CURRENT REVIEW

Regulation of Plant Immune Signaling by Calcium-Dependent Protein Kinases

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Activation of Ca^{2+} signaling is a universal response to stress that allows cells to quickly respond to environmental cues. Fluctuations in cytosolic Ca^{2+} are decoded in plants by Ca^{2+} -sensing proteins such as Ca^{2+} -dependent protein kinases (CDPKs). The perception of microbes results in an influx of Ca^{2+} that activates numerous CDPKs responsible for propagating immune signals required for resistance against disease-causing pathogens. This review describes our current understanding of CDPK activation and regulation, and provides a comprehensive overview of CDPK-mediated immune signaling through interaction with various substrates.

ANTI-MICROBIAL SIGNALING IN PLANTS

Plants have adapted to microbe-rich environments by evolving finely tuned immune systems to perceive and defend against potential pathogens. Typical of eukaryotic detection systems, plant cell surface receptors bind foreign ‘non-self’ molecules such as fungal chitin or bacterial flagellin (Couto and Zipfel 2016). These microbe-associated molecular patterns (MAMPs) are generally essential to microbial lifestyles and, therefore, are conserved across whole classes of microbes (Couto and Zipfel 2016). Immune signals are amplified through the detection of ‘infectious-self’ molecules known as damage-associated molecular patterns (DAMPs) such as cell wall fragments and small peptides released during infection or wounding (Gust et al. 2017). Recognition of MAMPs and DAMPs is mediated by plasma membrane-localized receptor kinases (RKs) or receptor-like proteins known as pattern recognition receptors (PRRs) (Couto and Zipfel 2016). Archetypal PRRs in plants include the RKs FLAGELLIN SENSING 2 and ELONGATION FACTOR-Tu (EF-Tu) RECEPTOR, which recognize the bacterial MAMPs flagellin and EF-Tu or their derived peptide epitopes flg22 and elf18, respectively (Boutrot and Zipfel 2017). Upon ligand-binding, several PRRs form heteromeric complexes with additional RKs, resulting in the activation of immune signaling (Couto and Zipfel 2016).

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Receptor-like cytoplasmic kinases (RLCKs) such as BOTRYTIS-INDUCED KINASE 1 (BIK1) and related PBS1-like kinases are direct convergent substrates of multiple PRRs in *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) (Bi et al. 2018). Phosphorylation of RLCKs by upstream PRRs results in catalytic activation (Liu et al. 2013; Lu et al. 2010), allowing subsequent phosphorylation of substrates such as the transmembrane NADPH-oxidase RESPIRATORY BURST OXIDASE D (RBOHD) (Kadota et al. 2014; Li et al. 2014). Immune signals are transduced via protein kinases of different classes, including mitogen-activated protein kinases (MAPKs) (Devendrakumar et al. 2018) and Ca^{2+} -dependent protein kinases (CDPKs) (Romeis and Herde 2014), ultimately leading to transcriptional activation of defense genes via different classes of transcription factors (Li et al. 2016).

Pathogen virulence strategies typically involve shutting down immune systems by disabling key signaling proteins through the activity of effector proteins deployed into the host cell during infection (Toruño et al. 2016). For example, pathogen effectors have been identified that target plant PRRs, PRR coreceptors, signaling kinases, transcription factors, and other immune proteins (Asai and Shirasu 2015; Macho and Zipfel 2015; Toruño et al. 2016). The presence or activity of effectors is recognized in resistant plants by intracellular nucleotide-binding domain and leucine rich repeat (NLR) receptors (Cui et al. 2015; Jones and Dangl 2006; Tsuda and Katagiri 2010). NLR activation results in a form of localized programmed cell death known as the hypersensitive response (HR) that is associated with a systemic increase in phytohormones that protect neighboring and distal tissues from further infection (Cui et al. 2015).

The release or production of second messengers such as Ca^{2+} is an important aspect of immune signal transduction (Aldon et al. 2018; Kudla et al. 2018). Transient and dynamic increases in cytosolic $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{\text{cyt}}$) constitute one of the earliest immune responses in plants (Yuan et al. 2017). Increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ are indispensable for local and systemic PRR- and NLR-mediated immune signaling (Seybold et al. 2014), as well as symbiotic interactions (Yuan et al. 2017; Zipfel and Oldroyd 2017). Ca^{2+} oscillations following immune induction have been experimentally determined via transgenic expression of luminescent or fluorescent Ca^{2+} sensors such as apoaequorin (Knight et al. 1991),ameleon fluorescent protein (Miyawaki et al. 1997), and R-GECO (Keinath et al. 2015). Although the channels remain elusive (Seybold et al. 2014), treatment with plasma membrane Ca^{2+} channel blockers suggests that an influx of extracellular Ca^{2+} is required for receptor-mediated defense responses (Blume et al. 2000; Grant et al. 2000; Jeworutzki et al. 2010; Lecourieux et al. 2002). Some pathogens, including *Xanthomonas campestris* pv. *campestris*, *Pseudomonas syringae* pv.

tomato, and *P. syringae* pv. *syringae*, can dampen MAMP-triggered responses by producing extracellular polysaccharides that sequester apoplastic Ca^{2+} (Aslam et al. 2008), highlighting the importance of Ca^{2+} in plant immune signaling.

Local changes in $[\text{Ca}^{2+}]_{\text{cyt}}$, known as Ca^{2+} signatures, vary in amplitude and duration in a stimulus-dependent manner and are thought to allow cells to elicit appropriate responses (Webb et al. 1996). This is achieved, in part, by Ca^{2+} sensor proteins (CSPs) that monitor changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and translate this information through downstream signaling cascades (Ranty et al. 2016). CSPs include calmodulin (CaM) and CaM-like proteins (CMLs) (Yang and Poovaiah 2003), calcineurin B-like proteins (Luan 2009), and CDPKs (Batistić and Kudla 2012;

Cheng et al. 2002), several of which are encoded in plants (Fig. 1A).

ROLE OF CDPKS IN IMMUNE SIGNALING

CDPKs represent a unique class of CSP found in plants and some protists, including ciliates and apicomplexan parasites (Billker et al. 2009; Hamel et al. 2014), which likely arose due to an ancestral fusion between CaM and CaM kinase (Chen et al. 2017; Zhang and Choi 2001). Since the initial purification and characterization of a CDPK from soybean (*Glycine max*) (Harmon et al. 1987), extensive CDPK families have been identified in all sequenced plant genomes, including early land

Fig. 1. Diagrammatic representation of plant Ca^{2+} sensor proteins (CSPs) and Ca^{2+} -mediated activation of Ca^{2+} -dependent protein kinases (CDPKs). **A**, Typical domain architecture of plant CSPs (modified from DeFalco et al. 2009). Calmodulin (CaM) and CaM-like proteins (CMLs), calcineurin B-like proteins (CBLs), and CDPKs contain Ca^{2+} -binding EF-hand motifs. CDPKs additionally contain a Ser/Thr kinase domain and a CDPK activation domain (CAD), composed of an autoinhibitory junction (AIJ) and a CaM-like domain containing EF-hands. Some CDPKs have been shown to autophosphorylate at Tyr residues, suggesting that they could be dual-specificity kinases (Bender et al. 2017; Oh et al. 2012). Myristoylation (Myr) sites at Gly2 are predicted for most CMLs (McCormack and Braam 2003). CBLs and CDPKs possess Myr sites at Gly2 and some contain additional palmitoylation (Palm) sites at Cys3-5 (Batistić et al. 2008; Martín and Busconi 2000). **B**, Relief-of-autoinhibition model for plant CDPK activation as proposed by Harmon et al. (1994), Harper et al. (1994), and Yoo and Harmon, (1996). At low $[\text{Ca}^{2+}]_{\text{cyt}}$, Ca^{2+} binds the C-terminal EF-hand lobe and the CAD blocks the active site of the kinase, rendering the protein inactive. An increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ allows Ca^{2+} to bind the N-terminal EF-lobe, resulting in an allosteric change that pulls the CAD away from the active site of the kinase, promoting the active conformation.

plants (Mohanta et al. 2015). Whereas lower eukaryotes have relatively small families (2 to 15 members), land plants have expansive families ranging from 11 to 53 members (Hamel et al. 2014; Mohanta et al. 2015; Valmonte et al. 2014). Plant CDPKs are divided among four well-resolved subgroups (I to IV) conserved across monocot and eudicot species (Hamel et al. 2014). Protists and plants have undergone independent expansion of CDPK gene families and, as such, ampicomplexan CDPKs divide into four subgroups that are distinct from those formed by plants (Valmonte et al. 2014). The high sequence similarity across phylogenetic subgroups suggests that CDPKs arose through recent duplication events and that paralogs likely share overlapping functions (Mohanta et al. 2015).

CDPKs regulate plant responses to environmental stress, including chilling, drought, salinity, infection, and herbivory, and are also important for long-term adaptive processes and development (Romeis and Herde 2014; Shi et al. 2018). CDPKs from several plant species are genetically required for defense responses or are upregulated following pathogen challenge, suggesting broad roles for CDPKs in immune signal transduction. In this review, we discuss recent advancements in our understanding of CDPK activation in the context of biotic stress and provide a detailed overview of CDPK-mediated immune signaling.

Biochemical and immune-induced activation of CDPKs.

The architecture of CDPKs is well-defined, containing amino (N)- and carboxyl (C)-terminal variable domains, a Ser/Thr kinase domain, an autoinhibitory junction (AIJ), and a regulatory CaM-like domain containing Ca^{2+} -binding EF-hands (Fig. 1A) (Oh et al. 2012). Notably, the ability to autophosphorylate at Tyr residues has been observed for some CDPKs, suggesting that they could be dual-specificity kinases (Bender et al. 2017; Oh et al. 2012). The presence of both a Ca^{2+} -binding domain and a protein kinase domain in a single protein allows CDPKs to both receive and propagate signals, suggesting that they are effective signal transducers (Harper et al. 2004; Wernimont et al. 2010, 2011). Analysis of *Arabidopsis* AtCPK1 (Harper et al. 1994) and apicomplexan CDPKs from *Toxoplasma gondii* (TgCDPK1) and *Cryptosporidium parvum* (CpCDPK1) (Wernimont et al. 2010) demonstrated that the catalytic activity of CDPKs is primarily controlled by the CaM-like domain and AIJ, known collectively as the CDPK-activation domain (CAD). The CaM-like domain is composed of up to four EF-hand motifs organized into two lobes (Cheng et al. 2002; Valmonte et al. 2014). Structural analysis of the CaM-like domain of AtCPK1 indicates that the C-terminal lobe has a high affinity for Ca^{2+} under resting conditions, whereas the N-terminal lobe is only bound in the presence of high $[\text{Ca}^{2+}]$ (Fig. 1B) (Chandran et al. 2006; Christodoulou et al. 2004).

Although there are no resolved structures for plant CDPKs, crystallization of active and inactive forms of CDPKs from the apicomplexan parasites *T. gondii* (Ingram et al. 2015; Wernimont et al. 2010), *C. parvum* (Wernimont et al. 2010), and *Plasmodium falciparum* (Wernimont et al. 2011) largely supports a “relief-of-autoinhibition” model for CDPK activation (Fig. 1B) (Harmon et al. 1994; Harper et al. 1994; Yoo and Harmon 1996). At resting $[\text{Ca}^{2+}]_{\text{cyt}}$ (0.1 to 0.2 μM), the AIJ and C-terminal lobe of the CaM-like domain act as a pseudosubstrate that occludes the active site of the kinase, rendering the protein inactive (Fig. 1B) (Chandran et al. 2006; Harper et al. 1994; Wernimont et al. 2010). This is facilitated by intramolecular bonding between the N-terminal portion of the kinase domain and the N-terminal EF-hand lobe at several contact points which “locks” the protein into an inactive state. An increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ to 1 to 5 μM promotes binding of Ca^{2+} to the N-terminal EF-hand lobe, resulting in a dramatic

conformational change that frees the pseudosubstrate from the active site of the kinase domain (Fig. 1B) (Wernimont et al. 2010). Consistent with the role of the CAD in autoinhibition under low $[\text{Ca}^{2+}]$ (Christodoulou et al. 2004), the deletion of this domain generates auto-active CDPKs (Harper et al. 1994; Yoo and Harmon 1996), which has been a valuable experimental tool (Boudsocq et al. 2010; Dubiella et al. 2013; Gao et al. 2013). However, this may not be a universal feature of CDPKs, as deletion of the CAD of TgCDPK1 abolishes kinase activity (Ingram et al. 2015). Molecular dynamics simulations with the crystal structure of TgCDPK1 indicates that interaction between the Ca^{2+} -loaded CAD and the N-terminal extension of the kinase domain acts as a molecular splint that stabilizes the active formation of the kinase (Ingram et al. 2015). Moreover, it was recently demonstrated that splice variants of rice OsCPK17 lacking the CAD are catalytically inactive (Almadanim et al. 2018). Therefore, it is possible that some plant CDPKs may not be regulated by a relief-of-autoinhibition mechanism as previously thought. This highlights the need for a solved crystal structure for plant CDPKs, which would allow more detailed structural and biochemical analysis of the regulatory mechanisms governing CDPK activation.

Although Ca^{2+} -mediated allosteric changes are typically required for activation, CDPKs vary in their Ca^{2+} dependency. For example, soybean CDPKs have $K_{0.5}$ values ($[\text{Ca}^{2+}]$ required for 50% optimal activity) that range between 0.06 and 1 μM (Lee et al. 1998), and *Arabidopsis* CDPKs have $K_{0.5}$ values between 0.2 and 20 μM (Boudsocq et al. 2012). A number of factors have been suggested to influence Ca^{2+} dependency such as the number of EF-hands in the CaM-like domain, the position of N-terminal EF-hands relative to the AIJ, or the presence of noncanonical EF-hand motifs, which has recently been well reviewed (Bender et al. 2018). Furthermore, some CDPKs show substrate-specific differences in $K_{0.5}$ values (Boudsocq et al. 2012; Lee et al. 1998; Szczegielniak et al. 2005), which may allow CDPKs to function in multiple pathways activated by different stimuli.

Regulation of CDPKs by posttranslational modifications.

The mechanisms underlying stimulus-dependent activation of CDPKs are not well understood, particularly for those involved in multiple pathways (Asano et al. 2012; Schulz et al. 2013). In addition to local changes in Ca^{2+} flux (discussed above), subcellular localization and tissue-specific expression may regulate CDPK activity by dictating substrate choice and limiting CDPKs to specific developmental pathways. Moreover, in order for cells to rapidly respond to environmental cues, reversible posttranslational modifications are needed to allow for dynamic changes in protein activity (He et al. 2017). Whereas typical RD (Arg-Asp)-type kinases require autophosphorylation in their activation loop for enzyme activity (Johnson et al. 1996), CDPKs contain an acidic residue in this region that bypasses this requirement (Harper et al. 2004). However, autophosphorylation of CDPKs has been shown to either activate or inhibit kinase activity, depending upon the biochemical context (Chaudhuri et al. 1999; Chehab et al. 2004; Oh et al. 2012; Saha and Singh 1995). For example, in vitro autophosphorylation of soybean GmCDPK β at Tyr24 attenuates enzyme activity whereas phosphorylation at other Ser and Thr residues enhances activity (Oh et al. 2012). Autophosphorylation may additionally be associated with Ca^{2+} sensitivity of some CDPKs, as demonstrated by *Arabidopsis* AtCPK28, which requires lower $[\text{Ca}^{2+}]$ for kinase activity when hyperphosphorylated in vitro (Bender et al. 2017). It has also recently been shown that Ca^{2+} -dependent autophosphorylation of tobacco (*Nicotiana tabacum*) NtCDPK1 at Ser6 and Thr21

disrupts phosphorylation of biological substrate REPRESSOR OF SHOOT GROWTH but enhances phosphorylation of generic substrate MYELIN BASIC PROTEIN in vitro, suggesting that site-specific phosphorylation may also dictate substrate choice (Ito et al. 2017).

Immune-induced activation of CDPKs has been observed using in-gel kinase assays (Boudsocq et al. 2010; Gao et al. 2013; Romeis et al. 2000; Seybold et al. 2017). Additionally, as phosphorylation increases the molecular weight of proteins, it is possible to qualitatively observe phosphorylation of CDPKs via mobility shift in western blots (Seybold et al. 2017). Although in vivo phosphorylated residues may not yet be known, Ca²⁺-dependent phosphorylation has been observed on several CDPKs. For example, following elicitation with the *Cladosporium fulvum* effector Avr9 in tobacco, NtCDPK2 is rapidly phosphorylated and activated (Romeis et al. 2000, 2001; Witte et al. 2010). Additionally, *Arabidopsis* AtCPK5 becomes phosphorylated following treatment with flg22 or elf18 (Dubiella et al. 2013), and AtCPK3 is activated following flg22 treatment in *Arabidopsis* protoplasts (Mehlmer et al. 2010). Rice OsCPK4 (Jiyang Wang et al. 2018) and orthologous AtCPK28 from *Arabidopsis* (Jinlong Wang et al.

2018) are also phosphorylated in response to chitin and flg22, respectively.

Large-scale phosphoproteomics analyses have identified CDPKs that are phosphorylated in response to immune elicitation. For example, *Arabidopsis* CDPKs from subgroups I and III were identified as immune-induced phosphoproteins following treatment with flg22 or xylanase (Table 1) (Benschop et al. 2007; Mattei et al. 2016; Rayapuram et al. 2014, 2017). Although powerful, large-scale approaches are not comprehensive, and targeted approaches are often necessary to identify phosphorylation sites on proteins of interest. For example, affinity purification of epitope-tagged tobacco NtCDPK2 and NtCDPK3 transiently expressed in *N. benthamiana* was used to identify in vivo immune-induced phosphorylation sites (Table 1) (Witte et al. 2010). Similarly, although not stress induced, in vivo phosphorylation sites were identified through affinity purification of StrepII-tagged *Arabidopsis* AtCPK28 expressed in stable transgenic lines (Table 1) (Matschi et al. 2013).

Interestingly, most of the immune-induced phosphorylation sites identified thus far on CDPKs map to the N- and C-terminal variable domains, with many residing in predicted Pro, Glu,

Table 1. Phosphorylation sites identified on calcium-dependent protein kinases involved in plant immunity

Species, Gene ID ^a	Protein	Phosphorylation sites ^b	Method	Reference
<i>Arabidopsis thaliana</i>				
830366 (At5g04870)	AtCPK1	Ser16, Ser32/Ser34, Thr64, Ser105, Thr107, Ser111, Ser129, Ser130, Ser137, Ser151, Ser163/Thr164, Ser180, Thr592, Ser605	In vitro autophosphorylation	Swatek et al. 2014
		Ser16/Ser18, Thr64, Ser83, Thr107, Ser137, Ser151	In vitro autophosphorylation	Hegeman et al. 2006
828465 (At4g23650)	AtCPK3	Thr96, Ser108, Ser134, Ser413	In vitro autophosphorylation	Swatek et al. 2014
829685 (At4g35310)	AtCPK5	Ser8, Thr98/Ser100	In vitro autophosphorylation	Hegeman et al. 2006
		Ser552	Phosphorylated following flg22 treatment in vivo	Rayapuram et al. 2014
816235 (At2g17290)	AtCPK6	Ser8, Ser19, Thr49, Thr86/Ser88, Ser420, Ser540	In vitro autophosphorylation	Swatek et al. 2014
		Ser37, Ser536	Differentially phosphorylated following flg22 treatment in vivo in one of two biological replicates	Benschop et al. 2007
831123 (At5g12480)	AtCPK7	Ser29	Differentially phosphorylated in vivo in response to xylanase and flg22 treatment in two of two biological replicates	Benschop et al. 2007
838470 (At1g18890)	AtCPK10	Thr38, Ser475/Ser478	In vitro autophosphorylation	Hegeman et al. 2006
		Ser523	Differentially phosphorylated in vivo following flg22 and xylanase treatment in two of two biological replicates	Benschop et al. 2007
840471 (At1g35670)	AtCPK11	Ser361	In vitro autophosphorylation	Hegeman et al. 2006
836753 (At5g66210)	AtCPK28	Ser24, Thr33, Ser36, Ser43, Ser213, Ser228, Ser318, Tyr463	In vitro autophosphorylation	Swatek et al. 2014
		Ser228, Ser318, Ser495	In vitro autophosphorylation	Hegeman et al. 2006
		Ser228, Ser318, Ser495	In vivo autophosphorylation	Matschi et al. 2013
		Ser6, Ser24, Thr33/Thr37, Ser36, Ser43, Ser198, Ser228, Ser318, Ser329, Ser360, Tyr463, Ser478, Ser495, Ser507/Ser510	In vitro autophosphorylation	Bender et al. 2017
<i>Oryza sativa</i>				
4328155 (Os02g0126400)	OsCPK4	Thr73, Ser210, Ser381	In vitro phosphorylation by RLCK176	Jiyang Wang et al. 2018
4336653 (Os04g0560600)	OsCPK12	Ser70	Phosphorylation reduced in response to <i>Xanthomonas oryzae</i> pv. <i>oryzae</i> treatment in three of three biological replicates	Hou et al. 2015
<i>Nicotiana tabacum</i>				
107827839	NtCDPK2	Ser40, Thr65	Phosphorylated in vivo following hypoosmotic stress	Witte et al. 2010
107783265	NtCDPK3	Ser54	Phosphorylated in vivo following hypoosmotic stress	Witte et al. 2010

^a *Arabidopsis thaliana* gene ID numbers were retrieved from The Arabidopsis Information Resource; *Oryza sativa* gene ID numbers were retrieved from the Rice Annotation Project database; *Nicotiana tabacum* gene ID numbers were retrieved from the National Center for Biotechnology Information gene database.

^b Ambiguously identified phosphorylation sites are denoted with a forward slash (example: Ser507/Ser510).

Ser, Thr (PEST) motifs which act as signals for protein degradation (Rechsteiner and Rogers 1996). Comparatively, in vitro investigation of autophosphorylation sites on *Arabidopsis* and *P. falciparum* CDPKs identified phosphorylated residues in all domains, with nearly one-half mapping to the N-terminal variable domain (Table 1) (Hegeman et al. 2006; Swatek et al. 2014). Based on functional data supporting a role for the N-terminal variable domain in CDPK localization and substrate specificity (Asai et al. 2013; Ito et al. 2010), phosphorylation in this domain may be an important regulatory mechanism in signal transduction (Asai et al. 2013; Ito et al. 2017; Witte et al. 2010). Interestingly, truncation of the N-terminal variable domain of castor (*Ricinus communis*) RcCDPK1 did not affect association with its biological substrate BACTERIAL-TYPE PHOSPHOENOLPYRUVATE CARBOXYLASE (Ying et al. 2017). However, the N-terminal extension was required for optimal auto- and transphosphorylation activity, suggesting a role in catalytic activation (Ying et al. 2017). This is in agreement with the proposed function of this domain as a “latch” that stabilizes the active conformation of TgCDPK1 (Ingram et al. 2015). Given the low conservation of this domain, further experiments are needed to determine its function in individual CDPKs, which may include domain truncation, mutation, or swap experiments. The role of phosphorylation in the C-terminal variable domain is even less clear; however, it is possible that phosphorylation could affect Ca^{2+} binding in nearby EF-hand motifs, which could also be experimentally determined through mutation of this domain. To determine the role played by residue-specific phosphorylation on CDPK function, phospho-ablative or phospho-mimetic mutations can be analyzed in the context of biochemical activation as well as pathway-specific signal transduction. Analysis may require the generation of higher-order mutants, because multiple phosphorylation sites could contribute to the regulation or activity of CDPKs.

The requisite protein kinase activities responsible for the phosphorylation or activation of CDPKs are largely unknown. CDPKs could be regulated through dynamic phosphorylation or dephosphorylation events by kinases or phosphatases; however, autophosphorylation may also be sufficient for their activation. Most CDPKs are localized to the plasma membrane through myristoylation and palmitoylation at positions 2 and 3 to 5, respectively (Chehab et al. 2004; Cheng et al. 2002; Martín and Busconi 2000; Resh 2006; Rutschmann et al. 2002; Witte et al. 2010) (Fig. 1A). However, CDPKs have also been identified in the cytosol, nucleus, peroxisome, endoplasmic reticulum, and plastid (Simeunovic et al. 2016). Genetic analysis indicates that CDPKs signal downstream of PRR complexes (Boudsocq et al. 2010); thus, membrane-bound protein kinases such as RKs and RLCKs as well as other Ca^{2+} -regulated proteins, including CaM and calcineurin B, have been suggested as likely CDPK-associating proteins that could regulate their phosphorylation status (Witte et al. 2010). Indeed, AtCPK28 was found to associate with CaM both in vitro and in vivo (Bender et al. 2017), and association with AtCaM reduced AtCPK28 autophosphorylation and in vitro kinase activity toward a synthetic peptide (Bender et al. 2017). Interestingly, it has recently been demonstrated that the AtCPK28 ortholog in rice, OsCPK4, is phosphorylated by the AtBIK1 ortholog OsRLCK176 (Jiyang Wang et al. 2018), suggesting that CDPKs may be directly regulated by RLCKs. CDPKs may also be regulated by 14-3-3 proteins that associate with phosphorylated proteins, influencing stability, activity, or association with binding partners (Ormancey et al. 2017). For example, 14-3-3 proteins have been shown to associate with and influence stability of AtCPK1 (Camoni et al. 1998) and AtCPK3 (Lachaud et al. 2013) in vivo, and can enhance Ca^{2+} -dependent kinase activity in vitro (Camoni et al. 1998). Moreover, AtCPK1, AtCPK24, and AtCPK28

possess predicted 14-3-3 binding motifs in their N-terminal domains (Cheng et al. 2002); however, association of 14-3-3 proteins with this motif could not be demonstrated for AtCPK1 (Pallucca et al. 2014). The identification of additional proteins responsible for the regulation of CDPKs is needed to gain deeper understanding of their in vivo activity.

CDPK IMMUNE SUBSTRATES

Standard approaches used to identify CDPK substrates, including in vitro kinase assays (Curran et al. 2011), yeast two-hybrid (Uno et al. 2009), or targeted co-immunoprecipitation (Asai et al. 2013; Monaghan et al. 2014), indicate that CDPKs are capable of associating with and phosphorylating multiple substrates (Table 2; Fig. 2). Furthermore, because several CDPKs appear to function in multiple pathways (Asano et al. 2012; Matschi et al. 2013; Monaghan et al. 2014; Schulz et al. 2013), it is likely that complex regulatory mechanisms exist that dictate CDPK activation and substrate choice in distinct pathways. Mass spectrometry has been used to identify phosphorylation sites on CDPK substrates and, although several phosphorylation motifs have been proposed (Cheng et al. 2002; Huang et al. 2001; Sebastia et al. 2004; Wu et al. 2012), predicting CDPK targets using standard approaches is difficult because CDPKs are capable of phosphorylating multiple distinct substrate motifs (Curran et al. 2011). Thus, it remains a challenge to identify and validate biologically relevant targets of CDPKs in signaling pathways.

CDPK-mediated regulation of NADPH oxidases.

The generation of extracellular reactive oxygen species (ROS) by NADPH oxidases is a conserved immune response required for disease resistance (Torres et al. 2002; Yoshioka et al. 2003) that is intimately tied to Ca^{2+} signaling (Ogasawara et al. 2008). NADPH oxidases are multispanning transmembrane proteins containing N-terminal EF hands and require Ca^{2+} binding for their activation (Oda et al. 2010; Ogasawara et al. 2008). In addition, NADPH oxidases require both Ca^{2+} -dependent and Ca^{2+} -independent phosphorylation for full activation. In *Arabidopsis*, the NADPH oxidase AtRBOHD becomes rapidly phosphorylated within 1 to 2 min following MAMP treatment by AtBIK1 in a Ca^{2+} -independent manner (Kadota et al. 2014; Li et al. 2014). Additional Ca^{2+} -dependent phosphorylation by CSPs such as CDPKs further activates NADPH oxidases (Dubiella et al. 2013; Kadota et al. 2014). AtRBOHD-catalyzed extracellular ROS is hypothesized to further activate plasma membrane Ca^{2+} channels, generating an activation circuit that amplifies immune signaling in local and distal tissues (Kadota et al. 2014).

CDPK-mediated ROS production is required for diverse signaling pathways, including drought and salinity tolerance, the production of secondary metabolites, and innate immune responses (Asano et al. 2012; Bulgakov et al. 2011; Dubiella et al. 2013; Zou et al. 2015). Ectopic expression of AtCPK1 in tomato protoplasts (Xing et al. 2001) and NtCDPK2 in *N. benthamiana* (Ludwig et al. 2005) stimulates ROS production. Moreover, in potato (*Solanum tuberosum*), StCDPK4 and StCDPK5 phosphorylate StRBOHB at Ser82 and Ser97 and are genetically required for StRBOHB-dependent ROS production (Kobayashi et al. 2007), providing resistance to the near-obligate hemibiotroph *Phytophthora infestans* (Kobayashi et al. 2012). Similarly, AtRBOHD is phosphorylated by a subset of group I CDPKs from *Arabidopsis* at several sites, including Ser133 (Gao et al. 2013) and Ser148 (Dubiella et al. 2013; Gao et al. 2013), corresponding to Ser82 and Ser97 of StRBOHB, respectively (Table 2; Fig. 2B).

Moreover, flg22-induced oxidative burst is strongly reduced in *Arabidopsis Atcpk4/5/6/11* loss-of-function plants compared with single, double, or triple mutants, suggesting overlapping roles in immune signal transduction and highlighting the importance of CDPKs in the local activation of RBOHD (Boudsocq et al. 2010). Several other *Arabidopsis* CDPKs are able to phosphorylate AtRBOH proteins or corresponding synthetic peptides, including AtCPK1, AtCPK2, AtCPK10, and AtCPK34 (Curran et al. 2011; Gao et al. 2013) (Table 2). Additional CDPKs associate with RBOH proteins, including BnaCPK2 from rapeseed (*Brassica napus* L.) (W. Wang et al. 2018), several CDPKs from turnip (*B. rapa* var. *rapa*) (Wang et al. 2016), and NbCDPK2 from *N. benthamiana* (Hyodo et al. 2017), further suggesting conserved activation of RBOH proteins by CDPKs.

In *Arabidopsis*, full activation of AtRBOHD requires additional phosphorylation by AtBIK1 (Kadota et al. 2015). Following MAMP treatment, activated AtBIK1 phosphorylates AtRBOHD at Ser39 and Ser343 in the presence of a Ca^{2+} chelator, suggesting that these sites are not regulated by CDPKs (Kadota et al. 2014; Li et al. 2014). Additionally, AtCPK4, AtCPK5, AtCPK6, and AtCPK11 but not AtBIK1 phosphorylate AtRBOHD at Ser133 and Ser163 in vitro (Dubiella et al. 2013; Kadota et al. 2014), while Ser347 is phosphorylated by CDPKs and AtBIK1 (Kadota et al. 2014). Thus, AtRBOHD activation could occur through phosphorylation by AtBIK1 and CDPKs at distinct sites in a two-step regulatory mechanism whereby immune-induced phosphorylation by AtBIK1 “primes” AtRBOHD for Ca^{2+} binding or phosphorylation by CDPKs (Kadota et al. 2014); however, this remains to be validated experimentally. Moreover, how phosphorylation contributes to RBOHD protein activation is not known.

Interestingly, treatment of *Arabidopsis* protoplasts with H_2O_2 induced phosphorylation of AtRBOHD as well as the rapid phosphorylation and activation of AtCPK5 (Dubiella et al. 2013). Phosphorylation of AtRBOHD at Ser39 was observed in tissues distal to those elicited with flg22 in *Arabidopsis* overexpressing *AtCPK5*, suggestive of long-distance signaling (Dubiella et al. 2013). Although AtRBOHD-Ser39 is phosphorylated in vivo by AtCPK5 and flg22-induced phosphorylation of this site could not be detected in *Atcpk5* mutants (Dubiella et al. 2013), phosphorylation at Ser39 was unaffected in *Atcpk5/6/11* triple mutants and was identified as a Ca^{2+} -independent AtBIK1-mediated phosphorylation site (Kadota et al. 2014). Therefore, it is plausible that the requirement of AtCPK5 for phosphorylation of Ser39 is indirect, possibly through activation of an RLCK, and that different kinases could phosphorylate the same site in different tissues. AtCPK5 was additionally found to associate with the NLR toll/interleukin-1 receptor nucleotide-binding sequence protein 2 (TN2) and phosphorylate the exocyst complex protein, EXO70B1, resulting in HR-like cell death and resistance to powdery mildew, underscoring the diverse substrates of single CDPKs (Liu et al. 2017).

Regulation of RLCK- and MAPK-mediated signaling by CDPKs.

Because constitutive immune signaling has been associated with compromised plant growth, development, and survival, control of immune output is critical (van Wersch et al. 2016). Immune pathways are attenuated via multiple mechanisms, including receptor-mediated endocytosis and proteasomal degradation, among others (Couto and Zipfel 2016). For example, unactivated AtBIK1 is polyubiquitinated and continually turned over through the 26S proteasome to optimize immune signaling potential upon pathogen challenge (Liang et al. 2016; Monaghan et al. 2014; Jinlong Wang et al. 2018). Analysis of orthologous group IV CDPKs (*Arabidopsis* AtCPK28 and rice OsCPK4) suggests a conserved role as negative regulators of

RLCK-mediated immune signaling (Fig. 2A) (Monaghan 2018). In *Arabidopsis*, loss-of-function *Atcpk28* mutants are characterized by increased pattern-triggered Ca^{2+} influx and ROS production, and enhanced resistance to *Pseudomonas syringae* pv. *tomato* strain DC3000 (Monaghan et al. 2014, 2015). Similarly, loss-of-function *Oscpk4* mutants display increased MAMP-triggered ROS and enhanced disease resistance to *X. oryzae* and *Magnaporthe oryzae* (Jiyang Wang et al. 2018). AtCPK28 associates with AtBIK1 and AtRBOHD and is able to phosphorylate AtBIK1 in vitro (Monaghan et al. 2014). Because AtBIK1 protein accumulation is increased in *Atcpk28* mutants and decreased in AtCPK28-OE lines, it was hypothesized that AtCPK28-mediated phosphorylation of AtBIK1 may influence subsequent polyubiquitination by E3 ligases (Monaghan et al. 2014). Similarly, appropriate accumulation of the rice AtBIK1 ortholog, OsRLCK176, is influenced by OsCPK4, and OsCPK4 interacts with and phosphorylates OsRLCK176 at Ser74, Ser209, Ser267, and Ser336 (Jiyang Wang et al. 2018) (Table 2). Interestingly, OsRLCK176 reciprocally phosphorylates OsCPK4 at Thr73, Ser210, and Ser381 in vitro, suggesting the possibility of a regulatory feedback loop (Jiyang Wang et al. 2018). Because AtBIK1 is similarly phosphorylated by AtCPK28 in vitro (Monaghan et al. 2014), it will be interesting to determine whether this phosphorylation contributes to AtBIK1 stability in vivo.

Immune-induced phosphorylation of AtBIK1/OsRLCK176 is independent of AtCPK28/OsCPK4 (Monaghan et al. 2014; Jiyang Wang et al. 2018), suggesting differential phosphorylation by PRRs and AtCPK28/OsCPK4. Although the relevance of AtCPK28/OsCPK4-mediated phosphorylation on AtBIK1/OsRLCK176 is unclear, a regulatory module controlling accumulation of AtBIK1 has recently been described in which AtCPK28 associates with and phosphorylates E3 ubiquitin ligases AtPUB25 and AtPUB26 at conserved residues Thr95 and Thr94, respectively, enhancing their ability to polyubiquitinate AtBIK1 (Jinlong Wang et al. 2018). AtPUB25, AtPUB26, and closely related AtPUB22, AtPUB23, and AtPUB24 negatively regulate immune responses (Trujillo et al. 2008; Jinlong Wang et al. 2018), and their overexpression results in enhanced AtBIK1 degradation (Jinlong Wang et al. 2018). Immune-activated AtBIK1 is protected from AtPUB25/26-mediated polyubiquitination (Jinlong Wang et al. 2018), suggesting interplay between phosphorylation and ubiquitination.

Interestingly, AtPUB22 is phosphorylated by AtMPK3 on Thr88, a site that corresponds to Thr95 and Thr94 on AtPUB25 and AtPUB26, respectively (Furlan et al. 2017), suggesting the possibility of overlapping MAPK- and CDPK-mediated regulation of these E3 ligases. Furthermore, another group IV CDPK in rice, OsCPK18, phosphorylates the AtMPK3 ortholog, OsMPK5, at Thr14 and Thr32, repressing immune responses against fungal blast disease (Xie et al. 2014). OsCPK18 is also capable of phosphorylating orthologous MAPKs from *Arabidopsis* (AtMPK3) and tobacco (NtWIPK and NtSIPK), suggesting that MAPK phosphorylation by CDPKs might be conserved in other species (Xie et al. 2014). Furthermore, OsMPK5 can phosphorylate OsCPK18 in vitro, which could indicate reciprocal regulation (Xie et al. 2014). Analysis of phospho-ablative or -mimetic point mutations, coupled with genetic analysis, will help resolve the feedback mechanisms between these kinases, if they exist.

Together, these studies indicate that group IV CDPKs phosphorylate a range of substrates, including RLCKs, PUB E3 ligases, and MAPKs, contributing to immune homeostasis. Because group IV CDPKs play additional roles in plant growth and development (Bundó and Coca 2016; Hettenhausen et al. 2013; Jin et al. 2017; Matschi et al. 2013, 2015; Yang et al. 2012), it is likely that these CDPKs have additional, currently unknown, substrates. How group IV CDPKs function in diverse pathways is

unknown; however, this could occur via site-specific phosphorylation. For example, another protein kinase with roles in plant immunity and development is BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1 (BAK1),

which is a co-receptor of multiple PRRs as well as the brassinosteroid receptor BRI1 (Ma et al. 2016). Interestingly, phosphorylation sites were identified on the C-terminal tail of AtBAK1 that were required for immune function but not

Table 2. Plant calcium-dependent protein kinases with known or putative roles in immunity^a

Subgroup, species, gene ID ^b	Protein	Immune function ^c	Substrates (P-site)
Subgroup I			
<i>Arabidopsis thaliana</i> 830366 (At5g04870)	AtCPK1	Genetically required for resistance against <i>Fusarium oxysporum</i> , <i>Botrytis cinerea</i> , and <i>Pseudomonas syringae</i> pv. <i>tomato</i> ¹ as well as effector-triggered cell death ² ; phosphorylates AtRBOHD leading to the production of ROS ²	AtRBOHD ² , AtRBOHF ² , PAL
820235 (At3g10660)	AtCPK2	Phosphorylates AtRBOHD, leading to the production of ROS, and contributes to effector-triggered cell death ²	AtRBOHD (Ser148) ² , AtRBOHF ²
826541 (At4g09570)	AtCPK4	Phosphorylation of AtWRKYs involved in the activation of effector-triggered immune genes ^{2,4} ; phosphorylates AtRBOHD, leading to the production of ROS, and contributes to effector-triggered cell death ² ; MAMP-induced phosphorylation of AtRBOHD ⁵ ; transcriptional activation of flg22-dependent immune genes ⁶	AtRBOHD (Ser133 ⁵ , Ser148 ² , Ser163 ⁵ , Ser347 ⁵), AtRBOHF ² , WRKY8 ² , WRKY28 ² (Thr199 ⁴), WRKY48 (Thr248) ²
829685 (At4g35310)	AtCPK5	Phosphorylation of AtWRKYs involved in the activation of effector-triggered immune genes ² ; transcriptional activation of flg22/Pep3/OG-dependent immune genes ^{6,7,8} ; required for resistance to <i>Pseudomonas syringae</i> pv. <i>tomato</i> and contributes to effector-triggered cell death ² ; MAMP-induced phosphorylation of AtRBOHD ^{5,9} ; genetically required for ROS-mediated signal propagation and resistance to <i>Pseudomonas syringae</i> pv. <i>tomato</i> in local and distal tissues ⁹ ; genetically required for TN2-dependent cell death and resistance to <i>Golovinomyces cichoracearum</i> ¹⁰	AtRBOHD ² (Ser39 ⁹ , Ser133 ⁵ , Ser148 ⁹ , Ser163 ^{5,9} , Ser347 ^{5,9}), AtRBOHF ² , AtWRKY8 ² , AtWRKY28 (Thr199) ² , AtWRKY48 (Thr248) ² , exo70B1 ¹⁰
816235 (At2g17290)	AtCPK6	Transcriptional activation of flg22/Pep3/OG-dependent immune genes ^{6,7,8} ; required for resistance to <i>Pseudomonas syringae</i> pv. <i>tomato</i> and contributes to effector-triggered cell death ² ; MAMP-induced phosphorylation of AtRBOHD ⁵	AtRBOHD ² (Ser133, Ser163, Ser347) ⁵ , AtRBOHF ² , AtWRKY8 ² , AtWRKY28 ² , AtWRKY48 ²
840471 (At1g35670)	AtCPK11	Transcriptional activation of flg22/Pep3/OG-dependent immune genes ^{6,7,8} ; phosphorylates AtRBOHD leading to the production of ROS and contributes to effector-triggered cell death ² ; MAMP-induced phosphorylation of AtRBOHD ⁵	AtRBOHD (Ser133 ⁵ , Ser148 ² , Ser163 ⁵ , Ser347 ⁵), AtRBOHF ² , AtWRKY8 ² , AtWRKY28 ² (Thr199 ⁴), AtWRKY48 ²
<i>Hordeum vulgare</i> HarvEST Unigene 12358	HvCDPK4	Transient over-expression increases <i>Blumeria graminis</i> host cell entry ¹¹	Unknown
<i>Oryza sativa</i> 818476 (Os03g0788500)	OsCPK10	Regulation of SA-/JA-related defense genes and resistance against <i>Magnaporthe grisea</i> and <i>P. syringae</i> pv. <i>tomato</i> ¹² , as well as resistance to <i>M. oryzae</i> ¹³	Unknown
<i>Nicotiana tabacum</i> 107827839	NtCDPK2	Genetically required for Cf-9/Avr9- and Cf4/Avr4-dependent cell death ¹⁴	Unknown
107783265	NtCDPK3	Genetically required for Cf-9/Avr9- and Cf4/Avr4-dependent cell death ¹⁴	Unknown
<i>Solanum tuberosum</i> 102604043	StCDPK4	In vivo phosphorylation of StRBOHB ¹⁵	StRBOHB (Ser82, Ser97) ¹⁵
102594309	StCDPK5	Expression of constitutively active variant stimulates ROS production and provides resistance against <i>Phytophthora infestans</i> and susceptibility to <i>Alternaria solani</i> ¹⁶ ; phosphorylation of StRBOH proteins leading to the production of ROS ^{15,16,17}	StRBOHB (Ser82, Ser97) ¹⁵ , StRBOHC ¹⁶
<i>S. lycopersicum</i> 101252935 (Solycl0g074570.1.1)	SlCDPK10	Required for resistance against <i>P. syringae</i> pv. <i>tomato</i> ¹⁸	Unknown
<i>Capsicum annuum</i>	CaCDPK15	Genetically required for resistance against <i>Ralstonia solanacearum</i> ¹⁹	Unknown

(Continued on next page)

^a Superscript numbers indicate literature cited, as follows: 1 = Coca and San Segundo 2010, 2 = Gao et al. 2013, 3 = Cheng et al. 2001, 4 = Gao and He 2013, 5 = Kadota et al. 2014, 6 = Boudsocq et al. 2010, 7 = Ma et al. 2013, 8 = Gravino et al. 2015, 9 = Dubiella et al. 2013, 10 = Liu et al. 2017, 11 = Freymark et al. 2007, 12 = Fu et al. 2013, 13 = Bundó and Coca 2017, 14 = Romeis et al. 2001, 15 = Kobayashi et al. 2007, 16 = Kobayashi et al. 2012, 17 = Asai et al. 2013, 18 = Wang et al. 2016, 19 = Shen et al. 2016, 20 = Wang et al. 2017, 21 = W. Wang et al. 2018, 22 = Geng et al. 2013, 23 = Perraki et al. 2017, 24 = Lachaud et al. 2013, 25 = Kanchiswamy et al. 2010, 26 = Asano et al. 2012, 27 = He et al. 2018, 28 = Curran et al. 2011, 29 = Fu et al. 2014, 30 = Wei et al. 2016, 31 = Monaghan et al. 2014, 32 = Monaghan et al. 2015, 33 = Jinlong Wang et al. 2018, 34 = Bundó and Coca 2016, 35 = Jiyang Wang et al. 2018, 36 = Xie et al. 2014, 37 = Yang et al. 2012.

^b *Arabidopsis thaliana* gene ID numbers were retrieved from The Arabidopsis Information Resource; *Oryza sativa* gene ID numbers were retrieved from the Rice Annotation Project Database; *Solanum lycopersicum* gene ID numbers were retrieved from EnsemblPlants; *Hordeum vulgare* gene ID numbers were retrieved from HarvEST Unigene; All other gene ID numbers were retrieved from the National Center for Biological Information Gene Database.

^c Avr = Avirulence; BIK1 = BOTRYTIS INDUCED KINASE 1; Cf proteins = Tomato resistance genes that protect against infection by *Cladosporium fulvum*; flg22 = 22 amino acid flagellin epitope; HsfB2a = Heat shock transcription factor B2AJA = jasmonic acid; MAMP = microbe-associated molecular pattern; MPK = Mitogen-Activated Protein Kinase; OG = oligogalacturonides; PAL = Phenylalanine ammonia lyase; Pep3 = Plant elicitor peptide 3; PUB = PLANT U-BOX PROTEIN; RBOH = RESPIRATORY BURST OXIDASE; REM1.3 = REMORIN 1.3; RLCK = Receptor-like cytoplasmic kinase; ROS = reactive oxygen species; SA = salicylic acid; TN2 = Toll/Interleukin-1 receptor nucleotide-binding sequence protein 2.

AtBRI1-dependent growth (Perraki et al. 2018). Phosphoproteomics analysis, in combination with directed mutagenesis, could be used to determine whether phosphosite-dependent uncoupling of CDPK signaling pathways is also possible.

Another example of potential interplay between CDPKs and MAPKs is in the production of ethylene (ET), a phytohormone associated with resistance to certain pathogens (Anver and Tsuda 2015; Zipfel 2013) as well as defense against herbivore

Table 2. (Continued from previous page)

Subgroup, species, gene ID ^b	Protein	Immune function ^c	Substrates (P-site)
<i>Brassica rapa</i> var. <i>rapa</i> 103858526	BrrCDPK4	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ; interacts with BrrRBOHD2 in yeast two-hybrid assays ²⁰	Unknown
103859857	BrrCDPK9	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ; interacts with BrrRBOHD2 in yeast two-hybrid assays ²⁰	Unknown
103842676	BrrCDPK10	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ; interacts with BrrRBOHD1 and BrrRBOHD2 in yeast two-hybrid assays ²⁰	Unknown
103855956	BrrCDPK17	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ; interacts with BrrRBOHD2 in yeast two-hybrid assays ²⁰	Unknown
<i>B. napus</i> 106452615	BnaCPK2	Interacts with BnaRBOHD in mating-based split ubiquitin system and bimolecular fluorescence complementation ²¹ ; overexpression stimulates ROS production and cell death ²¹	BnaRBOHD ²¹
<i>Triticum aestivum</i> 543461	TaCPK2-A	Genetically required for resistance to <i>Xanthomonas oryzae</i> pv. <i>oryzae</i> and <i>B. graminis</i> ²²	Unknown
Subgroup II			
<i>A. thaliana</i> 828465 (At4g23650)	AtCPK3	Over-expression of constitutively active variant limits the spread of infection by <i>Potato Virus X</i> by phosphorylating AtREM1.3, promoting callose deposition ²³ ; required for fumonisin B1-induced cell death ²⁴ ; phosphorylation of HsfB2a leading to the transcriptional activation of <i>Spodoptera littoralis</i> -dependent defense genes ²⁵	AtREM1.3 (Ser74, Thr86, Ser91) ²³ , HsfB2a ²⁵
<i>H. vulgare</i> HarvEST Unigene 5769	HvCDPK3	Transient over-expression increases <i>B. graminis</i> host cell entry ¹¹	Unknown
<i>O. sativa</i> (IIa) 4336653 (Os04g0560600)	OsCPK12	Over-expression suppresses ROS production and susceptibility to <i>M. grisea</i> ²⁶	Unknown
<i>O. sativa</i> (IIb) 4326638 (Os01g0622600)	OsCPK1	Genetically required for resistance against bacterial <i>X. oryzae</i> pv. <i>oryzae</i> ²⁷	Unknown
<i>S. lycopersicum</i> 101260391 (Solyc02g083850.2.1)	SICDPK18	Required for resistance against <i>X. oryzae</i> pv. <i>oryzae</i> ¹⁸	Unknown
<i>B. rapa</i> var. <i>rapa</i> 103846628	BrrCDPK7	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ; interacts with BrrRBOHD in yeast two-hybrid assays ²⁰	Unknown
Subgroup III			
<i>A. thaliana</i> 838470 (At1g18890)	AtCPK10	Phosphorylates AtRBOHD in in vitro kinase assays ²⁸	AtRBOHD ²⁸
<i>O. sativa</i> (IIIb) 4343644 (Os07g0568600)	OsCPK20	Over-expression increases resistance against <i>P. syringae</i> pv. <i>tomato</i> in <i>Arabidopsis</i> and <i>M. grisea</i> in rice ²⁹	Unknown
<i>B. rapa</i> var. <i>rapa</i> 28865434	BrrCDPK23	Upregulated in response to <i>P. syringae</i> pv. <i>tomato</i> ²⁰ ; interacts with BrrRBOHD in yeast two-hybrid assays ²⁰	Unknown
<i>Triticum aestivum</i> 100192130	TaCPK7-D	Genetically required for resistance against <i>Rhizoctonia cerealis</i> ³⁰	Unknown
Subgroup IV			
<i>A. thaliana</i> 36753 (At5g66210)	AtCPK28	Negatively regulates MAMP-induced signaling and resistance to <i>P. syringae</i> pv. <i>tomato</i> by controlling AtBIK1 accumulation via AtPUB25/26-mediated degradation ^{31,32,33}	AtBIK1 ³¹ , AtPUB25/26 (Thr95/Thr94) ³³
<i>O. sativa</i> 4328155 (Os02g0126400)	OsCPK4	Over-expression increases resistance against <i>M. oryzae</i> ³⁴ ; genetically required for resistance against <i>X. oryzae</i> and <i>M. oryzae</i> ³⁵ , the latter being in contradiction to a previous report ³⁴ ; phosphorylation of OsRLCK176 influences stability ³⁵	OsRLCK176 (Ser74, Ser209, Ser267, Ser336) ³⁵
4343002 (Os07g0409900)	OsCPK18	Negatively regulates resistance against <i>M. oryzae</i> ; represses defense gene expression through phosphorylation of OsMPK5 ³⁶	OsMPK5 (Thr14, Thr32) ³⁶
<i>Nicotiana attenuata</i> AF435451	NaCDPK4	Negatively regulates the production of JA and secondary metabolites associated with herbivore defense; ³⁷ silencing provides resistance against <i>Manduca sexta</i> feeding ³⁷	Unknown
AY971376	NaCDPK5	Negatively regulates the production of JA and secondary metabolites associated with herbivore defense; ³⁷ silencing provides resistance against <i>M. sexta</i> feeding ³⁷	Unknown

attack (von Dahl and Baldwin 2007). ET biosynthesis is catalyzed by the rate-limiting enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Gravino et al. 2015; Jin et al. 2017; Ludwig et al. 2005). Type I and type II ACS isoforms contain C-terminal CDPK phosphorylation motifs (Sebastià et al. 2004) and have been identified as direct targets of several CDPKs (Sebastià et al. 2004; Kamiyoshihara et al. 2010; Luo et al. 2014). Consistently, ectopic expression of a constitutively active *NtCDPK2* variant in *N. benthamiana* resulted in a 25-fold increase in basal levels of the ET precursor aminocyclopropane-1-carboxylic acid (Ludwig et al. 2005), although it is not known whether *NtCDPK2* directly regulates ACS. Type I ACS isoforms also contain predicted MAPK phosphorylation motifs (Kim et al. 2003), suggesting that ACS could be dynamically regulated by both CDPKs and MAPKs. Indeed, tomato (*Lycopersicon esculentum*) LeCDPK1/2 and *S. tuberosum* MAPKs StMPK1 and StWIPK can phosphorylate LeACS2 in vitro (Kamiyoshihara et al. 2010). Phosphorylation by all three kinases results in a band shift matching native LeACS2, suggesting that CDPKs and MAPKs phosphorylate at distinct sites (Kamiyoshihara et al. 2010). Treatment with either CDPK or MAPK inhibitors decreased LeACS2 accumulation in fruit subjected to wounding stress (Kamiyoshihara et al. 2010), indicating that phosphorylation by both kinase families is required for ACS2 stability.

Interestingly, constitutively active *NtCDPK2* compromised the expression of both wound-induced and Avr9-elicited MAPKs *NtSIPK* and *NtWIPK* (Ludwig et al. 2005). Treatment with pharmacological inhibitors of ET biosynthesis or ET perception restored MAPK activation (Ludwig et al. 2005), suggesting that *NtCDPK2*-induced ET production is responsible for attenuated MAPK activation. ET production is also reduced in *Arabidopsis Atcpk5/6/11* mutants, associated with decreased levels of AtACS2/6 and increased susceptibility to the necrotrophic pathogen *Botrytis cinerea* (Gravino et al. 2015). AtCPK5 and AtCPK6 are also required for wound-induced activation of ACS genes, which is dependent on MKK4/MKK5-MPK3/MPK6 signaling (Li et al. 2018). Further research will shed light on how these two signaling pathways fine tune ET production in response to wounding and pathogen attack.

CDPK-mediated transcriptional regulation.

WRKY transcription factors are involved in transcriptional reprogramming in response to biotic and abiotic stresses in plants (Rushton et al. 2010). A majority of immune-responsive genes contain W-boxes (C/TTGACT/C) in their cis elements, suggesting direct control by WRKY transcription factors which bind this motif (Eulgem and Somssich 2007). In *Arabidopsis*, AtCPK4, -5, -6, and -11 phosphorylate AtWRKY8, -28, and -48 (Fig. 2A), enhancing binding to the W-boxes in the promoter of

AtWRKY46, an early marker gene in NLR signaling (Gao and He 2013; Gao et al. 2013). AtCPK4, AtCPK5, AtCPK6, and AtCPK11 phosphorylate WRKY48 and WRKY28 at Thr247/Thr248 and Thr199, respectively (Gao and He 2013; Gao et al. 2013), indicating that CDPKs can regulate immune signaling through the activation of WRKY transcription factors.

CDPKs are required for PRR- and NLR-mediated transcriptional reprogramming. Genome-wide expression profiling identified a subset of closely related *Arabidopsis* CDPKs from subgroup I (AtCPK4, -5, -6, -11, and -26) and a CDPK from group II (AtCPK3) as important regulators of early MAMP-induced signaling (Boudsocq et al. 2010). It was found that 70% of genes upregulated by ectopic expression of constitutively active AtCPK5 and AtCPK11 variants were also upregulated in microarray datasets following flg22-treatment including *PHI1*, *NHL10*, *PER62*, and *PER4* (Boudsocq et al. 2010). Single *Atcpk* loss-of-function mutants did not display altered flg22-dependent transcriptional changes or susceptibility to *P. syringae* pv. *tomato* DC3000, whereas double, triple, and quadruple mutants showed progressive decreases in MAMP-triggered responses, indicative of functional redundancy (Boudsocq et al. 2010). AtCPK5-dependent gene expression also correlated with genes upregulated in *elf26*-, *harpin*-, chitin-, and NECROSIS-INDUCING PHYTOPHTHORA PROTEIN 1-activated cells, indicating that CDPKs function downstream of multiple PRRs (Boudsocq et al. 2010). Moreover, *Atcpk5/6/11* loss-of-function mutants display reduced expression of immune markers *MPK3* and *WRKY33* following PLANT ELICITOR PEPTIDE 3 treatment, and of *FRK1* and *PHI-1* following oligogalacturonide treatment (Gravino et al. 2015; Ma et al. 2013). A closely related CDPK, AtCPK1, regulates the expression of salicylic acid (SA)-dependent defense-related genes, including *PR1/2/3/5*, *PDF1*, *PAD4*, and *SID2*, providing resistance to the fungal pathogen *Fusarium oxysporum* (Coca and San Segundo 2010). Interestingly, comparison of global AtMKK4- and constitutively active AtCPK5-mediated signaling networks identified a subset of immune genes synergistically upregulated by both types of kinases or coregulated by CDPKs and MAPKs without any synergism (Boudsocq et al. 2010), suggesting overlapping roles between CDPKs and MAPKs in transcriptional regulation (Li et al. 2018; Ludwig et al. 2005; Wurzinger et al. 2011).

Interestingly, expression of constitutively active AtCPK3 and AtCPK13 variants in *Arabidopsis* increased *PDF1.2* expression in response to mechanical wounding or feeding by the generalist herbivore *Spodoptera littoralis* (Kanchiswamy et al. 2010). Both CDPKs can phosphorylate the transcription factor HsfB2a, which is genetically required for *PDF1.2* expression, suggesting a role for CDPKs in herbivore-induced gene expression (Kanchiswamy et al. 2010). However, *Atcpk3* and *Atcpk13* mutants did not

Fig. 2. CDPK-mediated immune signaling and phosphorylation of RESPIRATORY BURST OXIDASE (RBOH proteins). **A**, Model of CDPK immune signaling in plants. CDPKs are activated in response to pathogen infection through Ca^{2+} binding and phosphorylation (A). CDPKs from *Arabidopsis* (AtCPK2/4/5/11) (Gao et al. 2013; Kadota et al. 2014) and potato (StCDPK4/5) (Kobayashi et al. 2007) phosphorylate RBOH proteins promoting the accumulation of extracellular ROS (B). AtCPK28 and OsCPK4 regulate immune output by controlling the stability of AtBIK1 (Jinlong Wang et al. 2018) and OsRLCK176 (Jiyang Wang et al. 2018), respectively (C). In *Arabidopsis*, AtCPK28 phosphorylates the E3 ligase AtPUB25/26, which polyubiquitinates AtBIK1, resulting in degradation through the 26S proteasome (Jinlong Wang et al. 2018). OsRLCK176 and OsCPK4 undergo reciprocal phosphorylation, suggesting the possibility of a feedback regulatory mechanism (Jiyang Wang et al. 2018). Group I CDPKs from *Arabidopsis* (AtCPK4/5/6/11) phosphorylate AtWRKY8/28/48, resulting in transcriptional upregulation of defense genes (Gao and He 2013; Gao et al. 2013) (D). AtCDPKs upregulate three categories of target genes: those that are specific to CDPKs, those that are coregulated or synergistic with mitogen-activated protein kinases (MAPKs), and those that are coregulated but not synergistic with MAPKs (Boudsocq et al. 2010) and may also attenuate MAPK signaling through unknown mechanisms (Boudsocq et al. 2010; Ludwig et al. 2005). Rice OsCPK18 phosphorylates OsMPK5 suppressing defense gene expression (Xie et al. 2014) (E). OsMPK5 also transphosphorylates OsCPK18, indicating reciprocal phosphorylation (Xie et al. 2014). **B**, Phosphorylation of the cytoplasmic N-terminal tail of RBOHD by CDPKs (green), BIK1 (purple), and both CDPKs and BIK1 (red). Ser39, Ser339, and Ser343 on AtRBOHD are specifically phosphorylated by AtBIK1 (Kadota et al. 2014). AtRBOHD-Ser133 and -Ser163 are specifically phosphorylated by AtCPK4/5/6/11 (Dubella et al. 2013; Gao et al. 2013; Kadota et al. 2014) and AtRBOHD-Ser148 is specifically phosphorylated by AtCPK2/4/5/11 (Dubella et al. 2013; Gao et al. 2013). StCDPK4/5 phosphorylate StRBOHB at Ser82 and Ser97, corresponding to AtRBOHD-Ser133, and -Ser148, respectively (Kobayashi et al. 2007). AtRBOHD-Ser347 is phosphorylated by AtBIK1 (Kadota et al. 2014) as well as AtCPK4/5/6/11 (Dubella et al. 2013; Kadota et al. 2014).

display compromised resistance to herbivory or biosynthesis of jasmonic acid (JA) (Kanchiswamy et al. 2010), a phytohormone central to the response against herbivore attack signals (Chung et al. 2008; Wang and Wu 2013; Wasternack 2007). Additionally, virus-induced gene silencing of *NaCDPK4* and *NaCDPK5* in *N. attenuata* resulted in elevated JA levels in response to wounding or *Manduca sexta* feeding (Yang et al. 2012). *Nacdprk4* *Nacdprk5* mutants also displayed a significant reduction in herbivore feeding and one- to twofold higher concentrations of plant secondary metabolites associated with direct defense against

herbivores (Yang et al. 2012). Evidence provided from *Arabidopsis* and tobacco, described above, and several other species (Table 2), suggests conserved roles for CDPKs in the transcriptional regulation of immune signaling.

CURRENT CHALLENGES AND OUTLOOKS

CDPKs contribute to resistance against a broad range of microbes, including bacteria and fungi, while also protecting against herbivory and wounding stress. Several of these CDPKs

A

B

also play important roles in development and tolerance to abiotic stress. Additional functions in biotic stress responses have been uncovered, including antiviral immunity (Hyodo et al. 2017; Perraki et al. 2017) and symbiotic plant–microbe relationships (Gargantini et al. 2006; Ivashuta et al. 2005). How individual CDPKs recognize and respond to a broad range of environmental cues remains an outstanding question. Biochemical analyses indicate that CDPKs bind Ca^{2+} with different affinities, meaning that they could respond differently to changes in $[\text{Ca}^{2+}]_{\text{cyt}}$. It is tempting to speculate that stimuli-specific Ca^{2+} -signatures are decoded by CDPKs to activate distinct pathways, although this has yet to be demonstrated. The absence of a crystal structure for a plant CDPK has hindered our understanding of how Ca^{2+} influences kinase activation, particularly for those that do not appear to require Ca^{2+} for enzymatic activity. Phosphorylation has the potential to influence CDPK function by modulating Ca^{2+} dependency, kinase activity, or interaction with substrates; however, very few in vivo assays provide evidence for this. The identification of additional CDPK substrates and regulatory proteins will provide insight into the mechanisms underlying CDPK regulation and activity. Moreover, analysis of the mechanisms that activate CDPKs in response to stimuli-dependent signals and dictate substrate choice could theoretically allow immune responses to be upregulated without compromises to growth or development. The identification of additional CDPK-interacting proteins is needed to determine how substrate choice is regulated, which could be achieved through large-scale protein–protein interaction assays such as peptide arrays or immune-induced phosphoproteomics analysis of *cdpk* loss-of-function mutants. Additionally, given their ability to receive and propagate signals, CDPKs could be engineered to be activated under mild stress perception, making them excellent targets for biotechnological applications.

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