

Calcium-Dependent Protein Kinases: Hubs in Plant Stress Signaling and Development¹

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Calcium ions (Ca²⁺) have long been recognized as a major, conserved second messenger in eukaryotic signal transduction. In plants, the multigene family of calcium-dependent protein kinases (CDPKs) encodes structurally conserved, unimolecular calcium sensor/protein kinase effector proteins. CDPKs are ideal candidates for perceiving intracellular changes in Ca²⁺ concentration and translating them into specific phosphorylation events to initiate further downstream signaling processes. Accordingly, CDPKs have been predominantly characterized in rapid abiotic stress and immune signaling responses, with little focus upon CDPK function in long-term adaptive processes or plant development. The long-awaited identification of *in vivo/in planta* CDPK targets, including their respective phosphorylation sites, allows the elucidation of CDPK function on a molecular and biochemical level. Moreover, the resolution of kinase-specific phosphorylation patterns within a target protein provides mechanistic evidence suggesting that CDPKs act as signaling hubs in plant stress signaling and development. The CDPK gene family comprises Ser/Thr protein kinases organized in four subgroups (Harmon et al., 2000; Cheng et al., 2002); families of varying size have been characterized in *Arabidopsis* (*Arabidopsis thaliana*; 34), rice (*Oryza sativa*; 29), wheat (*Triticum aestivum*; 20), maize (*Zea mays*; 35), and poplar (*Populus trichocarpa*; 20; Harmon et al., 2000; Asano et al., 2005; Li et al., 2008; Ma et al., 2013; Zuo et al., 2013). CDPKs have a conserved molecular structure, consisting of a variable N-terminal domain, fused to a Ser/Thr kinase domain, and a CDPK activation domain (CAD). The CAD is formed from a pseudo-substrate region (sometimes referred to as the “inhibitory junction domain”) and a Ca²⁺-binding domain, highly homologous to calmodulin (also described as a calmodulin-like domain; Fig. 1A). The calcium-binding domain of a canonical CDPK contains up to four elongation factor (EF) hand motifs. These are organized in N-terminal and C-terminal EF lobes and may display

different Ca²⁺-binding affinities (Harper et al., 2004; Harper and Harmon, 2005; Boudsocq and Sheen, 2013; Liese and Romeis, 2013).

Based on this modular structure, a model for CDPK activation was proposed in which Ca²⁺ binding to the CAD induces conformational changes, leading to the release of the pseudosubstrate segment from the active site (Fig. 1B). This results in kinase activity, enabling CDPK autophosphorylation as well as the phosphorylation of target substrates (Liese and Romeis, 2013). With the recent availability of crystal structures from apicomplexan CDPKs, encompassing the kinase and CAD, this model has been refined (Wernimont et al., 2010, 2011). In the absence of Ca²⁺, the autoinhibitory region and the N-terminal lobe of the Ca²⁺-binding domain form two extended α -helices that block the active site, and likely bury the N-terminal part, of the kinase. In addition, one may speculate that the N-terminal variable domain (which is absent in the crystal structure of apicomplexan CDPKs) may be hidden, so that it has no access to the CDPK target. Upon Ca²⁺ binding, the structure of the helices is broken down, inducing a rotation of the entire regulatory domain around the kinase domain, probably releasing the N-terminal variable domain. It has been proposed that both the apo- and calcium-bound forms of the enzyme are stabilized by distinct contact sites between the kinase and regulatory domains (Wernimont et al., 2010). It is tempting to speculate that, in particular, some of the amino acids involved in forming these contact sites may be subjected to posttranslational modifications during the activation process (Liese and Romeis, 2013), modulating the stability of the active or inactive form.

CDPKs have been shown to function in many different aspects of plant biology, from environmental stress signaling upon the perception of abiotic and biotic stress stimuli to hormone-regulated processes in development (Cheng et al., 2002; Ludwig et al., 2004; Asano et al., 2012; Boudsocq and Sheen, 2013). While previously, CDPK characterization often relied on the biochemical activation proteins or the transcriptional induction of the respective genes in a given signaling response, recent studies draw a more comprehensive picture of CDPK function, including *in vivo/in planta* data concerning kinase/substrate interactions. Despite this significant progress, elucidating the impact of biochemical regulation on the *in vivo* function of a

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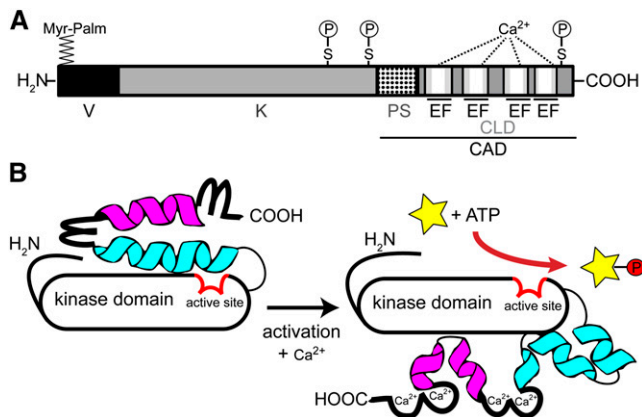


Figure 1. Scheme of CDPK modular structure and activation. A, Domain structure of Ca^{2+} -dependent protein kinase, drawn to scale for AtCPK28, consisting of 523 amino acids. V, N-terminal variable domain; K, kinase domain; PS, pseudosubstrate segment; CLD, calmodulin-like domain; EF, EF hand. The four white bars within the EF hand motifs represent the EF hand Ca^{2+} -binding loop. The position of myristoylation and palmitoylation (Myr-Palm) and the *in vivo* autophosphorylation sites (P) at Ser (S) residues are depicted. B, Model for CDPK activation (modified after Wernimont et al., 2010). Calcium binding to the EF hand motifs (loop) induces a conformational change, in which helix 1 (blue) and helix 2 (magenta) break into segments, rotating the calmodulin-like domain around the kinase domain. This rotation increases the accessibility of the N-terminal variable domain as well as of the active site (red) in the kinase domain (black), allowing phosphorylation activity toward its substrate proteins (yellow star, P).

distinct enzyme in a specific intracellular response remains a key challenge in CDPK research. The reasons for this challenge are, first, the lack of mechanistic understanding of *in planta* kinase activation (e.g. distinct stimulus- and/or pathway-specific posttranslational modifications of the kinase may exist) and, second, putative functional redundancy (Mori et al., 2006); no obvious, single *cdpk* mutant phenotype has been described for a long time, probably due to close homology within the CDPK subfamilies.

Here, we report on the current understanding of how single CDPK domains may provide the basis for CDPK isoform response specificity. We also summarize the latest progress in CDPK function, with a focus on *in vivo* substrate identification.

THE N-TERMINAL VARIABLE DOMAIN: SOLELY FOR LOCALIZATION?

The N-terminal variable domain differs in length and amino acid sequence even within the same species (Ito et al., 2010). With the majority of CDPKs harboring a predicted N-myristoylation and N-palmitoylation site, the N terminus was thought to be necessary for membrane association (Harmon et al., 2000; Cheng et al., 2002). In support of this idea, CDPK variants that carry amino acid substitutions G2A (myristoylation) and/or C4/5A (palmitoylation) were defective in the

membrane association demonstrated for AtCPK2 (Lu and Hrabak, 2002), AtCPK6, AtCPK9, and AtCPK13 (Benetka et al., 2008), AtCPK3 (Mehlmer et al., 2010), AtCPK5 (Lu and Hrabak, 2013), and AtCPK16 (Stael et al., 2011) from *Arabidopsis*, NtCDPK2/NtCDPK3 from *Nicotiana tabacum* (Witte et al., 2010), and StCDPK4/StCDPK5 from potato (*Solanum tuberosum*; Asai et al., 2013).

Additionally, the N-terminal variable domain is also subjected to *in vivo* phosphorylation. Both constitutively phosphorylated amino acids and stress stimulus-induced *in vivo* phosphorylation events, catalyzed either by the enzyme itself or by upstream kinases, were reported. In domain-swap experiments, in which the N-terminal variable domains were exchanged between NtCDPK2 and NtCDPK3 in *Nicotiana benthamiana*, the observed *in vivo* phosphorylation pattern was solely determined by the N-terminal variable domain, irrespective of the protein kinase domain used (Witte et al., 2010). This indicates that *in vivo* phosphorylation of CDPKs cannot simply be deduced by sequence alignment, either among close homologs within the same species or among orthologs from different species. Furthermore, introduction of the G2A amino acid substitution in NtCDPK2 resulted not only in a loss of membrane localization but, concomitantly, prevented phosphorylation by upstream kinases at sites within the N-terminal variable domain (Witte et al., 2010). Likewise, a requirement for correct CDPK localization for CDPK function was shown for potato StCDPK5 phosphorylating substrate protein NADPH oxidase StRBOHB (for respiratory burst oxidase homolog B) in plant immune signaling. A chimeric protein containing the variable domain, including myristoylation and palmitoylation sites, of tomato (*Solanum lycopersicum*) SlCDPK2 and the kinase domain and CAD from potato StCDPK5 is localized to the trans-Golgi system (as is SlCDPK2 itself) and is not able to phosphorylate StRBOHB (Asai et al., 2013). The requirement for the variable domain in directing target specificity was further shown by interspecies domain-swap experiments between NtCDPK1 and AtCPK9 (Ito et al., 2010).

The challenge: it is conceivable that a single CDPK isoform, which participates in various and independent stress signaling pathways, may show a differential acylation/phosphorylation pattern within its respective variable domain. However, only a very careful analysis of (stimulus-induced) variable domain-dependent *in vivo* localization and interaction with potential partner proteins, ideally in the absence of ectopic overexpression of kinase and/or substrate proteins, will elucidate the role of the variable domain for substrate specificity.

THE PROTEIN KINASE DOMAIN: WHAT REGULATES ACTIVITY TOWARD A SUBSTRATE IN VIVO?

Standard biochemical assays characterizing CDPK *in vitro* kinase activity have been conducted either with purified or recombinant proteins synthesized in

Escherichia coli and often using the synthetic peptide, syntide2, or histone protein as a model substrate. Kinase activity has been determined in the absence or presence of different Ca^{2+} concentrations, and the effect of further regulatory components such as lipids (Dixit and Jayabaskaran, 2012) or 14-3-3 proteins (Lachaud et al., 2013; Latz et al., 2013) was investigated. With the identification of stress- or pathway-specific CDPK isoforms, the biochemical characterization is now extended to in vivo kinase activation, mediated by stimulus-induced, posttranslational modification/phosphorylation of the CDPK, which may occur at all enzyme domains (Liese and Romeis, 2013). Kinase properties have been determined by in-gel kinase or immune complex kinase assays, in the absence or presence of model substrates or presumed in vivo targets, and autophosphorylation or substrate phosphorylation was visualized by autoradiography. CDPKs were shown to be activated as rapidly as 1 to 2 min after stress exposure; activation often occurred transiently, and activation and response duration were dependent on the isoform as well as the biological process under investigation (Romeis et al., 2000; Ludwig et al., 2005; Boudsocq et al., 2010). For example, in plant immune signaling, the bacterial 22-amino acid, flagellin-derived peptide flg22 elicited a transient activation for AtCPK5 and AtCPK6, peaking at 5 to 15 min (Boudsocq et al., 2010; Dubiella et al., 2013), while enhanced activation was observed between 2 and 3 h after the expression of the bacterial effector proteins AvrRpm1 and AvrRpt2 (Gao et al., 2013).

The rapid transient activation of CDPK kinase activity points to concerted action involving (upstream) protein kinases, phosphatases, and respective substrate proteins. Indeed, joint regulation of Slow Anion Channel1 (SLAC1) activity in the context of stomatal aperture has been demonstrated for the kinases Open Stomata1 (OST1) and AtCPK23/AtCPK21 and the phosphatase Abscisic Acid Insensitive1 (ABI1; Geiger et al., 2010, 2011; Brandt et al., 2012; Scherzer et al., 2012).

The identification of CDPK in vivo activity has long been hampered by the lack of bona fide in vivo substrate proteins. It became evident that some CDPKs, when purified or as recombinant enzymes, promiscuously phosphorylate proteins in vitro at many different Ser and Thr residues and far beyond the previously proposed CDPK phosphorylation motifs (Harper and Harmon, 2005). Selection based on protein-protein interaction screens such as the yeast two-hybrid assay has so far yielded a low success rate for the identification of in vivo CDPK targets, probably due to the complex mechanism of CDPK activation, combined with a transient kinase-substrate interaction. Significant progress in the identification of signaling pathways modulated by CDPKs was made by the ectopic expression of truncated, constitutively active CDPK variants, which lack the entire regulatory CAD (Sheen, 1996; Romeis et al., 2001; Ludwig et al., 2005). With respect to immune signaling, transient expression of these gain-of-function variants identified candidates AtCPK4, AtCPK5, AtCPK6, and AtCPK11 as triggering pathogen-associated molecular pattern-related

transcriptional reprogramming in Arabidopsis leaf mesophyll protoplasts (Boudsocq et al., 2010) and AtCPK5 and AtCPK6 as inducing a reactive oxygen species (ROS)-mediated cell death response in *Nicotiana benthamiana* leaves (Dubiella et al., 2013). Putative pathway and response specificity was confirmed in transgenic lines overexpressing the kinase and/or in loss-of-function mutants lacking (multiple) CDPK gene(s). The tool of transient ectopic expression has been developed even further, to directly investigate CDPK kinase/phosphorylation substrate relationships in vivo. Abscisic acid (ABA)-related anion channel activity was assessed in *Xenopus laevis* oocytes (Geiger et al., 2010, 2011), pollen tube growth-related cation channel activity in COS-7 cells (Zhao et al., 2013), and plant immune signaling-related responses in protoplasts and/or leaves of wild-type and mutant plants (Boudsocq et al., 2010; Dubiella et al., 2013; Gao et al., 2013). Despite this significant progress, however, one should remember that CDPKs may have been functionally assessed under conditions of overexpression (potentially promoting substrate promiscuity) and in the absence of the tissue- and cell type-specific localization.

Recently, state-of-the-art phosphoproteomics was shown to represent the next level in in vivo analysis of CDPK function. In particular, selected reaction monitoring mass spectrometry (SRM-MS) enabled the in vivo identification and quantification of kinase isoform-specific phosphorylation patterns in given substrate proteins. For example, six flg22-induced, AtCPK5-dependent, differentially phosphorylated amino acids in the N terminus of the NADPH oxidase RBOHD were identified and quantified, from both Arabidopsis plants and protoplasts (Dubiella et al., 2013). Amino acids Ser-39, Ser-148, Ser-163, and Ser-347 showed increased, and Ser-162 and Ser-343 showed reduced or unaltered, phosphorylation, which correlated with AtCPK5 activity. While all identified Ser residues were required for RBOHD-catalyzed ROS production, these data provide an unambiguous in vivo signature for AtCPK5 activity in a specific signaling response involving a distinct target protein. These data also indicate a functional differentiation (or hierarchy) at neighboring amino acids, suggesting that the RBOHD protein may be targeted by additional protein kinases in vivo. These kinases may be CDPKs, further calcium-regulated kinases such as CIPKs, or kinases from different classes. Interestingly, Ser-184 and Ser-185 in RBOHF are homologous to Ser-162 and Ser-163 in RBOHD (of which only Ser-163 is directly phosphorylated by CPK5; Dubiella et al., 2013). Ser-184 of RBOHF can be phosphorylated in vitro by the recombinant ABA-signaling-related SnRKII (for Snf1-related protein kinase)-type kinase OST1 (Sirichandra et al., 2009). Pending an in vivo proof, these data provide evidence for exquisite signaling pathway-specific regulation of identical substrate proteins through phosphorylation at neighboring amino acids by different protein kinases.

The challenge: modern mass spectrometry analysis combined with CDPK gain- and loss-of-function

approaches will allow the identification of CDPK- and pathway-specific substrate proteins. Future research is also expected to address the role and hierarchy of multiple *in vivo* phosphorylation events within a single target protein, which may be catalyzed by different protein kinases. In addition, SRM-MS may help to dissect sites subjected to CDPK autophosphorylation and to assess their contribution to stimulus-specific activating/inactivating modifications at the enzyme itself.

THE CDPK ACTIVATION DOMAIN: ARE ALL CDPKS REGULATED BY CALCIUM *IN VIVO*?

CDPKs were primarily perceived as enzymes that swap between a Ca^{2+} -free inactive and a Ca^{2+} -bound active enzyme form. While still valid, this model has been refined (Fig. 1). Experimental evidence indicates a differential contribution of N-terminal and C-terminal EF lobes within the CAD in either stabilizing or releasing the pseudosubstrate region (Christodoulou et al., 2004). Furthermore, CDPK variants, harboring site-specific amino acid substitutions in single or double EF hand motifs, demonstrated a differential requirement of the N- and C-terminal EF hand pairs in standard *in vitro* protein kinase assays (Zhao et al., 1994; Franz et al., 2011). Remarkably, a comparison between Ca^{2+} binding and phosphorylation activity identified Arabidopsis CDPKs that displayed apparent Ca^{2+} -insensitive kinase activity. These isoforms lacked up to three consensus EF hand motifs but were still able to bind Ca^{2+} *in vitro*, while lacking all four EF hand motifs like AtCPK25 abolished Ca^{2+} binding (Boudsocq et al., 2012). So far, it is entirely unknown how *in vivo* sequence variations in single EF hand motifs or different calcium-binding domains influence the translation of Ca^{2+} concentration changes into CDPK activation in a stimulus- and spatiotemporal-specific manner. With the availability of *in vivo* substrate proteins, a functional assessment of isoform-intrinsic Ca^{2+} dependency now appears more feasible. For example, the phosphorylation of SLAC1 by AtCPK21 displays activation kinetics corresponding with four Ca^{2+} -binding sites. Accordingly, constitutive phosphorylation coinciding with functional ion channel activation was only observed for truncated AtCPK21-VK (only variable and kinase domain) lacking the CAD. In contrast, AtCPK23 appears to be Ca^{2+} insensitive, and slow, constitutive SLAC1 activation was observed even with the full-length enzyme (Geiger et al., 2010). AtCPK11 and AtCPK24 have been identified as functional negative regulators of Arabidopsis pollen tube growth. AtCPK11, but not AtCPK24, displays Ca^{2+} sensitivity in *in vitro* kinase assays, and phosphorylates recombinant AtCPK24, but not vice versa. Furthermore, enhanced expression of AtCPK24, but not AtCPK11, was observed during pollen tube growth (Zhao et al., 2013). Taken together, in spite of the current lack of *in vivo* proof for the phosphorylation of either kinase and potential cation channel substrate, these results provide the first evidence for

a fascinating scenario in CDPK regulation: AtCPK11 perceives alternating changes in Ca^{2+} concentration, which may be relayed, through phosphorylation, to a second CDPK, AtCPK24, which finally phosphorylates the effector substrate, here probably a Shaker-type K^+ channel.

The challenge: under the hypothesis of distinct CDPKs being allocated to various biological processes and signaling pathways, and considering the increasing number of corresponding, even overlapping, *in vivo* phosphorylation substrates known for CDPKs, future research will be needed to determine Ca^{2+} -binding affinities and compare the calcium activation kinetics of CDPK isoforms *in vivo*.

CDPKs IN PLANT DEVELOPMENT

A major role for CDPK function in plant development has emerged in pollen tube growth, distinguished by an oscillating Ca^{2+} gradient. Twelve members of the Arabidopsis CDPK gene family are predominantly expressed in pollen (Myers et al., 2009). For AtCPK17/AtCPK34 as well as AtCPK11/AtCPK24, a role in pollen tube growth and fitness has been experimentally validated in double mutant lines (Myers et al., 2009; Zhao et al., 2013).

Furthermore, CDPKs are associated with the activity of phytohormones such as GA_3 . A well-documented example of a CDPK modulating GA_3 homeostasis is NtCDPK1 interacting *in vivo* with the bZIP transcription factor REPRESSOR OF SHOOT GROWTH, which directly regulates GA_3 biosynthesis and is inactivated by NtCDPK1-dependent phosphorylation (Ishida et al., 2008). Recently, a developmental phenotype in stem elongation and vascular development was described for a single CDPK (AtCPK28) loss-of-function mutant in Arabidopsis. Even more remarkably, this phenotype was strictly growth phase dependent. Specifically upon the transition to the generative growth phase, *cpk28* plants were characterized by shorter stems, increased secondary growth, and shortened petioles with enhanced anthocyanin production (Matschi et al., 2013). The stem phenotype correlated with reduced transcript levels of GA_3 biosynthesis genes and could be partially rescued by exogenous application of GA_3 . The wild-type phenotype could be fully restored in *cpk28* upon complementation with AtCPK28 protein expressed under the control of native AtCPK28, but also under the control of tissue-specific promoters, providing evidence for the participation of a mobile signal in mediating AtCPK28 protein function. Interestingly, transient silencing of the orthologous gene pair NaCDPK4 and NaCDPK5 in *Nicotiana attenuata* likewise resulted in plants of reduced height, based on compromised GA_3 -mediated stem elongation (Heinrich et al., 2013), accompanied by increased levels of the biologically active phytohormone, jasmonic acid, conjugate JA-Ile (Yang et al., 2012). Consequently, these silenced plants displayed enhanced resistance toward the herbivore *Manduca sexta*.

One may speculate that CPK28 and its orthologs form a branching point between GA₃ and JA-Ile, with the CDPK modulating the activity of these two phytohormone signaling pathways in order to balance plant growth and defense. The pleiotropic phenotypes raise the question of whether hormonal imbalance is an immediate consequence of reduced CDPK activity or a result of an as yet unknown underlying developmental process affected by these CDPKs. AtCPK28 was shown to display Ca²⁺-dependent kinase activity in vitro, suggesting an important role of Ca²⁺ sensing in developmental processes.

The challenge: future research is expected to identify in vivo features of developmentally induced changes in Ca²⁺ concentrations, including the nature of the inducing stimulus and the source of Ca²⁺ ions; for example, in the case of AtCPK28, activation correlating with the transition to the generative growth phase. Subsequently, the mechanism of the translation of Ca²⁺/CDPK activity toward a specific CDPK target into phytohormone-regulated growth processes remains to be elucidated.

CDPKs IN ABIOTIC STRESS SIGNALING

CDPKs have been known for many years to participate in Ca²⁺-related signal transduction induced by abiotic stress stimuli in the context of salinity, drought, and cold; in particular, isoforms were implicated in ABA-mediated signaling. CDPK-dependent changes in ion fluxes, metabolic changes, or alterations in gene expression were addressed. CDPKs were often identified as positive regulators of abiotic stress responses, and the overexpression of the respective kinase resulted in plants with enhanced stress tolerance (Asano et al., 2012; Boudsocq and Sheen, 2013).

Interestingly, with respect to abiotic stress signaling in Arabidopsis, CDPK substrates have so far been identified predominantly among either ABA Response Factor (ABF) transcriptional regulators or (guard cell-expressed) ion channel proteins and transporters. A positive regulatory effect of CDPKs in drought stress signaling may be explained by the enhanced expression of ABA-responsive genes (Xu et al., 2010). Consistently, in vivo interactions were shown between ABF2 and AtCPK4, AtCPK7, AtCPK10, and AtCPK30 (Lu et al., 2013), as well as between ABF1 and AtCPK11 (Lynch et al., 2012), using split-yellow fluorescent protein studies. Furthermore, in in vitro kinase assays, AtCPK4 and AtCPK11 were shown to phosphorylate ABF1 and ABF4 (Zhu et al., 2007; Lynch et al., 2012). Notably, other kinases, such as members of the SnRK gene families, also are able to interact with and to phosphorylate ABF2 in an ABA-dependent manner in vivo (Umezawa et al., 2013). In addition, CDPK phosphorylation substrates were found among cation and anion channels. CPK3 was reported as phosphorylating a two-pore K⁺ channel (TPK1), enabling 14-3-3 protein-mediated channel activation

(Latz et al., 2013). AtCPK6, AtCPK21, and AtCPK23 functionally interact with and phosphorylate the slow voltage-gated anion channel, SLAC1, as a prerequisite for channel activation (Geiger et al., 2010, 2011; Brandt et al., 2012; Demir et al., 2013), and anion currents mediated by SLAC1 and SLAC1 Homolog3 (SLAH3) were shown to be essential for ABA-mediated stomatal opening and closure (Hedrich, 2012). In addition, ABA-dependent, in planta interaction and colocalization in lipid rafts were shown for SLAH3 and AtCPK21 (Demir et al., 2013). While AtCPK21 was able to phosphorylate SLAH3 in vivo, response activation was suppressed in the presence of phosphatase ABI1 (Geiger et al., 2011).

The challenge: interestingly, single CDPK isoforms appear to participate in rapid short-term signaling reactions (e.g. AtCPK21 and AtCPK23 proteins function as positive regulators in ABA-induced stomatal closure) as well as in the regulation of long-term adaptive processes (e.g. *cpk21* and *cpk23* mutant lines display an accumulation of stress-related metabolite and marker genes, concomitant with an increase in stress tolerance; Ma and Wu, 2007; Franz et al., 2011). While some molecular CDPK targets in rapid signaling responses have been identified, the nature of the tissue- and/or cell type-specific CDPK target proteins mediating long-term responses still has to be determined.

CDPKs IN BIOTIC STRESS SIGNALING

CDPKs were recognized early as key players participating in the translation of pathogen signal-induced changes in the Ca²⁺ concentration into plant defense reactions. These reactions include the synthesis of ROS, altered gene expression, changes in phytohormone synthesis and signaling, and cell death (Tena et al., 2011; Boudsocq and Sheen, 2013). The rapid biochemical activation of CDPKs by pathogen stress and subsequent deactivation within 15 to 120 min (Romeis et al., 2000) suggests a role of CDPKs in the onset of plant immune reactions. Such rapid in vivo activation was first described for *Nicotiana tabacum* NtCDPK2, triggered by the fungal elicitor Avr9 in plants that harbored the corresponding tomato disease resistance gene Cf-9 (Romeis et al., 2000, 2001; Ludwig et al., 2005). Likewise, potato StCDPK4 and StCDPK5 were identified as modulators of early defense reactions, including StrBOHB-mediated ROS production in response to a *Phytophthora infestans* elicitor (Kobayashi et al., 2007, 2012). In Arabidopsis leaf mesophyll protoplasts, flg22-induced biochemical activation was shown for AtCPK5 and AtCPK6 (orthologs to StCDPK4 and StCDPK5; Dubiella et al., 2013) and for AtCPK4 and AtCPK11 (Boudsocq et al., 2010), whereas AtCPK1 and AtCPK2 (orthologs to NtCDPK2) were implicated predominantly during effector-triggered immunity (Gao et al., 2013). Functional studies also demonstrated a differing, partially overlapping pattern of defense gene expression triggered by these enzymes

(Boudsocq et al., 2010). In addition, the NADPH oxidase isoform RBOHD, as well as the WRKY transcriptional regulators WRKY8, WRKY28, and WRKY48, were reported as *in vitro* substrates phosphorylated by a subset of these CDPKs. Mutants lacking multiple CDPK or WRKY genes were unable to mount a full plant defense mediating resistance to bacteria (Gao et al., 2013). A recent study in Arabidopsis demonstrated AtCPK5-mediated changes in defense gene expression as well as long-lasting salicylic acid-dependent enhanced bacterial resistance. AtCPK5 was shown to rapidly phosphorylate specific Ser residues of RBOHD *in vivo* in response to bacterial flg22. Furthermore, AtCPK5 and AtRBOHD were shown to constitute a self-propagating mutual activation circuit, which provides the basis for ROS-mediated signal transduction from local to distal plant sites, resulting in the biochemical activation of AtCPK5 and AtCPK5-mediated defense responses in distal cells, ultimately driving resistance to pathogens (Dubiella et al., 2013). Interestingly, both AtCDPK5 and RBOHD contain EF hand Ca^{2+} -binding motifs and undergo posttranslational regulation, not only by phosphorylation but also by Ca^{2+} binding. While the precise role of Ca^{2+} within this mutual activation process has still to be elucidated, CDPKs seem to participate in a Ca^{2+} - and ROS-driven mechanism for cell-to-cell immune signal propagation, complementing a model that has been postulated previously (Torres et al., 2005; Miller et al., 2009).

The challenge: during the onset of the plant immune response, single CDPKs have been shown to activate both substrate proteins in the plasma membrane (e.g. proteins involved in rapid ROS-based signal propagation) and transcriptional regulators functioning in the nucleus (e.g. those responsible for long-lasting changes in gene expression). While subcellular localization has been reported for some CDPKs, a detailed cell biological analysis of CDPK localization or potential immune stimulus-dependent relocalization in a native plant system without overexpression or (heterologous) ectopic expression is still missing.

CDPKs AS SIGNALING HUBS?

The progress in recent CDPK research, and in particular the identification of *in vivo* substrates, points to two hallmark features of CDPK signaling: (1) a single enzyme family and even a single isoform may integrate signals triggered by different biological processes, suggesting a role of CDPKs as hubs in stress signaling and development; and (2) multiple CDPK isoforms not only share the same signaling pathway but even target an identical substrate protein.

A distinct CDPK may not only contribute to rapid and long-term responses within one signaling pathway but may control responses even in different signaling pathways. For example, guard cell-expressed AtCPK6 is implicated in ABA signaling, and AtCPK6-mediated ROS generation and direct phosphorylation

of SLAC1 was reported (Mori et al., 2006; Munemasa et al., 2011). When expressed in leaf protoplasts, AtCPK6 undergoes rapid flg22-induced biochemical activation and induces transcriptional reprogramming reminiscent of plant innate immune signaling (Gao et al., 2013). An even more stunning example is CPK11, reported to participate in plant immune and abiotic stress signaling (affecting transcriptional regulators) as well as in pollen tube growth (regulating a K^+ channel; Zhu et al., 2007; Zhao et al., 2013).

Furthermore, CDPKs apparently engage in joint actions. Arabidopsis AtCPK4, AtCPK5, AtCPK6, and AtCPK11, and recently also AtCPK1/AtCPK2, have been implicated in pathogen-induced plant defense signaling, with partially overlapping *in vitro* phosphorylation targets identified (Gao et al., 2013). AtCPK6, AtCPK21, and AtCPK23 participate in the activation of the abiotic stress-related anion channel SLAC1, which is also targeted by OST1 (Brandt et al., 2012). Although these data, in part, rely on ectopic expression, recombinant proteins, or heterologous experimental systems, the demand arises for a model outlining the molecular mechanism of such apparent multiple enzyme regulation. With the prerequisite of native coexpression in the same cell, does functional CDPK isoform specificity with respect to phosphorylation and subsequent intracellular response exist at all, and if yes, how can it be mechanistically explained?

One requirement is the identification of a distinct, stimulus-specific phosphorylation pattern for a defined *in vivo* substrate protein for each individual CDPK. This has been shown by SRM-MS analysis in the case of RBOHD phosphorylation by AtCPK5. Also, differential, site-specific *in vitro* phosphorylation of SLAC1 was reported for the kinases AtCPK23 and OST1. Such concerted regulation of phosphorylation by different protein kinases and dephosphorylation by phosphatases may be correlated with a positive or negative effect on the CDPK substrate (activity) itself as well as on induced downstream responses.

A second key may be CDPK regulation by Ca^{2+} binding within the CAD. It is conceivable that CDPK isoforms within a single cell may have distinct Ca^{2+} -binding affinities. Thus, depending on the subcellular localization with respect to a (stimulus-dependent) local change in the intracellular Ca^{2+} concentration, a distinct isoform may become activated or remain in its inactive form.

While technical advances in mass spectrometry facilitated the resolution of CDPK isoform-specific phosphorylation *in vivo* at the biochemical level, the deciphering of differential *in vivo* CDPK Ca^{2+} -binding affinities, as well as their requirement for triggering pathway-specific CDPK function, remains one of the biggest immediate challenges to be tackled in the field of CDPK research.

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