Genome wide identification and biochemical characterization of Calcineurin B-like calcium sensor proteins in *Chlamydomonas reinhardtii*

Manoj Kumar¹,²,³,#, Komal Sharma¹,²,#, Akhilesh K. Yadav¹,⁶#, Kajal Kanchan²,⁴#, Madhu Baghel⁵,⁷ Suneel Kateriya²,⁵,*, Girdhar K. Pandey¹,*

¹Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi, India
²School of Biotechnology, Jawaharlal Nehru University, 110067 New Delhi, India
³Amity Food and Agriculture foundation (AFAF), Amity University, Uttar Pradesh, Noida, 201313, India
⁴Amity Institute of Molecular Medicine and Stem Cell Research (AIMMSCR), Amity University Uttar Pradesh, Noida, 201313, India
⁵Department of Biochemistry, University of Delhi South Campus
⁶Shri Murli Manohar Town P. G. College, Ballia, India, 277001
⁷National Institute of Immunology, Aruna Asaf Ali Marg, Jawaharlal Nehru University, New Delhi, 110067, India.

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*# These authors contributed equally.

Corresponding authors: skateriya@jnu.ac.in, gkpandey@south.du.ac.in
Telephone no.: +91-11-24116615, +91-11-24113106 Ext. 7387
Fax no.: NA

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ABSTRACT

Calcium (Ca\(^{2+}\)) signalling is involved in the regulation of diverse biological functions through association with several proteins that enable them to respond to abiotic and biotic stresses. Though Ca\(^{2+}\)-dependent signalling has been implicated in the regulation of several physiological processes in *Chlamydomonas reinhardtii*, Ca\(^{2+}\) sensor proteins are not characterized completely. *Chlamydomonas reinhardtii* has diverged from land plants lineage, but shares many common genes with animals, particularly those encoding proteins of the eukaryotic flagellum (or cilium) along with the basal body. Calcineurin, a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, is an important effector of Ca\(^{2+}\) signalling in animals, while calcineurin B-like proteins (CBLs) play an important role in Ca\(^{2+}\) sensing and signalling in plants. The present study led to the identification of 13 novel CBL-like Ca\(^{2+}\) sensors in *Chlamydomonas reinhardtii* genome. One of the archetypical genes of the newly identified candidate, CrCBL-like1 was characterized. The ability of CrCBL-like1 protein to sense as well as bind Ca\(^{2+}\) were validated using two-step Ca\(^{2+}\)-binding kinetics. The CrCBL-like1 protein localized around plasma membrane, basal bodies and in flagella, and interacted with voltage gated Ca\(^{2+}\) channel protein (VGCC) present abundantly in the flagella, indicating its involvement in the regulation of the Ca\(^{2+}\) concentration for flagellar movement. The CrCBL-like1 transcript and protein expression was also found to respond to abiotic stresses, suggesting its involvement in diverse physiological processes. Thus, the present study identifies novel Ca\(^{2+}\) sensors and sheds light on key players involved in Ca\(^{2+}\) signalling in *Chlamydomonas reinhardtii*, that could further be extrapolated to understand the evolution of Ca\(^{2+}\) mediated signalling in other eukaryotes.
INTRODUCTION

Calcium ion (Ca\(^{2+}\)) is a versatile second messenger, which is involved in the regulation of diverse signalling pathways. It is involved in the direct regulation of several molecular and physiological processes in diverse organisms (1–4). In plants, Ca\(^{2+}\) signalling is reported to be involved in developmental and physiological processes including regulation of the abiotic and biotic stress responses (5–10). A highly dynamic Ca\(^{2+}\) signalling mechanism also exists in the chlorophyte algae, Chlamydomonas, particularly, with the flagellar activities like motility, mating, flagellation/deflagellation and flagellar length regulation (11–20). In addition to these activities, the role of Ca\(^{2+}\) signalling in the regulation of general stress responses as well as in processes pertaining to the cell body have not been thoroughly explored in Chlamydomonas. Some Ca\(^{2+}\) binding proteins have been characterized like Ca\(^{2+}\) dependent protein kinases, CDPK3 and CDPK4 and Ca\(^{2+}\) sensor, CAS in Chlamydomonas (11,21,22). In plants, major proteins that are involved in regulating Ca\(^{2+}\) associated stress responses are Ca\(^{2+}\) dependent protein kinases (CDPKs), calmodulin (CaMs) and calcineurin B-like proteins (CBLs) (23–25). During Ca\(^{2+}\) signalling, CBLs and CaMs act as sensor relay proteins, which interact with their effector proteins to transmit Ca\(^{2+}\) signals (23). However, CDPKs are sensor-effector proteins, which can directly phosphorylate and hence regulate their targets (26,27). These Ca\(^{2+}\) sensors can target proteins such as protein kinases, metabolic enzymes, transcription factors, channels/transporters or cytoskeleton associated proteins. CBLs interact and regulate the activity of protein kinases, known as CBL-interacting protein kinases (CIPKs) and form the CBL-CIPK module, which regulates diverse stress signalling pathways, intracellular ion homeostasis, germination and seed development (28).

It has been reported that the CBL-CIPK signalling module originated during early eukaryotic evolution (29–31). However, either component of this module was found to be missing in many green algae suggesting selective loss during evolution (29,30,32–34). CBLs have been shown to work independent of CIPKs in plants (35). Thus, owing to the importance of CBL-CIPK network in various signalling as well as in development and physiological processes in plants, we explored the existence and functional significance of this important Ca\(^{2+}\) signalling component in *C. reinhardtii*. CIPKs were not identified, however, 13 novel CBL-like proteins (CrCBL-likes) were identified in *C. reinhardtii* through genome wide analysis. They have characteristic EF-hand like Ca\(^{2+}\) binding motifs similar to those present in *Arabidopsis* CBLs. Furthermore, one of the members, CrCBL-like1, was characterized in detail for its
Ca\textsuperscript{2+} binding property, expression pattern and interacting partners. This is the first report that demonstrates the presence of CBL-like Ca\textsuperscript{2+} sensors in \textit{C. reinhardtii} and also provides functional relevance of one of the archetypical genes. In future, detailed functional characterization of the CrCBL-like proteins will pave the path for understanding their significance in different physiological as well as developmental processes of the green algae.

MATERIALS AND METHODS

\textit{In silico} analysis for identification of putative homologs of CBL in Chlamydomonas

Multiple sequence alignment of \textit{Arabidopsis} CBLs was performed and used as an input to generate HMM profile using HMMER software (36). The generated profile was used with default parameters in the HMM search program of the HMMER package against \textit{Chlamydomonas reinhardtii} proteome (Uniprot id: UP000006906). All the hits with significant positive scores were selected and then examined individually for the accessory domains. These hits were individually confirmed by BLASTP. The sequence analysis and motif detection were carried out using Geneious Version 7 (https://www.geneious.com) software with default parameters. The structural modeling of the CrCBL-like1 protein was performed using web based SWISS-MODEL program (37) and the structure was visualized using PyMOL X11Hybrid (Ver 2) (https://pymol.org).

\textit{Chlamydomonas reinhardtii} cell culture

\textit{Chlamydomonas reinhardtii} wild type strain CC-124 was procured from Chlamydomonas resource center (www.chlamycollection.org). Culture was grown using TAP (tris-acetate-phosphate media, pH 7.4 and supplemented with Hutner trace elements) at 25°C in a shaker incubator (120 rpm) in a synchronized manner (14 hrs light and 10 hrs dark condition). Light exposure was provided using white fluorescent light (30-40 \(\mu\)moles/m\textsuperscript{2}/s).

Cloning, Expression and Purification of the CrCBL-like1 protein

The ORF of Cre08.363750 (referred to as CrCBL-like1 in this study) was cloned into pET21a vector and the sequenced construct was transformed into \textit{E. coli} BL21\textdagger\textasciitilde DE3 cells. The transformed cells were grown at 37°C until OD of 0.5-0.6 after which the temperature was reduced to 28°C and 0.1 mM IPTG (isopropyl thiogalactosidase) was used for induction. The cells were pelleted after 6 to 7 hrs by centrifuging at 5000g for 10 minutes and stored at -80°C. The stored cells were thawed and
resuspended in 30 ml of resuspension buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM Imidazole, 5 mM 2-mercaptoethanol and 5% Glycerol) for purification of the expressed protein. The cells were lysed by sonication for 10 minutes with 20 seconds on/10 seconds off cycle in presence of 1 mM PMSF (phenylmethylsulfonyl fluoride) in the buffer. The sonicated cells were treated with 1% Triton X-100 for 30 minutes at 4˚C and centrifuged at 20,000g for 30 minutes. The supernatant was incubated with 1 ml of Ni-NTA beads (Qiagen) for 90 minutes. Further purification was performed according to Qiagen protocol with the following buffers- Wash buffer 1: Resuspension buffer with additional 150 mM NaCl; Wash buffer 2: Re-suspension buffer + 20mM Imidazole; Elution buffer: Resuspension buffer + 350 mM Imidazole). 1-2 µg of protein was visualized on 12% SDS PAGE and 1 µg of protein was used for western blot analysis.

Antibody against CrCBL-like1 protein

Affinity purified CrCBL-like1 protein was used for raising antibody in rabbit using commercial facility provided by Genei Bangalore, India. The antibody was used for CrCBL-like1 detection.

Native PAGE, SDS PAGE and Western Blotting

For protein expression analysis, the frozen cells were re-suspended in 1X PBS supplemented with 1mM PMSF and sonicated at 40% amplitude for 2 min. with 30 sec. on/off cycle. The prepared cell lysate was centrifuged at 10,000 rpm for 5 min. The clear supernatant containing the total cellular protein was dispensed and its concentration was estimated via nanodrop as well as Bradford assay. 30µg protein was mixed with 6X Laemmli buffer and then resolved on 12% SDS PAGE gel. Protein expression profile was visualized via western blot using the antibodies as mentioned previously. Anti-His antibodies and anti-mouse alkaline phosphatase (AP) conjugated secondary antibodies were used for detecting the recombinant proteins at a dilution of 1:1000 (Sigma H1029) and 1:10,000, respectively. For detecting endogenous CrCBL-like1 protein, rabbit anti-CrCBL-like1 antibody was used at a dilution of 1:3000 and anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody at a dilution of 1:5000, was used (Sigma, USA). Tubulin was visualized using mouse anti-tubulin antibody (Sigma) at a dilution of 1:5000 and anti-mouse HRP conjugated secondary antibody at a dilution of 1:5000. Western blot was performed according to the standard protocols. The blots were quantified using ImageJ tool (https://imagej.nih.gov/ij/).
**Gel shift assay:** For native PAGE, 6 µg of CrCBL-like1 was incubated with 2 mM CaCl₂ and 5 mM EGTA for 30 min. in 20 µl volume. 20 µl of 2X native PAGE dye was added and then separated on 12% polyacrylamide gel at 4°C for 3 hours at 160 volts. For SDS-PAGE, same set of samples were analyzed on 15% SDS-PAGE.

**Fluorescence spectroscopy**

Ca²⁺ binding experiments were performed via steady state intrinsic fluorescence spectroscopy (Cary Eclipse fluorescence spectrophotometer, Agilent). CrCBL-like1 didn’t have any tryptophan residue, therefore, tyrosine (Tyr) fluorescence was used for Ca²⁺ binding studies. 2 µM of CrCBL-like1 was used to measure the Tyr fluorescence. Change in fluorescence was detected upon adding Ca²⁺ and EGTA. 2 mM CaCl₂ and 5 mM EGTA was used in 50 mM Tris/HCl buffer pH 8 and 150 mM NaCl. Excitation wavelength - 280 nm, emission range - 285-400 nm, excitation bandwidth - 5 nm and emission bandwidth - 10 nm were used. For Ca²⁺ binding saturation curve, different concentrations of Ca²⁺ starting from 0.01 µM–3 mM were used. The saturation curve was plotted using fluorescence intensity measured at the wavelength 304.9 nm at Y-axis and free Ca²⁺ concentration in the X-axis. The data was fitted using non-linear regression in Graphpad Prism 5 and apparent Kd was calculated manually as midpoint of the sigmoidal curve assuming that 50% of CrCBL-like1 is bound to Ca²⁺. The experiment was performed thrice for statistical significance.

**Gel Exclusion Chromatography**

All the eluted fractions of proteins were pooled and concentrated to 20 mg/ml using 3 kDa Millipore filters. The protein was passed through Superdex 16/600 GL 75 Pg column at a 1-ml/min flow rate and the fractions containing the proteins were collected. Ca²⁺ binding was also observed via gel exclusion chromatography. 1.2 mg of CrCBL-like1 was incubated with 2 mM CaCl₂ and 5 mM EGTA for 30 minutes and run on Superdex 200 (10/300 GL) column. Gel filtration buffer: 50 mM Tris/HCl pH 8 and 150 mM NaCl. During gel filtration (GF) respective ligands (Ca²⁺/EGTA) were also added in the GF buffers.

**RNA extraction, cDNA preparation and Real Time Quantitative PCR**
Chlamydomonas reinhardtii was grown in 50 ml medium until mid log phase i.e., OD of 0.5. Different stress conditions including cold (4°C), oxidative stresses i.e., methyl viologen (1mM) and hydrogen peroxide (1µM), heat (37°C) and salt (200mM), were subjected by incubating the mid log phase culture from 30 min. to 4hrs. Samples were harvested at different time points and cells were freezed in liquid nitrogen and stored at -80°C. RNA was extracted via hot phenol method. The frozen cells were re-suspended into 300 µl of TES buffer (heated at 65 °C) and then aliquoted into 1.5 ml micro-centrifuge tube. Magna lyser beads (about 100 µl) were added to lyse the cells using hand held tissue grinder. Subsequently, 300 µl of hot phenol (1:1 ratio of acidic phenol pH 4.5 and chloroform) was added to the tube, mixed well by vortexing and incubated at 65°C for 30 minutes with constant shaking on dry bath heater. After 30 minutes, the tube was centrifuged at room temperature for 15 minutes at 12,000 rpm. The aqueous phase obtained after centrifugation was transferred to a new tube containing 240 µl of chilled isopropanol and 30 µl of sodium acetate (pH 5.2). The tubes were incubated at -80 °C for 15-30 minutes. Subsequently, the tubes were centrifuged at 15,000 rpm for 30 minutes, supernatant was discarded and the pellet was washed with 75% ethanol for 5 minutes. The pellet was air dried for approximately 30-40 minutes until residual ethanol evaporated and then, resuspended in 50 µl of nuclease free water. The integrity of the RNA was visualized via agarose gel electrophoresis. 1µg of RNA was used for cDNA preparation (High-capacity cDNA Reverse Transcription kit, Thermo Fischer) and subsequently qPCR (USB HotStart-IT SYBR Green qPCR Master Mix, Affymetrix) was performed according to manufacturer’s protocol. qPCR of CrCBL-like1 under salt and heat stress was performed as described above and data was evaluated to calculate the fold change in gene expression. The house keeping gene, Guanine nucleotide-binding protein subunit beta-like protein (Crgblp), was used as an internal control (38).

RNA-seq data analysis

The previously generated RNA-seq data were downloaded from chlamynet (http://viridiplantae.ibv.csic.es/ChlamyNet/ChlamyNet.html) and used to analyze expression patterns of CrCBL-like orthologs from Chlamydomonas. The expression values of different genes were plotted as heat maps using complex heat map package of Bioconductor using R software.

CD spectroscopy
Purified recombinant CrCBL-like1 was dialyzed in 10 mM sodium phosphate buffer of pH 7, which was compatible for CD spectropolarimeter. 2 mM CaCl_2/EGTA was added to buffer solution containing 0.5 mg/mL of CrCBL-like1 and spectra were collected on a J-815 (Jasco, Japan) CD spectropolarimeter continuously purged by N_2 and equipped with a temperature control system. Blank was set with 10 mM sodium phosphate buffer, pH 7. Spectral measurements were performed in far-UV (188–260 nm) range using quartz cell of path length 1 nm in a thermostatic cell holder. CD spectra were recorded using average of three scans and data was collected and plotted using IGOR software version 3.14.

**Immunofluorescence**

Cells in early log phase were harvested and re-suspended in 1X PBS. 200µL aliquot of cell suspension was seeded on acid washed cover slips that were coated with poly-L-lysine. Cells were fixed with 3.7% paraformaldehyde in 1X PBS and permeabilized by submerging in cold 100% ethanol at -20°C for 10 min. Cells were then washed with 1X PBS containing 0.25 M NaCl at room temperature for 5 min. Brief washings were done with 1X PBST (PBS containing 0.5% triton X-100) followed by incubation with rabbit anti-CrCBL-like1 antibody (1:250 dilutions) or pre-immune serum at 4°C overnight. Samples were then incubated with FITC conjugated anti-rabbit IgG antibody (Invitrogen), at 1:1000 dilutions. After three rounds of brief washings, cover slips were mounted on slides by applying antifade reagent (Slow Fade Gold, Molecular Probes). Slides were visualized by Leica TCS SP5 confocal microscope.

**Statistical analysis**

If not otherwise indicated, data were obtained from at least three independent experiments (n = 3). Mean values were calculated and used for the analysis of standard deviation (SD) or standard error (SEM). Data analysis was performed with Prism 6.0 software (GraphPad, La Jolla, California).

**RESULTS**

**Identification of putative CBL orthologs in Chlamydomonas reinhardtii**

To identify calcineurin B-like Ca^{2+} sensor proteins in C. reinhardtii, a multiple sequence alignment of Arabidopsis CBLs (AtCBLs) was performed and used as an input to generate HMM profile. This profile was used to search for CBL orthologs in Chlamydomonas proteome. A total of 35 proteins...
were identified, out of which 22 proteins were omitted because of high e-value or being the isoforms of other genes lacking EF-hand motif or having features of CDPKs. Thus, in total 13 proteins were identified as AtCBL orthologs and confirmed by BLASTP. Structural similarities and differences between these proteins were further analyzed. Multiple sequence alignments revealed sequence homology amongst these proteins especially at their EF-hand motifs, which is one of the major characteristics of the Ca$^{2+}$ sensors (Figure 1A). Majority of AtCBLs studied so far, contained four EF-hand motifs and a similar trend was observed in C. reinhardtii. Figure 1A shows the presence of conserved EF-hand like motifs in all the identified proteins. Further, structural modeling of Cre08.g363750 (CrCBL-like1) was performed using SWISS-MODEL. The PyMOL analysis revealed the presence of characteristic helix-loop-helix motif of EF-hands in the CrCBL-like1. EF-hand motif was more similar to calcineurin-B variant from Coccidioides immitis (PDB Id 5b8i) as the model showed one bound Ca$^{2+}$ with the EF-hand 3, while no bound Ca$^{2+}$ was detected using A. thaliana CBL2 crystal structure (PDB id 2zfd.1) as template. The structural model of CrCBL-like1 showed the typical EF-hand fold with Ca$^{2+}$ coordinated in a pentagonal-bipyramidal geometry with the side chain carboxylate of Asp-(X), Asp-(Z), Glu-(Z), the main chain carboxyl of Asp-(Y), and that of Phe-(Y), and serine with help of a water molecule (-X) (Figure 1B, C). CrCBL-like1 showed the presence of 3 canonical EF-hands (EF-hand 2, 3 and 4), while EF-hand 1 showed the EF-fold with some differences at 1st and 12th position, where Alanine and Threonine were present (Figure 1B, C). Thus, based on the presence of canonical and non-canonical EF-hands, the existence of CBL-like Ca$^{2+}$ sensors in green algae Chlamydomonas reinhardtii was established.

**Expression and purification analysis of CrCBL-like1 protein**

CrCBL-like1 was expressed and purified (Materials and methods section) through nickel NTA chromatography and subsequently through gel exclusion chromatography. A single highly pure fraction was obtained at the size of approximately 17 kDa. The identity of the protein was confirmed through western blotting using anti-Histidine antibody (Figure 2A). The oligomerisation of CrCBL-like1 was explored using gel exclusion chromatography. Protein eluted mostly as a dimer; however higher order oligomers were also visible but at lower concentrations (Figure 2B).

**CrCBL-like1 protein undergoes conformational change upon binding to calcium**
Ca\textsuperscript{2+} binding property of CrCBL-like1 and the associated conformational change was explored through multiple methods. We initially, confirmed the Ca\textsuperscript{2+} binding through native-PAGE and SDS-PAGE. Figure 2C demonstrates that CrCBL-like1 shows a shift in its position upon binding to Ca\textsuperscript{2+} and EGTA in native-PAGE confirming the conformational transitions and hence, indicating Ca\textsuperscript{2+} binding. These conformational differences were also visible on SDS-PAGE (Figure 2D). Glutathione-S-Transferase (GST) protein was used as a negative control, which didn’t show any Ca\textsuperscript{2+} and EGTA mediated mobility shift on both native- and SDS- PAGE (Suppl. Figure S1). Additionally, distinct conformation adopted by Ca\textsuperscript{2+} bound and unbound states was confirmed through gel exclusion chromatography, which aligned well with native-PAGE data. Ca\textsuperscript{2+} bound and unbound CrCBL-like1 proteins eluted at different elution volumes, with bound protein eluting earlier than the unbound protein suggesting that it was in an extended conformation when bound to Ca\textsuperscript{2+} (Figure 2G,H). Effect of Ca\textsuperscript{2+} binding on the secondary structure of CrCBL1 was analyzed by far UV circular dichroism spectroscopy. CrCBL-like1 CD spectra showed intense negative ellipticity at 208 nm and 222 nm depicting its significant alpha helical content, which is characteristic of most Ca\textsuperscript{2+} binding proteins. In the presence of Ca\textsuperscript{2+} ions, a decrease in helical content was observed as the peaks at 208 and 222 nm shift to lower negative values as compared to the control (Figure 2E). The conformational change in CrCBL-like1 protein due to Ca\textsuperscript{2+} binding was also investigated using Tyr fluorescence assay. Figure 2F shows that the Tyr fluorescence is altered in the Ca\textsuperscript{2+} bound and unbound complexes, strengthening the conclusion that CrCBL-like1 protein binds Ca\textsuperscript{2+} leading to conformational changes. In order to estimate the Ca\textsuperscript{2+} binding affinity of CrCBL-like1 protein, a Ca\textsuperscript{2+} titration assay using fluorescence spectroscopy was performed. A two-step Ca\textsuperscript{2+} binding saturation curve of CrCBL-like1 protein was observed (Figure 3). At nano-molar Ca\textsuperscript{2+} ion concentrations, a Ca\textsuperscript{2+} dependent decrease in fluorescence maxima of Tyr was observed. However, upon increasing the Ca\textsuperscript{2+} concentration further to the micro-molar range, a Ca\textsuperscript{2+} dependent increase in fluorescence maxima of Tyr was observed. Before performing the Ca\textsuperscript{2+} titrations, the protein was dialyzed in a buffer containing 10 mM EGTA and Chelex to remove any bound Ca\textsuperscript{2+} from the protein as well as trace amounts of Ca\textsuperscript{2+} present in buffer. The EGTA from the protein was removed by dialyzing it against the buffer treated with only Chelex. The apparent Kd estimated was 60 ± 2 nM and 30 ± 3 µM, respectively. The specificity of Ca\textsuperscript{2+} binding kinetics was confirmed by performing EGTA titration assay via fluorescence spectroscopy. A decrease in the Tyr fluorescence was observed thereby, confirming the specificity as well as reversibility of the Ca\textsuperscript{2+} binding reaction.
In vivo expression of CrCBL-like1 and light induced change in expression pattern

CBLs have been extensively studied in plant systems with involvement in several physiological and developmental processes (39). Most of the characterized CBLs are localized in plasma membrane and tonoplast where they regulate various proteins involved in sodium compartmentalization, potassium homeostasis and production of reactive oxygen species (39–43). Since CrCBL-like1 protein was identified using A. thaliana CBLs as template, therefore; it may be speculated that CrCBL-like1 protein might also localize in plasma membrane and perform similar functions. Immunostaining with CrCBL-like1 specific primary antibodies followed by FITC labeled secondary antibodies revealed the expression of this protein around plasma membrane as well as near basal bodies (Figure 4).

The presence of any signal sequence in the protein might be responsible for trafficking of the protein near cell membrane. It has been reported that dual fatty acyl modification via N-myristoylation and S-acylation determines the plasma membrane associated targeting of CBLs and CIPKs in Arabidopsis (44). Therefore, the protein sequences were analysed for the presence of these motifs. Interestingly, a myristoylation site with very high confidence was detected, however, palmitylation site was not detected using web based Expasy: Myristoylation palmitoylation tool (https://web.expasy.org/myristoylator/).

Further, there are reports showing differential gene expression and localization pattern of CBLs inside the cells in plants under different stress conditions (45). Since C. reinhardtii is a photosynthetic organism and needs light, therefore, any changes in CrCBL-like1 localization in response to changes in the light conditions were analyzed. As mentioned earlier, CrCBL-like1 predominantly localized near the plasma membrane and basal bodies (Figure 4A). However, in the middle of the light phase of 14:10 hrs light-dark cycle, CrCBL-like1 was found to be localized prominently in the flagella of C. reinhardtii cells (Figure 4B).

CrCBL-like1 interacts with Voltage Gated Calcium Channel (VGCC)

Ca\(^{2+}\) signaling is essential for flagellar movement and therefore, several voltage-gated Ca\(^{2+}\)-channels (VGCCs) have been reported to be present along the flagellar membrane of C. reinhardtii (46), which regulate appropriate Ca\(^{2+}\) concentration in the flagella for their movement. Many Ca\(^{2+}\) binding EF-hand containing proteins have been reported to regulate these VGCCs through certain Ca\(^{2+}\) feedback
mechanisms (47–49). Since the light exposure led to migration of CrCBL-like1 protein to flagella, it might have a role in flagellar movement by regulating VGCC. Consequently, the interaction of CrCBL-like1 protein with VGCC was also analysed. Indeed, the CrCBL-like1 protein co-localized with VGCC in the flagella during the mid-phase of light cycle (Figure 4C) as well as interacted with each other as established by co-immunoprecipitation analysis (Figure 4D).

**CrCBL-like genes are involved in stress response**

In order to understand the functional role of CrCBL-like genes in response to environmental stresses, the gene expression analysis of publically available RNA Seq data of *C. reinhardtii* under multiple stress conditions was analyzed. The heatmap revealed varying expression patterns for most of the genes, except Cre03.g178150, which showed consistently higher expression in all condition. As noted, *CrCBL-like1* as well as *Cre15.g641250* expressed similar patterns, showing higher expression in various nutrient deprived conditions, suggesting that these genes respond transcriptionally to extrinsic cues (Suppl. Figure S2). The changes in *CrCBL-like1* expression in response to additional stress conditions, such as heat, cold, salt, H2O2 and methyl viologen, (not included in the RNA seq data) were also analysed. The *CrCBL-like1* transcript as well as protein expression were tested by performing real-time PCR and western blot analyses, respectively. In all stress conditions, a decrease in protein expression was observed when compared to untreated sample except in cold condition(s) (Figure 5A, B, Suppl. Figure S3). The gene expression analysis of *CrCBL-like1* under the heat and salt stress conditions was performed and the results corroborated with the protein expression profiles (Figure 5C).

**DISCUSSION**

Ca2+ is considered a second messenger in eukaryotes. The temporally and spatially defined “Ca2+ signatures” are involved in signal transduction pathways regulating a number of biological processes. Ca2+-mediated signalling is widely employed in different physiological functions such as photobehavioural responses (phototaxis and photo-stop response), motility, chemotaxis and mating in *C. reinhardtii*, (10,12,13,50). A small variation in the intracellular Ca2+ concentration in the flagella of *C. reinhardtii*, leads to major changes in its flagellar beating patterns and hence, the photomovement (51). Based on these variations in intraflagellar Ca2+, *C. reinhardtii* confronts flagellar biogenesis or
deflagellation which involves various Ca\(^{2+}\) regulated proteins (14,15). Despite the prominent involvement of Ca\(^{2+}\) signalling in major biological functions, very few Ca\(^{2+}\) binding or Ca\(^{2+}\) signalling proteins have been reported in *C. reinhardtii*.

In recent years, Ca\(^{2+}\) sensor proteins have gained increasing attention due to their omnipresence in signalling pathways. The study presents the identification and molecular characterization of Ca\(^{2+}\) sensor proteins in *C. reinhardtii* using Arabidopsis CBLs as baits through genome-wide analysis. *In-silico* analysis revealed the presence of 13 orthologs of CBLs in *C. reinhardtii* (Figure 1A). Most of the characterized CBLs in other plant species contain typical Ca\(^{2+}\)-binding motif known as EF-hands. They are present mostly in two pairs. The identified proteins contained 4 EF-hands like motifs as observed by multiple sequence alignment. Structural modeling of the potential EF-hand like motifs revealed similar pentagonal-bipyramidal geometry with conserved Ca\(^{2+}\) binding residues (Figure 1B). In plants, CBLs have been reported to bind Ca\(^{2+}\) and regulate several physiological and developmental processes by interacting with its cognate kinases, known as CBL-interacting protein kinases (CIPKs) (28). CIPKs are specific kinases containing the NAF/FISL domains, along with kinase and phosphatase interaction motif. We did not find any CIPK orthologs in *C. reinhardtii*, which is in line with other reports (29,30). Given that, we identified these putative Ca\(^{2+}\) sensor proteins using CBLs as input, it’s intriguing to find the functioning of these identified proteins in the absence of CIPKs. However, CBL family members are reported to interact with other proteins as well, in addition to CIPKs. CBL3 interacts with AtMTAN, in addition to many CIPK family members through different regions, in Arabidopsis (52,53). CBL10 also interacts directly with AKTI that leads to inhibition of inward movement of K\(^{+}\) (54). CBL10 was also shown to be involved in reproductive development without the involvement of any of the CIPKs in Arabidopsis (55). These reports indicate that the interactions of the CBL or CBL-like proteins are not limited to CIPK members, suggesting that the identified proteins might have CIPK independent novel physiological functions in *C. reinhardtii*.

The CrCBL-like1 protein was further characterized based on structural modeling. Its EF-hand motif showed similarity to that of calcineurin-B variant from *Coccidioides immitis* (PDB Id: 5b8i) as well as CreinCBL8 in *C. reinhardtii* (56). CrCBL-like1 structural model showed the typical EF-hand fold with Ca\(^{2+}\) coordinated in a pentagonal-bipyramidal geometry as described in literature. The binding of Ca\(^{2+}\) to the EF-hand motifs is a functional feature of CBL proteins for relaying Ca\(^{2+}\) signals as shown in Figure 2. The binding of Ca\(^{2+}\) to CBLs using different methods like gel electrophoresis and mobility

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shift assays is well documented. The effect of Ca\textsuperscript{2+} binding on the mobility of the protein was assessed using native- and SDS-PAGE. Under both denaturing and native conditions, the Ca\textsuperscript{2+} bound and unbound protein showed differences in their mobility suggesting a change in the conformation of the protein. In the native condition, the Ca\textsuperscript{2+}-saturated protein had a larger apparent molecular mass compared to the Ca\textsuperscript{2+} depleted form, which suggests that Ca\textsuperscript{2+} binding leads to a more extended protein conformation (Figure 2C, D). This aligns well with the structural changes observed in most of the EF-hand containing proteins upon Ca\textsuperscript{2+} binding. It is well documented that the EF-hand motifs adopt an open conformation when bound to Ca\textsuperscript{2+}, with both the alpha helices lying perpendicular to each other. In contrast, it adopts a closed conformation, with both the helices lying parallel to each other in Ca\textsuperscript{2+} free state.

The Ca\textsuperscript{2+}-induced conformational changes in the protein observed using gel exclusion chromatography, were in agreement with the PAGE results. The data correlated well with the gel exclusion chromatography data wherein Ca\textsuperscript{2+} bound protein eluted earlier compared to the unbound protein. However, under the denaturing conditions, the Ca\textsuperscript{2+} bound protein migrated faster compared to EGTA bound protein suggesting vice versa (Figure 2E, F). Such faster movement has been observed in a few Ca\textsuperscript{2+}-binding proteins when associated with Ca\textsuperscript{2+}, in comparison to Ca\textsuperscript{2+} depleted proteins (57–60). Since EGTA has a higher molecular mass compared to Ca\textsuperscript{2+}, therefore, the net mass of the protein when bound to EGTA is higher, hence its mobility is retarded. Ca\textsuperscript{2+} binding often leads to change in the secondary structure of the protein. The distinct conformers of Ca\textsuperscript{2+} bound and unbound forms were confirmed through UV circular dichroism spectroscopy. Taken together, we established that Ca\textsuperscript{2+} associates with CrCBL-like1 protein.

In literature, the CBL proteins have been commonly referred to as Ca\textsuperscript{2+} sensors, however, there are very few reports about the estimation of Ca\textsuperscript{2+}-binding affinity. Therefore, we performed Ca\textsuperscript{2+} binding kinetics of CrCBL1-like1 protein by monitoring changes in the steady state intrinsic fluorescence of Tyr upon Ca\textsuperscript{2+} titrations. This method has been used previously to calculate the dissociation constant of Ca\textsuperscript{2+} binding proteins (61,62). Since, CrCBL-like1 lacked tryptophan residues as also observed in most of the CBL proteins, we used the change in Tyr fluorescence upon Ca\textsuperscript{2+} titration to obtain a dose response curve. CrCBL-like1 has 4 EF-hand motifs, of which, all except EF-hand 1 have Tyr residue(s) in their motif; therefore, change in the intrinsic fluorescence of Tyr could be easily used to study Ca\textsuperscript{2+} binding kinetics. CrCBL-like 1 showed fluorescence maxima at 304.6 nm without any bound Ca\textsuperscript{2+},
thus, to calculate the Ca\textsuperscript{2+}-binding affinity, different concentrations of free Ca\textsuperscript{2+} were added and the change in the fluorescence at 304.6 nm was plotted. One high affinity Ca\textsuperscript{2+} binding site with apparent Kd of 60 ± 2 nM and a second site with Kd of 30 ± 3 µM were observed (Figure 3). Cells are known to maintain an approximately 20,000-fold gradient between their intracellular and extracellular Ca\textsuperscript{2+} concentrations, which range from nM to mM (4,63). Our results indicate association around Kd of 60 nM, which is well within the physiological intracellular range of Ca\textsuperscript{2+}, suggesting the intracellular role of CrCBL-like1. A higher Kd indicates a potential role of CrCBL-like1 under cellular conditions in which the Ca\textsuperscript{2+} levels are increased like a sudden change in the environment due to stress.

The CrCBL-like1 protein has a myristoylation motif at its extreme N-terminus similar to that of other well characterized CBLs (44). This strengthens the localization pattern of the CBL-like1 protein near membrane as in our study. However, on exposure to light, specific redistribution and localization of CrCBL-like1 protein in flagella of \textit{C. reinhardtii} was observed. This is an interesting result considering the fact that several VGCCs are reported to be present along the flagellar membrane of \textit{C. reinhardtii} (46). Many Ca\textsuperscript{2+} binding proteins containing EF-hands regulate VGCCs, directly or indirectly, through a Ca\textsuperscript{2+} feedback mechanism. Some EF-hand containing superfamilies like calcineurin, calmodulin or CaBPs (Calcium Binding Protein) are involved in the regulation of the activity of VGCCs (47,64). Thus, we observed an interaction between CrCBL-Like1 protein and VGCC in \textit{C. reinhardtii} (Figure 4). VGCCs are involved in the regulation of Ca\textsuperscript{2+} levels in the flagella required for the movement. It is possible that CrCBL-like1 is involved in the regulation of VGCC and hence, maintenance of optimal concentration of Ca\textsuperscript{2+} in the flagella of \textit{C. reinhardtii}. It has been reported that high Ca\textsuperscript{2+} concentration (>10\textsuperscript{-6} M Ca\textsuperscript{2+}) is required along the flagella for its reverse movement and deflagellation occurs if Ca\textsuperscript{2+} ion concentration exceeds 10\textsuperscript{-6} M at basal bodies in \textit{C. reinhardtii} (65). This suggests the requirement of a Ca\textsuperscript{2+} ion sensor at such important location to regulate the Ca\textsuperscript{2+} concentration. Considering this, the presence of CrCBL-like1 in the basal bodies and its migration to flagella explains the requirement of CrCBL-like1 to potentially monitor the Ca\textsuperscript{2+} concentration. The photo-perception by the eyespot is followed by the activation of VGCCs localized along the flagella. Interaction between CrCBL-like1 and VGCC might also suggest Ca\textsuperscript{2+} dependent direct or indirect activation of VGCC. Further, gene and protein expression analysis of CrCBL-like1 showed a decrease in overall expression pattern in response to various kinds of stresses (Figure 5), which is obvious, as \textit{Chlamydomonas} tend to deflagellate under stress.
condition. Due to deflagellation, most of the CrCBL-like1 expression might be reduced as CrCBL-like1 is expressed in basal bodies and flagella.

This work opens a new avenue to study stress signalling in Chlamydomonas, considering that CrCBL-like1 could be used as a tool to study the role of Ca^{2+} in stress signalling as well as to explore the diversity of lesser-known CIPK-independent functions of CBL-like proteins.

**Abbreviations:**

- Ca^{2+}  Calcium
- CBL  Calcineurin B-like protein
- CIPK  CBL-interacting protein kinase
- VGCC  Voltage-gated Ca^{2+}-channel
- CDPK  Ca^{2+} dependent protein kinases
- CAS  Calcium sensor
- CaM  Calmodulin
- CaBP  Calcium binding protein

**AUTHOR CONTRIBUTION:** GKP and SK conceptualized and designed the research plan. MK, KS, AKY, KK and MB conducted the experiments. MK, KK, SK and GKP interpreted and analyzed the data. MK, KK and KS prepared the Figures. MK, KS, KK, SK and GKP wrote the manuscripts with active inputs from others. All authors have reviewed the manuscript.

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**REFERENCES:**

1. Sanders D, Pelloux J, Brownlee C, Harper JF. Calcium at the crossroads of signaling. Plant Cell [Internet]. 2002 [cited 2019 Feb 7];14 Suppl:S401-17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12045291

2. Trewavas A, Read N, Campbell AK, Knight M. Transduction of Ca^{2+} signals in plant cells and compartmentalization of the Ca^{2+} signal. Biochem Soc Trans [Internet]. 1996 Nov 1 [cited 2019 Feb 8];24(4):971–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8968494

3. Kudla J, Batistic O, Hashimoto K. Calcium Signals: The Lead Currency of Plant Information
1. Lee SC, Pan J. Regulation of Flagellar Biogenesis by a Calcium Dependent Protein Kinase in Chlamydomonas reinhardtii. PLoS One. 2013; 8(4):e58141.

2. Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim B-G, et al. Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. Plant J. [Internet]. 2007 Aug 21 [cited 2019 Feb 10];52(2):232–39. Available from: http://doi.wiley.com/10.1111/j.1365-313X.2007.03236.x

3. Eckert C, Offenborn JN, Heinz T, Armarego-Marriott T, Schütteke S, Zhang C, et al. The vacuolar calcium sensors CBL2 and CBL3 affect seed size and embryonic development in Arabidopsis thaliana. Plant J [Internet]. 2014 Apr 1 [cited 2019 Feb 8];78(1):146–56. Available from: http://doi.wiley.com/10.1111/jpc.12456

4. Hochmal AK, Schulze S, Trompelt K, Hippler M. Calcium-dependent regulation of photosynthesis. Biochim Biophys Acta - Bioenerg [Internet]. 2015 Sep 1 [cited 2019 Feb 8];1847(9):993–1003. Available from: https://www.sciencedirect.com/science/article/pii/S0005272815000353#bb0530

5. Lee SC, Lan W-Z, Kim B-G, Li L, Cheong YH, Pandey GK, et al. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci [Internet]. 2007 Oct 2 [cited 2019 Feb 7];104(40):15959–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17898163

6. Pande G, Reddy MK, Sopor SK, Lata Singla-Pareek S. Calcium Homeostasis in Plants: Role of Calcium Binding Proteins in Abiotic Stress Tolerance [Internet]. Vol. I, Indian Journal of Biotechnology. 2002 [cited 2019 Feb 8]. Available from: http://nopr.niscair.res.in/bitstream/123456789/19858/1/IJBT 1(2) 135-143.pdf

7. Liang Y, Pan J. Regulation of Flagellar Spoke Protein 2 Is a Calmodulin Binding Protein Required for Motility in Chlamydomonas reinhardtii. Eukaryot Cell. 2004; 3(4):766–75.

8. Dolle R, Pfau J, Nultsch W. Role of Calcium Ions in Motility and Phototaxis of Chlamydomonas reinhardtii. J Plant Physiol. 1987;126(4–5):467–73.

9. Nultsch W, Pfau J, Dolle R. Effects of calcium channel blockers on phototaxis and motility of Chlamydomonas reinhardtii. Arch Microbiol [Internet]. 1986 May [cited 2019 Feb 10];144(4):393–7. Available from: http://link.springer.com/10.1007/BF00409890

10. Wakabayashi K, Misawa Y, Mochiji S, Kamiya R. Reduction-oxidation poise regulates the sign of phototaxis in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A [Internet]. 2011 Jul 5 [cited 2019 Feb 10];108(27):11280–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21690384

11. Okita N, Isogai N, Hirono M, Kamiya R, Yoshimura K. Phototactic activity in Chlamydomonas "non-phototactic" mutants deficient in Ca"-dependent control of flagellar dominance or in inner-arm dynein. J Cell Sci [Internet]. 2005 Feb 1 [cited 2019 Feb 10];118(Pt 3):529–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15657081

12. Goodenough U, Shames B, Small L, Saito T, Crain R. The role of calcium in the Chlamydomonas reinhardtii mating reaction. J Cell Biol. 1993;121.

13. Quaromy LM, Criss Hartzell H. Two Distinct, Calcium-mediated, Signal Transduction Pathways Can Trigger Deflagellation in Chlamydomonas reinhardtii. [cited 2019 Feb 10]; Available from: http://doi.org/10.1083/jcb.124.5.807

14. Cheshire JL, Evans JH, Keller LR. Ca2+ signaling in the Chlamydomonas flagellum regeneration system: cellular and molecular responses. J Cell Sci [Internet]. 1994 Sep [cited 2019 Feb 10];107 (Pt 9):2491–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7844166

15. Cao M, Li G, Pan J. Regulation of cilia assembly, disassembly, and length by protein phosphorylation. Methods in cell biology. 2009.

16. Motiwalla MJ, Sequeira MP, D’Souza JS. Two Calcium-Dependent Protein Kinases from Chlamydomonas reinhardtii are transcriptionally regulated by nutrient starvation. Plant Signal Behav [Internet]. 2014 Jan 29 [cited 2019 Feb 8];9(1):e27969. Available from: http://www.tandfonline.com/doi/abs/10.4161/psb.27969
22. Petroutos D, Busch A, Janßen I, Trompelt K, Berger SV, Weim S, et al. The Chloroplast Calcium Sensor CAS Is Required for Photoacclimation in *Chlamydomonas reinhardtii*. Plant Cell. 2011;
23. Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W. Calmodulins and calcineurin-B-like proteins: Calcium sensors for specific signal response coupling in plants. Plant Cell. 2002;14(SUPPL.).
24. Kudla J, Becker D, Grill E, Hedrich R, Hippler M, Kummer U, et al. Advances and current challenges in calcium signaling. New Phytol [Internet]. 2018 Apr [cited 2019 Nov 12];218(2):414–31. Available from: http://doi.wiley.com/10.1111/nph.14966
25. Zhu JK. Abiotic Stress Signaling and Responses in Plants. Vol. 167, Cell. Cell Press; 2016. p. 313–24.
26. Kolukisaoglu Ü, Weim S, Blazevic D, Batistic O, Kudla J. Calcium Sensors and Their Interacting Protein Kinases: Genomics of the Arabidopsis and Rice CBL-CIPK Signaling Networks. Plant Physiol. 2004 Jan;134(1):43–58.
27. Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford N, et al. The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol. 2003 Jun 1;132(2):666–80.
28. Sanjal SK, Pandey A, Pandey GK. The CBL-CIPK signaling module in plants: A mechanistic perspective. Vol. 155, Physiologia Plantarum. Blackwell Publishing Ltd; 2015. p. 89–108.
29. Beckmann L, Edel KH, Batistic O, Kudla J. A calcium sensor-protein kinase signaling module diversified in plants and is retained in all lineages of Bikonta species. Sci Rep. 2016 Aug 19;6.
30. Jamisheer K M, Jindal S, Laxmi A. Evolution of TOR–SnRK dynamics in green plants and its integration with phytohormone signaling networks. J Exp Bot [Internet]. 2019 Apr 15 [cited 2019 Nov 12];70(8):2239–59. Available from: https://academic.oup.com/jxb/article/70/8/2239/5380873
31. Brawley SH, Blouin NA, Ficko-Blean E, Wheeler GL, Lohr M, Goodson H V., et al. Insights into the red algae and eukaryotic evolution from the genome of *Porphyra umbilicalis* (Bangiophyceae, Rhodophyta). Proc Natl Acad Sci U S A; 2017;
32. Luan S. The CBL-CIPK network in plant calcium signaling. Trends in Plant Science. 2009.
33. Mohanta TK, Mohanta N, Mohanta YK, Parida P, Bae H. Genome-wide identification of Calcineurin B-Like (CBL) gene family of plants reveals novel conserved motifs and evolutionary aspects in calcium signaling events. BMC Plant Biol. 2015;
34. Mohanta TK, Kumar P, Bae H. Genomics and evolutionary aspect of calcium signaling event in calmodulin and calmodulin-like proteins in plants. BMC Plant Biol. 2017 Feb 3;17(1).
35. Grefen C, Blatt MR. Do calcineurin B-like proteins interact Independently of the Serine Threonine kinase CIPK23 with the K+ channel AKT1? Lessons Learned from a Ménage à Trois. Plant Physiol. 2012 Jul;159(3):915–9.
36. Eddy SR. Accelerated profile HMM searches. PLoS Comput Biol. 2011 Oct;7(10).
37. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res [Internet]. 2018 Jul 2 [cited 2019 Nov 12];46(W1):W296–303. Available from: https://academic.oup.com/nar/article/46/W1/W296/5000024
38. López-Paz C, Liu D, Geng S, Umen JG. Identification of *Chlamydomonas reinhardtii* endogenous genomic flanking sequences for improved transgene expression. Plant J [Internet]. 2017 Dec 18 [cited 2019 Nov 12];92(6):1232–44. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/tpj.13731
39. Pandey GK, Kanwar P, Pandey A. Global comparative analysis of CBL-CIPK gene families in plants [Internet]. [cited 2019 Feb 10]. 91 p. Available from: https://books.google.co.in/books?id=P71ABAAQBAJ&pg=PA62&lpg=PA62&dq=pandey+potassium+homeostasis&source=bl&ots=U8pYi6i_HP&sig=ACfU3U0cAbVJPju3jSoL_31dZhPZ263OwbQ&hl=en&sa=X&ved=2ahUKEwif5sK21LDgAhtuU7t00KHYm2C0Q6AEwDoECAUQAQ#v=onepage&q=pandey+potassium+h
40. Steinhorst L, Kudla J. Calcium - a central regulator of pollen germination and tube growth. Biochim Biophys Acta - Mol Cell Res [Internet]. 2013 Jul 1 [cited 2019 Feb 10];1833(7):1573–81. Available from: https://www.sciencedirect.com/science/article/pii/S0167488912002911
41. Pandey GK, Grant JJ, Cheong YH, Kim B-G, Li LG, Luan S. Calcineurin-B-Like Protein CBL9 Interacts with Target Kinase CIPK3 in the Regulation of ABA Response in Seed Germination. Mol Plant • [Internet]. 2007 [cited 2019 Feb 10];1(2):238–48. Available from: https://www.cell.com/molecular-plant/pdf/S1674-2052(14)60432-9.pdf
42. Kim B-G, Waadt R, Cheong YH, Pandey GK, Domínguez-Solis JR, Schüttke S, et al. The
calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis. Plant J [Internet]. 2007 Sep 6 [cited 2019 Feb 8];52(3):473–84. Available from: http://doi.wiley.com/10.1111/j.1365-313X.2007.03249.x

43. Amtmann A, Armengaud P. The role of calcium sensor-interacting protein kinases in plant adaptation to potassium-deficiency: new answers to old questions. Cell Res [Internet]. 2007 Jun 14 [cited 2019 Feb 10];17(6):483–5. Available from: http://www.nature.com/articles/cr200749

44. Batistić O, Waadt R, Steinhorst L, Held K, Kudla J. CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. Plant J [Internet]. 2009 Nov 13 [cited 2019 Feb 8];61(2):211–22. Available from: http://doi.wiley.com/10.1111/j.1365-313X.2009.04045.x

45. Kleist TJ, Laun S. Constant change: dynamic regulation of membrane transport by calcium signalling networks keeps plants in tune with their environment. Plant Cell Environ [Internet]. 2016 Mar [cited 2019 Nov 20];39(3):467–81. Available from: http://doi.wiley.com/10.1111/pcen.12599

46. Beck C, Uhl R. On the localization of voltage-sensitive calcium channels in the flagella of *Chlamydomonas reinhardtii*. J Cell Biol. 1994 Jun;125(5):1119–25.

47. Dick IE, Tadross MR, Liang H, Tay LH, Yang W, Yue DT. A modular switch for spatial Ca2+-selectivity in the calmodulin regulation of CaV channels. Nature. 2008 Feb 14;451(7180):830–4.

48. Zühlke RD, Pitt GS, Tsien RW, Reuter H. Ca2+-sensitive inactivation and facilitation of L-type Ca2+ channels both depend on specific amino acid residues in a consensus calmodulin-binding motif in the α(1C) subunit. J Biol Chem. 2000 Jul 14;275(28):21121–9.

49. Pitt GS, Zühlke RD, Hudmon A, Schulman H, Reuter H, Tsien RW. Molecular Basis of Calmodulin Tethering and Ca2+-dependent Inactivation of L-type Ca2+ Channels. J Biol Chem. 2001 Aug 17;276(33):30794–802.

50. Liang Y, Pan J. Regulation of Flagellar Biogenesis by a Calcium Dependent Protein Kinase in *Chlamydomonas reinhardtii*. Roy S, editor. PLoS One [Internet]. 2013 Jul 25 [cited 2019 Nov 12];8(7):e69902. Available from: https://dx.plos.org/10.1371/journal.pone.0069902

51. Schmidt JA, Eckert R. Calcium couples flagellar reversal to photostimulation in *Chlamydomonas reinhardtii*. Nature. 1976;262(5570):713–5.

52. Oh SI, Park J, Yoon S, Kim Y, Park S, Ryu M, et al. The arabidopsis calcium sensor calcineurin B-like 3 inhibits the 5′-methylthioadenosine nucleosidase in a calcium-dependent manner. Plant Physiol. 2008 Dec;148(4):1883–96.

53. Ok SH, Cho JH, Oh SI, Choi MN, Ma JY, Shin JS, et al. Calcineurin B-like 3 calcium sensor associates with and inhibits 5′-methylthioadenosine nucleosidase 2 in Arabidopsis. Plant Sci. 2015 Sep 1;238:228–40.

54. Ren XL, Qi GN, Feng HQ, Zhao S, Zhao SS, Wang Y, et al. Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K+ homeostasis in Arabidopsis. Plant J. 2013 Apr;74(2):258–66.

55. Monihan SM, Magness CA, Yadegari R, Smith SE, Schumaker KS. Arabidopsis CALCINEURIN B-LIKE10 functions independently of the SOS pathway during reproductive development in saline conditions. Plant Physiol. 2016 May 1;171(1):369–79.

56. Mohanta TK, Yadav D, Khan AL, Hashem A, Abd_allah EF, Al-Harrasi A. Molecular players of EF-hand containing calcium signaling event in plants. Vol. 20, International Journal of Molecular Sciences. MDPI AG; 2019.

57. Garrigos M, Deschamps S, Viel A, Lund S, Champeil P, Moller J V, et al. Detection of Ca2+-binding proteins by electrophoretic migration in the presence of Ca2+ combined with 45Ca2+ overlay of protein blots. Anal Biochem [Internet]. 1991 Apr [cited 2019 Oct 23];194(1):82–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1831012

58. Zeng H, Zhang Y, Zhang X, Pi E, Zhu Y. Analysis of EF-hand proteins in soybean genome suggests their potential roles in environmental and nutritional stress signaling. Front Plant Sci. 2017 May 24:8.

59. Wu X, Qiao Z, Liu H, Acharya BR, Li C, Zhang W. CML20, an Arabidopsis Calmodulin-like Protein, Negatively Regulates Guard Cell ABA Signaling and Drought Stress Tolerance. Front Plant Sci [Internet]. 2017 May 23 [cited 2019 Oct 16];8. Available from: http://journal.frontiersin.org/article/10.3389/fpls.2017.00824/full

60. Vanderbeld B, Snedden WA. Developmental and stimulus-induced expression patterns of Arabidopsis calmodulin-like genes CML37, CML38 and CML39. Plant Mol Biol [Internet]. 2007 Aug [cited 2019 Oct 23];64(6):683–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17579812
Figure legends

Figure 1: Identification of CBL orthologs in Chlamydomonas reinhardtii. a Amino acid sequence alignment of the Chlamydomonas CBL orthologs showing conserved EF-hands is shown. b Structural modeling of the Cre08.g363750 (CrCBL-like1), EF-hands 1, 2, 3 and 4 are shown in blue, green, red and magenta colors, respectively. c. Ca\(^{2+}\)-bound EF-hand 3 motif, Ca\(^{2+}\) is shown as an orange sphere. Amino acid residues involved in the Ca\(^{2+}\) coordination are shown in single letters.

Figure 2: Heterologous expression, purification and Ca\(^{2+}\) binding studies of the CrCBL-like1. a Purification of CrCBL-like1, P: Pellet after sonication and 20,000g Spin, S: Supernatant after 20,000g spin, E1 and E2: Elutions (1 µg, 2 µg), M: Marker (precision plus protein dual color standards), WB: Western blot with penta-anti-Histidine monoclonal antibody. b Gel filtration profile of CrCBL-like1, 20 mg/ml protein was applied to Superdex 16/600 GL 75 Pg column at a 1ml/min flow rate. Profile shows the presence of dimer and trace amounts of the higher order oligomers. c and d Gel Shift assay upon Ca\(^{2+}\) binding via native- and SDS- PAGE, 6 µg of CrCBL-like1 was incubated with 2 mM CaCl\(_2\) and 5 mM EGTA for 30 min. in 20 µl volume. The 20 µl of 2X native PAGE dye was added and then run on 12% polyacrylamide gel at 4°C. Same sets of the samples were run on 15% SDS PAGE at RT; e and f Detection of the Ca\(^{2+}\) binding through gel filtration (GF). 1.2 mg of protein was incubated with 2 mM CaCl\(_2\) and 5 mM EGTA for 30 min. and applied to superdex 200 (10/300 GL) column. e GF profile of CrCBL-like1 without any treatment and with 5 mM EGTA. f GF profile of CrCBL-like1 with 2 mM Ca\(^{2+}\) and with 2 mM Ca\(^{2+}\) + 5 mM EGTA. GF buffer: 50 mM Tris/HCl pH8 and 150 mM NaCl. During GF, respective ligands (Ca\(^{2+}\) and EGTA) were also added in the GF buffers. g CD Spectroscopy of CrCBL-like1 in presence of Ca\(^{2+}\) and EGTA. 0.5 mg/ml of recombinant CrCBL-like1 protein was incubated with buffer containing indicated concentrations of Ca\(^{2+}\) and EGTA or both for 5
min. on ice followed by CD analysis. Fluorescence Spectroscopy method for 2 µM of CrCBL-like1 was used to measure the tyrosine (intrinsic) fluorescence. Change in the fluorescence was detected upon adding Ca\(^{2+}\) and EGTA. The 2 mM CaCl\(_2\) and 5 mM EGTA was used in 50 mM Tris/HCl buffer pH8 and 150 mM NaCl. Excitation wavelength 280 nm, Emission range 285-400 nm, Excitation bandwidth 5 nm and emission bandwidth 10 nm. N = 4.

**Figure 3: Ca\(^{2+}\) binding kinetics of the CrCBL-Like1.** The 2 µM of protein was used to measure the tyrosine fluorescence. Change in the fluorescence was detected upon adding different concentration of Ca\(^{2+}\) and EGTA. a The graph shows the dose dependent change in tyrosine fluorescence upon Ca\(^{2+}\) titration. Ca\(^{2+}\) concentrations used were from 0.001-10 µM. The Kd calculated was 60 ± 2 nM. b Second dose response curve was seen upon increasing the Ca\(^{2+}\) concentration from 0.001-5 mM. The Kd calculated was 30 ± 3 µM. c Specificity of the Ca\(^{2+}\) dependent change in tyrosine fluorescence was monitored by adding different concentration of EGTA as a chelator. EGTA concentration corresponds to (0.001-10 mM). Buffer used was 50 mM TRIS/HCl pH 8 and 150 mM NaCl. Excitation wavelength 280 nm, Emission wavelength 305 nm, Excitation bandwidth 5 nm and emission bandwidth 10 nm, N=3.

**Figure 4: Expression pattern of the CrCBL-like1 in Chlamydomonas and its interaction with voltage-gated Ca\(^{2+}\)channel (VGCC).** a CrCBL-like1 protein expression was observed near plasma membrane and basal bodies of the Chlamydomonas cells. b CrCBL-L1 seen to be expressed in the flagella probed by CrCBL-like1 antibody. Cells showing the localization in flagella have been presented along with the focus on a single cell (Inset). c CrCBL-like1 co-localized with VGCC in the flagella. Antibody used: CrCBL-like1 (anti-rabbit, red) and VGCC (anti-goat, green). d VGCC was present in immunoprecipitation (IP) elutions of CrCBL-like1. VGCC band detected at the expected molecular weight (300 kDa) corresponding to the input (Total cell lysate; CrTCL). IP of Pre-immune serum (PIS) serves as negative control where no bands corresponding to VGCC were detected. N=3.

**Figure 5: Expression analysis of the CrCBL-like1 in response to abiotic stress.** a CrCBL-like1 protein expression upon Cold, Methyl viologen, Hydrogen peroxide (H\(_2\)O\(_2\)) and Heat treatments. b CrCBL-like1 protein expression upon Salt stress. The expression of CrCBL-like1 protein changed differentially due to these treatments as detected by western blot using CrCBL-like1 antibody. c CrCBL-like1 gene expression analysis upon heat and salt stress.
The expression of CrCBL-like1 decreased after two hours similar to protein expression trend. N=3.
A) Gel electrophoresis with bands labeled P, S, E1, E2, M, and WB.

B) Chromatogram showing peaks at different volumes.

C) Western blot analysis with lanes labeled 1, 2, 3, and 4.

1. CrCBL-Like1
2. CrCBL-Like1 + 2 mM EGTA
3. CrCBL-Like1 + 2 mM Ca^{2+}
4. CrCBL-Like1 + 2 mM Ca^{2+} + 5 mM EGTA

D) Gel image showing bands labeled 1, 2, 3, and 4.

E) Absorbance at 280 nm versus volume (ml) for CrCBL-Like1 and CrCBL-Like1 + EGTA.

F) Absorbance at 280 nm versus volume (ml) for CrCBL-Like1 + Ca^{2+} and CrCBL-Like1 + Ca^{2+} + EGTA.

G) Molar ellipticity versus wavelength for CrCBL-Like1, CrCBL-Like1 + EGTA, CrCBL-Like1 + EGTA + Ca^{2+}.

H) Fluorescence intensity versus wavelength (nm) for CrCBL-Like1, CrCBL-Like1 + EGTA, CrCBL-Like1 + Ca^{2+}, CrCBL-Like1 + Ca^{2+} + EGTA.
|       | COLD | METHYL VILOGEN | H₂O₂ | HEAT |
|-------|------|----------------|------|------|
|       | Control | 0.5 | 1h | 2h | 0.5 | 1h | 2h | 0.5 | 1h | 2h |
| CrCBL-Like 1 | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| α-tub   | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |

**B**

|       | SALT | HEAT |
|-------|------|------|
|       | Control | 1h | 2h | 4h | 1h | 2h |
| CrCBL-Like 1 | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) | ![Image](image25.png) | ![Image](image26.png) |
| α-tub   | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) | ![Image](image31.png) | ![Image](image32.png) | ![Image](image33.png) | ![Image](image34.png) |

|       | 0.0 | 0.5 | 1.0 | 1.5 | 0.0 | 0.5 | 1.0 | 1.5 |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
|       | Control | 1h | 2h | 1h | 2h | 1h | 2h | 1h | 2h |