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Review

Biochemical regulation of *in vivo* function of plant calcium-dependent protein kinases (CDPK)☆

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ABSTRACT

Calcium (Ca^{2+}) is a major second messenger in plant signal transduction mediating stress- and developmental processes. Plant Ca^{2+}-dependent protein kinases (CDPKs) are mono-molecular Ca^{2+}-sensor/protein kinase effector proteins, which perceive Ca^{2+} signals and translate them into protein phosphorylation and thus represent an ideal tool for signal transduction. This review focuses on recent developments in CDPK structural analysis and CDPK *in vivo* phosphorylation substrate identification. We discuss mechanisms implicated in the *in vivo* regulation of CDPK activity including Ca^{2+} binding to the CDPK EF-hands, Ca^{2+}-triggered intra-molecular conformation changes, and CDPK (auto)-phosphorylation. Moreover, we address regulation and integration into signaling cascades of selected members of the plant CDPK family, for which *in vivo* function and phosphorylation in abiotic and biotic stress signaling have been demonstrated. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. Introduction

The calcium ion (Ca^{2+}) plays a central role as second messenger in eukaryotic signal transduction. In plants, modulation of cytoplasmic Ca^{2+}-concentration occurs in response to various endogenous and external signals, including changes in hormone status, abiotic stress stimuli such as drought, high and low temperature or light, biotic stress stimuli such as microbial elicitors, symbiotic nodulation factors, as well as mechanical disturbances [1–5]. One of the most fascinating questions is the subsequent regulation of response specificity during Ca^{2+}-initiated signal transduction. It has been shown that specific stimuli lead to distinct Ca^{2+}-signals, and differences in amplitude, frequency, oscillation duration, and cellular distribution of the intracellular Ca^{2+}-concentration changes have been discussed [2,3,5–11]. An additional degree of specificity is conferred by the presence or absence of specific Ca^{2+}-binding proteins in different cell types and their localization to distinct subcellular compartments. In this review we will focus on how Ca^{2+}-signals in plants initiate rapid signal transduction processes by the activation of phosphorylation cascades. In plants, Ca^{2+}-sensor proteins which directly regulate kinase activity belong to three families: calmodulin interacting with calcium/calmodulin regulated kinases (CCaMKs), the Ca^{2+}-sensor protein calcineurin B-like (CBL) regulating CBL-interacting protein kinases (CIPKs), and the Ca^{2+}-dependent protein kinases (CDPKs). CDPKs are chimeras containing a Ca^{2+}-sensor domain and a protein kinase effector domain within the same molecule. This review focuses on CDPKs and their structural and functional properties particular with focus on the Ca^{2+} binding via EF-hand motifs. Ca^{2+} binding to the CDPK regulatory domain triggers an intra-molecular conformational change, in which an inactive protein kinase conformation is converted to an active one and as a consequence subsequent signal transduction processes are initiated. Thus, the analysis of the biochemical regulation and function of CDPK calcium sensor/protein kinase effector proteins will give insight into the mechanism of initiation of Ca^{2+}-regulated signal transduction.

2. Structure of CDPKs

CDPKs comprise a gene family of protein kinases restricted to plants and some protists encompassing 34 members in *Arabidopsis thaliana*. The conserved CDPK structure is composed of three domains, namely the N-terminal variable domain, the serin/threonin protein kinase domain and a CDPK activation domain (CAD) (Fig. 1). The CAD consists of a pseudosubstrate segment and a Ca^{2+}-binding domain which is highly homologous to calmodulin, and thus encompasses what in previous reports had been described as inhibitory junction domain and the calmodulin-like domain (CLD). It was proposed that CDPKs developed during evolution via fusion of a calmodulin and a CaMK. The CLD contains, generally four Ca^{2+}-binding EF-hand motifs. The EF-hands act pairwise, so that an N-terminal EF-hand pair (N-EF lobe) and a C-terminal EF-hand pair (C-EF lobe) can be considered [12–16].

Protein sequence comparison of the 34 *Arabidopsis* CDPKs revealed a high degree of conservation of the kinase domain (44%–95% identity

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Fig. 1. Domain structure of a Ca^{2+}-dependent protein kinase (CDPK). V, N-terminal variable domain; K, kinase domain; CAD, CDPK activation domain; PS, pseudosubstrate segment; CLD, calmodulin-like domain; EF, EF-hand. The four white bars within the EF-hand motifs represent the EF-hand Ca^{2+}-binding loop. The scheme is drawn to scale exemplary for *At*CPK21, consisting of 531 amino acids.

and 60%–98% similarity), the pseudosubstrate segment (23%–100% identity and 42%–100% similarity) and the CLD (27%–97% identity and 50%–98% similarity) [17]. In contrast, the N-terminal variable domain shows a high variability in length and sequence between these 34 CDPK isoforms. Interestingly, the N-terminal domain was shown to be subjected to phosphorylation by upstream kinases and has been discussed in the context of mediating substrate specificity [18,19].

The modular structure of CDPKs taken together with published biochemical and functional data suggest that *in vivo* activation of CDPKs is driven by a conformational change induced by Ca^{2+}-binding (for example, as consequence of increased cytoplasmic calcium concentrations following plant stress exposure) leading to a release of the pseudosubstrate segment from the active site of the kinase domain. This does not rule out that additional regulatory mechanisms such as CDPK protein (auto-) phosphorylation [13,15,16,19–25] (discussed below), lipid binding or interaction with 14-3-3 proteins are involved [24,26].

3. Regulation of CDPKs by Ca^{2+}

Understanding of the mechanism of CDPK activation upon Ca^{2+}-binding is a major goal in CDPK research since many years. A breakthrough was recently accomplished with the crystal structure of a Ca^{2+}-bound active state of apicomplexan protist CDPKs from *Cryptosporidium parvum* (*Cp*CDPK1, *Cp*CDPK3) and *Toxoplasma gondii* (*Tg*CDPK1) and Ca^{2+}-free inactive state of *Tg*CDPK1, *Tg*CDPK3 [15,16,27]. This deduced model may also be representative for most plant CDPKs because on one hand the sequence alignment of apicomplexan CDPKs reveals a conserved consensus domain order, in which a kinase domain is followed by four EF hand binding motifs, and on the other hand a high sequence conservation exists within the autoinhibitory pseudosubstrate segment including additional conserved amino acids required for adopting the active and inactive conformations. Here, we briefly summarize the major hallmarks resulting from this analysis (Fig. 2).

In the CAD of the CDPK N- and C-terminal EF lobes contain two EF hands each (EF1/EF2 and EF3/EF4) whereby each single EF hand consists of an (E-)helix–loop–(F-)helix of the consensus Ca^{2+}-binding motif. In the Ca^{2+}-free inactive form of the CDPK N- and C-terminal EF lobes are connected via two antiparallel α-helices (Fig. 2A). The longer N-terminal α-helix named CH1 (CDPK activating domain α-helix 1) directly adjacent to the kinase domain is comprised of the autoinhibitory pseudosubstrate segment (previously also described as an independent junction domain) and is extended up to the E-helix of EF-hand 1 of the

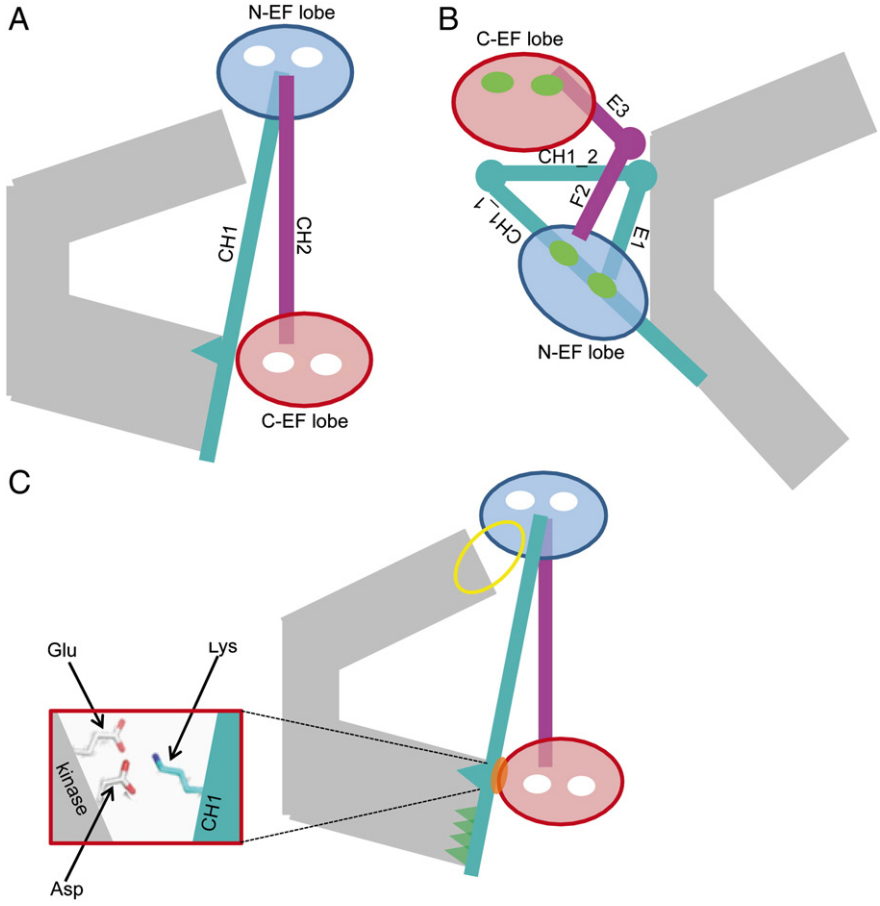

Fig. 2. Scheme of an inactive (A) and active (B) CDPK conformation based on data from [15,16]. The kinase domain is depicted in gray and the CAD (CDPK-activating domain) in color. The CH1 helix is shown in cyan and CH2 in pale magenta. The N-EF lobe is represented as blue circles, the C-EF lobe as red circles. The E-helix of EF-hand 1, F-helix of EF-hand 2, and E-helix of EF-hand 3 are indicated as E1, F2 and E3. Ca^{2+} is represented by green circles. (C) Contact points and areas which contribute to the stabilizing the inactive CDPK conformation are indicated. This includes the Lys–Glu–Asp triad, important for holding the kinase in its inactive state (highlighted), hydrophobic residues within CH1 inserting in the C-terminal lobe of the kinase domain (indicated by green spikes), a hydrophobic pocket composed of the interface of CH1 and the C-EF lobe (indicated by orange oval) and the N-EF lobe of the CAD interacting with the N-lobe of the kinase domain (indicated by yellow oval).

N-terminal EF lobe. In the inactive CDPK conformation a basic amino acid, mostly lysine in few CDPKs also arginine, from CH1 blocks the substrate binding site by interacting with glutamic acid and aspartic acid from the kinase domain (indicated as triangular thorn protruding from CH1 in Fig. 2A). As a consequence of this autoinhibitory Lys–Glu–Asp triad (Fig. 2C) Glu is retrieved from interaction with ATP in the ATP binding site required for phosphotransfer of the CDPK. A second shorter helix of the CAD (named CH2) connecting the N- and C-terminal EF lobes extends from the second helix of EF-hand 2 (F2) up to the first helix of EF-hand 3(E3). The Ca^{2+}-free inactivated CDPK conformation is further stabilized by additional kinase-CAD interactions: These include contact sites between (i) hydrophobic residues within CH1, which reach into an amphipathic cleft in the C-terminal lobe of the kinase domain (indicated by green spikes in Fig. 2C), (ii) an isoleucine from the kinase domain inserting in a hydrophobic pocket composed of the interface of CH1 and the C-EF lobe (hydrophobic pocket indicated by orange oval in Fig. 2C) and (iii) the N-EF lobe of the CAD interacting with the N-lobe of the kinase domain (indicated by yellow oval in Fig. 2C). In several apicomplexans CDPKs an insertion of few amino acids exists between the first and second EF-hand in the N-EF lobe, creating an interacting site with the N-lobe of the kinase domain. This insertion is absent in plants. However, for plasmodium *Pf*CDPK3, which contains only a short insertion and thus is closer to plant CDPKs, nevertheless a similar overall structure to other apicomplexans CDPKs was identified. Therefore, it is likely that plant CDPKs accommodate a similar structural fold [16].

After Ca^{2+} binding of all four EF hands, a substantial refolding of the CAD is induced in which the entire activation domain translocates to a new position about 135° clockwise on the other side of the kinase domain and is stabilized by the formation of new contact sites (Fig. 2B). This results in subsequent refolding and exposure of the active site of the kinase domain [15]. Also, the CH1 and CH2 helices, no longer antiparallel, are broken into defined subsegments, and form hydrophobic contacts with the Ca^{2+}-loaded EF-lobes. Thereby, CH1 is unfolded in three segments, CH1_1, CH1_2, and CH1_3 (corresponding to E1, E helix of EF hand1). Interestingly, while in the Ca^{2+}-free conformation CH1 shared contact sites with the C-EF lobe, in the Ca^{2+}-bound conformation, subsegment CH1_1 interacts with the hydrophobic surface of the N-EF lobe. Furthermore, the CH1_2 subsegment interacts in the Ca^{2+}-bound conformation with the hydrophobic surface of the C-terminal EF lobe [13,16,28]. This is contrary to previously discussed models of CDPK activation, where both C- and N-terminal EF lobes enclose CH1_2 by their hydrophobic surfaces [12,24,29].

3.1. Do N- and C-terminal EF lobes mediate different roles in the Ca^{2+}-induced conformational change?

While the above described structural analysis focused on CDPKs in either their Ca^{2+}-free or their Ca^{2+}-bound form, biochemical studies with purified CDPKs suggested that N- and C-terminal EF lobes confer different Ca^{2+}-binding affinities. For *Arabidopsis*, tomato but also *Plasmodium falciparum* CDPKs a pair of high-affinity Ca^{2+}-binding sites at the C-terminal EF lobe and low-affinity sites at the N-terminal EF lobe was reported [12,14,30]. It was discussed that both C-terminal EF-hands due to their high Ca^{2+}-binding affinity may already be Ca^{2+}-loaded under basal Ca^{2+}-levels in their respective organism [12]. Based on the scheme of the structural analysis depicted in Fig. 2 this basal Ca^{2+} binding to the C-terminal EF-hands can be interpreted as a decomposition of helix CH2 into the segments F2 (F-helix of EF2), a linker segment, and E3 (E-helix EF3). An enhanced flexibility of F2 may result in an increase in the Ca^{2+} binding affinity of EF2. Thus, Ca^{2+}-binding to the C-terminal EF-hands may be crucial for enhancing the Ca^{2+}-affinity of the N-terminal EF-hands.

For *At*CPK1 from *Arabidopsis* the interaction between the amphiphatic α-helix CH1_2 and the CLD was analyzed based on a crystal structure in the presence of Ca^{2+} using a truncated version of *At*CPK1 containing the CH1_2 and the CLD (CH1_2-CLD) [13]. In this study, a symmetric dimer of Ca^{2+} bound CH1_2-CLD with domain-swap interactions was formed, in which CH1_2 interacts with the C-EF lobe of the partner CH1_2-CLD. Besides structural differences, probably emerging from missing parts of the CDPK or the artificial dimer formation, an interaction between CH1_2 and CLD similar to those described by Wernimont et al. [15,16] was identified. Thus, an important role of the C-terminal EF-hands could be the stabilization of the active conformation via interaction with CH1_2.

In the absence of Ca^{2+}-binding to the N-terminal EF-hands, CH1_2 is part of the long CH1 helix. It was discussed that the last helix-collapse leading to an active conformation via pulling CH1 helix out of the catalytic domain is the rotation at the center of CH1_1 and CH1_2 [16]. The collapse of CH1 potentially produces the interacting target peptides CH1_1 and CH1_2, which could stabilize the CAD in its active conformation [16]. This proposed function of the pseudosubstrate segment in stabilizing the CAD could explain the observed increase of Ca^{2+}-affinity of the EF-hands in presence of the pseudosubstrate segment [12]. Even when the C-terminal EF-hands are Ca^{2+}-saturated, the interaction between CH1_2 and the C-terminal EF-hands could be prevented by inaccessibility of CH1_2. This is probably the case when CH1_2 is part of the CH1 helix, suggesting that Ca^{2+}-binding to the N-terminal EF-hands is necessary for decomposition of CH1. This may indicate that Ca^{2+}-binding to the N-terminal EF lobe is the initiating event for CDPK activation. Coinciding, the N-terminal EF-hands were identified as the crucial EF-hands for calcium-regulated kinase activity. Mutated CDPK variants of *Arabidopsis* (*At*CPK21) and *P. falciparum* (*Pf*CDPK1), which carried non-functional N-terminal EF-hand motifs, only showed reduced Ca^{2+}-regulated kinase activity [30,31].

3.2. Does substrate specificity modulate calcium-dependency?

It has been shown for some CDPKs that Ca^{2+}-dependency of *in vitro* kinase activity is modulated via the substrate [32–34]. For example, *At*CPK10 and *At*CPK32 were almost Ca^{2+}-independent in *in vitro* experiments with the artificial substrate syntide-2, while in contrast a clear Ca^{2+}-dependent kinase activity could be observed with histone as substrate. This indicates that also the substrate, as an interaction partner, may influence the stability of the active kinase conformation.

4. EF-hands as Ca^{2+}-binding motif of CDPKs

According to the consensus conserved structure, CDPKs contain four Ca^{2+} binding EF-hand motifs. An EF-hand motif consists of an (E-) helix–loop–(F-)helix structure with the Ca^{2+} binding loop consisting of twelve amino acids (Fig. 3). Ca^{2+}-binding is accomplished via negatively charged amino acids [35,36]. From these twelve amino acids of the loop region six are actively engaged in Ca^{2+} coordination (highlighted in the EF-hand loop consensus pattern below). In Fig. 3 the consensus sequence

1 2 3 4 5 6 7 8 9 10/11 12 13
D-{W}-[**DNS**]-{ILVFYW}-[**DENSTG**]-[DNQGHRK]-{**GP**}-[LIVMC]-[**DENQSTAGC**]-x(2)-[**DE**]-[LIVMFYW]
[acceptable amino acids for a given position]; {not accepted amino acids for a given position};
x= any amino acids; (number of position with the same condition).

Fig. 3. Consensus sequence of the Ca^{2+}-binding loop from amino acid one to twelve starting with the N-terminal amino acid.

Table 1
Degenerated EF-hand motifs of *A. thaliana* CDPKs. The analysis is based on the twelve amino acids of the EF-hand Ca^{2+}-binding loop.

CDPK	No. of degenerated EF-hand	Position of altered amino acid in Ca^{2+}-binding-loop	Ca^{2+}-dependency of kinase activity [a,b]
CPK7	1	5 (R)	No [33]
CPK8	1	3 (K)	No [33]
CPK10	3	6 (T)	No [33]
CPK13	2,3	Hand 2: 3 (K) and 5 (K) hand 3: 9 (L)	No [33]
CPK14	1	5 (R)	Not analyzed
CPK23	1	12 (Q)	No (SLAC1-NT) [38]
CPK25	1, 2, (3, 4 missing)	Hand 1: 3 (G) hand 2: 3 (H) and 4 (L)	No [33]
CPK32	1	5 (R)	No [33]

[a] An absence of Ca^{2+}-dependency of kinase activity is considered when less than 50% increase in maximum kinase activity was observed in the presence of calcium in *in vitro* kinase assays.
[b] Substrate syntide-2 except for CPK23 where SLAC1-NT was used.

of an EF-hand motif is shown according to PROSITE-documentation PDOC00018. This documentation draws an additional consensus hydrophobic amino acid in position 13, which is not part of the loop [37].

Sequence analysis of the *Arabidopsis* CDPK gene family with 34 members revealed that not all isoforms contain four consensus EF-hand motifs, but deviation from the consensus sequence in one or more EF motifs is observed (Table 1). In general, a non-functional EF-hand remains as a degenerated EF-hand motif in the protein. Remarkably, in *At*CPK25 not only EF1 and EF2 are degenerated, but the entire C-terminal EF lobe appears to be truncated. Accordingly, it was reported that *At*CPK25 is unable to bind Ca^{2+}. However, the enzyme shows constitutive enzymatic activity *in vitro* [33]. On the basis of the model in Fig. 2 it is tempting to speculate that *At*CPK25 is frozen in the activated CDPK conformation: one contact site between the kinase domain and a hydrophobic pocket composed of the C-EF lobe and CH1, which is required for holding the enzyme in the inactivated state (Fig. 2C), is lost, whereas at the same time modifications in the N-EF lobe may cause a structural arrangement of CH1 segments, which could result in the stabilization of the activated state. However, also other regulatory mechanisms may account for the constitutive activity of *At*CPK25 [33].

4.1. Are degenerated EF-hand motifs functional?

It was shown that CDPKs with degenerated EF-hand motifs such as CPK23 and CPK13 are catalytically active but have no or only weak Ca^{2+}-dependency in *in vitro* kinase assays [38,39]. Recently, a comprehensive analysis addressing the Ca^{2+}-dependency of CDPK kinase activity of *Arabidopsis* CDPKs containing degenerated EF-hand motifs showed that not only CPK25 but also several analyzed CDPKs of subgroup III (CPK7, 8, 10, 13, 30 and 32) displayed *in vitro* kinase activity independent of a Ca^{2+}-activation [33]. Five of seven CDPKs carrying one or two altered EF-hand motif(s) have a degenerated EF-hand 1 (Table 1). This identifies EF-hand 1 as being most frequently modified. Furthermore, EF1 amino acid substitution always occurs in a position engaged in calcium coordination. In contrast, alterations in EF-hand 3 affect a position not directly engaged in Ca^{2+}-binding, potentially leading to an incorrect structure. This coincides with results of *in vitro* protein kinase assays, in which enzyme variants with amino acid substitutions directly affecting Ca^{2+}-binding in the N-terminal EF-hands showed a more severe impact on the Ca^{2+}-dependency of kinase activity as in the C-terminal EF-hands [30,31].

It would be interesting to investigate, whether amino acid substitutions at degenerated EF-hand motifs to the consensus sequence is sufficient to restore Ca^{2+}-dependency. This is in particular important, because CPK30 which also belongs to CDPK subgroup III, lacks Ca^{2+}-dependency with syntide-2 as substrate, despite of having four consensus EF-hand motifs [33]. This indicates that also other CDPK regions, for example within the CH1 pseudosubstrate segment or the kinase N- or C-lobes, which are involved in the formation of contact site formation between kinase domain and CAD to stabilize either the inactive or active conformation, contribute to the Ca^{2+} dependency of CDPKs.

4.2. What is the conformation of degenerated EF-hand motifs?

In *P. falciparum Pf*CDPK3, EF-hand 1 motif is degenerated compared to the EF consensus sequence and contains K and Q in position 5 and 12, respectively. Based on the crystal structure of the active CDPK, no bound Ca^{2+} in the EF-hand 1 site could be detected [16]. Interestingly, the CAD, even with just three Ca^{2+} loaded EF-hands, still adopts the same topology as a CAD with four functional Ca^{2+} loaded EF-hand motifs, analyzed in parallel. Despite lacking Ca^{2+} as a stabilizing force the EF-hand 1 adopts an active conformation. It is discussed that the EF-hand 1 is held in the active conformation by a glutamine which inserts in the Ca^{2+}-binding loop, and two coordinated water molecules creating a series of hydrogen bonds which stabilize the charged loop [16]. This demonstrates that even a degenerated EF-hand can support the active protein conformation, and suggests that by pairing, the degenerated EF-hand is stabilized via the non-degenerated partner EF-hand.

5. Modification of CDPK function by phosphorylation

In addition to activation by Ca^{2+}-binding, CDPKs were shown to be subjected to phosphorylation, which can occur at different amino acids throughout the entire protein, and which is catalyzed either by the enzyme itself (auto-phosphorylation) or by upstream kinases. The function of CDPK phosphorylation is still a matter of debate and both substrate accessibility, as well as regulation of kinase activity, is discussed. Ca^{2+}-dependent CDPK auto-phosphorylation was, for example, reported for enzymes from *Arabidopsis*, groundnut, tomato or soybean in *in vitro* kinase assays [14,31,40,41]. Ca^{2+}-independent auto-phosphorylation was observed with a CDPK from winged bean [42]. Surprisingly, this enzyme showed clear Ca^{2+} requirement for downstream substrate phosphorylation, which was inhibited by auto-phosphorylation. In contrast, for tobacco *Nt*CDPK2, an enzyme involved in early plant immune signaling, a correlation between pathogen stimulus-dependent *in vivo* phosphorylation and enhanced CDPK kinase activity was observed [25,43].

With the recent progress in mass spectrometry-based phosphoproteomics CDPKs are identified as proteins subjected to differential *in vivo* phosphorylation in response to various endogenous or external stimuli [19,44–46]. In addition, CDPK auto-phosphorylation sites were assessed by mass spectrometry following *in vitro* protein kinase assays with recombinant CDPKs [47]. So far, phosphorylated serin or threonine amino acid residues were observed in all protein domains of CDPKs. Interestingly, it was shown in domain swap experiments of the variable domain of two closely related tobacco CDPKs *Nt*CDPK2 and *Nt*CDPK3 that the phosphorylation pattern was directed by the sequence of the respective phosphorylation motifs in the variable domain, but independent of the kinase domain [19]. Amino acid substitutions of phosphorylation sites within CDPKs to alanine blocking the *in vivo*/*in vitro* phosphorylation in the N-terminal variable domain may cause a decrease in CDPK kinase activity and reduction in CDPK functional response. In *in vitro* kinase assays with *Mc*CDPK1 from *Mesembryanthemum crystallinum* single amino acid substitutions of auto-phosphorylation sites (one in the N-terminal variable domain, one in the CLD) to alanine resulted in a slight increase of kinase activity, whereas the simultaneous substitution of both sites caused a dramatic decrease in substrate phosphorylation [48].

So far, no example is known to the authors, where a phosphomimetic mutation in the N-terminal variable domain to aspartate or glutamate resulted in increased *in vitro*/*in vivo* kinase activity or significant stimulus-independent constitutive response activation. This suggests that phosphorylation at the CDPK may fulfill structural roles or support substrate accessibility rather than trigger kinase activation [19].

5.1. Upstream kinases and phosphatases modify the CDPK phosphorylation status

For *Nt*CDPK2, a transient, stimulus-dependent differential *in vivo* phosphorylation was reported to involve both CDPK autophosphorylation as well as trans-phosphorylation [19]. This suggests a coordinated regulation through an intra-molecular phosphorylation and upstream kinase-dependent phosphorylation, as well as de-phosphorylation by protein phosphatases. Additionally elicitation-stimulus-dependent *in vivo* phosphorylation of CDPK proteins was shown to correlate with an increased enzymatic activity [25,43]. Nevertheless, the nature of upstream protein kinases and/or phosphatases as well as their potential regulation by Ca^{2+} is still unknown.

5.2. Does phosphorylation directly control CDPK activity?

For some protein kinases, such as members of the MAP kinase or SnRK kinase families, a stimulus-dependent phosphorylation within the kinase activation loop was shown to be prerequisite for kinase activation [49–52]. Accordingly, amino acid substitutions of respective phosphorylation sites to negatively charged aspartic acid resulted in stimulus-independent, constitutive active kinase variants [50,53]. Such regulation by phosphorylation within a kinase activation loop does not exist for CDPKs. And although *in vivo* phosphorylation at the CDPK protein may contribute to enhanced or reduced CDPK function and response regulation, the precise role of phosphorylation at distinct sites is yet unknown.

In the context of this review it is tempting to speculate that CDPKs, and in particular those CDPKs for which a Ca^{2+}-independent *in vitro* enzyme activity due to alterations in the CAD or EF hands was observed, are still subjected to regulation through *in vivo* phosphorylation: the introduction of negative charges will lead to localized changes of the surface properties and may thus interfere with contact sites that stabilize/destabilize the active or inactive CDPK conformation. Interestingly, Wernimont et al. discuss potential phosphorylation sites within the pseudosubstrate segment (CH1) of apicomplexan CDPKs, but the original data are not included in their structural analysis [16]. Remarkably besides one serin and threonin residue also a tyrosin was identified as a putative auto-phosphorylation site of these CDPKs. Since CDPKs are so far known as serin/threonin kinases it will be required to confirm these data based on a functional (*in vivo*) analysis. Thus, the answer to the question for a direct phosphorylation control of CDPK activity will have to await further experimentation addressing enzyme *in vivo* function and phosphorylation status combined with structure-deduced mutational enzyme analysis.

6. *In vivo* function of CDPKs

CDPKs are implicated in many different processes of plant biology, from hormone-regulated developmental processes to abiotic and biotic stress signaling (for review see [17,24,54–56]). Often, however, CDPK function was simply deduced from differential gene expression of a distinct CDPK member within a given time period (few minutes to several hours) in a specific biological context of interest. Furthermore, function of CDPKs was correlated with their tissue-specific expression. Some CDPKs occur ubiquitously in the whole plant, whereas for example 5 *At*CPKs out of 34 are highly expressed in various stages during pollen development [32]. Also, a CDPK isoform may be expressed in different cell types: so is *At*CPK23 expressed in guard cells, where the enzyme is functionally integrated in the control of stomata closure (see below), but also in leaf mesophyll cells. Thus, different *At*CPK23-mediated phosphorylation substrates mediating distinct signaling responses are likely.

Furthermore, CDPK function is dependent on specific sub-cellular localization. For most members of the plant CDPK gene family an N-terminal acylation (myristoylation with or without palmitoylation) allowing membrane-targeting was predicted and for some isoforms also experimentally confirmed [17,33,48,57]. Deletion of the N-terminal myristoylation site results in a reduction of plasma membrane localization [48]. Subsequently, the CDPK is compromised in its *in vivo* regulation (for example being prevented from phosphorylation catalyzed by a membrane-bound upstream kinase) and biological function [19]. However, it was also reported that despite carrying functional acylation motifs, CDPKs are located not only at membranes but also in the cytosol, whereas for some fully or partially soluble isoforms a cytosolic and nuclear localization was shown [48,58–61]. Furthermore, a stimulus-induced change in CDPK subcellular localization was reported for some isoforms [48,58–60].

In this review we will focus on latest developments, in which CDPK *in vivo* function was addressed in the context of pathway-specific subcellular localized *in vivo* phosphorylation substrates of CDPK. In a comprehensive approach, CDPK phosphorylation specificity was addressed by *in vitro* protein kinase assays using 274 peptide substrates, which encompassed previously *in vivo* mapped phosphorylation sites, and the correlation between *in vivo* phosphorylation and CDPK *in vitro* kinase activity was assessed [62]. In addition, CDPKs have been functionally characterized by gain- and loss-of-function lines *in planta*, either by investigating *cdpk* mutant-lines, stable CDPK overexpressing transgenic plant lines, upon transient silencing based on co-suppression, or upon expression of constitutive active or dominant negative CDPK enzyme variants in *Arabidopsis* leaf mesophyll protoplasts or *Nicotiana benthamiana* leaves. CDPK function was then investigated on the molecular level either with respect to direct (Ca^{2+}-dependent) reversible protein phosphorylation or to response regulation. Among the most comprehensively characterized CDPKs are enzymes which participate in the activation of early plant defence responses, for example after elicitation with microbial-derived peptide elicitors, or in abiotic stress signaling, for example in response to plant hormone ABA [25,38,63–66].

With respect to the plant immune response, members of the CDPK family have been shown to participate in the activation of early defence responses, including homologous kinase pairs *Nt*CPDK2/3 in tobacco or *St*CDPK4/5 in potato [25,63–65]. The expression of constitutive active tobacco *Nt*CDPK2-VK variants, lacking the regulatory calcium-binding domain and pseudosubstrate segment, in *N. benthamiana*, correlated with the induction of plant defense reactions including Reactive Oxygen Species (ROS) production, changes in phytohormone levels, defense gene expression and cell death development, whereas in plants suppressed for *NtCPDK2* expression these responses were compromised [25]. These data provided evidence that a plant NADPH-oxidase, responsible for ROS production, may be among CDPK substrates. Alongside this, in *Arabidopsis*, analysis of *cpk* multiple mutant plants, for example quadruple mutant lacking functional *CPK4*, *CPK5*, *CPK6*, *CPK11* genes, showed reduced elicitation-induced ROS production [61]. In potato, homologous enzymes *St*CDPK4 and *St*CDPK5 were selected from an expression library as proteins capable of phosphorylating the N-terminus of recombinant potato Respiratory Burst Oxidase Homologue *St*(RBOHB), coding for potato NADPH-oxidase. Phosphorylation at distinct sites was subsequently investigated upon transient overexpression of *St*RBOHB in *N. benthamiana* [64,65]. However, a confirmation of this protein as *in vivo* substrate of this CDPK, based on both a direct link between CDPK *in vivo* biochemical activity and target phosphorylation, for example, determined by *in vivo* phosphoproteomics, and on functional studies by mutant analysis of kinase and kinase target *in planta*, is still missing. In contrast, with respect to abiotic stress signaling, a similar comprehensive approach has successfully identified *At*CPK21 and *At*CPK23 as *in vivo* regulators for

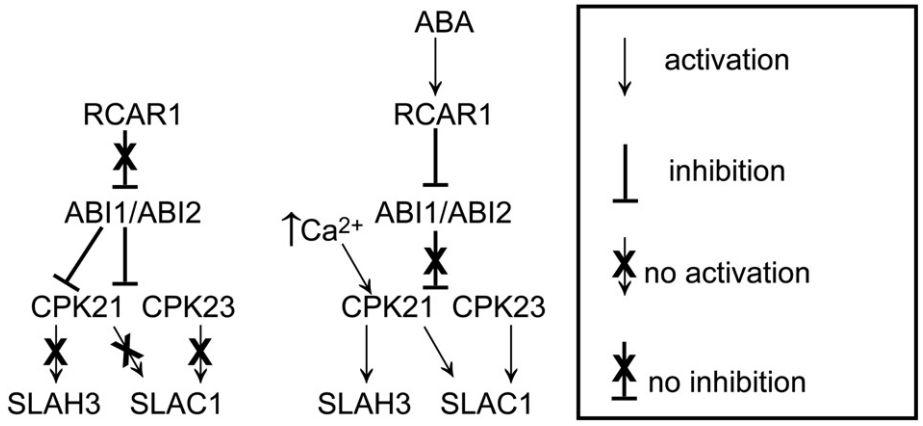

Fig. 4. Model of CDPK-dependent signal transduction for ion channel regulation in guard cells, modified after [66]. Perception of plant hormone ABA by the ABA receptor RCAR1 causes a repression of ABI1/ABI2 phosphatase activity and concomitant activation of *At*CPK21 and *At*CPK23. CPK21, as a Ca^{2+}-dependent kinase, and CPK23 as a Ca^{2+}-independent kinase, control anion channels SLAC1 and SLAH3.

plasma membrane bound slow-voltage gated ion channels Slow Anion Channel-Associated 1 (SLAC1) and SLAC1 Homolog 3 (SLAH3) [38,66]. Upon co-expression of constitutively active CPK21 with SLAC1 or SLAH3 and CPK23 with SLAC1 in *Xenopus* oocytes a slow voltage S-type anion current could be monitored. Furthermore, patch clamp measurements with guard cells protoplast derived from *Arabidopsis cpk23* [38] or *slac1* [67] mutant plants, displayed a dramatic reduction of anion current compared to Col-0 wild type, confirming the *in vivo* relevance of the CPK23-dependent SLAC1 activation. *In vivo* interaction between SLAH3 and CPK21 and SLAC1 and CPK23 was observed based on in BIFC (bi-molecular fluorescence complementation) assays in *Arabidopsis*. Furthermore, *in vivo* interaction of CPK23 and the N-terminal cytosolic part of SLAC1 was shown via co-immunoprecipitation. *In vitro* protein kinase assays, revealed a CPK21- and CPK23-dependent phosphorylation of the cytosolic domain of SLAC1, and a CPK21-dependent phosphorylation of SLAH3 [38,66]. Even more, a phytohormone ABA-induced and CPK21-dependent *in vivo* phosphorylation of the cytosolic N-terminal domain of SLAH3 in *Arabidopsis* protoplasts was shown [66].

Taken together, these data lead to a model for CDPKs integrated in a regulatory network for anion channel opening, which includes ABA-receptor Regulatory Components of ABA Receptor 1 (RCAR1) [68,69] and phosphatases ABA insensitive 1/2 (ABI1/2) [70–73] (Fig. 4). For SLAC1 and SLAH3 this model was further experimentally supported by an *in vitro* reconstitution of the entire signal transduction pathway from ABA binding to CDPK-dependent substrate phosphorylation [38,66].

Recently, also *At*CPK3 and *At*CPK6 were implicated in this process. Based on heterologous and recombinant expression systems, CPK6 was shown to phosphorylate SLAC1, to trigger SLAC1 currents in oocytes, and subsequent SLAC1 de-phosphorylation was catalyzed by phosphatase ABI1 [74]. Also, a CPK3-catalyzed phosphorylation of SLAC1 was determined in *in vitro* kinase assays, and CPK3-dependent activation of and interaction with SLAC1 was observed in *Xenopus* oocytes [75]. Furthermore, patch clamp measurements with *Arabidopsis* guard cells derived from *cpk3 cpk6* single and double mutant lines displayed a reduced ABA- and Ca^{2+}-, and for *cpk6* also methyl jasmonate-dependent, S-type anion current [76,77]. Despite a missing *in vivo* proof demonstrating SLAC1 as a biochemical *in vivo* phosphorylated CPK3 and CPK6 substrate, these results provide evidence that members of the plant CDPK family have partially overlapping functions. Thus, CDPKs are integrated in complex signaling networks, which may allow a fine tuning of response regulation.

With the availability of CDPK crystal structures on one hand, and the introduction of highly sensitive mass spectrometry-based *in vivo* phosphoproteomics on the other hand, we envisage a rapid future progress in the molecular understanding of CDPK *in vivo* regulation and function, addressing (i) CDPK regulation by calcium with respect to CDPK isoform specific Ca^{2+} binding affinities (ii) CDPK regulation by phosphorylation and de-phosphorylation, and (iii) the interplay between the activated CDPK and CDPK/response-specific phosphorylation substrates.

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