proteins in stress responses

Ca^{2+} plays a major role in the response to environmental stresses, both biotic and abiotic (Reddy, 2001; Reddy and Reddy, 2002, 2004). CBPs have been shown to be involved in both responses, some are specific to biotic, some to abiotic and others are involved in both responses. Six WRKY transcription factors and two TGA transcription factors that are involved in activation of stress or defense pathways interacted with CaM/CMLs in the microarray overlay assays done by Popescu et al. (2007). The CaM/CML interaction was verified by immunoprecipitation for two of the WRKYs (43 and 53) and TGA3. TGA3 has been shown to bind Ca/CaM and as a transcription factor, binds to the CaM3 promoter (Reddy et al., 2002; Popescu et al., 2007; Szymanski et al., 1996). TGA3 binding to the CaM3 promoter is enhanced in the presence of Ca/CaM (Szymanski et al., 1996). Another transcription factor, WRKY7, a WRKY group IId member was isolated in a labeled CaM screen. A CaM overlay assay with members of the WRKY group IId family showed CaM-binding by all tested members while representative members of the other WRKY families did not bind CaM (WRKY43 and WRKY53 proteins were not tested) (Park et al., 2005).

Receptor-like kinases (RLKs) have been shown to be involved in developmental and defense-related processes. The CaM/CML microarrays also identified (RKLs) and RLK-like proteins that bind CaM/CML (Popescu et al., 2007). It has been reported that BT genes mediate diverse hormone, stress and metabolic responses (Mandadi et al., 2009). Accumulation of *BT2* (At3g48360) mRNA was affected by a variety of hormones, nutrients and stresses, and *BT2* was required for response to many of these same factors (Mandadi et al., 2009). Here we have discussed the functions of just a few CBPs. A detailed discussion on functions of CBPs in stress responses can be found in recent reviews (Galon et al., 2010; Kim et al., 2009b).

6.2.1. Biotic stresses

As reviewed by Bouche et al. (2005), a number of CBPs have been identified with a role in response to pathogens. A protein first identified in tobacco as a CaM-binding protein involved in pathogen response, NAD kinase (NADK) catalyzes the phosphorylation of NAD to NADP (Harding et al., 1997). Two Arabidopsis NADKs were isolated later but only one is CaM-binding (Turner et al., 2004). Other CBPs involved in biotic stress response include CaM-regulated catalase (Yang and Poovaiah, 2002b), GAD (MacGregor et al., 2003), a receptor-like protein MLO (Kim et al., 2002), and two cyclic-nucleotide gated channels, AtCNGC2 (Clapham, 1995) and AtCNGC4 (Babu et al., 1988). Some CBPs found to be induced by pathogens include SCA1- a Ca^{2+}-ATPase in soybeans (Chung et al., 2000), several isoforms of CBP-60 in bean (Ali et al., 2003) and PICBP in Arabidopsis (Reddy et al., 2003). Mutations in an Arabidopsis CBP60 (CBP60g, At5g26920) that abolish CaM binding prevent complementation of SA production and bacterial growth defects of *cbp60g* mutants (Wang et al., 2009). CaM binding is Ca^{2+} dependent, which demonstrates that CBP60g constitutes a Ca^{2+} link between MAMP recognition and SA accumulation in resistance to *Pseudomonas syringae* (Wang et al., 2009).

A protein involved in production of glucosinolates, IQD1 was shown to bind CaM in a Ca^{2+}-dependent manner and overexpression of IQD1 reduces insect herbivory (Levy et al., 2005). A family of 33 IQD genes was found in Arabidopsis with a conserved CaM-binding domain and CaM-binding was confirmed for one other family member, IQD20 (Levy et al., 2005). Two cell death-related proteins have been shown to bind CaM. A family of proteins containing a BAG (BCL-2-associated athanogene) domain has seven members in Arabidopsis, three of which have a CaM-binding domain—AtBAG5, 6 & 7 (Kabbage and Dickman, 2008). AtBAG6, isolated in a screen with labeled CaM, binds CaM through an IQ motif in a Ca^{2+}-independent manner and induces cell death (Kang et al., 2006). A cell death suppressor protein, Bax inhibior-1 (BI-1) was shown to bind CaM using the split-ubiquitin system, overlay assay and bimolecular fluorescence complementation analysis (Ihara-Ohori et al., 2007).

The transcription factor CAMTA3 has also been shown to be a suppressor of biotic defense responses in Arabidopsis with mutants showing enhanced resistance to bacterial and fungal pathogens (Du et al., 2009; Galon et al., 2008). Plant–pathogen interactions lead to local and systemic acquired resistance. Salicylic acid is required for both of these responses, but whether and how salicylic acid level is regulated by Ca^{2+} signaling during plant–pathogen interaction is unclear. A mechanistic connection of a Ca^{2+} signal to salicylic-acid-mediated immune response through CaM, has been shown for AtSR1 (CAMTA3), a Ca^{2+}/CaM-binding transcription factor and EDS1, an established regulator of salicylic acid level (Du et al., 2009). In loss-of-function alleles of Arabidopsis *SR1* there is constitutive disease resistance and elevated levels of salicylic acid, suggesting that it is a negative regulator of plant immunity. Interaction of SR1 with the promoter of *EDS1* resulted in repressesion of its expression. The CaM-binding domain is required to function as a negative regulator.

6.2.2. Abiotic stresses

A variety of abiotic stresses can induce a transient cytosolic Ca^{2+} increase and the CaM and CML sensors are often induced in response to these stresses (Knight, 2000; Reddy, 2001). CBPs are involved in osmotic stress, cold and heat stress, oxidative stress and xenobiotic stress (Bouche et al., 2005). Some CAMTA family members are induced by salt (Yang and Poovaiah, 2002a), the Ca^{2+}-ATPase, ACA4 plays a role in salt tolerance (Geisler et al., 2000), and AtCaMBP25 is induced by osmotic stress (Perruc et al., 2004). A MYB transcription factor cloned from a library from dehydrated Arabidopsis was later shown to bind CaM and regulate the expression of salt- and dehydration-responsive genes in Arabidopsis (Yoo et al., 2005). A Ca^{2+}/CaM-regulated receptor-like kinase (CRLK) was shown to be a positive regulator of cold tolerance in plants (Yang et al., 2010b). The *crlk1* knockout mutants showed delayed induction of cold-responsive genes and exhibited increased sensitivity to chilling and freezing temperatures. Northern analysis showed that the induction of cold-responsive genes, including CBF1, RD29A, COR15a, and KIN1 in crlk1 mutants, is delayed as compared with wild-type plants. The interaction of CRLK with a MAP kinase kinase kinase (MEKK1) suggests that it acts through MAP kinase pathway (Yang et al., 2010a).

CBPs involved in heat shock are Arabidopsis homologs of wheat FKBPs including TWD1 which bind CaM and are induced by heat shock (Kamphausen et al., 2002; Reddy et al., 2002) and Arabidopsis homologs of wheat TCB60. Recently, a Ser/Thr phosphatase AtPP7 was found to interact with CaM (Liu et al., 2007). A *pp7* knockout impaired the thermotolerance of Arabidopsis seedlings and overexpression increased thermotolerance (Liu et al., 2007). Most recently, a CaM-binding protein kinase AtCBK3 was shown to be a part of a heat-shock signal transduction pathway and a

knockout mutant and an overexpressor again impaired or increased respectively thermotolerance (Liu et al., 2008).

Two CaM-binding transcription factors AtBT and some CAMTAs are induced by H_2O_2, indicating their involvement in oxidative stress (Du and Poovaiah, 2004; Yang and Poovaiah, 2002a). CAMTA3 has been show to bind a CGCG element in cold-induced genes and induced their expression. AtCNGC1, apyrase, catalase and a multidrug resistance gene AtMRP1 are involved in tolerance to xenobiotic compounds (Bouche et al., 2005).

In addition to the above described CBPs with known functions there are other CBPs for which functions have not been elucidated. Some of these are described below. A transcription factor CBNAC (CaM-binding NAC protein), a member of NAC family transcription factors, that binds to a GCTT core sequence was isolated using HRP-labeled CaM screen (Kim et al., 2007). CBNAC was found to bind to its cognate DNA sequence and repress transcription (Kim et al., 2007). Another HRP CaM-labeled screen identified a protein kinase, AtCK whose phosphorylation of myelin basic protein was enhanced by Ca^{2+}-bound CaM (Jeong et al., 2007). AtMKP1, a MAPK phosphatase was also identified in a HRP labeled CaM screen and the phosphatase activity was increased by CaM in a Ca^{2+}-dependent manner (Lee et al., 2008). The plasma membrane protein PCaP1 interacts with CaM and the phosphatidylinositol phosphates Ptdins (3,4,5) P(3) and Ptdins (3,5) P(2) and CaM association with PCaP1 weakened the interaction of PCaP2 with PtdInsPs (Nagasaki et al., 2008). Calmodulin was found to interact with and regulate the RNA-binding activity of an Arabidopsis polyadenylation factor subunit (AtCPSF30) (Delaney et al., 2006). Other confirmed CaM-binding proteins include a non-specific lipid transfer protein-1 (nsLTP1) (Wang et al., 2005), a ubiquitin protease 6 (AtUBP6) (Moon et al., 2005), and ACA11, a Ca^{2+}-ATPase that localizes to the vacuole membrane (Lee et al., 2007).

7. Conclusions and future perspectives

CaMs and CMLs constitute a large group of calcium of sensors in Arabidopsis and other plants. These Ca^{2+}-binding proteins, with no activity of their own, bind target proteins and modulate their activity. Screening of expression libraries and protein chips containing a partial proteome with labeled CaM and CMLs has resulted in identification of a large number of target proteins. A large interactome of CaMs and CMLs and their interacting proteins is emerging. The probing of a protein chip containing the full complement of Arabidopsis proteins with all CaMs and CMLs will identify all the possible interactions between CaM/CMLs and their targets. A prototype protein microarray with over 1100 proteins identified about 15% of the proteins on the chip as targets of one of the three CaMs or four CMLs, suggesting that a large number CBPs exists in plants (Popescu et al., 2007). This high percentage of targets is likely a reflection of biased selection of proteins, mostly transcription factors and signaling proteins, which are likely to participate in Ca^{2+} signaling pathways. Application of a computational pipeline that we developed predicted another 600 putative CaM interactors (Hamilton et al., 2010). A major challenge in the future will be to experimentally verify these predictions, determine their specificity, and test their biological significance. Given the large number of CaM and CML targets, it appears that the calcium sensor interactome is highly complex. However, not all the interactions that are found in these *in vitro* assays may be physiologically relevant as some observed interactors may not be expressed in the same cell type/tissue or developmental stage. Even if they are expressed, they may be in different cellular compartments, which precludes their interaction. Hence, analysis of the interaction data with integrative methods, which take into consideration expression at the RNA and protein level, subcellular localization and other parameters, will yield more meaningful information. A complete understanding of calcium signaling pathways will require us to investigate these interactions using system-level approach.

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