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CDPKs in immune and stress signaling

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Abstract

Ca^{2+} has long been recognized as a conserved second messenger and principal mediator in plant immune and stress responses. How Ca^{2+} signals are sensed and relayed into diverse primary and global signaling events is still largely unknown. Comprehensive analyses of the plant-specific multigene family of Ca^{2+}-dependent protein kinases (CDPKs) are unraveling the molecular, cellular and genetic mechanisms of Ca^{2+} signaling. CDPKs, which exhibit overlapping and distinct expression patterns, sub-cellular localizations, substrate specificities and Ca^{2+} sensitivities, play versatile roles in the activation and repression of enzymes, channels and transcription factors. Here, we review the recent advances on the multifaceted functions of CDPKs in the complex immune and stress signaling networks, including oxidative burst, stomatal movements, hormonal signaling and gene regulation.

Ca^{2+} sensor protein kinases in immune and stress signaling networks

Despite long-standing knowledge that Ca^{2+} mediates plant responses to a wide range of developmental and environmental stimuli through variations of its intracellular concentrations, the molecular, cellular and genetic links between Ca^{2+} signatures and the multiple downstream signaling events are largely obscure. Calcium signatures are defined by spatio-temporal features, including amplitude, frequency, duration and sub-cellular location, that are likely to contribute to Ca^{2+} signaling specificity along with the diverse proteins able to sense and decode these signals [1–9]. Plants possess three main families of calcium sensors: calmodulin (CaM), calcineurin B-like (CBL) and calcium-dependent protein kinases (CDPKs). Unlike CaM and CBL that must relay the Ca^{2+}-induced conformational change to protein partners, CDPKs have the unique feature of both Ca^{2+} sensing and responding activities within a single protein to directly translate Ca^{2+} signals into phosphorylation events [2,6,10,11]. Recently, major progress has been made to uncover the central roles of CDPKs in triggering appropriate and diverse downstream responses in

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the plant immune and stress signaling networks. In this review, we examine the recent advances on the molecular activation mechanism of CDPKs, as well as their versatile roles in immune and stress signaling, such as the regulation of oxidative burst, cell death, stomatal movements, hormonal signaling and gene expression.

CDPK structure, regulation and Ca^{2+} signal relay

CDPK structure and regulation

CDPKs harbor an N-terminal variable domain, a Ser/Thr kinase domain, an auto-inhibitory junction region and a regulatory calmodulin-like domain (CaM-LD) [1,2,10,11]. The four EF-hand Ca^{2+}-binding motifs of the CaM-LD are organized into two lobes that have distinct Ca^{2+} affinities resulting in different roles in CDPK regulation [12,13]. In the basal state, the intramolecular interaction between the junction region and the catalytic center maintains the kinase in an inactive state by a pseudosubstrate mechanism [1,2]. The C-terminal lobe of the CaM-LD exhibiting high Ca^{2+} affinity interacts with the auto-inhibitory region at low Ca^{2+} level to stabilize the structure [14]. Ca^{2+} binding to the low-affinity N-terminal lobe of the CaM-LD induces a conformational change that releases the auto-inhibition [1,2]. As a result, deleting both the autoinhibitory domain and the CaM-LD generates a constitutively active form that constitutes a powerful tool for studying CDPK functions *in vivo* [15–17].

The activation model, based on the *in vitro* characterization of recombinant proteins, is now supported by the *in planta* analysis of EF-hand-mutated variants of *Arabidopsis* (*Arabidopsis thaliana*) AtCPK21 [18]. Interestingly, the recent crystal structure of full-length apicomplexan (*Toxoplasma gondii* and *Cryptosporidium parvum*) CDPKs in both apo and Ca^{2+}-bound forms confirms the essential role of the N-lobe of the CaM-LD in triggering CDPK activation [19]. Importantly, multiple CDPK isoforms from soybean (*Glycine max*) and *Arabidopsis* exhibit distinct Ca^{2+} sensitivities, which is consistent with their roles in decoding different Ca^{2+} signals [20,21]. However, several CDPKs, including *Arabidopsis* AtCPK13 and AtCPK23, were recently shown to be weakly or not sensitive to Ca^{2+} (Table 1) [21–23]. Despite alterations in some EF-hand motifs, the CDPKs with apparently lower Ca^{2+}-sensitivity are able to bind Ca^{2+} *in vitro* [21], which triggers the same conformational change as in canonical CDPKs [24]. Moreover, most reported CDPK assays used generic but not specific and biologically relevant endogenous substrates to determine the Ca^{2+} sensitivity of CDPK activities, which may vary with different substrates [1,20,21]. Thus, it remains possible that all CDPKs exhibit Ca^{2+} activation *in vivo* with appropriate substrates. A high-throughput screen of synthetic peptides has identified many potential CDPK substrates for further characterization [25]. Nonetheless, Ca^{2+} also regulates other aspects of CDPKs, such as protein interactions [26,27] or sub-cellular localization [24]. Thus, CDPKs can sense various Ca^{2+} signals through the amplitude and sub-cellular location of Ca^{2+} rise, but how they may distinguish frequency and duration requires further investigation, such as elucidating the molecular mechanism of CDPK deactivation.

Besides Ca^{2+}, phosphorylation, lipids and interaction with 14-3-3 proteins have been reported to further modulate CDPKs *in vitro* [1,2,4,10,11]. Recent *in vivo* studies, including phosphoproteomic approaches [28,29], are starting to reveal the biological significance of these regulatory mechanisms in response to various signals. For instance, stress-dependent phosphorylations correlated with kinase activation have been reported for the tobacco (*Nicotiana tabacum*) NtCDPK2 and NtCDPK3 [30]. In maize (*Zea mays*), the lipid activation of ZmCPK11 by direct binding of phosphatidic acid probably occurs in response to wounding [31].

CDPK expression and localization

CDPKs are encoded by multigene families of 34 members in *Arabidopsis* [1,2,10], 31 in rice (*Oryza sativa*) [32,33] and at least 20 in wheat (*Triticum aestivum*) [34], and are divided into four subgroups (Figure 1, Table 1). Extensive transcriptomic analyses have revealed different expression patterns for each isoform, which contributes to the functional specificity of the CDPKs [1,33–35]. Some CDPKs are expressed in most organs whereas others are specific to some tissues. For example, AtCPK17 and AtCPK34 are preferentially expressed in mature pollen and regulate pollen tube growth [36]. Differential expression of CDPKs has been observed in response to diverse stimuli, including abscisic acid (ABA), cold, drought, salinity, heat, elicitors and pathogens [16,33–35], which correlates with the presence of stress-responsive *cis*-elements in rice *CDPK* gene promoters [35]. CDPK protein accumulation has also been reported after cold or ABA treatment, resulting in enhanced CDPK kinase activity [37–39].

Most CDPKs have a predicted N-myristoylation site involved in membrane targeting (TermiNator, http://www.isv.cnrs-gif.fr/terminator2/index.html), which has been confirmed *in vitro* for some of them [12,40–45]. This irreversible co-translational acylation requires a second post-translational signal to maintain the membrane association, such as reversible palmitoylation [30,41], a polybasic domain that may be modified by phosphorylation [43] or protein interaction. As a result, most CDPKs are membrane anchored (Table 1) and the reversibility of the second signal may allow CDPKs to shuttle between membranes and the cytosol or nucleus. Diverse cellular localizations of CDPKs have been observed (Table 1), including the cytosol, nucleus, plasma membrane, endoplasmic reticulum (ER), tonoplast, mitochondria, chloroplasts, oil bodies and peroxisomes [17,27,34,36,38–40,42,44,46–48], indicating that CDPKs have access to a plethora of potential substrates throughout the cell. Moreover, stress-induced nuclear accumulation has been observed for the ice plant (*Mesembryanthemum crystallinum*) McCPK1 [43,49] and the groundnut (*Arachis hypogaea*) AhCPK2 [24].

Substrate specificity

Biochemical analyses based on known substrates have identified four distinct motifs that define CDPK target sites [2]. An extensive survey of 534 synthetic peptides tested for *in vitro* kinase assays with four recombinant *Arabidopsis* CDPKs (AtCPK1, AtCPK10, AtCPK16 and AtCPK34) revealed that CDPK specificity relies either on the substrate itself or on kinetic parameters for a common substrate [25]. This could result from different Ca^{2+} affinities that can affect substrate accessibility, as suggested by the crystal structure of apicomplexan CDPKs. Indeed, the N-lobe of the CaM-LD is located near the N-terminus of the kinase domain in resting conditions while the entire CaM-LD rotates around the kinase domain to release the substrate-binding site in the Ca^{2+}-bound form [19]. Moreover, a domain-swap analysis between NtCDPK1 and AtCPK9 demonstrates the key role of the N-terminal variable domain in controlling substrate specificity [50].

Identifying *in vivo* substrates is an important challenge to unravel CDPK functions. To date, most substrates have been described using *in vitro* assays and are involved in diverse cellular processes, such as primary and secondary metabolism, stress responses, ion and water transport, transcription and signaling [2,4,10,25]. Interestingly, an analysis of 274 synthetic peptides harboring *in vivo*-mapped phosphorylation sites showed a 27% match with AtCPK substrates in an *in vitro* kinase reaction, suggesting potential biological relevance [25]. Yeast-two-hybrid screens with CDPK variants exhibiting altered kinase activity have facilitated the identification of putative substrates that require *in vivo* confirmation [49,51,52]. Several transcription factors, including ABF4 (ABA-responsive element-binding factor 4), RSG (repression of shoot growth) and HsfB2a (heat shock factor

B2a), have been further characterized as *in vivo* CDPK substrates involved in ABA [47], gibberellin [26,50] and herbivore-induced signaling [23], respectively. Recently, an *in planta* random screen coupling bimolecular fluorescence complementation and flow cytometry has identified new AtCPK3 interactors that could not be revealed by yeast-two-hybrid assays, suggesting the potential to identify plant-specific protein interactions [53]. Future challenges include correlating the mutant phenotypes of particular CDPKs and their corresponding substrates to establish their biological significance.

In summary, the multigene family of CDPKs encodes key Ca^{2+} sensor protein kinases that differ by their expression pattern, sub-cellular localization, substrate specificities, Ca^{2+} sensitivities and regulation by lipids, phosphorylation and protein interactions. This huge diversity in molecular and biochemical properties of CDPKs is likely to provide functional specificity and redundancy in mediating plant Ca^{2+} signaling in immune and stress responses.

CDPKs in immune signaling

Plants sense potential pathogens through the recognition of microbe-associated molecular patterns (MAMPs) by cell-surface pattern-recognition receptors (PRRs) or effector proteins via intracellular nucleotide-binding leucine-rich repeat (NB-LRR) immune sensors to initiate overlapping and distinct signaling cascades, including protein kinase activation, Ca^{2+} influx, hormone biosynthesis, oxidative burst and transcriptional reprogramming. Recent studies have provided compelling evidence for the involvement of CDPKs in most of these signaling events (Figure 2).

Hormonal signaling and gene regulation in plant defense

The tobacco NtCDPK2 from subgroup I is the first CDPK identified for its role in race-specific plant defense. The extracellular Avr9 fungal effector strongly activates NtCDPK2 in tobacco leaves expressing the corresponding tomato (*Lycopersicon esculentum*) resistance gene Cf9 as a LRR receptor-like protein on the plasma membrane [16]. This activation requires both NtCDPK2 autophosphorylation and phosphorylation by an upstream kinase, revealing the complexity of fine-tuning defense signaling cascades [30]. Transient expression of constitutively active NtCDPK2 triggers jasmonic acid (JA) and ethylene (ET) accumulation, and subsequent induction of JA- and ET-regulated genes [54]. ET production could occur through stabilizing the rate-limiting enzyme ACC synthase (ACS) of ET biosynthesis by direct phosphorylation, as observed for the closest homolog LeCDPK2 in tomato [55] and purified maize CDPKs [56]. Interestingly, constitutively active NtCDPK2 blocks the Avr9–Cf9 activation of mitogen-activated protein kinases (MAPKs) in an ET-dependent manner, potentially serving as a negative feedback to reset the system after elicitation [54] (Figure 2). In *Arabidopsis*, overexpressing the closest homolog AtCPK1 confers broad-spectrum resistance to bacteria and fungi [48]. However, unlike NtCDPK2, which reduces salicylic acid (SA) levels and the expression of SA-regulated *pathogenesis-related* genes (*PR1a* and *PR2a*) [54], long-term AtCPK1 overexpression triggers SA accumulation through the induction of SA regulatory and biosynthesis genes, *PAD4* (*phytoalexin-deficient 4*) and *SID2/ICS1* (*SA induction-deficient 2/isochorismate synthase 1*), and consequently SA-regulated genes without affecting JA or ET [48]. Interestingly, AtCPK1 specifically phosphorylates phenylalanine ammonia-lyase (PAL) *in vitro*, which is a key enzyme in plant defense notably involved in an alternative pathway to produce SA [57]. Although the role of PAL phosphorylation in defense is not clear, this finding further supports the notion that AtCPK1 plays a crucial role in SA accumulation (Figure 2). Although we could expect that protein kinase orthologs play similar roles in various plant species, these results suggest that some closely related CDPK homologs might have evolved independently in different plant species or in different biological contexts. However, it is

also crucial to distinguish transient and long-term CDPK overexpression and activation, which may lead to distinct direct and indirect physiological consequences.

In *Arabidopsis* leaf cells, the bacterial MAMP flg22 (a 22-amino acid peptide of flagellin) transiently activates multiple CDPK activities. Interestingly, a cell-based functional genomic screen with 25 constitutively active AtCPKs using a flg22-responsive reporter *NHL10-LUC* (*NDR1/HIN1-like10-luciferase*) identified four related CDPKs from subgroup I, AtCPK4, AtCPK5, AtCPK6 and AtCPK11, as early transcriptional regulators in MAMP signaling [17]. Unlike NtCDPK2, these four AtCPKs do not affect MAPK activation by flg22. Unexpectedly, CDPKs and MAPK cascades differentially regulate flg22-induced early genes in at least four regulatory programs, displaying CDPK-specific, MAPK-specific, CDPK/MAPK parallel or CDPK/MAPK synergistic regulation, which imply independent or co-regulation of common targeted transcription factors, transcription machinery and/or chromatin remodeling complexes [58,59] (Figure 2). Correlated with genome-wide analysis of redundant and specific target genes modulated by multiple MAMPs and by transiently expressed active AtCPK5 and AtCPK11, *cpk5 cpk6* double mutant, *cpk5 cpk6 cpk11* triple mutant and *cpk4 cpk5 cpk6 cpk11* quadruple mutant exhibit gradually reduced flg22 responsiveness for gene expression and pathogen resistance, demonstrating the key positive roles of these CDPKs in convergent MAMP signaling [17].

In summary, various CDPKs from subgroup I mediate transient and sustained transcriptional reprogramming in plant innate immune responses, and play key roles in the regulation of SA, ET and JA hormonal signaling.

Oxidative burst and cell death

Transient expression of active NtCDPK2 also induces ROS (reactive oxygen species) production and HR (hypersensitive response)-like cell death upon exposure to a non-symptom-producing stress [54]. Consistently, silencing its orthologs in *Nicotiana benthamiana* reduces the HR elicited by the Avr4–Cf4 and Avr9–Cf9 interactions [16]. Interestingly, the closest homolog AtCPK1 also triggers ROS production by stimulating the NADPH oxidase activity in tomato protoplasts [60]. Active forms of potato (*Solanum tuberosum*) StCDPK4 and StCDPK5, close homologs of AtCPK5/AtCPK6, induce ROS production by directly phosphorylating the NADPH oxidase RBOHB (respiratory burst oxidase homolog B) *in vivo* at the site targeted by infection with *Phytophthora infestans* [61]. As a consequence, transgenic potato plants overexpressing the active variant of StCDPK5 display increased ROS production, HR-like cell death and resistance to the hemibiotrophic pathogen *P. infestans*, but higher susceptibility to the necrotrophic pathogen *Alternaria solani* [62]. Coherently, the *Arabidopsis cpk5 cpk6* double mutant, *cpk5 cpk6 cpk11* triple mutant and *cpk4 cpk5 cpk6 cpk11* quadruple mutant show reduced early ROS production in response to flg22 [17]. These results suggest that multiple CDPKs from subgroup I play a key role in the defense-induced oxidative burst by activating NADPH oxidases through direct phosphorylation (Figure 2). Homologs of AtCPK5/AtCPK6 also mediate plant defense in monocots. The rice OsCPK13 induces cell death, accumulation of PR proteins and up-regulation of some defense genes when ectopically expressed in sorghum (*Sorghum bicolor*) [63], whereas the active form of barley (*Hordeum vulgare*) HvCDPK4 triggers cell death [64]. Thus, CDPKs display conserved defense functions among plant species in promoting cell death. However, genetic manipulation of CDPKs in crop protection may need specifically tailored strategies considering the site and duration of CDPK activation and the targeted pathogens.

By contrast, several CDPKs have been shown to play negative roles in plant defense. In barley, HvCDPK3 from subgroup II promotes host cell entry of the powdery mildew fungus during both compatible and incompatible interactions [64]. In rice, overexpressing

OsCPK12 from subgroup II confers susceptibility to both virulent and avirulent blast fungus, potentially through ABA hypersensitivity and reduction in ROS production [65]. Interestingly, Ca^{2+}- and CaM-regulated protein kinase (CCaMK) is a central signaling hub in root nodule and arbuscular mycorrhiza symbioses in plants [66]. However, plant defense must be shut down in the initial phase of the plant-microbe interaction, so that the symbiont is not recognized as a pathogen. In *Medicago truncatula*, MtCDPK1 from subgroup IV is likely to be involved in this process because the *MtCDPK1*-silenced plants are compromised in establishing symbiotic interactions and display enhanced ROS production induced by Nod factors and increased expression of cell wall biosynthesis and defense genes [67].

Responses to wounding and herbivore attacks

During insect attack, wounded tissues constitute entry sites for herbivory elicitors, making wounding perception a part of herbivore recognition. A recent screen of 19 *cpk* T-DNA insertion mutant lines identified two CDPKs, AtCPK3 and AtCPK13, as positive regulators of *PDF1.2* induction by *Spodoptera littoralis* caterpillars [23]. This regulation probably occurs through phosphorylation-dependent activation of the transcription factor HsfB2a, without affecting the level of ET, JA or ABA. Moreover, AtCPK3, but not AtCPK13, also triggers a negative feedback on herbivore-induced Ca^{2+} signals, indicating that CDPKs can play redundant as well as specific functions in plant defense. Given that AtCPK3 can be activated by flg22 in protoplasts [44] and induce the flg22-responsive gene *NHL10* [17], it may also be involved in MAMP signaling. By contrast, the tomato LeCDPK2 phosphorylates the ethylene biosynthesis enzyme LeACS2 at the site phosphorylated *in vivo* after wounding, suggesting that LeCDPK2 contributes to ethylene production in response to wounding [55]. Interestingly, MAPKs also phosphorylate LeACS2 at a different site, and both CDPK and MAPK phosphorylations are required simultaneously to stabilize LeACS2 *in vivo* [55]. Wounding also induces extracellular alkalinization through the inhibition of the plasma membrane H^+-ATPase, which may be mediated in tomato by the membrane-anchored LeCPK1 [12] (Figure 2). In maize, ZmCPK11, which is closely related to AtCPK4/AtCPK11, is activated by wounding in a JA-dependent pathway; however, its precise biological function remains to be determined [68]. Recently, two redundant CDPKs from subgroup IV in coyote tobacco (*Nicotiana attenuata*), NaCDPK4 and NaCDPK5, were proposed to negatively regulate herbivore resistance by blocking JA and defense metabolite accumulation, without affecting the expression of JA biosynthesis enzymes [69].

Thus, various CDPKs from different subgroups mediate plant responses to wounding and herbivores through the regulation of hormone biosynthesis, such as ET and JA, and gene expression, either with a positive or a negative effect.

CDPKs in hormonal and abiotic stress signaling

Gene regulation in ABA, drought and salt stress signaling

ABA is a key stress hormone that mediates plant responses to drought and salinity. AtCPK10 and AtCPK30 from subgroup III are the first CDPKs identified as positive regulators of the barley stress- and ABA-inducible *HVA1* promoter in maize protoplasts [15]. Constitutively active AtCPK10 also induces endogenous stress- and ABA-inducible genes in *Arabidopsis* leaf cells (Y. Niu and J. Sheen, unpublished), suggesting a conservation of specific CDPK functions in dicots and monocots. This transcriptional induction is likely to occur through the ABA-responsive transcription factors, ABFs, which were identified as *in vitro* substrates for several CDPKs from subgroups I and III [39,47] (Figure 3). In particular, AtCPK32 activates ABF4 *in vivo*, resulting in the induction of ABF4 target genes [47]. AtCPK4 and AtCPK11 regulate both ABF1 and ABF4 and induce gene expression several hours after ABA elicitation, suggesting a role in long-term

adaptation [39]. This modified gene expression correlates with ABA hypersensitivity and increased tolerance to drought and salt in plants overexpressing AtCPK4 or AtCPK11, whereas the *cpk4 cpk11* double mutant exhibits the opposite phenotypes. Given that only selected ABA-responsive genes are partially affected in the *cpk4 cpk11* double mutant, other regulators also contribute to ABA signaling [39]. Similar results were observed in rice transgenic plants overexpressing OsCPK21 [70] and OsCPK13 (OsCDPK7) [71], suggesting that ABA-induced transcriptional reprogramming via ABFs is likely to be a key feature of CDPK signaling in both monocots and dicots.

Although the role of CDPK phosphorylation has not been thoroughly investigated, other transcription factors involved in stress-induced gene regulation have also been identified as CDPK substrates *in vitro*. They include the *Arabidopsis* dehydration-inducible gene family AtDi19, which encodes nuclear zinc-finger proteins [25,51], and the ice plant pseudo-response regulator transcription factor CSP1 [49] (Figure 3). By contrast, AtCPK12, the closest homolog of AtCPK4/AtCPK11, inhibits ABA responses by stimulating the negative regulator ABI2 (ABA insensitive 2), although the role of ABI2 phosphorylation by AtCPK12 requires further studies [72].

Metabolic and transport regulation in drought and salt stress signaling

Drought and salinity trigger the production of ROS, which must be detoxified. Soybean GmCDPKα and GmCDPKγ have been shown to phosphorylate *in vitro* the serine acetyltranferase 2;1 (GmSerat2;1) involved in cysteine biosynthesis, at the site phosphorylated *in vivo* after oxidative stress [73]. Since the phosphorylation releases the feedback inhibition by cysteine, CDPKs may participate in anti-oxidant responses by providing cysteine for glutathione production. In rice, OsCPK12 regulates ROS homeostasis under salt stress conditions by inducing the expression of ROS scavenger genes *OsAPX2/OsAPX8* and repressing the NADPH oxidase gene *OsRBOHI*, leading to increased salt tolerance [65]. Overexpressing AtCPK6 confers drought tolerance, correlated with enhanced gene expression and accumulation of the compatible osmolyte proline, but reduces lipid peroxidation, probably through decreased ROS production [74]. However, the *cpk6* single mutant does not exhibit any stress phenotype, as observed in pathogen responses because of functional redundancy among CDPKs [17]. By contrast, AtCPK21 is a negative regulator of osmotic responses and inhibits proline accumulation [18]. The subtle enhanced drought tolerance in the *cpk21* mutant is likely to result from the compensated overexpression of the closest homolog AtCPK23 [18], which also negatively regulates drought and salt resistance by inhibiting K^+ uptake [75] (Figure 3). Interestingly, unlike in defense responses [17,23], AtCPK3 does not regulate gene expression in salt stress signaling but modulates the phosphoproteome independently of MAPK cascades [44]. In particular, two putative substrates, a glutathione-*S*-transferase and a subunit of a potassium channel, suggest a role of AtCPK3 in anti-oxidant responses and K^+ uptake, respectively.

Limiting water loss is crucial during dehydration conditions and may occur through the down-regulation of aquaporin water channels, as observed in spinach (*Spinacea oleracea*) with the CDPK-stimulated aquaporin PM28A [76,77]. Regulating Ca^{2+} signals is also a key component of stress signaling to ensure that specific responses are induced by each stress. The *Arabidopsis* auto-inhibited calcium ATPase ACA2 has been shown to play a crucial role in generating the appropriate Ca^{2+} signal in yeast exposed to high salinity [78]. Interestingly, the active form of AtCPK1 inhibits the basal activity of ACA2 and blocks stimulation by CaM in yeast [79]. However, AtCPK1 is not localized in the ER, unlike its closest homolog AtCPK2 and ACA2, suggesting that AtCPK2 might regulate ACA2 *in planta* [40,48]. Thus, multiple CDPKs differentially modulate transcription factors, metabolic enzymes, ion and water transport to positively or negatively regulate drought and salt responses.

Regulation of stomatal movements

Ca^{2+} is an essential component of guard cell signaling, and regulates ion fluxes involved in stomatal movements, notably through CDPK-dependent phosphorylation of channels. The *cpk3 cpk6* double mutant is impaired in ABA-activation of slow-type anionic channels and Ca^{2+} permeable channels, resulting in decreased ABA-induced stomatal closure [80]. Despite a weak *in vivo* interaction with the major guard cell Cl^- channel SLAC1 [22], AtCPK6 but not AtCPK3 was recently shown to phosphorylate SLAC1 at S59 and to activate the channel in Xenopus (*Xenopus laevis*) oocytes [81]. Similarly, two close homologs from subgroup II, AtCPK21 and AtCPK23, also phosphorylate and activate SLAC1 and its related NO_3^- channel SLAH3 in response to ABA [22,82]. AtCPK23 preferentially regulates SLAC1 independently of Ca^{2+} whereas AtCPK21 mainly regulates SLAH3 in a Ca^{2+}-dependent manner (Figure 3). Whether the three CDPKs target the same phosphosite remains to be determined to understand the biological relevance of such a redundancy. Surprisingly, *cpk21* and *cpk23* single mutants do not exhibit any stomata phenotype, unlike *cpk3* and *cpk6* [22,80,82]. It is thus possible that the stronger phenotypes observed in *cpk3* and *cpk6* result from the inhibition of Ca^{2+} currents that would impact global Ca^{2+}-regulated responses rather than only inhibiting anionic channels. Interestingly, unlike other *cpk* mutants, *cpk6* is also impaired in methyl jasmonate (MeJA)-induced S-type anionic current and stomatal closure [83]. Since MeJA and ABA differentially mediate pathogen and abiotic stress responses, distinct hormonal pathways may regulate stomatal closure upon biotic and abiotic stresses.

The *cpk4 cpk11* double mutant is also partially compromised in ABA-induced stomatal closure with enhanced leaf water loss [39], whereas its closest homolog in grapevine (*Vitis vinifera*) ACPK1 confers the opposite phenotype when overexpressed in *Arabidopsis* [84]. The inhibition of K^+ inward channels, such as KAT1, also contributes to stomatal closure. In the broad bean (*Vicia faba*), a CDPK phosphorylates KAT1 *in vitro* [85], which inhibits the channel activity [86]. Moreover, the ABA inhibition of K^+ inward channels is abolished in the *Arabidopsis cpk10* mutant, leading to reduced stomatal closure and drought hypersensitivity, suggesting that AtCPK10 may down-regulate KAT1 *in vivo* [27]. AtCPK1 stimulates a vacuolar Cl^- channel, resulting in Cl^- uptake into the vacuole and stomatal opening [87]. Despite some discrepancies in ABA-induced stomatal closure in some *cpk* mutants, the impaired Ca^{2+}-induced stomatal closure in multiple *cpk* mutants from subgroups I, II and III, *cpk4 cpk11* and *cpk3 cpk6* double mutants, *cpk10* single mutant and *cpk7 cpk8 cpk32* triple mutant, demonstrates the crucial role of CDPKs in regulating stomatal movements, even though the molecular mechanism remains to be elucidated [88].

Cold tolerance

Ca^{2+}-mediated early cold induction of the key transcriptional regulators, the CBFs (cold-responsive element-binding factors), is crucial for cold tolerance. Several CaM-interacting transcription activators (CAMTAs) have been shown to bind to the *CBF2* promoter and the *camta3* mutant is impaired in cold-induction of *CBF1* and *CBF2*. The reduced freezing tolerance of the *camta1 camta3* double mutant further supports the notion that CAMTAs play a role in cold-stimulated Ca^{2+} signaling [89]. However, the functions of CDPKs in cold stress signaling remain mostly elusive. Unlike in salt or drought signaling, OsCPK13 (OsCDPK7) confers cold resistance without affecting transcriptional regulation [71]. Similarly, AtCPK1 does not affect *CBF* induction but regulates the phosphoproteome after cold treatment [90]. A membrane-bound rice CDPK is activated by cold after 18–24h, suggesting a role in the adaptive process rather than early responses [91]. Likewise, overexpressing OsCPK7 (OsCDPK13), which is preferentially expressed in cold-tolerant rice varieties, triggers cold resistance and the accumulation of the cold-responsive chaperone

calreticulin [37,92]. Thus, CDPKs are clearly involved in cold tolerance, but their molecular functions remain to be explored (Figure 3).

CDPKs at the crossroad of stress signaling networks

Recent advances have revealed CDPKs as central regulators of Ca^{2+}-mediated immune and stress responses that are crucial for plant survival. Some key players, including AtCPK1, AtCPK3, AtCPK4, AtCPK6, AtCPK11, OsCPK12 and OsCPK13, represent crucial signaling nodes that mediate plant responses to both abiotic stress and pathogens (Table 1). Beside some specificity, several CDPKs also show functional redundancy that may provide robust plant responsiveness and adaptability to adverse environmental conditions. For instance, AtCPK4, AtCPK5, AtCPK6 and AtCPK11 co-regulate gene expression in early MAMP signaling [17], whereas AtCPK4, AtCPK11, AtCPK10, AtCPK30 and AtCPK32 all mediate ABA responses through ABFs [15,39,47]. There is also functional redundancy with other protein kinase families such as the SNF1-related kinases 2 (SnRK2s) [93,94] and MAPKs [17] (Figures 2 and 3). In particular, several effector proteins of guard cell signaling such as KAT1, SLAC1, RBOHs and ABFs are targeted by both CDPKs and SnRK2s, potentially at different sites, although the co-regulation has not been investigated [95]. Furthermore, analogous to the roles in antagonizing SnRK2s [95], the protein phosphatase 2C (PP2C) ABI1, ABI2 and/or related PP2Cs inhibit CDPKs in stress and ABA signaling, such as AtCPK10/AtCPK30-mediated gene regulation [15,96], or AtCPK21/AtCPK23 activation of anionic channels [22,82].

More complex interactions have been observed between CDPKs and MAPK cascades, from synergism [17,55] to independence [44] and antagonism [54]. The multiple cross-talks between CDPKs and SnRK2s or MAPKs provide additional layers of regulation to fine-tune plant immune and stress responses. Strikingly, some CDPKs may play opposite roles in different cell types. For instance, AtCPK21/AtCPK23 activate SLAC1/SLAH3 in guard cells to promote stomatal closure [22,82], whereas *cpk21* and *cpk23* mutants are drought tolerant at the whole plant level [18,75]. Distinct CDPK substrates are likely to be responsible for different physiological functions in diverse cellular contexts.

Concluding remarks and perspectives

Extensive research efforts with integrative approaches have provided conclusive evidence that CDPKs are versatile and evolutionarily conserved Ca^{2+}-sensors/transducers that function in a diverse array of plant processes in response to environmental challenges. Combined with expression patterns, intracellular localizations/translocations and modulation by lipids, phosphorylation and interacting proteins, the broad ranges of Ca^{2+} sensitivity and substrate specificity of CDPKs dictate complex and sophisticated Ca^{2+} signaling networks via protein phosphorylation to coordinate the dynamic plant cellular processes. Future molecular, cellular, genetic, genomic and phosphoproteomic studies should lead to more precise understanding of specific and redundant roles of CDPKs in the immune and stress signaling networks in cooperation with other Ca^{2+} sensors and protein kinases, such as RLKs (receptor-like kinases), MAPKs, SnRK1s, SnRK2s and SnRK3s/CIPKs (CBL-interacting protein kinases) [5–7,9,17,25,88,93,94,97,98]. Considering that Ca^{2+} signals are restricted and localized inside the cells, establishing molecular, cellular and genetic links with specific Ca^{2+} channels, pumps and transporters [8] in a spatio-temporal analysis of CDPK activation and translocation is crucial to decipher Ca^{2+}-mediated signaling networks in plants. Unlike the Ca^{2+}-independent SnRK2s and MAPKs, analyzing the *in vivo* activation of CDPKs has been limited because of the difficulty in maintaining precise Ca^{2+} levels reflecting the physiological states in cell extracts. Developing specific anti-phosphopeptide antibodies raised against active CDPKs or their immediate substrates should

facilitate the monitoring of CDPK activation *in planta* [17,25,30,99,100]. A complementary genetic approach by mutating the EF-hands should establish a functional link between CDPKs and Ca^{2+} signaling [18,36]. Finally, identifying *in vivo* substrates and the unique regulatory features of each isoform should provide new insights into CDPK signaling to understand their integrated roles in diverse biological responses, a prerequisite for genetic manipulation of agronomically valuable traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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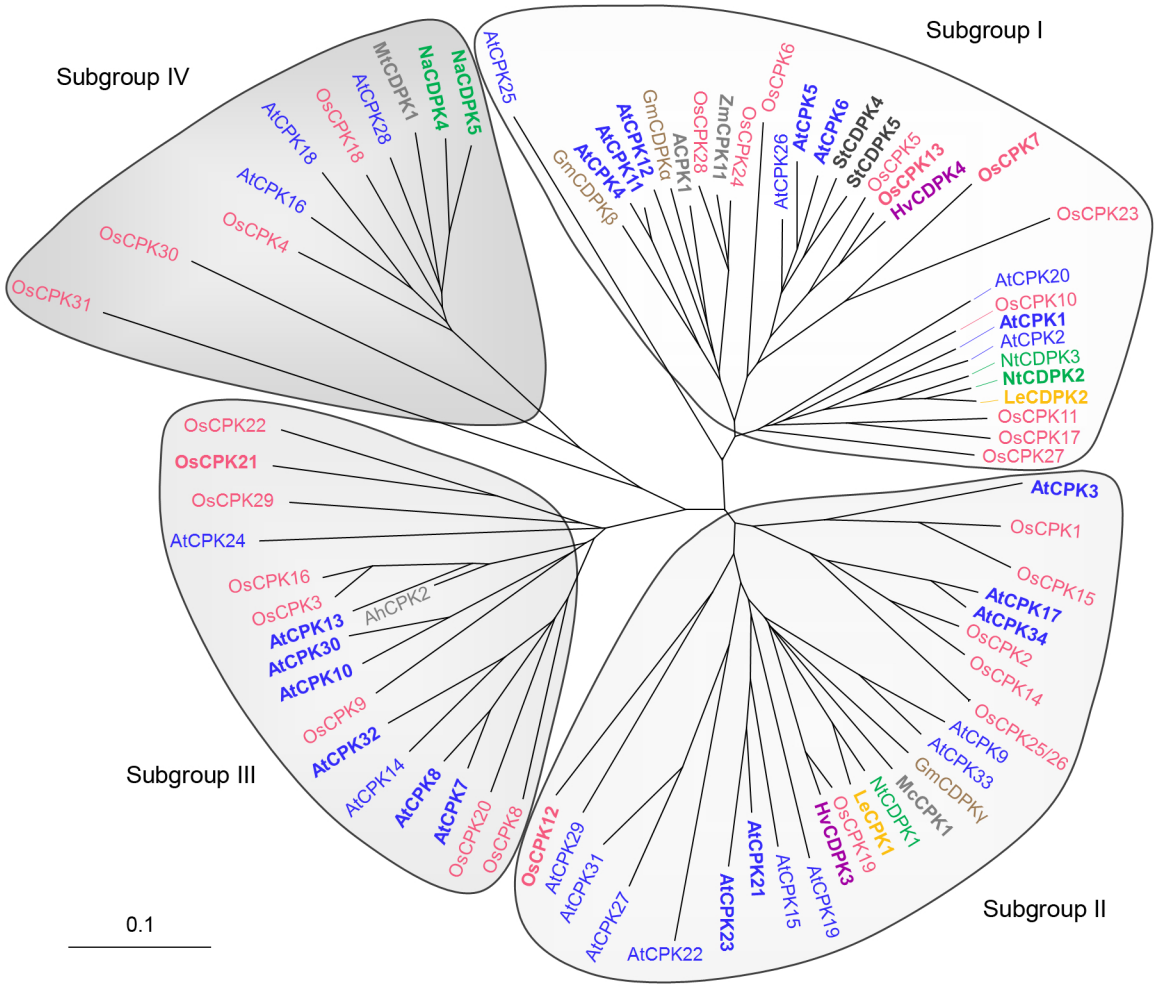

Figure 1.

Relation tree of selected plant CDPKs. The full-length amino acid sequences of CDPKs (see supplementary material online) from *Arabidopsis* (At, blue), rice (Os, pink), soybean (Gm, brown), potato (St, black), barley (Hv, purple), tobacco (Nt, green), coyote tobacco (Na, green), tomato (Le, yellow) and grapevine (ACPK1, gray), maize (Zm, gray), alfalfa (Mt, gray), ice plant (Mc, gray) and peanut (Ah, gray) were aligned and analyzed with ClustalX and TreeView algorithms. The CDPK family is divided into four major subgroups (I–IV). The branched lengths are proportional to divergence and the scale of 0.1 represents 10% change. The CDPKs with known biological functions are highlighted in bold.

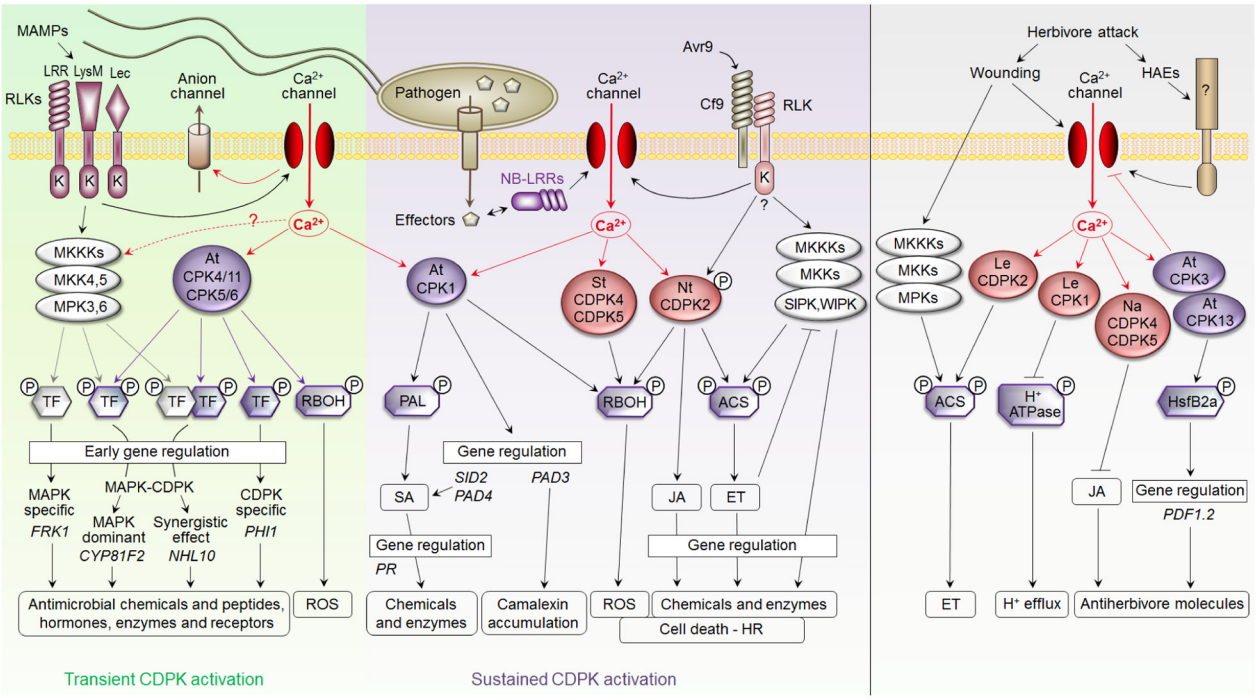

Figure 2.

CDPK signaling network in immune responses. Microbe-associated molecular pattern (MAMP) perception by different cell-surface receptor kinases (RLKs) with distinct extracellular domains triggers transient CDPK activation to regulate transcription factors and early gene expression either independently or in coordination with MAPK cascades. Several CDPKs also activate NADPH oxidases (respiratory burst oxidase homologs, RBOHs) to induce early reactive oxygen species (ROS) production. By contrast, the sustained CDPK activation by extracellular (Avr9) or intracellular effector proteins leads to biosynthesis of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) through regulatory gene induction or enzyme activation such as phenylalanine ammonia-lyase (PAL) and ACC synthase (ACS). CDPKs also trigger a prolonged oxidative burst involved in cell death and hypersensitive response (HR). Constitutively active NtCDPK2 inhibits MAPK [salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK)] activation by Avr9–Cf9 in an ET-dependent manner. Herbivores can be sensed through wounding or herbivore-associated elicitors (HAEs) by unknown receptors to activate MAPKs and Ca^{2+} influx. The co-regulation of ACS by MAPKs and CDPKs leads to ET production, whereas LeCPK1 inhibits the plasma membrane H^{+}-ATPase to induce extracellular alkalinization. AtCPK3 and AtCPK13 mediate herbivore-induced gene expression by phosphorylating the transcription factor HsfB2a whereas only AtCPK3 negatively regulates Ca^{2+} channels. NaCDPK4 and NaCDPK5 negatively regulate defense against herbivores by inhibiting JA accumulation and subsequent production of defense metabolites. Abbreviations: MKKs, mitogen-activated protein kinase kinases; MKKKs, mitogen-activated protein kinase kinase kinases; MPKs, mitogen-activated protein kinases; TF, transcription factor.

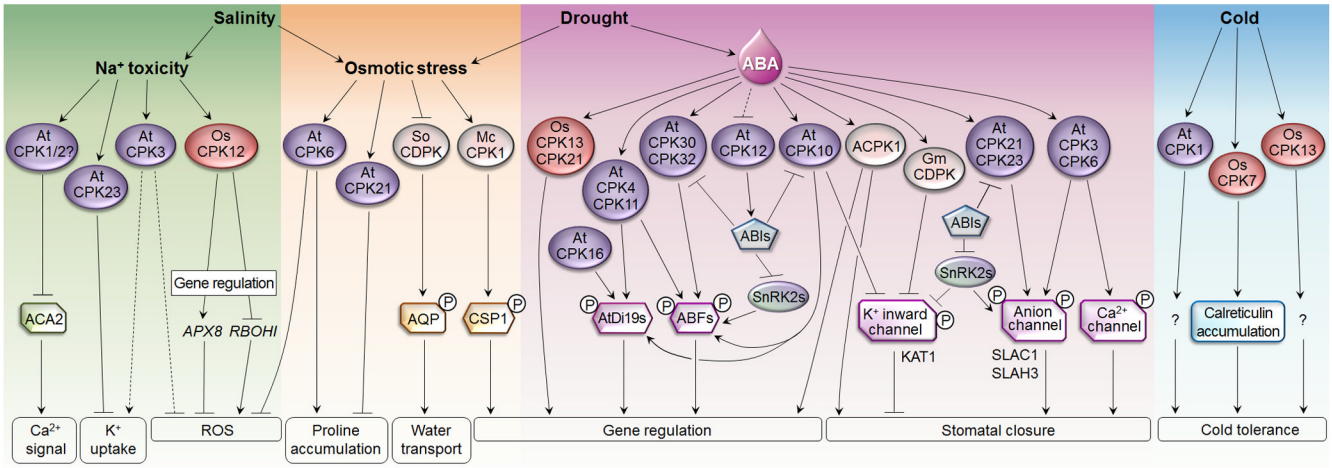

Figure 3.

CDPK signaling network in abiotic stress responses. Plants sense drought and salinity through Na^+ toxicity, osmotic stress and ABA synthesis to activate CDPKs, which regulate K^+ uptake, ROS production, accumulation of compatible osmolytes (proline), water transport (aquaporin, AQP) and gene expression. Redundant CDPKs modulate gene expression by activating the transcription factors ABFs and AtDi19s. AtCPK12 is a negative regulator that stimulates the protein phosphatase ABIs, inhibiting SnRK2- and CDPK-dependent transcriptional regulation. CDPKs also promote stomatal closure by inhibiting the K^+ inward channel (KAT1), and activating slow-type anionic channels (SLAC1 and SLAH3). CDPKs and SnRK2s share common substrates (ABFs and channels) and common down-regulators (ABIs). Some CDPKs also inhibit permeable Ca^{2+} channels or Ca^{2+}-pumps (ACA2) in a negative feedback loop. Several CDPKs have been shown to trigger cold tolerance; however, the molecular mechanism is not understood.

Table 1

Functional characterization of *Arabidopsis* CPKs

Protein (synonym)	Gene	Sub-group	Expression [a]	*N*-acylation prediction[b]	Localization[c]	Ca^{2+} - dep[d]	Function[c]	Refs
CPK1 (AK1)	At5g04870	I	R, S, GC	N-Myr	Peroxisomes, oil bodies	Yes	SA and defense, cold	[13,25,46,48,57,60, 79,87,90]
CPK2	At3g10660	I	R, P	N-Myr	ER and other membranes	Yes	ND	[21,40,42]
CPK3 (CDPK6)	At4g23650	II	R, S, GC	N-Myr	Soluble, PM, tonoplast	Yes	Herbivore, salinity, stomata	[21,23,44,46,53,80, 88]
CPK4	At4g09570	I	R, S, P, GC	–	Cytosol, nucleus	Yes	MAMP, ABA, drought, salinity, stomata	[17,21,39,46,51,52, 88]
CPK5	At4g35310	I	R, S, GC	N-Myr	Membrane, cytosol, nucleus	Yes	MAMP	[17,21]
CPK6 (CDPK3)	At2g17290	I	R, S, P, GC	N-Myr	Membrane, cytosol, nucleus	Yes	MAMP, ABA, drought, salinity, stomata	[17,42,74,80,81,83, 88]
CPK7	At5g12480	III	R, S, GC	N-Myr-Palm	PM	Unclear	Stomata	[21,46,88]
CPK8 (CDPK19)	At5g19450	III	R, S, GC	N-Myr-Palm	PM	Unclear	Stomata	[21,46,88]
CPK9	At3g20410	II	R, S, GC	N-Myr-Palm	PM	Yes	ND	[21,42,46]
CPK10 (CDPK1)	At1g18890	III	R, S, GC	N-Myr-Palm	PM	Unclear	ABA, drought, stomata	[15,21,25,27,88]
CPK11 (CDPK2)	At1g35670	I	R, S, P, GC	–	Cytosol, nucleus	Yes	MAMP, ABA, drought, salinity, stomata	[17,21,39,51,52,88]
CPK12 (CDPK9)	At5g23580	I	R, S	–	Cytosol, nucleus	Yes	ABA	[72]
CPK13	At3g51850	III	R, S, GC	N-Myr-Palm	Membrane, PM	Unclear	Herbivore	[21,23,42]
CPK14	At2g41860	III	P	N-Myr-Palm	ND	ND	ND	
CPK15	At4g21940	II	S	N-Myr-Palm	ND	ND	ND	
CPK16	At2g17890	IV	P	N-Myr-Palm	PM	ND	ND	[25,45,46]
CPK17	At5g12180	II	P	N-Myr-Palm	PM	ND	Pollen tube growth	[36]
CPK18	At4g36070	IV	P	N-Myr-Palm	ND	ND	ND	
CPK19	At1g61950	II	–	–	Membrane	Yes	ND	[21]
CPK20	At2g38910	I	P	N-Myr	ND	ND	ND	
CPK21	At4g04720	II	R, S, GC	N-Myr-Palm	PM	Yes	Osmotic stress, stomata	[18,22,46,82]
CPK22	At4g04710	II	R, S, GC	N-Myr-Palm	ND	ND	ND	
CPK23	At4g04740	II	–	N-Myr-Palm	PM	Unclear	Drought, salinity, stomata	[22,75,82]
CPK24	At2g31500	III	P	N-Myr-Palm	ND	ND	ND	
CPK25	At2g35890	I	P	N-Myr	Membrane	No	ND	[21]

Protein (synonym)	Gene	Sub-group	Expression [a]	*N*-acylation prediction[b]	Localization[c]	Ca^{2+} - dep[d]	Function[c]	Refs
CPK26	At4g38230	I	P	–	ND	ND	ND	
CPK27	At4g04700	II	R, S, GC	N-Myr-Palm	ND	ND	ND	
CPK28	At5g66210	IV	R, S, GC	N-Myr-Palm	PM	ND	ND	[46]
CPK29	At1g76040	II	R, S	–	ND	ND	ND	
CPK30 (CDPK1a)	At1g74740	III	R, S, GC	N-Myr-Palm	Membrane	Unclear	ABA and abiotic stress	[15,21]
CPK31	At4g04695	II	–	N-Myr-Palm	ND	ND	ND	
CPK32	At3g57530	III	R, S, P, GC	N-Myr-Palm	Membrane, nucleus	Unclear	ABA, stomata	[21,47,88]
CPK33	At1g50700	II	R, S	N-Myr-Palm	ND	ND	ND	
CPK34	At5g19360	II	P	N-Myr-Palm	PM	Yes	Pollen tube growth	[25,36]

[a] The expression of CPKs in root (R), shoot (S), pollen (P) and guard cell (GC) was compiled from diverse microarray data (http://www.weigelworld.org/resources/microarray/AtGenExpress/). Several CPKs exhibit a low expression level in all organs (−).

[b] The absence (−) or presence of acylation at the N-terminus, either a myristate (N-Myr) and/or a palmitate (Palm), was predicted by TermiNator program (http://www.isv.cnrs-gif.fr/terminator2/index.html).

[c] Abbreviations: ER, endoplasmic reticulum; PM, plasma membrane; ND, not determined; SA, salicylic acid; MAMP, microbe-associated molecular pattern; ABA, abscisic acid.

[d] Some CPKs are clearly Ca^{2+}-dependent for their activity (Yes) whereas others exhibit no or low calcium stimulation on general and unspecific substrates despite effective calcium binding (Unclear). Only CPK25 lacking EF-hands is truly Ca^{2+}-independent (No). ND, not determined.