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Two Calcium-Dependent Protein Kinases from *Chlamydomonas reinhardtii* are transcriptionally regulated by nutrient starvation

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We report here, the transcriptional regulation of 2 Calcium Dependent Protein Kinases in response to nutrient starvation of *Chlamydomonas reinhardtii* vegetative cells. The CDPK proteins, CDPK1 and CDPK3; share 53% identity among themselves, a maximum of 57% and 52% to higher plants respectively and 42% to apicomplexan protozoans. We expressed a CDPK1-GFP fusion protein in the *C. reinhardtii* vegetative cells and showed its distribution both in the cell body and the membrane-matrix fraction of the flagella. The fusion protein exhibits mobility shift in the presence of Ca^{2+}, confirming its Ca^{2+}-binding properties. To the best of our knowledge, this is the first report of transcriptional regulation of CDPKs from a unicellular chlorophyte in response to nutrient starvation namely acetate (A), phosphorus (P), and nitrogen (N).

Introduction

Plants lead a sedentary lifestyle and are exposed to high salt, extreme temperatures, drought, heavy metals, excessive light and radiation, and oxidative stress. They have developed response mechanisms that lead to their survival or death.[1] For such purposes, plants use Ca^{2+} as a second messenger and harbor Ca^{2+}-binding proteins as effector proteins leading to a signal transduction pathway.[2] Plants possess 3 main families of Ca^{2+} sensors: the Calcium-dependent Protein Kinases (CDPKs), Calmodulin (CaM), and Calceneurin B-like (CBL-like) proteins.[3] CaM and CBL-like proteins do not possess any enzymatic activities; however, CDPKs exhibit a protein kinase activity and calcium-sensing abilities present on the same polypeptide. CDPKs are specific to plants, algae, and some apicomplexan protozoans, but are absent in animals.[4]

Based on the homology they share with each other, the multigene family of CDPKs has now been classified into 4 subgroups. All of these differ by their pattern of expression in response to the stimuli used, sub-cellular localizations, substrate specificities, Ca^{2+} sensitivities, and, regulation by phosphorylation and lipids, all this resulting in functional specificity and redundancy.[5] CDPKs consist of 4 domains: an N-terminal variable domain, a serine-threonine protein kinase domain, an auto-inhibitory junction domain, and a C-terminal calmodulin-like Ca^{2+}-binding domain with EF hands, that bind Ca^{2+} to activate the serine-threonine kinase activity.[6] Besides their involvement in the normal growth and development of plants, CDPKs have been associated with a response to biotic and abiotic stress agents and therefore may be involved in various signal transduction pathways. The array of abiotic stress agents that have shown CDPK regulation include abscisic acid, cold tolerance, drought, high salt, oxidative bursts, wounding, heavy metal, osmotic stress, etc.[5,7-12] They are also known to play an important role in parasite motility, gamete formation, host cell attachment and invasion, exocytosis, and parasite transmission in apicomplexans.[13]

Calcium is known to exert a regulatory effect on the cellular activities of the unicellular, biflagellated green alga *Chlamydomonas reinhardtii*, in particular, with the flagellar related activities of motility, phototaxis, mating, flagellation/deflagellation, and flagellar length regulation.[14-17] In the membrane-matrix fraction of the flagella there are kinases which get activated in the presence of calcium.[18] A recent study, using in silico methods of analyses, recently reported the presence of 14 CDPKs in *C. reinhardtii*, of which CDPK3 (*Mw* 53 kDa) has been shown to be localized in the flagella and plays a role in flagellar biogenesis.[19] CDPK1 has been annotated as FAP223 (*Mw* 67 kDa) in the flagellar proteome analysis and thus far, there have been no studies performed on this enzyme.[20] The effect of some stresses on the physiology of *C. reinhardtii* shows that depending on the type of stress, the dose, and the

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length of exposure, the flagella can be paralyzed or lost and later, cells manifest "palmelloids" or apoptose or form actively motile gametes.[21-27] As mentioned earlier, since Ca^{2+} is known to be involved in several stress induced phenomenons of *Chlamydomonas*, we set out to explore the regulation of the 2 CDPKs, CDPK1 and CDPK3, under varying stress conditions by studying their transcript profiles using RT-PCR.

Results and Discussion

In silico analyses of *C. reinhardtii* CDPK1 and CDPK3

CDPK1 is a protein of 613 amino acids (4786 bp) while CDPK3 is a protein of 484 amino acids (3615 bp). CDPK1 and CDPK3, like other canonical CDPKs have a protein kinase domain, autoinhibitory-junction domain, and a C-terminal CaM-like binding domain and 4 EF domains (**Fig. 1A**). In addition to these domains, CDPK1 also exhibits an N-terminal C2 domain (aa 1 to 95; **Fig. 1A**) that is absent from any known CDPKs in higher plants. Our in silico analysis have revealed the presence of the C2 domain in CDPKs of at least 2 other unicellular algae such as *Dunaliella tertiolecta* and *Volvox carteri f. nagariensis*. It may be noted that the C2 domain was

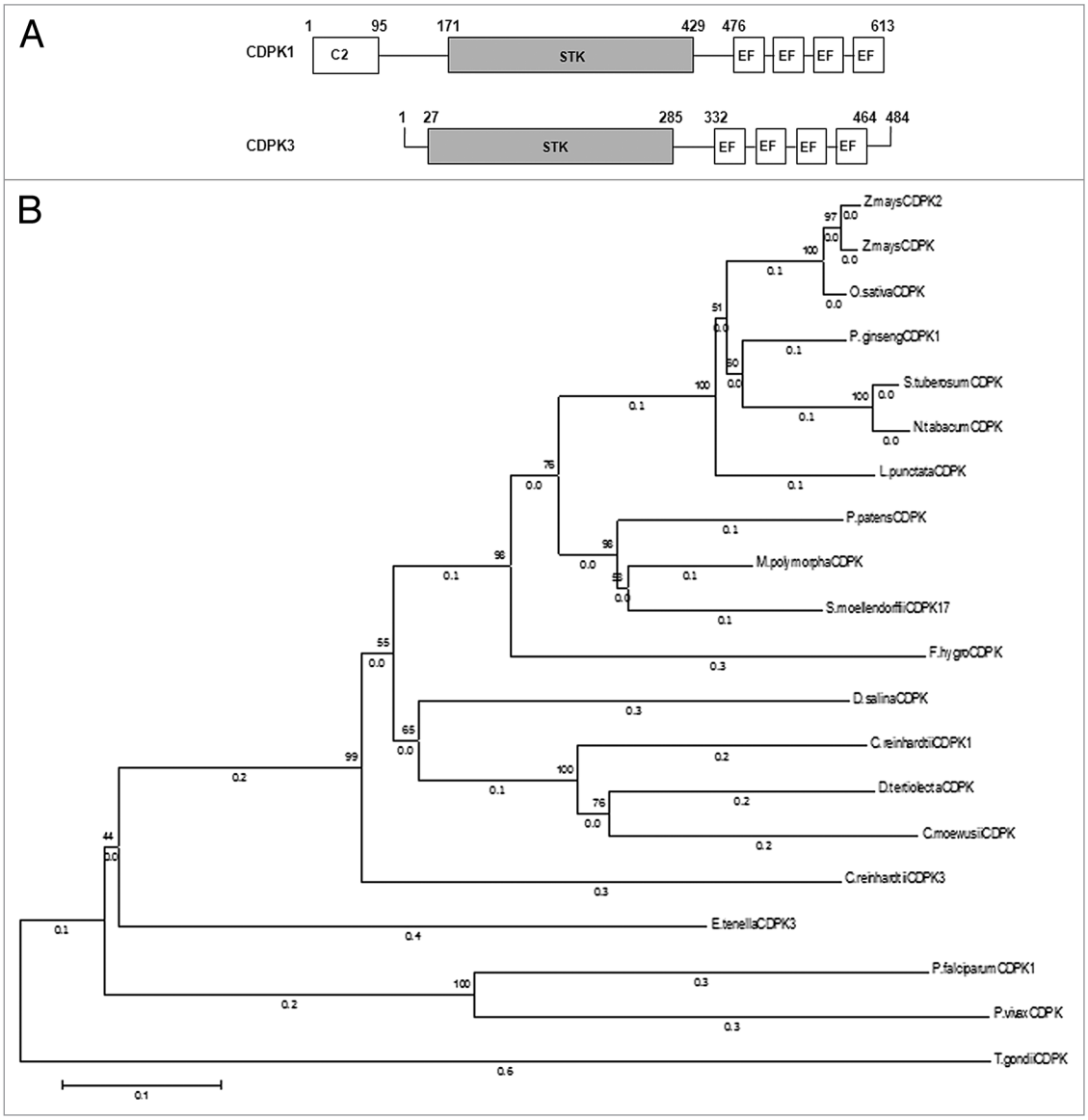

Figure 1. CDPKs from *Chlamydomonas reinhardtii*. (**A**) Schematic diagram of CDPK1 and CDPK3 showing the C2 domain from 1–95 aa in CDPK1, protein kinase catalytic domains (STK), and the 4 EF hands in both the CDPKs. (**B**) Phylogenetic relationship between *C. reinhardtii* CDPK1, CDPK3, and other CDPKs from algae, moss, higher plants, and apicomplexans. The phylogenetic tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 4.60159844 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 371 positions in the final data set. Evolutionary analyses were conducted in MEGA6.

first identified as a calcium-binding motif in protein kinase C and has now been identified in a number of eukaryotic signaling proteins involved in membrane trafficking, protein phosphorylation, and activation of GTPases.[28-32] Upon binding to calcium, C2 domain is known to bind to substrates such as phospholipids and intracellular proteins.[33,34] In silico analysis using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) tool for multiple alignment of the entire CDPK1 and CDPK3 protein sequences with that of sequences obtained from NCBI's Protein tool (http://www.ncbi.nlm.nih.gov/protein) showed that there is a 52–57% identity to higher plants and, both the CDPKs show a maximum of 42% identity to apicomplexan protozoans. Of the 500 sequences that were obtained in this blast, 20 have been chosen that showed > 30% identity with the CDPK1 and CDPK3 and used to generate a Phylogenetic tree using MEGA6: Molecular Evolutionary Genetics Analysis version 6.0 software (**Fig. 1B**).[35-38] Care was administered to represent at least one sequence from the major phyla of higher plants, lower plants, algae, and apicomplexans. Also, the full-length protein sequences were accepted provided these harbored the consensus Ser/Thr protein kinase domain, conserved Asp and Lys residues with the active site (D[L/I/V]K motif), and the calmodulin domain comprising of the EF-hand motif. The tree showed 4 clusters each containing algal, higher plants, apicomplexans, and a mixed cluster containing lower plants (mosses, Lichens, *Selaginella*; **Fig. 1B**). Although the identity between CDPK1 and CDPK3 is 53%, they seem to have diverged. Algal and apicomplexan CDPKs seem to share a common node but have diverged or parallelly evolved, and that might be the reason that they share a lower homology.

We too noted the non-clustering of the algal and plant CDPKs as observed in a recently reported study.[39] In addition, algae such as *D. tertiolecta* and *C. moewusii* shared 63% and 58% identity to the CrCDPK1 while a 53% and 51% identity to CrCDPK3, respectively. As of now, these algal CDPKs have been characterized as Ca^{2+}-binding and Ca^{2+}-dependent Protein Kinases.[40,41]

Expression analysis of *CDPK1* and *CDPK3* transcripts under nutrient starvation

The vegetative cells of *C. reinhardtii* when exposed to acetate, phosphorus, and nitrogen starvation showed a significant change in their *CDPK* transcript expression profiles. In the case of acetate starvation, only *CDPK3* showed a significant (11-fold) increase at 1 h which continued until 3h and then decreased to ~4-fold at 6 h after which it remained constant until 18 h. However, the transcript levels of *CDPK1* did not show any significant changes (**Fig. 2A**). On the other hand, in cells starved of phosphorus, the transcripts of both *CDPK1* and *CDPK3* respectively showed an 8- and 6-fold increase at 3 h, after which it remained constant until 18 h (**Fig. 2B**). For cells starved of nitrogen, transcripts of *CDPK1* showed a 14-fold increase at 1 h followed by a decrease and the same trend was observed for *CDPK3* transcripts with ~11-fold increase at 1 h (**Fig. 2C**). Taken together, our results clearly demonstrate an almost equal regulation of *CDPK1* and *CDPK3* in response to media starved of P and N. *CDPK1*, on the other hand, is not

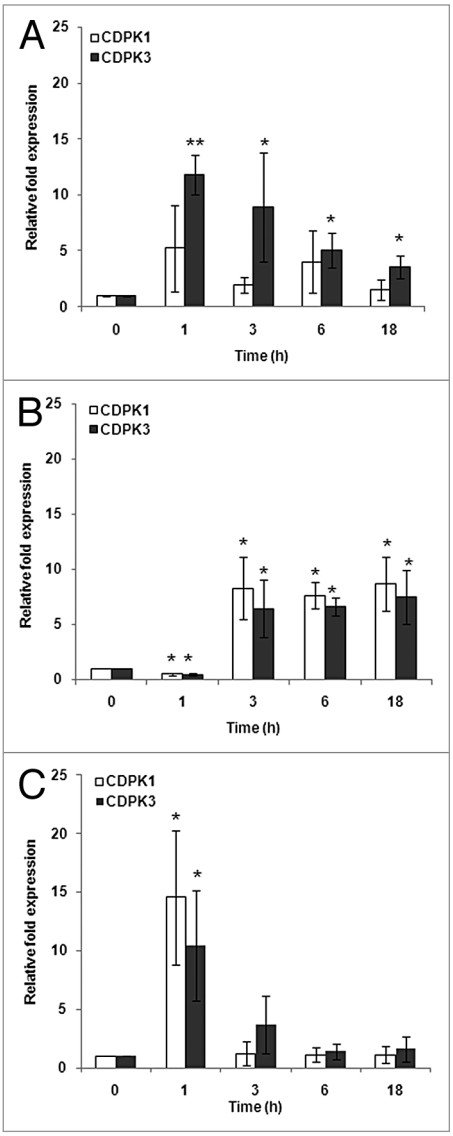

Figure 2. Expression patterns of *CDPK1* and *CDPK3* transcripts in response to nutrient starvation at 0, 1, 3, 6, and 18 h by real-time RT-PCR analysis. (**A**) Acetate starvation. (**B**) Phosphorus starvation. (**C**) Nitrogen starvation. The single and double asterisks indicate a statistically significant difference at $P < 0.05$, $P < 0.005$ respectively.

significantly regulated under conditions of acetate starvation. In essence, there seems to be an important role for CDPKs when the vegetative cells of *C. reinhardtii* cells are nutritionally starved; whether Ca^{2+} is invoked as a second messenger needs to be addressed. Earlier reports of phosphate starvation leading to the accumulation of polyphosphate and Ca^{2+} in *C. reinhardtii* cells have been observed.[42] These polyphosphate stores have been shown to be calcium stores and the subsequent potential of Ca^{2+} as a second messenger has been provided in these studies. When correlated with the current study, we suggest an accumulation of Ca^{2+} during P starvation thereby activating CDPKs (**Fig. 2B**). Ca^{2+} is released into the medium by cells that undergo mating and plays a vital role in this process.[14] Therefore, there might be a rapid (within minutes) rise in the

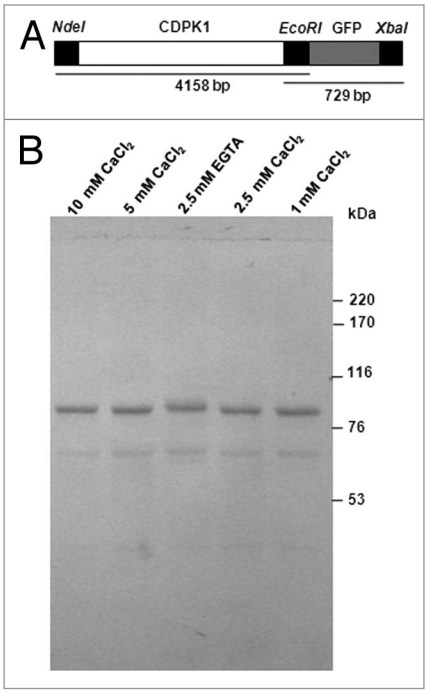

Figure 3. Ca^{2+} mobility of *C. reinhardtii* CDPK1-GFP. (**A**) CDPK1-GFP construct used to transform *C. reinhardtii* cells and (**B**) Ca^{2+} mobility shift of CDPK1-GFP fusion protein. Lanes 1, 2, 4, and 5 are treatments with different concentrations of $CaCl_2$; lane 3 is the lysate treated with EGTA only. CDPK1-GFP cell lysate treated with $CaCl_2$ (10 mM, 5 mM, 2.5 mM, and 1 mM) and EGTA (2.5 mM) were run on a 7% SDS -PAGE followed by Western Blotting using anti-GFP antibody. CDPK1 showed a mobility shift in presence of Ca^{2+}.

Ca^{2+} levels inside the cells post N starvation, leading to increase in the effector proteins and in the current scenario, both the CDPKs (**Fig. 2C**). As for the regulation of CDPK in other organisms is concerned, studies on *Funaria* and *Arabidopsis* have reported an increase in the expression of *CDPK* at the gene level in response to phosphate starvation using northern analysis and microarray, respectively.[43,44] Also, the same study on *Funaria* has shown an upregulation of *CDPK* gene in response to nitrogen starvation using northern analysis.[43] The findings on rice suggest that the *OsCDPK1* is involved in the signal transduction pathway(s) in the low-nitrogen stress response.[45] In addition, a recent study using in silico analyses predicted a few CDPKs as important regulators of phosphate deficiency-induced root hair remodeling in *Arabidopsis*.[46] We see a substantial increase (~6- to 14-fold over the controls) in the transcript levels of the *CDPKs*, as against those reported for *Funaria* or *Arabidopsis*.

While, the enzymatic activity of CDPK1 remains to be tested, we outline a brief account of Ca^{2+}-dependent protein kinase activity reported in other algal systems. The first report for the presence of a CDPK-like enzyme was performed in 1990 by Guo and Roux who partially purified and characterized the enzyme from *D. salina*. The enzyme, identified using an in-gel kinase assay and shown to be activated at free Ca^{2+} concentrations above 10^{-7} M, was not sensitive to phospholipids and was found to be inhibited by calmodulin antagonists.[47] Almost a decade later, a 40 kDa Ca^{2+}-dependent protein kinase was found to be activated in *Dunaliella tertiolecta* by heat shock, acidic stress, and H_2O_2 treatment.[48]Using an in-gel kinase assay, 3 CDPKs (62, 54, and 47 kDa) were identified in *D. tertiolecta* cell extracts.[40] When the same technique was extended to homogenates of another green alga, *Closterium ehrenbergii* using histone H1, myelin basic protein, and casein as substrates, CDPKs ranging in relative molecular mass from 47–60 kDa were observed. Of these, the 55 kDa CDPK was found to be immunologically related to the 62 kDa *D. tertiolecta* CDPK.[49] Another CDPK was found to share epitopes with the 62 kDa *D. tertiolecta* CDPK that was present in the internodal cells of the brackish water inhabiting *Lamprothamnium succinctum* charophyte. This enzyme of 53 kDa was found to be involved in processing turgor regulation in response to hypoosmotic treatment.[50] The predicted *Mw* of CDPK1 and CDPK3 is 67 and 53 kDa, respectively, and seems to fall in the range of those observed for algal CDPKs, which belong to one cluster (**Fig. 1B**).

Extensive work on CDPK3 has already been reported and since the C2 domain appears to be novel to *C. reinhardtii* CDPK1, the current interest was pursued by the overexpression of a recombinant CDPK1-GFP protein for all further analyses (**Fig. 3A**).[19]

Calcium-dependent mobility shift of CDPK1

Studies have shown that the binding of Ca^{2+} to EF-hands of a CDPK results in the protein undergoing a conformational change. Due to this conformational change, a mobility shift is seen in the presence of calcium.[49,51-54] The electrophoretic mobility of the CDPK1-GFP fusion protein was compared in the presence and absence (EGTA) of Ca^{2+}. As expected, the mobility shift of the fusion protein was clearly seen in presence of Ca^{2+} when compared to that treated with EGTA, and a 4 kDa difference was observed (**Fig. 3B**), proving that CDPK1 is a calcium binding protein. Similar mobility shifts have been observed earlier, a difference of 3 kDa with soybean CDPK, 5 kDa with *Vicia faba* CDPK, ~5 kDa with *Closterium ehrenbergii* CDPK[49,52,53]

CDPK1 is a flagellar membrane-matrix protein

The CDPK1-GFP *C. reinhardtii* clones were used to study the sub-cellular localization of CDPK1. Although a strong promoter (PSAD) was used to express the CDPK1-GFP fusion protein, repeated attempts at directly observing GFP fluorescence using live or fixed cells were unsuccessful. Similar experience in the field prompted us to switch over to immunostaining experiments.[55] Immunostaining of *C. reinhardtii* cells with anti-GFP antibody revealed the presence of CDPK1-GFP fusion protein in both the cell body as well as the flagella (**Fig. 4A**). For sub-flagellar localization experiments, flagellar isolation was performed. The flagella were further fractionated into the membrane-matrix and axonemal fractions; protein extracts were subjected to denaturing gel electrophoresis; followed by western analysis and immuno-probing using anti-GFP antibody. CDPK1 was present in the whole cells, cell body, flagella, and the membrane-matrix fraction, but was absent in

the axonemal fraction (**Fig. 4B**). A recent study of CDPK3 being distributed in both the cell body and flagellar membrane-matrix fraction of *C. reinhardtii* cells has been shown.[19] Moreover, whether the protein re-localizes upon an exposure to stress needs to be addressed. As far as the sub-cellular localization of CDPKs is concerned, they have been shown to occupy several parts of the plant cell, such as cytoplasm, peroxisomes, nucleus, endoplasmic reticulum, mitochondrial outer membrane, and plasma membrane.[56-62] Studies on protozoans have shown that CDPKs are localized in the schizonts and pellicle in *Plasmodium*, in sporozoites in *Eimeria*, and also in the cilia and body of *Paramecium*.[63-67] CDPK3 is localized in the *C. reinhardtii* membrane-matrix fraction of the flagella.[19] Another 65 kDa CDPK has been shown to be localized in the *C. moewusii* flagella.[41]

In conclusion, the 2 CDPKs from *C. reinhardtii* cells are regulated by nutrient starvation (P and N); however, it was only CDPK3 that was upregulated upon acetate starvation. This might suggest a plausible Ca^{2+}-dependent pathway in response to nutrient stress.

Materials and Methods

Plant materials and abiotic stress treatment

The wild-type *C. reinhardtii* (strain *cc124*, mt-) cells were grown in Tris-Acetate Phosphate (TAP) medium at 24 °C in continuous light until the cell density reached $3x10^6$ cells/ml.[68] Acetate, phosphorus, and nitrogen deficient media was prepared as follows. Glacial acetic acid was omitted in the acetate-free medium and the pH was adjusted to 7.0 using HCl. K_2HPO_4 and KH_2PO_4 was replaced with 1.4 mM KCl in the phosphorus-free medium and NH_4Cl was replaced with 7.4 mM KCl in the nitrogen-free medium.[69] For the acetate, phosphorus, and nitrogen starvation experiments, cells were washed with TAP and divided into 2 sets (treated and control). The treated sets were washed with nutrient-starved medium (TAP-A, TAP-P, and TAP-N) and the control set with TAP. The cells were inoculated in the respective media to a cell density of 10^5 cells/ml. Cells were harvested at 0, 1, 3, 6, 18 h.

RNA isolation and cDNA synthesis

RNA was extracted as per standard protocol using Trizol reagent (Invitrogen, Cat. No. 15596–026). Nucleic acid was treated with DNaseI (Thermo Scientific, Cat. No. #EN0521) containing buffer to remove DNA. The total RNA was quantified using the Infinite® 200 PRO NanoQuant (TECAN). cDNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, Cat. No. K1621). The absence of DNA was confirmed by running a PCR with forward and reverse actin primers (**Table 1**) followed by a 2% Agarose Gel Electrophoresis.

Real Time PCR

The gene expression analysis was performed using real-time-PCR. The reaction mixture (10 µl) contained 10 ng of prepared cDNA, 5 µl of 2 X Sso Fast Eva Green Supermix (BIORAD, Cat. No. 172–5201), and 0.5 µl of 5 picomoles/µl of forward and reverse primers each (See **Table 1** for the list of primers). All reactions carried out in duplicates were performed in a CFX96™ Thermocycler (BIO-RAD) under the following conditions: 3 min at 95 °C, 40 cycles of 10 s at 95 °C, and 30 s at 60 °C and melt curve (65–95 °C) analysis. The *C. reinhardtii* β subunit-like polypeptide (*Cblp*) gene, which is constitutively expressed was used as the reference gene.[70] Data was analyzed using the 2(-Delta Delta C(T)).[71] The results of the RT-PCR experiments are represented as a fold increase over the controls, calculated from the Ct values. It may be noted that while sample duplicates were maintained for each Real Time PCR reaction, the entire experiment was conducted thrice.

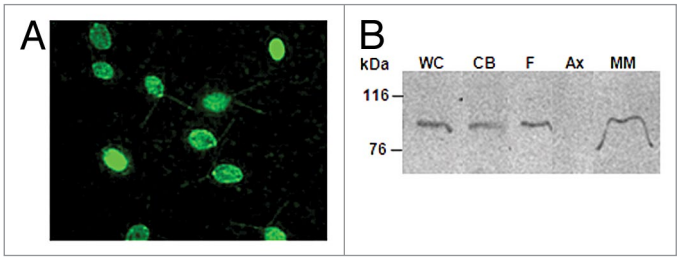

Figure 4. *C. reinhardtii* CDPK1 is localized in the cell body and flagella. (**A**) Immunostaining of cells expressing CDPK1-GFP with anti-GFP antibody. CDPK1-GFP fusion protein was localized in *C. reinhardtii* flagella and cell body. (**B**) Whole cell (WC), cell body (CB), isolated flagella (F), axoneme (Ax), and membrane-matrix (M-M) fractions of CDPK1-GFP clone were analyzed by immunoblotting using anti-GFP antibody. CDPK1- GFP fusion protein was present in the cell body and the membrane-matrix fraction of the flagella. The bar on (**A**) indicate 10 µm.

Construction of CDPK1-GFP fusion protein

CDPK1 was amplified from *C. reinhardtii* gDNA using the KOD Xtreme kit (Calbiochem,Cat. No. 71975–3) and primers CIGUF and CIGR (**Table 1**). This was followed by a secondary amplification using primers with RE sites C1GUFNdeI and CIGREcoRI (**Table 1**). It was cloned in pUC19 and transformed in *E. coli* DH5α cells. The plasmid was isolated, purified, and RE digested with NdeI and EcoRI, ligated with pChlamiRNA3 (RE digested with NdeI and EcoRI), and transformed in *E. coli* DH5α cells. The GFP plasmid (pCRGFP) was obtained from Chlamy Resource Centre (http://www.chlamy.org). It was RE digested with BamHI and subsequently the 717 bp product of GFP gene was gel purified. The GFP gene was amplified using KOD Xtreme kit (Calbiochem,Cat. No. 71975–3) and primers GFPfwdEcoRI and GFPrevXbaI and cloned in pUC19 followed by transformation in *E. coli* DH5α cells (**Table 1**). The GFP amplicon was transformed into *E. coli* DH5α cells. The plasmid was isolated, purified, and RE digested with EcoRI and XbaI and ligated with pChlamiRNA3-CDPK1 plasmid (RE digested with EcoRI and XbaI). This resulting plasmid (henceforth called pCrCDPK1GFP) was transformed in *E. coli* DH5α cells (**Fig. 3A**). Sequencing of CDPK1-GFP plasmid was done using nested primers C1seqF1 to C1seqR9 (**Table 1**). After sequence confirmation, the vector was transformed into *C. reinhardtii* cells.

Transformation in *C. reinhardtii* cells and screening for CDPK1-GFP clones

The pCrCDPK1GFP plasmid was isolated, purified, and linearized with NotI. Transformation of pCrCDPK1GFP in

Table 1. Primer sequences used in this study

Name	Primer Sequence (5′-3′)
Primers for sequencing pCCDPK1GFP	
C1seqF1	ACTCGTTGTGCATTCTAGGACC
C1seqR1	AGCTTCAGCGTCTGGTCG
C1seqF2	TGTGGCGTTGCACACAGAT
C1seqR2	TTGTTGGTGGCGGAGTAAGT
C1seqF3	GGACTGCTGGAAGGACTACG
C1seqR3	TCCGACAGCAGGAAGTTCTC
C1seqF4	CACAACATGGGCGTCATTC
C1seqR4	CTGCACCGTACGTCTGCTG
C1seqF5	CCGCAGAGTGTATGACATGG
C1seqR5	ACGCGACGGAAACACATAG
C1seqF6	CTGCGTCCACGCGTCTAC
C1seqR6	TGCCGTTGATCTCGTCCA
C1seqF7	CCTGCTGGTGTGTTTGCAAC
C1seqR7	TCAATCACACGGCACATCG
C1seqF8	GGAAGCGTCTGGTGAAGATG
C1seqR8	GTCTTGTAGTTGCCGTCGTCC
C1seqF9	AGCAGCACGACTTCTTCAAGTC
C1seqR9	CACACCGACCCTGGTCAC
Primers for generating CDPK1-GFP	
ClGUF	GAGGCCTAGGAGACTGTGCATGTGAGGC
C1GR	CATATGGAGGCCTAGGAGACTGTGCA
C1GUFNdeI	CAACCCGCGCATCATTGCCACGAA
ClGREcorI	GAATTCCAACCCGCGCATCATTGCC
GFPfwdEcoRI	GAATTCATGGCCAAGGGCGAGGAGC
GFPrevXbaI	TCTAGATTACTTGTACAGCTCGTCCATGCCG
Primers for Actin PCR	
CreActin fwd	CGCTGGAGAAGACCTACGAG
CreActin rev	GGAGTTGAAGGTGGTGTCGT
Primers for Real Time PCR	
Cblp fwd	CTTCTCGCCCATGACCAC
Cblp rev	CCCACCAGGTTGTTCTTC
CDPK1RT1F	AGCCAACTTGGAATGAGGTG
CDPK1RT1R	CGTCTTGCCCTCTGTAAAGC
CDPK3RT1F	TTCTCGGTTGCAAACATCCT
CDPK3RT1R	ACGTCCTTCACGTCCTCCTT

C. reinhardtii was performed as per the protocol of Kindle.[72] Selection for positive clones were done on (10 µg/ml) paromomycin in TAP agar plates. Clones were picked up from the plates and maintained on TAP agar plates. Transformants were screened for the presence of CDPK1-GFP fusion protein by PCR using primers GFPfwdEcoRI and C1seqR8 (**Table 1**), immunocytochemistry, and by western blotting using an anti-GFP antibody (Sigma, Cat. No. G 1544). Twelve positive clones were obtained.

Calcium-mobility gel shift assay

To determine if the protein binds to Ca^{2+}, calcium mobility shift assay was performed. The cell pellet of CDPK1-GFP clone was resuspended in lysis buffer (50 mM Tris, 10% glycerol and 1 mM PMSF, pH 7.4) and sonicated. The cell lysate was centrifuged at 13,400 g for 5 min. The supernatant was incubated with 2.5 mM EGTA, 1 mM, 2.5 mM, 5 mM, and 10 mM $CaCl_2$ on ice for 30 min followed by addition of 5 X SDS sample buffer and incubation for 15 min at 100 °C. Proteins were electrophoresed on 7% SDS-polyacrylamide gel by the method of Laemmli and electro-transferred to a nitrocellulose membrane.[73] The membrane was blocked with 3% (w/v) BSA (Bovine Serum Albumin) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 1 h and then incubated with anti-GFP antibody (1:1,000) (Sigma, Cat. No. G 1544) for 2 h at room temperature. The membrane was washed thrice (15 min each) with PBS containing 0.05% Tween 20 (PBST) and incubated with goat-anti rabbit IgG conjugated with HRP (horseradish peroxidase) (1:5,000) (Santa Cruz Biotechnology, INC, Cat. No. sc-2004) for 1h. The membrane was washed as above and developed using peroxide buffer and HRP substrate, DAB (3,3′-diaminobenzidine tetrahydrochloride) (Roche Applied Science, Cat. No.11718096001).

Immunofluorescence microscopy

To localize CDPK1-GFP protein, *C. reinhardtii* cells expressing the CDPK1-GFP fusion protein were grown until logarithmic phase. The transformed cells were resuspended in microtubule stabilizing buffer (MTSB-30 mM HEPES, 15 mM KCl, 5 mM $MgSO_4$, 5 mM EGTA, 100 µM dithiothreitol, pH 7.0 with KOH) and allowed to adhere to polyethyleneimine-treated coverslip. They were washed with PBS and fixed in chilled methanol for 10 min. The cells fixed on the coverslip were washed with PBS and incubated with 3% (w/v) BSA in PBS blocking solution for 1 h. This was followed by a wash with PBS and incubation with primary antibody, anti-GFP (1:100) (Genscript, Cat No. A01388) for 1 h. The cells were washed briefly as before, followed by incubation with secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (1:100) (Invitrogen, Cat No. A-11008) for 1 h. The cells were finally washed with PBST and PBS and then mounted in ProLong® Gold Antifade Reagent (Invitrogen, Cat No. P36934). The cells were observed under a Nikon Eclipse 90i microscope with the Imaging Software NIS-Elements BR.

Flagella isolation and fractionation

Flagella were isolated from CDPK1-GFP clone as previously described.[74] An aliquot of flagella was stored separately at –20 °C. The remaining flagella were extracted in 1% Igepal for 10 min on ice. After centrifugation (31,000 g for 20 min at 4 °C), the membrane-matrix (M-M) was obtained in the soluble phase while the insoluble phase (axoneme) was washed with HMDEK buffer (10 mM HEPES, pH 7.2, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM EDTA, and 25 mM KCl) followed by resuspension in the same buffer. The M-M and axonemal fractions were stored at –20 °C until further use.

Sub-flagellar localization of CDPK1

Whole cells, cell body, flagella, axonemal proteins, and membrane-matrix fraction of the CDPK1-GFP clone were separated on an 8% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 3% (w/v) BSA in PBS for 1 h and then incubated with anti-GFP antibody (1:1,000) (Sigma, Cat. No. G 1544) for 2 h at room temperature. The membrane was washed thrice (15 min each) with PBST and incubated with goat-anti rabbit IgG conjugated with HRP (1:7,500) (Calbiochem, Cat. No. DCO3L) for 1h. This was followed by washes with PBST and development of the blot using peroxide buffer and HRP substrate, DAB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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