Characterization of Arabidopsis calcium-dependent protein kinases: activated or not by calcium?

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CDPKs (calcium-dependent protein kinases), which contain both calmodulin-like calcium binding and serine/threonine protein kinase domains, are only present in plants and some protozoans. Upon activation by a stimulus, they transduce the signal through phosphorylation cascades to induce downstream responses, including transcriptional regulation. To understand the functional specificities of CDPKs, 14 Arabidopsis CPKs (CDPKs in plants) representative of the three main subgroups were characterized at the biochemical level, using HA (haemagglutinin)-tagged CPKs expressed in planta. Most of them were partially or mainly associated with membranes, in agreement with acylation predictions. Importantly, CPKs displayed highly variable calcium-dependencies for their kinase activities: seven CPKs from subgroups 1 and 2 were clearly sensitive to calcium with different intensities, whereas six CPKs from subgroup 3 exhibited low or no calcium sensitivity to two generic substrates. Interestingly, this apparent calcium-independence correlated with significant alterations in the predicted EF-hands of these kinases, although they all bound calcium. The noticeable exception, CPK25, was calcium-independent owing to the absence of functional EF-hands. Taken together, the results of the present study suggest that calcium binding differentially affects CDPK isoforms that may be activated by distinct molecular mechanisms.

Key words: Arabidopsis thaliana, calcium binding, calcium-dependent protein kinase (CDPK), calcium sensitivity, EF-hand, myristoylation.

INTRODUCTION

Calcium is a ubiquitous second messenger in all eukaryotes which mediates a variety of developmental and environmental stress responses. In plants, the free calcium concentration is maintained at low levels in the cytosol (100–200 nM) to avoid toxicity, whereas the organelles and the cell wall can accumulate concentrations up to several millimolar [1]. This compartmentalization allows signal-induced calcium elevations in the cytosol, which are characterized by their amplitude, duration and frequency and calcium stores. The precise combinations of these spatiotemporal features constitute calcium signatures that seem to be specific to each stimulus [1–3]. These signals must be decoded by calcium sensors to trigger the appropriate downstream responses. Plants possess three main families of calcium sensors: CaM (calmodulin), CBL (calcineurin B-like) and CDPKs (calcium-dependent protein kinases). CaM and CBLs do not possess any intrinsic enzymatic activity and need to transmit the calcium-induced conformational change to target proteins [1–3]. In contrast, CDPKs that are specific to plants and some protists display both calcium-sensing and protein-kinase-responding activities within a single polypeptide [4,5]. As a result, CDPKs are proposed to be directly activated by calcium binding, with different affinities, to regulate target proteins by phosphorylation. Despite the involvement of CDPKs in various signalling networks [4,6], only a few studies have reported the activation of CDPKs by specific stimuli, potentially due to technical barriers [7–10]. Deciphering the molecular mechanisms underlying CDPK activation by calcium constitutes a prerequisite to analyse CDPK activation by diverse stimuli.

The CDPKs consist of a variable N-terminal domain, a conserved serine/threonine kinase domain followed by an autoinhibitory junction region and a C-terminal regulatory CaMLD (CaM-like domain). The N-terminal domain often contains acylation prediction sites (myristoylation with/without palmitoylation) that are involved in membrane targeting [4,5]. The CaMLD comprises two pairs of EF-hand calcium-binding motifs grouped into two lobes, N- and C-terminal, that display low and high calcium affinity respectively [11,12]. Crystal structure and biochemical analyses revealed different roles for the two lobes in CDPK activation [9,11,13]. The current model proposes that the junction domain maintains the CDPK in an inactive state by a pseudosubstrate mechanism, although the C-terminal lobe already loaded with calcium is prebound to the autoinhibitory domain in resting conditions (100–200 nM free calcium). Upon a rise in calcium (several hundred nanomolar to a few micromolar), calcium binding to the N-terminal lobe triggers the conformational change to disengage the autoinhibitory domain from the active site [4]. This model based on calcium-activation of CDPKs has been confirmed with a few isoforms from different plant species, mostly using recombinant proteins produced in bacteria [9,14–19]. The Arabidopsis genome contains 34 CPK genes divided into four subgroups [4,5]. Interestingly, divergent sequences of EF-hand motifs in some isoforms could alter calcium binding, resulting in either distinct calcium affinities or calcium insensitivities. In particular, two Arabidopsis.

Abbreviations used: CaM, calmodulin; CaMLD, CaM-like domain; CBL, calcineurin B-like; CPK/CDPK, calcium-dependent protein kinase; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GST, glutathione transferase; HA, haemagglutinin; PA, phosphatidic acid; qRT-PCR, quantitative real-time PCR; WT, wild-type.

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isofoms, CPK13 and CPK23, were shown to be weakly sensitive to calcium when produced in bacteria [17,18]. Thus determining the calcium sensitivity of multiple CDPKs is essential to understand their activation mechanism. Considering the current model, it is important to maintain physiological conditions using proteins produced in planta and purified in resting conditions when calcium ions can already bind the C-lobe EF-hands.

To investigate the calcium-dependence of Arabidopsis CPKs, we selected 14 CPKs expressed in roots as representative of the three main subgroups. Indeed, subgroup 4 contains only three isofoms, including CPK16, which is pollen-specific, and CPK18 which is expressed at very low levels (Genetevigator) [20]. Without specific antibodies against each isoform, a transgenic approach with HA (haemagglutinin)-tagged proteins was carried out. Using CPK–HA produced in planta, we provide biochemical evidence for distinct calcium sensitivities of 14 Arabidopsis CPKs, seven of them being almost calcium-independent in vitro. The ability of CPKs to bind calcium was analysed and the role of calcium in CDPK regulation is discussed.

**EXPERIMENTAL**

**Plant growth and materials**

All experiments were performed with Arabidopsis thaliana ecotype Columbia (Col0) WT (wild-type) or CPK-overexpressing transgenic lines. For seedling assays, seeds were sterilized and grown in 0.5 × Murashige and Skoog liquid medium (Sigma) containing 0.5 % sucrose for 9 days at 21 °C with a 16 h photoperiod. For root assays, Arabidopsis plants were grown for 3 weeks in hydroponic conditions using the Araponics growing system and the nutrient solution described previously [21], in a controlled chamber at 22 °C and 60 % relative humidity, with a 16 h photoperiod.

**Generation of CPK-overexpressing lines**

To generate CPK-overexpressing lines, the full-length cDNA of each CPK was cloned from Arabidopsis Col0 plants using the primers listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/447/bj4470291add.htm) and inserted into a pCB302-derived plant expression mini-binary vector [22], containing an HA epitope tag at the C-terminus, between a 3SS-derived promoter and a NOS (nopaline synthase) terminator. The integrity of the constructs was confirmed by sequencing before introduction into Agrobacterium tumefaciens GV3101 to transform WT Arabidopsis Col0 plants using the drop-by-drop method [23]. Transgenic plants were selected on basta resistance and expression of the transgene was monitored by Western blot analysis. Segregation of the basta resistance was analysed in T1 and T2 generations to select transgenic lines with one T-DNA (transfer DNA) insertion at the homozygous state.

**Production of recombinant proteins GST (glutathione transferase)–CPK**

The full-length cDNA of each CPK was cloned into pGEX-2T (GE Healthcare) allowing a GST fusion at the N-terminus. Protein expression was induced in Escherichia coli BL21(DE3)pLys cells (Stratagene) with 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside) at 18 °C for 15 h. The bacterial pellet was resuspended in PBS-T [PBS (pH 7.3) containing 1 % Triton X-100 and anti-protease cocktail (Roche)] and incubated with 0.5 mg/ml lysozyme at 4 °C for 20 min. After addition of 2.75 mM DTT (dithiothreitol) and 1 % N-dodecanoylsarcosine, cells were lysed by sonication. After centrifugation at 8000 g at 4 °C for 10 min, the bacterial lysate was incubated with a 50 % slurry of glutathione–Sepharose 4B (GE Healthcare) at 4 °C overnight. Beads were washed four times with PBS and recombinant GST–CPKs were eluted with 20 mM reduced glutathione (Sigma) in 50 nM Tris/HCl (pH 8). For GST–CPKs 7, 8, 10 and 32, the low amount of proteins was directly eluted in SDS/PAGE sample buffer.

**qRT-PCR (quantitative real-time PCR)**

Total RNA was isolated from seedlings using the NucleoSpin® RNA II kit (Macherey-Nagel). cDNA was synthesized from 4 μg of total RNA using 0.5 μg of oligo(dT) primer and H-Minus MMLV (Moloney murine leukaemia virus) reverse transcriptase (Fermentas). qRT-PCR analysis was carried out with a LightCycler® 480 real-time PCR system using SYBR Green I master mix (Roche). TUB4 (tubulin β-chain 4; At5g44340) and EIF4a (eukaryotic translation initiation factor 4A; At3g13920) were used as control genes. All primers used for qRT-PCR have been described previously [24].

**Cellular fractionation**

Seedlings were grown in liquid nitrogen and homogenized in 50 nM Tris/HCl (pH 8), 300 mM sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM DTT and protease inhibitor cocktail (Roche). The protein extract was centrifuged at 53 000 rev./min (Beckman rotor TL100.3) for 1 h at 4 °C. The supernatant was diluted in SDS/PAGE sample buffer and constitutes the soluble fraction. The pellet, which constitutes the microsomal fraction, was resuspended in SDS/PAGE sample buffer in the same final volume as the soluble fraction. Equal volumes were analysed by Western blotting.

**Protein extraction and Western blot analysis**

To monitor CPK–HA expression levels in transgenic lines, seedlings were grown in liquid nitrogen and homogenized in 50 mM Hepes/KOH (pH 7.5), 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 mM DTT, 5 μg/ml leupeptin, 5 μg/ml antipain and 1 % Triton X-100. The protein extract was recovered after centrifugation at 21 100 g for 15 min at 4 °C.

For EMSAs (electrophoretic mobility-shift assays), ground seedlings were homogenized in 50 mM Tris/HCl (pH 8), 1 mM PMSF, 1 mM DTT, 5 μg/ml leupeptin, 5 μg/ml antipain and 1 % Triton X-100 with either 1 mM CaCl₂ or 10 mM EGTA. The protein concentration was determined using the Bradford method [24a]. Proteins were separated by SDS/PAGE on 10 % gels (for regular detection) or 8 % gels (for EMSAs) and transferred on to PVDF membranes (Millipore). After blocking with 5 % non-fat skimmed milk in TBS-T [Tris-buffered saline (10 mM Tris/HCl, pH 7.5, and 154 mM NaCl) with 0.1 % Tween 20], blots were probed with a 1:12 000 dilution of a monoclonal anti-HA antibody (Sigma) followed by a 1:12 000 dilution of horseradish peroxidase-conjugated anti–mouse IgG (Sigma). The reaction was visualized using the ECL® (enhanced chemiluminescence) Plus kit (GE Healthcare).

**Protein extraction and CPK activity**

Experiments were performed with calcium-chelated buffers to control [Ca²⁺]free. The amount of total CaCl₂ required to reach the desired [Ca²⁺]free in each buffer was calculated...
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Prediction of calcium-binding motifs

The presence of EF-hand calcium-binding motifs in CDPK sequences (Supplementary Table S1) was predicted using six different methods: Plam [26], SMART [27], Prosite [28] and PatternScan through the software InterProScan [29] (http://www.ebi.ac.uk/Tools/pfa/iprscan/), Blocks Scanner [30] (http://blocks.thecr.org/blocks/blocks_search.html) and a prediction method recently developed, based on a structural alphabet [31,32]. From the prediction results, a consensus score s has been assigned for each calcium-binding site as follows: s = 0 if a maximum of two methods predict the binding site, s = 2 if three or four methods predict the binding site, and s = 5 if five or six methods predict the binding site.

RESULTS

Characterization of the CPK transgenic lines

In order to characterize Arabidopsis CPKs from the three main subgroups, transgenic lines expressing individually each HA-tagged kinase (CPK–HA) were generated: CPKs 2, 4, 5, 11 and 25 from subgroup 1, CPKs 3, 9 and 19 from subgroup 2, and CPKs 7, 8, 10, 13, 30 and 32 from subgroup 3. The transgenic lines were characterized for the expression level of the HA-tagged CPK in seedlings, at the transcript level by qRT-PCR to compare with the endogenous expression level in WT and at the protein level by Western blot analysis with an anti-HA antibody (Figure 1). In most cases (CPKs 4, 5, 11, 3, 9, 7, 8, 10, 13 and 32), CPK–HA was overexpressed 3–30-fold compared with the endogenous CPK level, whereas a strong overexpression from 80- to 2800-fold was measured in the other cases (CPKs 2, 25, 19 and 30). At the protein level, each kinase could be detected by the anti-HA antibody, confirming the expression of the transgene in all lines. Variable protein levels were observed, being strong (CPKs 2, 4, 5, 11, 3 and 9), moderate (CPKs 25, 7, 13 and 32) or weak (CPKs 19, 8, 10 and 30). It is of note that the CPK–HA transcript level did not correlate with the protein level intensity (Figure 1). This can be explained by the highly variable expression levels of endogenous CPKs, whereas all transgene expressions are driven by the 35S promoter. Nevertheless, these Arabidopsis CPK transgenic lines constitute valuable tools to study each kinase individually in planta. The amount of protein and exposure time of immunoblots were adjusted for each kinase in subsequent experiments to allow sufficient detection.

Fractionation of the Arabidopsis CPKs

Most Arabidopsis CPKs have predicted N-terminal acylation sites that could target them to membranes (TermiNator [33], http://www.isv.cnrs-gif.fr/terminator3/index.html). It was thus interesting to analyse the solubility of each CPK in resting conditions. Using subcellular fractionation of protein extracts from seedlings, soluble and microsomal fractions were recovered after ultracentrifugation and CPK–HA was detected by immunoblot analysis (Figure 2). CPKs 4 and 11 that have no acylation prediction site were present only in the soluble fraction, as expected. Surprisingly, CPK19, which is not predicted to be acylated, was even more abundant in the microsomal fraction than in the soluble fraction, suggesting an association with membranes. CPKs 2, 5, 25 and 3, which are predicted to be N-myristoylated, were all associated with membranes, either mainly (CPKs 2 and 25) or in addition to their soluble localization (CPKs 5 and 3). In contrast, all other CPKs (CPKs 9, 7, 8, 10, 13, 30 and 32) displayed a prediction for both N-myristoylation and palmitoylation.
Figure 1 Characterization of the CPK–HA transgenic lines

CPK overexpression (ratio between the expression level of endogenous CPK + CPK–HA in transgenic seedlings and the expression level of endogenous CPK in WT seedlings) was determined by qRT-PCR. Values are means±S.E.M. for three independent experiments performed in duplicate (n = 6). Proteins from seedlings (15 μg) were analysed by Western blotting with an anti-HA antibody and CPK–HA was detected with either a short (top panel) or a long (middle panel) exposure time, reflecting variable expression levels. WT seedlings were used as a control. The same protein samples were stained with Coomassie Blue (bottom panel) for loading control. Molecular mass (kDa) is indicated on the right-hand side. WB, Western blot.

CPK overexpression (ratio between the expression level of endogenous CPK + CPK–HA in transgenic seedlings and the expression level of endogenous CPK in WT seedlings) was determined by qRT-PCR. Values are means±S.E.M. for three independent experiments performed in duplicate (n = 6). Proteins from seedlings (15 μg) were analysed by Western blotting with an anti-HA antibody and CPK–HA was detected with either a short (top panel) or a long (middle panel) exposure time, reflecting variable expression levels. WT seedlings were used as a control. The same protein samples were stained with Coomassie Blue (bottom panel) for loading control. Molecular mass (kDa) is indicated on the right-hand side. WB, Western blot.

Figure 2 Subcellular fractionation of CPK–HA

Proteins from seedlings were fractionated by ultracentrifugation at 53 000 rev./min and equal volumes of soluble (S) and membrane-associated (M) fractions were analysed by Western blotting with an anti-HA antibody. Molecular mass (kDa) is indicated on the right-hand side.

Calcium-dependence of CPK activity

The specific activity of each CPK–HA was first evaluated in vitro on syntide-2 substrate in the presence of 100 μM free calcium using syntide-2 as a substrate. The phosphorylation activity (cpm) was normalized to the amount of protein of each CPK–HA immunopurified from Arabidopsis roots. The phosphorylation activity was normalized to the amount of protein of each CPK–HA monitored by Western blot analysis (arbitrary unit) to determine the specific activity. Values are means±S.E.M. for two independent experiments performed in duplicate (n = 4).

CPK13 significantly phosphorylated the substrate 2–3-fold over the background.

To investigate the calcium-dependence of CPK phosphorylation activity, each immunopurified CPK–HA was assayed for syntide-2 phosphorylation at various Ca2+free. The assay at 1 mM Ca2+free was equivalent to EGTA conditions (results not shown). Interestingly, strong differences in calcium-dependence were observed between the kinases, with quite similar behaviours within each subgroup (Figure 4). All CPKs from subgroup 1, except CPK25, displayed enhanced activity with increasing Ca2+free. CPKs 2 and 5 were highly sensitive to calcium (K0.5 = 250 and 210 nM respectively), whereas CPKs 4 and 11 exhibited a more moderate sensitivity (K0.5 = 3.1 and 4.5 μM respectively). The three CPKs from subgroup 2 were clearly calcium-dependent but with lower sensitivities (K0.5 = 18.3, 8.4 and 6.5 μM for CPKs 3, 9 and 19 respectively). Surprisingly, in subgroup 3, CPKs 7, 13 and 30 were calcium-independent, whereas CPKs 8, 10 and 32 were only weakly dependent, with a 60–75% basal activity even at very low calcium concentrations.

Because CPK sensitivity to calcium can vary with substrates [14], we investigated whether the strong differences observed in calcium-dependence with syntide-2 were specific, using another artificial substrate, histone. Each CPK–HA was immunopurified from Arabidopsis roots and assayed in vitro for histone phosphorylation in the presence of 2 mM EGTA or 100 μM free calcium. The calcium stimulation of CPK activity was calculated for both syntide-2 and histone assays (Figure 5). In most cases, the calcium-dependences observed with syntide-2 were confirmed with histone: seven CPKs (CPKs 2, 4, 5, 11, 3, 9 and 19) were clearly sensitive to calcium, whereas five CPKs (CPKs 25,
Two CPKs from subgroup 3 (CPKs 10 and 32) that were nearly calcium-independent on syntide-2 exhibited a significant calcium stimulation on histone (3.4- and 5.7-fold respectively). This suggests that calcium-dependence of CPK activity might be revealed for more CDPKs with appropriate substrates.

Calcium binding of CPKs

The surprising discovery that approximately one-third of the CPKs studied were calcium-independent raised the question of their ability to bind calcium. Calcium-binding-induced electrophoretic mobility shifts in denaturing SDS/PAGE have been described previously for several CDPKs [10,34]. This approach was carried out on the 14 Arabidopsis CPKs using seedling proteins extracted in the presence of 1 mM calcium or 10 mM EGTA and analysed by immunoblotting (Figure 6). A strong increase in CPK–HA mobility in the presence of calcium was observed only in a few cases (CPKs 2, 4 and 11). Slighter mobility shifts were also observed for CPKs 3, 9, 19, 7 and 32. However, no evidence of calcium binding could be seen for the other CPKs, especially CPK5 which is clearly calcium-dependent for its activity (Figure 4). This suggests that calcium-binding might not always modify the electrophoretic mobility. Indeed, the two lobes of EF-hand pairs were recently shown to differently contribute to the conformational change and kinase activation induced by calcium: CPK21 mutated in the C-terminal lobe no longer displayed the calcium-induced mobility shift, while retaining most calcium activation [9].

To determine further the ability of CPKs to bind calcium, a direct binding assay with 45CaCl2 was carried out using CPK–HA immunopurified from transgenic seedlings; however, the low amount of immunopurified proteins was not sufficient to detect any signal. To overcome the low sensitivity of the assay, recombinant proteins with GST fusions were produced in E. coli. Only ten GST–CPKs, representative of the three subgroups, could be produced at the expected molecular mass (70–100 kDa). We first confirmed on five kinases that recombinant GST–CPKs retained their Ca2+-dependence for kinase activity (Supplementary Figure S1 at http://www.BiochemJ.org/bj/447/bj4470291add.htm). However, the relative intensities of specific activity among CPKs was modified compared with CPK–HA produced in planta (Figure 3), suggesting that endogenous regulatory mechanisms lacking in...
bacteria further modulate CPK activity in vivo. The $^{45}$Ca-binding assay was then carried out on GST–CPKs, using BSA and GST as negative controls (Figure 7A and Supplementary Figure S2 at http://www.BiochemJ.org/bj/447/bj4470291add.htm). All CPKs from subgroups 1 and 2 (CPKs 2, 5, 11, 3 and 9) that are activated by calcium (Figures 4 and 5) were able to bind calcium, whereas CPK25 could not. Interestingly, four CPKs from subgroup 3 (CPKs 10, 13, 30 and 32) that are weakly or not sensitive to calcium in in vitro kinase assays (Figures 4 and 5) could clearly bind calcium (Figure 7A). Moreover, GST–CPK7 and GST–CPK8 could also be produced, but as cleaved proteins displaying two bands on SDS/PAGE (Supplementary Figure S2). GST–CPK7 was analysed in more detail owing to a sufficient amount of protein. MS analysis confirmed that the upper band corresponded to CPK7, whereas the lower band mainly contained GST in addition to a few peptides of CPK7 (results not shown). Importantly, the upper band strongly bound calcium compared with the lower band, whereas GST alone could not (Supplementary Figure S2). Similar results were observed for CPK8 with lower intensities owing to the lower amount of protein. Taken together, these results indicate that CPKs displaying weak or no calcium sensitivity can also bind calcium, with the noticeable exception of CPK25.

To correlate the calcium binding and sensitivity results with potential modifications in the calcium-binding motifs, the predictions of EF-hands were compared, using six different programs (Supplementary Figure S3 at http://www.BiochemJ.org/bj/447/bj4470291add.htm). A consensus score of prediction was computed for each EF-hand of the 14 CPKs (Figure 7B). In subgroups 1 and 2, four calcium-binding sites were well-predicted for all calcium-dependent CPKs, either strongly (CPKs 4, 5, 11, 9 and 19) or with a more moderate prediction on the third EF-hand (CPKs 2 and 3). In contrast, CPK25, which lacks half of the CaMLD, was predicted as a non-calcium-binding protein. Interestingly, more divergent results were obtained for CPKs from subgroup 3. Only two EF-hands were predicted well for four CPKs (CPKs 7, 8, 13 and 32), whereas three and four motifs were identified in CPK10 and CPK30 respectively. However, none of the EF-hands of CPK30 was predicted by all six methods, unlike in calcium-dependent CPKs (Supplementary Figure S3). Thus the lack of one EF-hand or the alteration of two motifs are sufficient to compromise calcium sensitivity.
DISCUSSION

Understanding the specific functions of *Arabidopsis* CPKs requires the deciphering of their activation mechanisms in planta, which depends on calcium sensitivity and subcellular localization. In the present study, the generation of CPK-overexpressing lines that depend on calcium sensitivity and subcellular localization was achieved by deciphering their activation mechanisms.

McCDPK1 (Arabidopsis) DISCUSSION site that stabilizes the membrane interaction as reported for seven CPKs harbouring an additional predicted palmitoylation site. Of these, seven CPKs (2, 3, 9 and 13) were shown to be myristoylated in vitro [19,37,38], indicating the accuracy of the predictions. Interestingly, full membrane association was observed for the seven CPKs harbouring an additional predicted palmitoylation site that stabilizes the membrane interaction as reported for OSCP2 (OsCPK2; *Os is Oryza sativa*) [39]. The four CPKs lacking the predicted palmitoylation site were mainly or partially membrane-associated. Although the soluble localization may be due to strong overexpression of the kinases, it is consistent with the reversibility of the second signal (unknown for these proteins) which allows shuttling between the cytosol and the membranes [35]. Previous studies have reported the subcellular localizations for ten out of the 14 CPKs investigated in the present work, and are in agreement with our observations (Supplementary Table S2) [10,17,19,24,37,38,40–42]. Unexpectedly, CPK19 also targeted to membranes, despite the absence of any predicted acylation motif, probably due to the isoleucine residue at position 6 that does not match the consensus motif [43]. Yet CPK19 displays a glycine residue in position 2 available for myristoylation, the other residues in positions 3, 4, 5 and 7 match the consensus motif [43] and a polylysine sequence at the N-terminus would stabilize the membrane interaction. Thus the non-N-myristoylation of CPK19 may be a false prediction that would be interesting to test in vitro. CPK19 is also present in the soluble fraction, but with a lower molecular mass than the membrane-associated form, and may correspond to degradation products. Interestingly, the relationship tree based on the variable N-terminal domains of CPKs correlates with the observed solubilities, suggesting an important role of the N-terminus in CPK localization (Supplementary Figure S4 at http://www.BiochemJ.org/bj/447/bj4470291add.htm). Although CPKs of subgroups 2 and 3 that are mostly membrane-bound form homogenous subfamilies, subgroup 1 is split into two branches: one comprising CPKs 2 and 25, mainly anchored to membranes, and the other one comprising CPKs 4, 5 and 11, which are fully or partially soluble. Thus most CPKs are associated with membranes in control conditions, allowing the regulation of membrane target proteins, such as NADPH oxidase [44] or the anionic channel SLAC1 [18]. Interestingly, CPKs can also be translocated in response to stress stimuli to ensure additional cellular functions, such as gene expression regulation, as reported for AhCPK2 (*Arachis hypogaea* CPK2) [45] and MeCDPK1 (*Mesembryanthemum crystallinum* CDPK1) [46] that move to the nucleus upon osmotic stress.

Recent in planta studies have shown that a few CPKs were almost inactive in the absence of calcium, including CPKs 4 and 11 from subgroup 1 [10], and CPKs 3 and 21 from subgroup 2 [9,19]. Using CDPKs produced in planta, a high calcium-dependence of the kinase activities is demonstrated in the present study, for seven of the 14 CPKs studied. They all belong to subgroups 1 and 2 and include CPKs 3, 4 and 11. The concentrations of calcium required for half-maximal activity (K_{0.5}) on syntide-2 strongly vary among the seven CPKs from 0.2 to 20 μM calcium. Similarly, a 10-fold difference in K_{0.5} values was reported between three recombinant soya bean (*Glycine max*) CDPKs, from 0.06 to 1 μM [14]. These various sensitivities to calcium are consistent with their expected role in sensing different calcium signatures and the K_{0.5} measured are within the physiological range. Interestingly, the comparison of K_{0.5} values on different generic substrates revealed substrate-specific calcium sensitivities for soya bean CDPKc [14] and maize (*Zea mays*) ZmCPK11 [8], whereas other CDPKs exhibited comparable sensitivities [15,16]. Similarly, we observed different levels in Ca^{2+} stimulation of some CDPK activities using histone and syntide-2 substrates. These differences may be related to the variable N-terminal domain of CDPKs, which was recently shown to be important for substrate recognition [47]. It would also allow a fine-tuned regulation of calcium signalling, ensuring that the CDPK will phosphorylate the appropriate substrate only in response to the correct calcium signal.

On the other hand, six CPKs from subgroup 3 were shown to be poorly or not affected by calcium concentrations. Similarly, weak calcium-dependence has been recently reported for two recombinant proteins acting on plant-specific substrates, CPK13 [17] and CPK23 [18]. However, we cannot exclude that these kinases could exhibit some calcium stimulation with appropriate substrates, as observed in the present study for CPKs 10 and 32 on histone. Indeed, a recent large-scale analysis of 103 peptides has revealed significant differences in substrate specificities for four *Arabidopsis* CPKs [48], and CPK sensitivity to Ca^{2+} has been shown to depend on substrate [14]. Thus it raises the question of the true ability of CDPKs to bind calcium. Using calcium-interacting experiments, we showed that both calcium-dependent and calcium-independent CDPKs could bind calcium. Importantly, bioinformatics tools predicted EF-hands with strong probabilities for all CDPKs displaying clear calcium sensitivities, whereas predictions were weaker for the other kinases. In particular, the N-lobe, which was shown to be essential for CDPK activation [9], is badly predicted for CPKs 7, 8 and 32 (Figure 7B). Moreover, the EF-hands within each lobe of CaMLD were shown to function in a co-operative way [11,15], suggesting that non-canonical calcium-binding motifs would further reduce calcium affinity. Thus the absence or alteration of some of the EF-hands could explain the observed differences in calcium sensitivities. Although calcium binding alone seems not to be sufficient to activate CDPKs of subgroup 3, it may be a prerequisite for additional regulatory processes, such as lipid binding [6].

Alternatively, the conformational change induced by calcium binding may regulate other aspects of CDPKs than activity, such as the ability to interact with proteins. For example, CPK10 interacts with HSP (heat-shock protein) 1 in a calcium-dependent manner to recruit the partner at the plasma membrane [41]. Interestingly, the analysis of groundnut (*A. hypogaea*) AhCPK2 revealed that atypical EF-hand motifs in CaMLD were indispensable for acquiring a functional NLS (nuclear localization signal) in the junction domain [45]. Indeed, the interaction of AhCPK2 with nuclear importins occurred only at a specific subsaturating [Ca^{2+}]_{free} that would trigger full conformational change blocking the importin interaction in canonical CDPKs. This indicates that Ca^{2+} binding regulates the subcellular localization of AhCPK2 to ensure that the kinase does not become fully active before reaching the final location. Thus the non-canonical calcium-binding motifs...
of CPKs from subgroup 3 may allow additional regulations for fine-tuning of calcium-mediated responses.

Unlike all other CPKs, CPK25 is deleted in its C-terminal region and displays weak sequence conservation in its truncated CaMLD. As a result, CPK25 has no predicted EF-hand, does not bind calcium and exhibits a constitutive activity in vitro. Thus CPK25 could play a role in housekeeping processes. Alternatively, CPK25 activity could be modulated in vivo by other regulatory mechanisms, including phosphorylation, lipid binding or interaction with 14-3-3 proteins, which are known to affect CDPK activity [5,6]. In particular, some studies reported that these regulations could be calcium-independent, such as autophosphorylation [17,49] and PA (phosphatidic acid) stimulation [8]. Since PA is also recognized as a second messenger such as autophosphorylation [17,49] and PA (phosphatidic acid) binding or interaction with 14-3-3 proteins, which are known other regulatory mechanisms, including phosphorylation, lipid deserve more attention in future studies to precisely decipher yet fully understood, and additional regulatory mechanisms will

Taken together, these results reveal a great heterogeneity among CPKs towards calcium sensitivity and subcellular localization that are essential for the diverse cellular functions of the family. By compiling our results with the literature on Arabidopsis CPK activities (Supplementary Table S2), subgroup 3 appears to be clearly different from the others, displaying weak or no calcium sensitivity on generic substrates, despite effective calcium binding. Thus the role of calcium in CDPK regulation is not yet fully understood, and additional regulatory mechanisms will deserve more attention in future studies to precisely decipher CDPK activation mechanisms in vivo.

AUTHOR CONTRIBUTION
Marie Boudsocq and Christiane Lariére designed the study. Marie Boudsocq generated the transgenic lines. Marie Boudsocq and Marie-Jo Drouillard performed the experiments. Leslie Regad performed the bioinformatic analysis. Marie Boudsocq and Christiane Lariére wrote the paper with input from all co-authors.

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SUPPLEMENTARY ONLINE DATA

Characterization of Arabidopsis calcium-dependent protein kinases: activated or not by calcium?

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Figure S1 Specific activity of GST–CPKs on syntide-2

(A) Recombinant GST–CPK proteins and negative control GST (120 ng) were assayed for syntide-2 phosphorylation in vitro, in the presence of 100 μM free calcium (+) or 2 mM EGTA (−). The background measured with GST was subtracted from CPK phosphorylation activity and the amount of CPK–GST protein was further quantified by SDS/PAGE after Coomassie Blue staining. The specific activity was calculated as the ratio between the CPK activity (c.p.m.) and the protein amount (arbitrary units). Values are means ± S.E.M. from two independent experiments performed in duplicate (n = 4). The GST–CPKs retain their calcium-dependence as CPK–HA produced in planta, but the relative intensities of specific activities are modified. For example, CPK25 displays a 8-fold higher specific activity than CPK11 when produced in bacteria, whereas their activities are comparable when produced in planta. This suggests that additional regulatory mechanisms occur in planta.

(B) Recombinant GST–CPK proteins and negative controls, GST and BSA (1 μg), were analysed by Coomassie Blue staining to evaluate the level of purification. The molecular mass in kDa is indicated on the left-hand side. Except for GST–CPK25 which displays an additional degraded band at approximately 50 kDa, all GST–CPKs exhibit only one band at the expected size, indicating a good level of purity.

Figure S2 Calcium binding of GST–CPK7 and GST–CPK8

Recombinant GST–CPK proteins and the negative control GST were separated by SDS/PAGE (10 % gel) and assayed for 45Ca binding on nitrocellulose membranes (left-hand panel). The amount of protein was monitored by Ponceau staining (right-hand panel). The molecular mass in kDa is indicated on the left-hand side. Both kinases are produced as a cleaved protein, the upper band corresponding to CPK and the lower one mainly to GST. The ‘CPK’ band clearly binds calcium, despite a much lower amount of protein compared with the ‘GST’ band.

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Figure S3  Detailed predictions of EF-hand calcium-binding motifs

The presence of EF-hands (one to four sites per kinase) was predicted in the 14 CPKs using six methods (+). The predictions are consistent among the six programs for CPKs from subgroups 1 and 2, whereas more variable predictions are observed for CPKs from subgroup 3, especially for sites 1 and 3. A, Pfam; B, SMART; C, Blocks searcher; D, Prosite; E, PatternScan; and F, Reynès et al. [19].

Figure S4  Relationship tree of the 14 Arabidopsis CPKs

Full-length protein sequences or N-terminal domains of the 14 CPKs were aligned with ClustalX and analyzed with TreeView. Although subgroups 2 and 3 display similar organizations in both trees, the tree based on N-terminal regions splits subgroup 1 into two branches: one branch contains the fully membrane-bound isoforms (CPKs 2 and 25), whereas the other one contains the partially or fully soluble isoforms (CPKs 4, 5 and 11).
Table S1  Oligonucleotide sequences for full-length CPK cDNA cloning

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Table S2  Summary of biochemical characterization of CPKs

| Subgroup | Protein | Gene             | N-acylation prediction* | Localization† | Calcium-dependence‡ | Calcium binding§ | K_{50} on syntide-2 (μM) || Reference(s) |
|----------|---------|------------------|-------------------------|---------------|---------------------|-----------------|------------------|--------------------------|
| Subgroup 1 | CPK1   | A5g04670         | N-myristoylated         | Membrane/peroxisomes, lipid bodies | Y              | Y                | n.d.             | [1–4]                    |
| CPK2     | A8g10660 | N-myristoylated   | Membrane/endoplasmic reticulum | Y              | Y                | 0.25             | The present study and [5,6] |
| CPK4     | A4g05670 | N-myristoylated   | Soluble/cytosol, nucleus | Y              | Y                | 3.1              | The present study and [7–9] |
| CPK5     | A4g35310 | N-myristoylated   | Membrane, soluble/cytoysol, nucleus | Y              | Y                | 0.21             | The present study and [8] |
| CPK11    | A1g35670 | N-myristoylated   | Soluble/cytosol, nucleus | Y              | Y                | 4.5              | The present study and [7,8,10] |
| CPK12    | A5g3580 | N-myristoylated   | Cytosol, nucleus        | Y              | n.d.             | n.d.             | [11]                     |
| CPK25    | A1g2680 | N-myristoylated   | Membrane                | N              | N                | --               | The present study        |
| Subgroup 2 | CPK3   | A4g23650         | N-myristoylated         | Membrane, soluble/PM, tonoplast, cytosol, nucleus | Y              | Y                | 18.3             | The present study and [3,12,13] |
| CPK9     | A3g20410 | N-myristoylated/palmitoylated | Membrane/PM             | Y              | Y                | 8.4              | The present study and [3,6] |
| CPK19    | A1g61050 | Met excised      | Membrane                 | Y              | Y                | 6.5              | The present study        |
| CPK21    | A4g04720 | N-myristoylated/palmitoylated | Membrane/PM             | Y              | Y                | n.d.             | [3,14,15]                |
| CPK23    | A4g04740 | N-myristoylated/palmitoylated | PM                      | Strong        | n.d.             | n.d.             | [15]                     |
| CPK34    | A5g19360 | N-myristoylated/palmitoylated | PM                      | Y              | Y                | n.d.             | [16]                     |
| Subgroup 3 | CPK7   | A5g12480         | N-myristoylated         | Membrane/PM    | N                | Y                | --               | The present study and [3] |
| CPK8     | A5g19450 | N-myristoylated/palmitoylated | Membrane/PM             | Weak           | Y                | --               | The present study        |
| CPK10    | A1g19800 | N-myristoylated/palmitoylated | Membrane/PM             | Weak           | Y                | --               | The present study and [17] |
| CPK13    | A3g51650 | N-myristoylated/palmitoylated | Membrane/PM, cytosol, nucleus | Weak           | Y                | --               | The present study and [6,13] |
| CPK30    | A1g74740 | N-myristoylated/palmitoylated | Membrane                | N              | Y                | --               | The present study        |
| CPK32    | A5g57530 | N-myristoylated/palmitoylated | Membrane/cell periphery, nucleus | Weak           | Y                | --               | The present study and [18] |

*N-acylation was predicted with TermiNator. Proteins were predicted to be N-myristoylated, palmitoylated, excised of the first methionine residue (Met excised) or without any acylation motif (–).
†Solubility was determined by cellular fractionation, whereas subcellular localization was determined with GFP (green fluorescent protein) fusion proteins.
‡Calcium-dependence was clearly observed (Y), or with any tested substrate (N), or some calcium stimulation was observed for at least one substrate despite a strong basal activity (weak).
§Calcium binding was determined either by the 45Ca-binding assay, calcium-induced mobility shift or crystallography.
∥Calcium binding was determined either by the 45Ca-binding assay, calcium-induced mobility shift or crystallography.
REFERENCES


