Ectopic expression of finger millet calmodulin confers drought and salinity tolerance in Arabidopsis thaliana

Gautam Jamra1,3 · Aparna Agarwal1 · Nidhi Singh3 · Sibaji K. Sanyal3 · Anil Kumar1,2 · Girdhar K. Pandey3

Received: 4 April 2021 / Accepted: 24 June 2021 / Published online: 11 July 2021
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract

Key message Overexpression of finger millet calmodulin imparts drought and salt tolerance in plants.

Abstract Drought and salinity are major environmental stresses which affect crop productivity and therefore are major hindrance in feeding growing population worldwide. Calcium (Ca2+) signaling plays a crucial role during the plant’s response to these stress stimuli. Calmodulin (CaM), a crucial Ca2+ sensor, is involved in transducing the signal downstream in various physiological, developmental and stress responses by modulating a plethora of target proteins. The role of CaM has been well established in the model plant Arabidopsis thaliana for regulating various developmental processes, stress signaling and ion transport. In the current study, we investigate the CaM of Eleusine coracana (common name finger millet, known especially for its drought tolerance and superior Ca2+ content). In-silico analysis showed that Eleusine CaM (EcCaM) has greater similarity to rice CaM as compared to Arabidopsis CaM due to the presence of highly conserved four EF-hand domains. To decipher the in-planta function of EcCaM, we have adopted the gain-of-function approach by generating the 35S::EcCaM over-expression transgenic in Arabidopsis. Overexpression of EcCaM in Arabidopsis makes the plant tolerant to polyethylene glycol (PEG) induced drought and salt stress (NaCl) as demonstrated by post-germination based phenotypic assay, ion leakage, MDA and proline estimation, ROS detection under stressed and normal conditions. Moreover, EcCaM overexpression leads to hypersensitivity toward exogenously applied ABA at the seed germination stage. These findings reveal that EcCaM mediates tolerance to drought and salinity stress. Also, our results indicate that EcCaM is involved in modulating ABA signaling. Summarizing our results, we report for the first time that EcCaM is involved in modulating plants response to stress and this information can be used for the generation of future-ready crops that can tolerate a wide range of abiotic stresses.

Keywords Abiotic stress · Calcium signaling · CaM · Drought response · Salinity response · Overexpression

Introduction

Every year, crops lose their growth and productivity due to various abiotic stresses, fluctuating temperature, a perturbation in soil water content, high salt salinity and others (Wang et al. 2013; Arunanondchai et al. 2018). Plants elicit a wide range of physiological and biochemical defense mechanism through the plethora of signaling pathways to adapt to these adverse environments (Pandey et al. 2016). Nearly all plant reactions to stress stimuli lead to alteration in cellular calcium (Ca2+) concentration, which is termed as Ca2+ signatures (Pandey, 2008; Pandey and Sanyal, 2021). These Ca2+ signatures are sensed by several Ca2+-binding proteins divided into—Ca2+ sensors and Ca2+ sensor—relay proteins (Hashimoto and Kudla 2011; Ranty et al. 2016). Binding with Ca2+ ion causes conformational changes in

Communicated by Aryadeep Roychoudhury.

* Anil Kumar
directoreducation.rlbcau@gmail.com

* Girdhar K. Pandey
gkpandey@south.du.ac.in

1 Department of Molecular Biology and Genetic Engineering, GBPUA&T, Pantnagar, Uttarakhand, India

2 Rani Lakshmi Bai Central Agriculture University, NH-75, Near Pahuj Dam, Gwalior Road, Jhansi 284003, Uttar Pradesh, India

3 Lab No. 302, Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Marg, South Campus, South Moti Bagh, Dhaula Kuan, New Delhi 110021, India
these sensor proteins in a \( \text{Ca}^{2+} \)-dependent manner which evokes downstream signaling cascades. The majority of plant \( \text{Ca}^{2+} \)-sensor proteins including calmodulins (CaMs), calmodulins-like proteins (CMLs), calcineurin B-like proteins (CBLs) and \( \text{Ca}^{2+} \)-dependent protein kinases (CDPKs) contain acidic EF-hand \( \text{Ca}^{2+} \) binding motifs (DeFalco et al. 2010; Mohanta et al. 2019; Sanyal et al. 2019).

CaM is a small, ubiquitous, highly conserved protein found in almost all eukaryotic organisms, whereas CMLs are present only in higher plants (Luan et al. 2002; Tuteja and Mahajan 2007). CaM contains four EF-hands with a high affinity to bind with four \( \text{Ca}^{2+} \) (Zielinski 1998), while CML protein has 1–6 EF-hands. CaM and CMLs serve as sensor relays, controlling the wide range of cellular pathways through influencing their target proteins by protein–protein interactions or change in gene expressions (Virdi et al. 2015; He et al. 2018). It is well established that various plant CaM and CML proteins were involved in physiochemical plant responses and induced by different type of stimuli and hormones (Townley and Knight 2002; Ali et al. 2003; Zeng et al. 2015; Gao et al. 2019). *Glycine max* GmCaM4 enhanced tolerance to high salinity conditions through interaction with Myb2 transcription factor, which is the regulator of salt responsive genes in soybean (Rao et al. 2014). In *Arabidopsis thaliana* (henceforth At), binding of CaM activates CaM-binding transcription factor CAMTA3, which decreases salicylic acid levels and provides disease resistance through negative regulation of EDS1 (Du et al. 2009). *ArCML8* and *ArCML9* alter the expression of many stress-responsive genes and knockout mutants of *AtCML9* provide salt tolerance to plant through ABA-mediating signaling (Magnan et al. 2008; Park et al. 2010). Moreover, *ArCaM5* (also known as *ArCML18*) interacts with the *AtNHX1* c-terminus in a \( \text{Ca}^{2+} \)- and pH-dependent manner. This interaction suggests the availability of \( \text{Ca}^{2+} \)-pH-dependent signaling encounters, which are involved in salt tolerance (Yamaguchi et al. 2005). Expression of *Solanum habrochaites* ShCML44 is highly upregulated under high salt, cold and drought stresses, and overexpression of *ShCML44* improved plant growth and tolerance to multiple abiotic stresses through regulation of many downstream genes (Munir et al. 2016).

In soybean and Arabidopsis, overexpression of CaM-binding transcription factor *GmCAMTA12* improved the growth of plants under manitol induced drought conditions (Noman et al. 2019). In grapevine, the expression of CML21 is positively regulated by heat, cold, high salinity and drought conditions (Aleynova et al. 2020). Abiotic stress-treated transcriptome data of *Brassica napus* revealed the altered expression of *BnCaMs* and *BnCMLs* genes (He et al. 2020). Overall, these examples of CaM and CMLs (and their interacting partner CAMTA) suggest their crucial role in \( \text{Ca}^{2+} \) signaling mediated modulation of plant growth and adaptation to abiotic stress.

Finger millet (*Eleusine coracana* L) is rich in minerals and nutrients, and this cereal crop is grown in semi-arid and subtropics region of the world (Fakrudin et al. 2004; Kumar et al. 2016a, b). It is an agronomically viable crop that can grow in a wide variety of conditions, including drought, salt, waterlogging while maintaining optimal yields, as it is one of the best germplasms for abiotic stress-tolerant genes (Dida et al. 2007; Ramakrishna et al. 2018). Therefore, the adaptability capacity of finger millet to survive under different abiotic stress conditions considered as an attractive crop for the identification of genes and pathways involved in adaptation against adverse environmental conditions (Sood et al. 2016). The United States national academies consider finger millet to be a possible “super cereal” (National Research Council 1996), as it has ten times more \( \text{Ca}^{2+} \) content than wheat, maize or brown rice (a fact that qualifies it as a good source for \( \text{Ca}^{2+} \) nutrient compared to other crops). It is also a good source of iron, zinc, fiber and essential amino acids (Vadivoo et al. 1998; McDonough et al. 2000; Gupta et al. 2017).

In an earlier study, a finger millet CaM (*EcCaM*) was reported to be involved in high grain \( \text{Ca}^{2+} \) accumulation in high \( \text{Ca}^{2+} \) containing genotypes (Kumar et al. 2014a, b). It was reported that *EcCaM* transcripts were expressed strongly in developing spikes (Singh et al. 2015) and the protein is localized more in embryo and aleurone layer of grains of high \( \text{Ca}^{2+} \) finger millet genotype, GP-45 (Kumar et al. 2014a, b). It was hypothesized that higher expression and accumulation of *EcCaM* played a role in the drought tolerance of the GP-45 genotype (Jamra et al. 2020). Therefore, in the present study, we elucidate the *in planta* functioning of this finger millet CaM, *EcCaM*. We have used the Arabidopsis heterologous system for gain-of-function and tested the candidate gene for its functional role in different stress conditions.

**Materials and methods**

**Plant and growth condition**

Finger millet GP-45 genotype was used for transcript profiling after abiotic stress treatment. For this, GP-45 seeds were surface-sterilized for 5 min. in 2% (v/v) bleach, rinsed and soaked in autoclave milliQ (MQ) water for 1 h and plated on \( \frac{1}{2} \) MS media followed by dark incubation for 3 days. Finger millet was grown at a temperature of 27 ± 1 °C with a relative humidity of 70% with photoperiod 80 μmol m\(^{-2}\) s\(^{-1}\) with a 16/8-h day/night cycle. For expression analysis, 12-day-old seedlings grown on \( \frac{1}{2} \) MS medium were transferred to 20% PEG and 200 mM NaCl and samples were harvested with 0, 3 and 6 h. Transgenic Arabidopsis plants, harboring the *EcCaM*, were grown on \( \frac{1}{2} \) MS medium containing 1% (w/v)
sucrose and 0.8% (w/v) agar in growth room maintained at 22 ± 2 °C and 60% relative humidity under a photoperiod of 16 h light (light intensity 100 μmol m−2 s−1) and 8 h darkness.

**In-silico characterization of EcCaM**

The nucleotide sequence of CaM EcCaM gene was fetched from de novo assembled transcriptome data of developing spikes of finger millet genotypes used in a previous study (Kumar et al. 2015; Singh et al. 2015). A protein blast of EcCaM protein sequence as a query sequence was performed by NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments of EcCaM protein sequence with their closely related protein sequences were analyzed through a tool of CLC genomic server (https://digitalights.qiagen.com) and the phylogenetic tree was constructed through MEGA7 (Larkin et al. 2007; Kumar et al. 2016a, b).

**RNA isolation and quantitative RT-PCR analysis**

Total RNA was isolated from tissue of PEG (finger millet 20% PEG and Arabidopsis 5% PEG) and NaCl (finger millet 200 mM and Arabidopsis 125 mM) for varying exposure time 0, 3 and 6 h of 12-day-old seedlings by Hot-Phenol methods according to (Sanyal et al. 2017). 1 μg of RNA was used to synthesis cDNA synthesis using Prime script© RT reagent Kit (TaKaRa, Japan). RT-qPCR was performed using Agilent AriaMx Real-Time PCR system using Agilent SYBR qPCR Master Mix Kit according to manufacturer’s instructions and finger millet EcCaM-RT and EcTUBULIN-RT and ArCaM-RT and ArACTIN-RT primers sets listed in Table 1. Relative expression was determined according to (Sanyal et al. 2017). For finger millet and Arabidopsis, Tubulin and Actin was used as an internal control, respectively.

**Generation of overexpression construct and EcCaM in Arabidopsis transgenic**

To generate EcCaM overexpressing plants, the coding region (ORF) of EcCaM was introduced in between NcoI and BstEII restriction sites of the plant transformation vector pCAMBIA1301 under the control of the CaMV35S promoter. The constructs were confirmed by sequencing and then transformed into Arabidopsis wild-type plants (Col-0) by the floral dip method (Clough and Bent 1998). T0 seeds harvested from these plants were screened on selection media (1/2 MS media containing 30-μg/ml hygromycin [HiMedia, India]) to obtain T1 plants. Subsequently, T1 seeds were plated on MS medium (containing 30-μg/ml hygromycin) for the confirmation of segregation ratios and then transferred to soil till maturity to generate T2 and T3 generation, which were screened as homozygous transformants and used for physiological analysis.

**Semi-quantitative PCR for validation of overexpression lines**

Total RNA was isolated from leaf tissues of Col-0 and overexpression lines using the protocol mentioned in (Sanyal et al. 2017). The total RNA was reverse transcribed into first-strand cDNA with Prime script© RT reagent Kit (TaKaRa, Japan). EcCaM Semi-quantitative PCR was performed in a thermocycler with profiling 95 for 4 min; 95 for 30 s; 58 for 30 s; 72 for 40 s; 72 for 7 min at 27 cycles using EcCaM

### Table 1 Primers used in experiments

| S. No | Genes     | Forward 5'----3'                           | Reverse 5'----3'                          |
|-------|-----------|-------------------------------------------|------------------------------------------|
| 1     | EcCaM-RT  | ATGATCAATGAGGGTGATGCTG                    | TCTCTCATGGTATCGCTCTCT                   |
| 2     | EcTUBULIN-RT | TAC TTT GTC GAG TGG ATC CC                   | GCG GAA CAT CTC CTG GAT G               |
| 3     | EcCaM-FL  | CATGCATGGATGCGCGACGATGTACC                  | GGTNACC TCACCTGGCCATCACC                      |
| 4     | ArCaM     | GGTTAGATGATGATGATGATGA                     | CACACAAAGATCACAACGAG                      |
| 5     | AtACTIN   | CTTGCACCACAGACATGAA                       | CACCCGATCCAGACATGCTT                      |
| 6     | ArACTIN2  | TGGACACAGATGAA                           | AACACCCGATCCAGACATGAA                     |
| 7     | ArRD29A-RT| GTGCCCAAGCGATTTGAC                      | CTGATGCTACGCTACCTGAC                     |
| 8     | AtKIN47-RT| CACACGCGTTGGTGTACAC                     | CACCCGATCCAGACATGCTT                      |
| 9     | ArAKIN1-RT| GGC AGC GGG AGG TGT TAA C                  | TGACCCGCTAGCTGGCTGT                      |
| 10    | ArRD22-RT | CATGATCCGAGGAGAA                      | CGGTGGGTAAGAAAGTTGGTC                    |
| 11    | AtCBL4-RT | GCTTCTGGCAAGCAGGACCG                    | GATAGGCAATCGGACTGTCT                      |
| 12    | AtCBLI0-RT| AGATCAAGCTCTCCTCCTG                     | CAATCGAAGGACTGGGACTCG                     |
| 13    | AtCIPK24-RT| ATAAAAAGTTTTGTAAGAATG               | GCAAACTTACCTGGAC                       |
| 14    | AtNHX-RT  | GGAGACCATTTGGATGACTC                    | CTTACTAAGATCGACAGGG                      |
| 15    | AtNCED3-RT| TACGCCGTTAGCTTAGAGG                    | ACCTGCTCGCAGAACCATC                      |
and AtACTIN RT-PCR forward and reverse primers listed in Table 1. RT-PCR product was visualized by electrophoresis on a 1.2% agarose gel.

Post-germination-based phenotypic assays under various abiotic conditions (PEG and NaCl)

For the root growth assay, surface-sterilized and cold-stratified of Col-0 and Arabidopsis transgenic seeds were germinated on ½ MS agar medium for 4 days, followed by transfer to ½ MS growth medium containing various PEG-6000 concentrations (0, 10, or 15%) and also subjected to various NaCl concentrations (0, 150 mM, or 175 mM). The plates were kept vertically to observed root elongation and salt-sensitive chlorosis phenotypes for daily observation. The root length was measured by ImageJ software.

Fresh weight and total chlorophyll content

To determined fresh weight, 5 seedlings were measured with three biological replicates. Total chlorophyll content was measured from seedlings harvested, weighted and extracted in DMSO (Barnes et al. 1992). The absorbance of supernatant was recorded at wavelength 664 nm and 647 nm and calculation was done using equation with formula: [chlorophyll a + chlorophyll b] = 17.90 × A647 + 8.08 × A664 (Arnon 1949).

MDA, proline and ion leakage quantification

MDA estimation was done by quantifying thiobarbituric acid reactive substances (TBARS) following the protocol by (Heath and Packer 1968). 100 mg of samples (12-day-old seedlings treated with PEG and NaCl) were homogenized in 500 μl of 0.1% TCA and centrifuged at 12,000 rpm at 4 °C for 10 min. Supernatant (500 μl) was mixed with 1.5 ml of 0.5% (w/v) TBA in 20% TCA (w/v) and incubated at 95 °C for 30 min. The reaction was stopped by keeping the tubes on ice followed by centrifugation for 5 min. at 12,000 rpm at 4 °C. The absorbance of the resultant supernatant was measured at 532 and 647 nm. OD600 values were subtracted from MDA-TBA complexes values at 532 nm and MDA calculation is calculated using the Lambert–Beer law with an extinction coefficient εM = 155 mM−1 cm−1. Values presented as μMMDA g−1 FW.

Proline estimation was done using (Bates et al. 1973). To quantify proline content, 100 mg (12-day-old seedlings treated with PEG and NaCl) were extracted in 2.0 ml of 3% sulphosalicylic acid and centrifuged at 12,000 rpm for 10 min at 4 °C. 100 μl supernatant was reacted with 100 μl 3% sulphosalicylic acid by subsequently added 200 μl glacial acetic acid and 200 μl acid Ninhydrin mixtures boiling at 100 °C for 1 h. The reaction was stopped by keeping it on ice for 30 min and 1.2 ml toluene was added to the reaction mixture vortexed and centrifuged. The absorbance of the chromophore was taken at 520 nm using toluene as a blank. Proline concentration was determined by plotted standard curve and values expressed in μM g−1 FW.

Electrolyte leakage was determined following the method by (Murray et al. 1989). 12-day-old seedlings were treated with PEG and NaCl. After 12 h ion leakage (IL initial) was measured using a conductivity meter. The samples were then heated in a boiling water bath for 1 h and complete ion leakage (IL final) of the solution was measured. Relative ion leakage was calculated by the following formula: IL initial/IL final × 100.

NBT and DAB staining for ROS detection

3,3′-Diaminobenzidine (DAB) and nitrotetrazolium blue chloride (NBT) staining was performed using (Kumar et al. 2014b). Arabidopsis seedlings of wild-type Col-0 and EcCaM overexpression lines were grown on ½ MS plates for 15 days at 22 °C under long-day conditions (16 h-light/8 h-dark cycle) with 200 μ E-m−2 s−1 and 75% humidity. 15-day-old seedlings were treated with a 10% PEG (drought) and 150 mM NaCl for 6 h. The untreated seedlings that were grown under the same conditions were served as the experimental control. Following stress treatment in assay plates, the seedlings were washed with distilled water. These seedlings were then immersed in DAB or NBT staining solution for detection of H2O2 or O2−, respectively. After a staining/de-staining protocol, photographs were documented.

Germination-based phenotypic assays under ABA

For the ABA seed germination sensitivity assay, Col-0 and Arabidopsis transgenic seeds were germinated on ½ MS agar medium for 7 days, followed by transfer to ½ MS growth medium-containing various ABA concentrations (0, 0.5, 0.75 or 1 μM) (Pandey et al. 2004). The plates were kept vertically to observed sensitive phenotype.

Expression profiling of stress marker genes for drought and salinity tolerance

For expression analysis, 12-day-old seedlings of Col-0 and transgenic lines treated with 5% PEG and 125-mM NaCl and harvested at 0-, 3- and 6-h time interval. Stress-responsive genes, SOS pathway and ABA biosynthesis genes were analyzed by qRT-PCR. AtACTIN2 was used as an internal control. RT-qPCR was performed using Agilent AriaMx Real-Time PCR system using Agilent SYBR qPCR Master Mix Kit according to manufacturer’s instructions and gene-specific primers listed (Table 1) in the table to analyze the expression pattern of genes responsible for drought and salt.
stress. Relative expression levels of genes were normalized with *AtACTIN2* and calculated according to (Sanyal et al. 2017).

**Statistical analysis**

Statistical analysis was performed by one-way ANOVA with three triplicates and each of which contain three plants using. The mean comparison was analyzed by Tukey’s multiple compression tests.

**Results**

*In-silico* analysis of EcCaM indicates conservation of important domains in the protein and its relatedness to rice CaM

We identified a 450 bp long EcCaM gene from the finger millet transcriptome using the rice CaM1 gene (Genebank Accession no. XM_015766855.2) as a reference sequence (Table 2). Transcriptome sequencing has revealed the presence of four copies of CaM genes in *Eleusine coracana*, EcCaM1, EcCaM11, EcCaM14 and EcCaM17 that were validated by Southern hybridization (Kumar et al. 2014a, b). Our particular sequence was identical to previously identified EcCaM1 (Kumar et al. 2014a, b). Using *In-silico* analysis, we identified that the 149 amino acid residue containing EcCaM shows a high probability of having four conserved Ca²⁺-binding EF-hands. In Fig. 1a, the multiple alignment of EcCaM with six other CaM protein sequences revealed that the EcCaM shared 100% sequence identity with all cereals CaM protein sequences, except AtCaM1, with which it showed 88% similarity. The 1st–24th amino acids of AtCaM1 differed from the EcCaM and other CaMs, and as a result, the first EF-hand indicated gaps in our analysis. Based on the similarity of sequences, we posit that the EcCaM protein would have similar properties like the compared monocot CaMs, especially OsCaM. Our hypothesis is supported by the fact that in earlier analysis, EcCaM was similar to CaMs from the Poaceae family (Kumar et al. 2014a, b). Some other amino acids substitutions were also found in AtCaM1 at the 75th (Arginine to Lysine), 123rd (Aspartate to glutamate) and 145th (Valine to Isoleucine) positions—but all these falls outside the predicted EF-hands. The phylogenetic tree analysis of EcCaM with the following CaM sequences also placed the EcCaM in the same group of cereals but distinct to the AtCaM1 (Fig. 1b). The distribution of conserved motifs among CaMs also suggested that CaM is conserved among cereals.

CaM is differentially expressed under PEG and salt treatment in *Eleusine coracana* and Arabidopsis

Our previous studies have established that (1) GP-45 is a drought-tolerant genotype (Jamra et al. 2020) and (2) CaM genes are highly expressed in GP-45 (Kumar et al. 2014a, b). Therefore, we wondered if the EcCaM, we were investigating in this study, had a role in drought stress. Therefore, we performed qRT-PCR to analyze the expression profile of EcCaM under 20% PEG-induced drought stress condition we had previously used (Jamra et al. 2020) and salinity stress (200 mM NaCl) with the time frame of 3 and 6 h after treatment. EcCaM transcript was increased within 3 h after exposure of the seedlings to PEG medium and reached a maximum to 6 h (Fig. 2a), while exposure to NaCl medium EcCaM transcripts were initially elevated (within 3 h), and then, we observed a decline in the expression (6 h) (Fig. 2a). This finding suggested that EcCaM transcripts were induced by both osmotic and salinity stress with different time intervals. One of the important questions raised here is how the distant relative of EcCaM, i.e., AtCaM1 performed under similar stimuli. To address this question, we performed qRT-PCR in Arabidopsis with slight modification in the stimuli (5% PEG and 125 mM NaCl) and similar time intervals (3 and 6 h) (Fig. 2b). AtCaM1 transcript showed a similar elevation profile under PEG-mediated perturbation in expression. However, under NaCl stress, the AtCaM1 transcript showed an enhanced expression profile even at 6 h.

| Table 2  | CaM sequences from different plant species used in multiple sequence alignment study |
|----------|-----------------------------------------------------------------------------------|
| Gene name          | Species                  | Genbank accession numbers |
| EcCaM: calmodulin | *Eleusine coracana*         | ACX56274.1                 |
| OsCaM1: calmodulin-1 | *Oryza sativa Japonica* | XP_015622341.1             |
| OsCaM: calmodulin  | *Oryza sativa Japonica*     | XP_015631102.1             |
| ZmCaM: calmodulin1 | *Zea mays*                | NP_001281081.1             |
| SiCaM: calmodulin  | *Setaria italica*          | XP_004984568.1             |
| ShCaM: calmodulin  | *Sorghum bicolor*          | XP_002467948.1             |
| AtCaM1: calmodulin1 | *Arabidopsis thaliana*  | NP_001330399.1             |
Overexpression of EcCaM confers drought tolerance in Arabidopsis linking it to drought stress response

As our expression analysis indicated that EcCaM can be perturbed during drought stress, we asked if this change in expression could be linked to plants physiological response. Since transformation of finger millet is a cumbersome process, we took the heterologous expression approach to investigate our hypothesis. Therefore, we generated Arabidopsis transgenic lines with overexpression of EcCaM under the control of 35S CaMV constitutive promoter. Semi-quantitative PCR/qRT-PCR was performed to determine the expression levels of EcCaM, and all three transgenic lines showed expression of EcCaM transcripts compared with WT-Col-0 (Fig. 3b), and these lines were selected for further phenotypic analysis. To elucidate the role of EcCaM under drought condition post-germination-based phenotypic assay was performed on ½ MS supplemented with different PEG-6000 concentrations (0, 10 and 15%). The increase in the PEG-6000 drastically affected the growth of WT-Col-0 seedlings.

Fig. 1 Sequence alignment, phylogenetic tree and motif analysis of EcCaM protein sequence. A Multiple sequence alignment of EcCaM protein with related plant CaM indicating conservation among sequences done by CLC genomic sequence viewer. Black boxes indicating presence of characteristic 4 EF-hands in all proteins. B Phylogenetic tree based on the CaM protein sequences created by MEGA7
The transgenic EcCaM overexpression lines in comparison grew noticeably better than WT-Col-0 (Fig. 3a). Moreover, while compared with WT-Col-0, EcCaM overexpression lines exhibited significantly better fresh weight; longer root length and better root morphology and more chlorophyll levels under PEG-6000-induced drought stress (Fig. 3c–e). However, we did observe difference in root length among the transgenic lines after subjecting to stress. We believe that this may be due to difference in expression of EcCaM in the transgenic lines. This finding reveals that all three transgenic lines exhibited more tolerance toward PEG-induced drought stress than WT-Col-0.

**Overexpression of EcCaM also confers salinity tolerance in Arabidopsis indicating it could be node for communicating abiotic stress stimuli**

As we have observed high expression of EcCaM even under salt stress, we also investigated the phenotype of transgenic lines under salt stress. Similar to our analysis performed for PEG, we performed a post-germination-based phenotype assay on different ½ MS supplemented with various NaCl concentrations (0, 150, 175 mM). Similar to our observed tolerance under PEG treatment, all three overexpression lines exhibited remarkably enhanced tolerance to salinity stress compared with WT-Col-0 (Fig. 4a). We did observe some photo-bleaching (chlorosis) and stunted growth of seedling at 175 mM NaCl for all the tested lines. As expected, the EcCaM overexpression lines displayed significant fresh weight, root growth and chlorophyll content compared to WT-Col-0 under NaCl-induced salinity stress (Fig. 4b, c). This indicates that overexpression of EcCaM can also account for enhanced salinity tolerance in transgenic lines.

**Overexpression of EcCaM affected membrane damage, proline accumulation and ion leakage**

To investigate the potential physiological mechanism for better drought and salinity tolerance of EcCaM overexpression lines, we estimated the MDA and proline content in the WT-Col-0 and EcCaM overexpression seedlings under normal, PEG and NaCl-mediated stress conditions. We also measured ion leakage in all the genotypes under similar condition. Under normal growth conditions, the MDA, proline and ion leakage levels of EcCaM overexpression lines and Col-0 were similar. MDA levels were significantly reduced and proline was significantly higher in transgenic lines compared to WT-Col-0 under both PEG- and NaCl-mediated stresses (Fig. 5a, b). We also observed that less ion leakage was exhibited by transgenic lines in comparison to WT-Col-0 under both PEG and NaCl stress conditions (Fig. 5c). These parameters further indicate that EcCaM can regulate these physiological parameters to enhance the plant’s defense response against abiotic stress.

**Reduced accumulation of reactive oxygen species in overexpression lines under drought and salinity stress conditions**

Reactive oxygen species (ROS) also plays a crucial role during plants exposure to stress (abiotic or biotic), both as
a signaling molecule and molecular effector (Baxter et al. 2014). Out of many ROS species, $O_2^\cdot$ and $H_2O_2$ are some of the main players that contribute to the cellular ROS pool during drought and salt stress condition. No significant difference was observed between WT-Col-0 and overexpression lines under normal condition in the detection of $O_2^\cdot$ by NBT and $H_2O_2$ by DAB staining, respectively. Compared to WT-Col-0, overexpression lines showed significantly
CBL4/SOS3]], salinity [majorly salt overly sensitive (SOS) path-
stresses were observed (Fig. 6b). These results suggested
under osmotic and salinity
Col-0
lines as compared to WT-
stresses (Fig. 6a). Similarly, weaker DAB staining and,
P
by one-way ANOVA
sion lines (* \(P < 0.01\); *** \(P < 0.05\); ** \(P < 0.001\)) by one-way ANOVA

weaker NBT staining, and hence, it can be inferred that
they have less \(O_2^-\) level under both osmotic and salinity
stresses (Fig. 6a). Similarly, weaker DAB staining and,
consequently, a lesser amount of \(H_2O_2\) in overexpression
lines as compared to WT-Col-0 under osmotic and salinity
stresses were observed (Fig. 6b). These results suggested
that EcCaM overexpression shows a reduction in oxidative
stress level and confers enhanced tolerance to drought and
salinity stress.

**EcCaM probably communicate the abiotic stress stimuli through the ABA signaling pathway**

Since drought perception by plants is routed through the
ABA signaling pathway, we wanted to assess the response of
EcCaM transgenic lines to ABA. Therefore, we subjected the
seeds from EcCaM transgenic lines and WT-Col-0 to growth
on \(\frac{1}{2}\) MS supplemented with different ABA concentration
(0, 0.5, 0.75, or 1 \(\mu\)M). In the absence of ABA, both EcCaM
overexpression lines and WT-Col-0 had similar germination
profile. However, the hypersensitivity was observed in
the presence of ABA and this hypersensitivity was more
prominent on increasing ABA concentration. Green cotyle-
don and better root morphology were apparent in WT-Col-0
as compared to EcCaM overexpression seedlings on higher
ABA concentration (Fig. 7a, b). These results indicated that
at least during germination stage, EcCaM overexpression
seeds were more sensitive to ABA than WT-Col-0 seeds.

**Overexpression of EcCaM in Arabidopsis affects transcript levels of abiotic stress-responsive, SOS pathway and ABA biosynthesis genes**

Our results indicated that the EcCaM may also modulate
other important players involved in the regulation of plants
response to stress stimulus. Therefore, we examined the
transcript profile of different stress-related and ABA bio-
synthesis marker genes for drought (RD29A, RD22, COR47,
KIN), salinity [majorly salt overly sensitive (SOS) path-
way genes-CBL4/SOS3, CIPK24/SOS2, NHX1/SOS1 and
CBL10] and ABA (NCED3) responses (Qiu et al. 2002;
Pandey et al. 2004; Kim et al. 2007). After exposure to
drought stress, RD29A transcripts reached a maximum at
3 h further declined at 6 h. However, RD29A transcripts
were expressed more in the EcCaM overexpression line as
compared to WT-Col-0 at 3 h (Fig. 8a). Contrastingly, a sig-
nificant difference was observed in the transcripts of COR47,
RD22 and KIN between EcCaM overexpression line and
WT-Col-0 (Fig. 8b–d). For these transcripts, we observed
lesser induction of transcripts in the EcCaM overexpression
line compared to WT-Col-0. We also analyzed the NCED3
transcript accumulation in WT-Col-0 and EcCaM overex-
pression lines under drought stress. When we subjected the
plants to drought stress, we observed that NCED3 transcripts
were significantly higher in the overexpression line as com-
pared to WT-Col-0 (Fig. 8e).

Next, we analyzed the SOS pathway genes to monitor
their perturbation during salt stress. In general, after salt
stress, the transcripts of CBL4/SOS3 enhanced, but we did
not observe any significant difference in CBL4/SOS3 tran-
script levels between EcCaM overexpression line and WT-
Col-0 (Fig. 9a). In contrast, the transcript levels of CBL10
were significantly enhanced in the EcCaM overexpression
line as compared to WT-Col-0 even in the control condi-
tion. Under salt stress, although the transcripts of CBL10
decreased, yet we could observe EcCaM overexpression
maintained comparatively higher transcripts of CBL10 com-
pared to WT-Col-0 (Fig. 8b). The transcripts of CIPK24/
SOS2 did not significantly change in the WT-Col-0 for
the duration of our treatment. In the EcCaM overexpression
line, overall, a lower transcript level for CIPK24/SOS2 was
observed (compared to WT-Col-0), and a subtle expression
perturbation on the higher side was observed after salt stress
(but yet lower than WT-Col-0) (Fig. 9c). Transcripts of
NHX1/SOS1 were perturbed on the higher side for both WT-
Col-0 and EcCaM overexpression line after salt stress, but
the EcCaM overexpression line maintained an overall higher
level of NHX1/SOS1 after salt stress (Fig. 9d). Finally, we
analyzed the transcript profile of NCED3 in WT-Col-0 and
EcCaM overexpression line after salt stress. There was sig-
nificant NCED3 transcript accumulation under salinity stress
between the overexpression lines and WT-Col-0 (Fig. 9e).

**Discussion**

Plants are equipped with four major gene families of Ca\(^{2+}\)
sensor proteins (besides these four gene families more of
these Ca\(^{2+}\) sensor are being discovered), and the CaM family
of Ca\(^{2+}\) sensors are very well studied till date (Pandey and
Sanyal 2021). Drought and high salinity are the major envi-
ronmental cues frequently experienced by plants and both
impose osmotic stress on plant cells. Osmotic stress induces
adverse responses at molecular and cellular levels, and a primary event as increase in the cytosolic Ca\(^{2+}\) concentration, and subsequent transduction of Ca\(^{2+}\) signals that promote appropriate cellular responses in efforts to mitigate potential damages (Xiong and Zhu 2002). Major works on CaMs have been reported from the model plant Arabidopsis, and we only have a few studies that functionally characterizing these proteins from cereals (Magnan et al. 2008; Vadassery et al. 2012). For instance, earlier reports had indicated that CaM from *Glycine max* (Park et al. 2004), *Oryza sativa* (Saeng-ngam et al. 2012), *Vigna radiata* (Botella and Arteca 1994) and *Hordeum vulgare* (Shen et al. 2020) are involved in plants salt stress response. Our present effort is on the *in-planta* characterization of CaM from *Eleusine coracana*, also known as finger millet, a crop that holds promise for the future. To the best of our knowledge, this is the first effort to evaluate the functional aspects of the finger millet EcCaM gene for its role in drought and salinity stresses.

In the present study, we identified EcCaM in finger millet using sequence-based search from in-house database

**Fig. 4** Overexpression of EcCaM in Arabidopsis enhanced salt tolerance. A 4-day-old seedling grown on 1/2 MS transferred to 1/2 MS containing various concentration of NaCl (0, 150 and 175 mM). B Quantification of fresh weight of 4-day-old seedlings exposed to various NaCl concentration and grown vertically for 6 days. C Quantification of chlorophyll content of 4-day-old seedlings exposed to various NaCl concentration and grown vertically for 6 days. The data were presented as the mean ± SD of three independent experiments. Asterisks above each column indicate statistical difference between WT-Col-0 and overexpression lines (*P* < 0.05; **P** < 0.01; ***P** < 0.001) by one-way ANOVA.
recently developed through a high-throughput transcriptome project. This particular sequence was similar to the one reported by Kumar and colleagues, who had used RACE-PCR to amplify this gene (Kumar et al. 2014a, b). We have further through our analysis shown that important EF-hand domains are present in EcCaM and the protein is similar (based on amino acid sequence) to *Oryza sativa*, *Zea mays*, *Setaria italica*, *Sorghum bicolor* CaMs, and stipulates conserved behavior of CaM proteins in plants especially among monocots. Furthermore, as reported by Kumar and colleagues, our analysis also shows that EcCaM is closer to *O. sativa* CaM1 (in terms of protein sequence). This result affirms the conserved co-linearity of the finger millet and rice genomes, which were also revealed by the genome-wide comparative, study (Srinivasachary et al. 2007).

As stated earlier, the high drought tolerance of GP-45 and higher expression of EcCaM was the basis of our hypothesis to check the expression of EcCaM under drought and salt stress. Our results on EcCaM and AtCaM indicate that their expression profile is conserved even in distantly related species. As the EcCaM transcript in finger millet seedling was highly induced by drought and salinity stress, it suggested

---

**Fig. 5** MDA content, proline content and ion leakage under drought (10% PEG) and salinity (150 mM) of EcCaM overexpression lines and Col-0. **A** MDA content comparison between Col-0 and transgenic lines after PEG and NaCl treatment. **B** Proline content comparison between Col-0 and transgenic lines after PEG and NaCl treatment. **C** Ion leakage comparison between Col-0 and transgenic lines after PEG and NaCl treatment. The data were presented as the mean ± SD of three independent experiments. Asterisks above each column indicate statistical difference between overexpression lines and Col-0 (*P* < 0.05; **P** < 0.01; ***P*** < 0.001) by one-way ANOVA.
Fig. 6 ROS detection under PEG and NaCl stress conditions. A Superoxide (O$_2^-$) accumulation detected by NBT staining. B Peroxide (H$_2$O$_2$) accumulation detected by DAB staining.

Fig. 7 Overexpression of EcCaM in Arabidopsis increased ABA sensitivity. A EcCaM overexpression and WT-Col-0 seeds were germinated on $\frac{1}{2}$ MS medium supplemented various ABA concentration (0, 0.5, 0.75 and 1 µM). B Cotyledon emergence % after ABA treatment.
Fig. 8 Relative expression profiling of stress-responsive genes under drought stress. A RD29A, B COR47, C KIN1, D RD22, E NCED3. The data were presented as the mean ± SD of three independent experiments. Asterisks above each column indicate statistical difference between Col-0 and overexpression lines (*P < 0.05; **P < 0.01; ***P < 0.001) by one-way ANOVA.
that this gene is probably involved in these stimuli. Similarly, prior findings have reported that AtCAMTA was involved in drought stress response (Pandey et al. 2013) and AtCML9 is induced by salt, cold and ABA stress (Magnan et al. 2008). Recently, one more report conferred the role of MdCaM and MdCMLs in apples under salt stress (Li et al. 2019). The EcCaM-overexpressing seedlings displayed a more tolerant phenotype under drought and salinity stress as compared to WT-Col-0. Under PEG-mediated drought stress and NaCl-mediated salinity, stress overexpression lines exhibited better fresh weight, chlorophyll content and root adaptability which suggests that overexpression of EcCaM might help

Fig. 9 Relative expression profiling of stress-responsive genes under salt stress. A CBL4, B CBL10, C CIPK24, D NHX1, E NCED3. The data were presented as the mean ± SD of three independent experiments. Asterisks above each column indicate statistical difference between Col-0 and overexpression lines (*P < 0.05; **P < 0.01; ***P < 0.001) by one-way ANOVA.
in the maintenance of growth through the improvement of root development. Moreover, overexpression of EcCaM in Arabidopsis could also defend against stress-induced oxidative damage and damage to the photosynthetic system under stress. Thus our findings were similar to the previous reports that overexpression of CaM/CML enhanced tolerance to abiotic tolerance in Arabidopsis (Larkindale and Knight 2002; Xu et al. 2011), tobacco (Li and Gong 2009; Zeng et al. 2015), M. truncatula (Wang et al. 2013). We further explored the physiological mechanisms by which the overexpressing lines show tolerance to drought and salt stress than WT-Col-0. Our findings also revealed that overexpression lines accumulated more proline, less MDA content and less ion leakage than WT-Col-0 under drought and salt treatment conditions. The higher accumulated proline might be allowing the overexpression lines for better effective osmoregulation, thus conferring them the observed tolerant phenotype to drought stress by minimizing the water loss and maximizing better water uptake. Proline and other solutes function in lowering cellular osmotic potential and restoring intracellular solute concentration which prevents water loss from cells (Farooq et al. 2009; Zhang et al. 2011). In agreement with prior reports that in MtCaM1 (Wang et al. 2013) overexpressing lines significant higher level of proline than that in WT-Col-0 under abiotic stress, which contributed to improved tolerance to drought, salt and cold stress. Overexpressions of CBL interacting protein kinases (CIPK), TaCIPK23 (Cui et al. 2018) and OsCIPK03 and OsCIPK12 (Zhang et al. 2011) have also shown similar effects. Several effector molecules/proteins are involved in processes like ROS scavenging and maintenance of ion homeostasis. MDA, 

$\text{H}_2\text{O}_2$ and ion leakage are also considered an indicator of oxidative damage and membrane injury (Hasegawa et al. 2000). Following prior reports, our findings also indicate that the EcCaM overexpression lines contain lesser MDA and show lesser ion leakage as reported for StCaM2 (Raina et al. 2021) and another Ca$^{2+}$ sensor MsCBL4 (An et al. 2020). We posit that overexpression of EcCaM leads to elevated proline associated osmotic potential balance under stress and lesser membrane damage and ion leakage, thereby resulting in the observed tolerant phenotype.

EcCaM overexpression lines also exhibited lower ROS accumulation as compared to WT-Col-0 under drought and salt stress. This suggested that EcCaM overexpression enhances the antioxidant enzyme activity which enhances the ROS scavenging activity and as a result lowers the accumulation of ROS. A similar finding has been demonstrated for StCaM2, and it was reported that StCaM2 modulated the levels of antioxidant enzymes and reduced the accumulation of ROS under drought and salt stress (Raina et al. 2021). ABA plays critical roles in regulating root growth, seed germination, stomatal movement and stress responses (Bu et al. 2009; Seo et al. 2014). In our study, the detailed phenotypic analysis revealed that EcCaM overexpression lines were hypersensitive to ABA with delayed seed germination after exogenous ABA treatment. An earlier report on ectopic expression of OsMSR and CPK32 had also indicated ABA-hypersensitive phenotypes during seed germination assay (Xu et al. 2011; Choi et al. 2000). We are currently unable to explain this phenotype and this requires further molecular investigation in near future.

The expression of RD29A was strongly induced under drought conditions in EcCaM overexpressing lines, while the lesser transcript of RD22, COR47 and KIN1 was observed when compared to WT-Col-0. Accordance with one of the prior report supported ours findings that overexpression of AtCPK6 does not significantly affects RD22 and COR47 expression under drought stress (Xu et al. 2010). For the latter three, it was expected as the transgenic lines with better stress adaptability (due to better performance of other physiological parameters) may not induce other stress pathway genes. For RD29A, we know that high drought induces its expression and posit that its upregulation in overexpression lines confer further tolerance toward drought stress (Yamaguchi-Shinozaki and Shinozaki 1994; Cheong et al. 2003; Pandey et al. 2004). Transcript of NCED3 was higher in EcCaM overexpressing lines as compared to WT-Col-0 under drought stress. Prior reports speculated that higher NCED3 expression in overexpression of MycFOF2ox Arabidopsis plants than wild type involved in ABA catabolism and responses to drought stress (Qu et al. 2020). SOS pathway is one of the most in-depth studied Ca$^{2+}$ signaling networks under salt stress (Luan 2009; Qi et al. 2002). CBL4/SOS3 transcript was marginally higher in WT-Col-0 (compared to transgenic lines) and CIPK2/SOS2 transcripts were not significantly perturbed, probably indicating that EcCaM did not crosstalk with the SOS pathway. However, the transcripts of AtNHX1 were enhanced significantly in EcCaM-overexpressing lines as compared to WT-Col-0 under salt stress. To the best of our knowledge, there is no report indicating the crosstalk of CaM and SOS pathway (SOS are modulated majorly by the CBL-CIPK module) (Sanyal et al. 2020). However, there is one report, indicating that AtCML18 was involved in salt signal transduction through interaction with AtNHX1 transporter (Yamaguchi et al. 2005). Therefore, there might be a possibility that overexpression of EcCaM target SOS1 directly or indirectly as per our experiments showing enhanced expression of SOS1. Interestingly, CBL10 expression was higher in EcCaM overexpression lines. CBL10 and SOS2/CIPK24 in Arabidopsis can also regulate vacuolar salt sequestration (Sanyal et al. 2015). We propose that the EcCaM might regulate some sodium transporters such as SOS1. EcCaM overexpression harbors higher NCED3 accumulation as compared to WT-Col-0 when exposed
to salinity stress. For salt stress, *EcCaM* works in ABA-dependent pathway to provide tolerance to the plants. *EcCaM* overexpression might be an important regulatory player for the regulation of ABA biosynthesis and signaling under drought and salt stress; however, our phenotype in ABA indicates that this simple explanation will not suffice to fully explain the phenomenon and requires further investigation.

Therefore, based on our findings, we proposed a hypothetical model which exhibits the functional role of *EcCaM* during drought and salinity stresses (Fig. 10). Previous studies suggested that finger millet is rich in Ca$^{2+}$ and harboring higher expression of CaM. Our hypothesis is that during abiotic stress conditions increase in cytosolic Ca$^{2+}$ occur which leads to disturbance of cytosolic ionic levels. Increase in cytosolic Ca$^{2+}$ sensed by *EcCaM* and activate Ca$^{2+}$-CaM signal transduction, which leads to activate and regulates DREB pathways genes, SOS pathways genes, osmo-protectant accumulation, ABA biosynthesis genes and also ROS homeostasis. All these activated pathways and stress-responsive genes lead to develop drought and salinity stress tolerance.

### Conclusion

Our findings have revealed that *EcCaM* play an important role in drought and salinity stresses responses. Furthermore, we have shown *EcCaM* transgenic lines have lesser ROS accumulation, better metabolite balance and stress-responsive and ABA biosynthesis gene profile under drought and salt stresses. In nutshell, *EcCaM*, a novel CaM protein identified from finger millet holds the potential for its use in the biotechnological improvement of crops for developing stress tolerance trait. Exploring the detailed protein function of *EcCaM* and further research to clarify the molecular mechanism that resulted in the stress-tolerant phenotype will improve our understanding of the Ca$^{2+}$ signaling component’s regulatory mechanism in crop plants.

### Acknowledgements

We acknowledge Department of Plant Molecular Biology, University of Delhi South Campus and G. B. Pant Agriculture and Technology University for providing facilities to conduct this research work. This study was supported by Department of Biotechnology (Project code 7069) to AK. Research work in GKP’s lab is supported by Delhi University (IoE/FRP grant), Board of Research in Nuclear Sciences (BRNS), Department of Biotechnology (DBT), Science and Engineering Research Board (SERB), Council for Scientific and Industrial Research (CSIR), India. GJ, AA and SKS acknowledges DBT fellowship and NS is thankful to UGC, India for D. S. Kothari postdoctoral fellowship.
Author contribution statement GKP and AK conceived and planned the research. GJ, AA and NS conducted experiments. GJ, AA, NS, SKS, AK and GKP analyzed the data. GJ and GKP wrote and revised the manuscript.

Declarations

Conflict of interest Authors declares no conflict of interest.

References

Aleyanova OA, Kiselev KV, Ogneva ZV, Dubrovina AS (2020) The grapevine calmodulin-like protein gene CML21 Is regulated by alternative splicing and involved in abiotic stress response. Int J Mol Sci 21:7939

Ali GS, Reddy VS, Lindgren PB et al (2003) Differential expression of genes encoding calmodulin-bindingproteins in response to bacte-

rrial pathogens and inducers of defense responses. Plant Mol Biol 51:803–815

An Y, Yang XX, Zhang L et al (2020) Alfalfa MsCBL4 enhances calcium metabolism but not sodium transport in transgenic tobacco under salt and saline-alkali stress. Plant Cell Rep 39:997–1011

Arunanondchai P, Fei C, Fisher A et al (2018) How does climate change affect agriculture? The Routledge handbook of agricul-
tural economics. Routledge, London, pp 191–210

Arnon DI (1949) Copper enzymes in isolated chloroplasts- Polype-

noxidase in Beta vulgaris. Plant Physiol 24:1–15

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free

proline for water-stress studies. Plant Soil 39:205–207

Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling. J Exp Bot 65:1229–1240

Botella JR, Arteca RN (1994) Differential expression of two calmodu-

lin genes in response to physical and chemical stimuli. Plant Mol Biol 24:757–766

Bu Q, Li H, Zhao Q et al (2009) The Arabidopsis RING finger E3 ligase RHA2a is a novel positive regulator of abscisic acid signal-

ing during seed germination and early seedling development. Plant Physiol 150:463–481

Cheong YH, Kim KN, Pandey GK (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis. Plant Cell 15:1833–1845

Choi H, Hong J, Ha J et al (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275:1723–1730

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agro-
bacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743

Cui XY, Du YT, Fu JD et al (2018) Wheat CBL-interacting pro-
tein kinase 23 positively regulates drought stress and ABA

responses. BMC Plant Biol 18:93. https://doi.org/10.1186/s12870-018-1306-5

DeFalco TA, Bender KW, Snedden WA (2010) Breaking the code: Ca++
sensors in plant signalling. Biochem J 425:27–40

Dida MM, Ramakrishnan SS, Benetzen JL et al (2007) The genetic

expression of finger millet, Eleusine coracana. Theor Appl Genet 114:321–332

Du LQ, Ali GS, Simons KA et al (2009) Ca++/calmodulin regulates salicylic-acid-mediated plant immunity. Nat 457:1154-U1116

Fakrudin B, Kulkani RS, Shashidhar HE, Hittalman S (2004) Genetic diversity assessment of finger millet, Eleusine coracana (Gaertn.), germplasm through RAPD analysis. Biodivers Int Newsl 138:50–54

Farooq M, Wahid A, Lee DJ et al (2009) Advances in drought resistance of Rice. Crit Rev Plant Sci 28:199–217

Gao QY, Xiong TT, Li XP et al (2019) Calcium and calcium sensors in fruit development and ripening. SciHortic 253:412–421

Gupta SM, Arora S, Mirza N et al (2017) Fingerprint Miller: a “certain” crop for an “uncertain” future and a solution to food insecurity and hidden hunger under stressful environments. Front Plant Sci 8:643

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499

Hashimoto K, Kudla J (2011) Calcium decoding mechanism in plants. Biochimie 93:2054–2059

He M, He HCQ, Ding NZ (2018) Abiotic stresses: General defenses of land plants and chances for engineering multistress tolerance. Front Plant Sci 9:1771

He X, Liu W, Li W et al (2020) Genome-wide identification and expression analysis of CaM/CML genes in Brassica napus under abiotic stress. J Plant Physiol 255:153251

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198. https://doi.org/10.1016/S0003-9865(02)00037-7

Jamra G, Shah P, Agarwal A et al (2020) Elucidating the physio-
morphological and biochemical responses towards PEG-induced drought stress in finger millet genotypes. Int J Curr Microbiol Adv Sci 9:1672–1687

Kim BG, Waadt R, Cheong YH et al (2007) The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabi-
dopsis. Plant J 52:473–484

Kumar A, Mirza N, Charan T et al (2014a) Isolation, characterization and immunolocalization of a seed dominant CaM from Finger Millet (Eleusine coracana L. Gartin.) for studying its functional role in differential accumulation of calcium in developing grains. Appl Biochem Biotechnol 172:2955–2973

Kumar D, Yusuf MA, Singh P et al (2014b) Histochemical detection of superoxide and H2O2 accumulation in Brassica juncea seedlings. Bio-Protoc 4(8):1108

Kumar A, Gaur VS, Goel A, Gupta AK (2015) De novo assembly and characterization of developing spikes transcriptome of finger mil-
let (Eleusine coracana): a minor crop having nutraceutical proper-
ties. Plant Mol Biol Rep 33:905–922

Kumar A, Metwal M, Kaur S et al (2016a) Nutraceutical value of finger millet [Eleusine coracana (L.) Gaertn.], and their improvement using omics approaches. Front Plant Sci 7:934

Kumar S, Stecher G, Tamura K (2016b) MEGA7: molecular evolution-
ary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874

Larkin MA, Blackshields G, Brown NP et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol 128:682–695

Li ZG, Gong M (2009) Involvement of calcium and calmodulin in mechanical stimulation-induced heat tolerance in tobacco (Nicotia-
tiana tabacum L.) suspension cultured cells. Plant Physiol Com-
mun 45:363–365

Li C, Meng D, Zhang J, Cheng L (2019) Genome-wide identification and expression analysis of CaM/CML genes in response to bacte-

rial pathogens and inducers of defense responses. Plant Mol Biol 51:463–499

Luan S (2009) The CBL-CIPK network in plant calcium signaling. Trends Plant Sci 14:37–42

Luan S, Kudla J, Rodriguez-Concepcion M et al (2002) Calmodulins and calcineurin B–like proteins: calcium sensors for specific sig-

nal response coupling in plants. Plant Cell 14:389–400
Magnan F, Ranty B, Charpenteau M et al (2008) Mutations in AtCML9, a calmodulin-like protein from Arabidopsis thaliana, alter plant responses to abiotic stress and abscisic acid. Plant J 56:575–589

McDonough CM, Rooney LW, Saldivar S (2000) The millets. In: Kulp K, Ponte JG Jr (eds) Handbook of cereal science and technology. Marcel Dekker Inc., New York, pp 177–195

Mohanta TK, Yadav D, Khan AL et al (2019) Molecular players of EF-hand containing calcium sensing event in plants. Int J Mol Sci 20:1476

Munir S, Liu H, Xing Y et al (2016) Overexpression of calmodulin-like (ShCML44) stress-responsive gene from Solanum habrochaites enhances tolerance to multiple abiotic stresses. Sci Rep 6(1):1–20

Murray MB, Cape JN, Fowler D (1989) Quantification of frost damage in plant tissues by rates of electrolyte leakage. New Phyto 113:307–311

National Research Council (1996) Lost Crops of Africa: grains, vol I. The National Academies Press, Washington, DC

Noman M, Jameel A, Qiang WD et al (2019) Overexpression of GmCAMTA12 enhanced drought tolerance in Arabidopsis and soybean. Int J Mol Sci 20:4849

Pandey GK, Cheong YH, Kim KN et al (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in Arabidopsis. Plant Cell 16:1912–1924

Pandey N, Ranjan A, Pant P et al (2013) CAMTA 1 regulates drought responses in Arabidopsis thaliana. BMC Genomics 14:216

Pandey GK, Pandey A, Prasad M, Böhm M (2016) Abiotic stress signaling in plants: functional genomic intervention. Front Plant Sci 7:681

Park HC, Kim ML, Kang YH et al (2004) Pathogen- and NaCl-induced expression of the ScAmp4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol 135:2150–2161

Park HC, Park CY, Koo SC et al (2010) AtCML8, a calmodulin-like protein, differentially activating CaM-dependent enzymes in Arabidopsis thaliana. Plant Cell Rep 29:1297–1304

Qiu QS, Guo Y, Dietrich MA et al (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc Natl Acad Sci USA 99:8436–8441

Qu L, Sun M, Li X et al (2020) The Arabidopsis F-box protein FOF2 regulates ABA-mediated seed germination and drought tolerance. Plant Sci 301:110643

Raina M, Kumar A, Yadav N et al (2021) StCaM2, a calcium binding protein, alleviates negative effects of salinity and drought stress in tobacco. Plant Mol Biol 106:85

Ramakrishna C, Singh S, Raghavendrarao S et al (2018) The membrane tethered transcription factor EchZIP17 from finger millet promotes plant growth and enhances tolerance to abiotic stresses. Sci Rep 8:2148

Ranty B, Aldon D, Cotelle V et al (2016) Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. Front Plant Sci 7:327

Rao SS, El-Habhab MH, Havens WM et al (2014) Overexpression of GmCaM4 in soybean enhances resistance to pathogens and tolerance to salt stress. Mol Plant Pathol 15:145–160

Saeng-ngam S, Takiprom W, Buaboocha T et al (2012) The role of the OsCam1-1 salt stress sensor in ABA accumulation and salt tolerance in rice. J Plant Biol 55:198–208

Sanyal SK, Pandey A, Pandey GK (2015) The CBL-CIPK signaling module in plants: a mechanistic perspective. Physiol Plant 155:89–108

Sanyal SK, Kanwar P, Samtani H et al (2017) Alternative splicing of CIPK3 results in distinct target selection to propagate ABA signaling in Arabidopsis. Front Plant Sci 8:1924

Sanyal SK, Mahiwall S, Pandey GK (2019) Calcium signaling: a communication network that regulates cellular processes. In: Sopory S (ed) Sensory biology of plants. Springer, Singapore, pp 279–309

Sanyal SK, Mahiwall S, Nambiar DM, Pandey GK (2020) CBL-CIPK module-mediated phosphoregulation: facts and hypothesis. Biochem J 477:853–871

Seo KI, Lee JH, Nezamas CD et al (2014) ABD1 is an Arabidopsis DCAF substrate receptor for CUL4-DDB1-based E3 ligases that acts as a negative regulator of abscisic acid signaling. Plant Cell 26:695–711

Shen Q, Fu L, Su T et al (2020) Calmodulin HvCaM1 negatively regulates salt tolerance via modulation of HvHKT1s and HvCAMTA4. Plant Physiol 183:1650–1662

Singh UM, Metwal M, Singh M et al (2015) Identification and characterization of calcium transporter gene family in finger millet in relation to grain calcium content. Gene 566:37–46

Sood S, Kumar A, Babu BK et al (2016) Gene discovery and advances in finger millet [Eleusine coracana (L.) Gaertn.] genomics: an important nutri-cereal of future. Front Plant Sci 7:1–17

Srinivasachary DMM, Gale MD, Devos KM (2007) Comparative analyses reveal high levels of conserved colinearity between the finger millet and rice genomes. Theor Appl Genet 115:489–499

Townley HE, Knight MR (2002) Calmodulin as a potential negative regulator of Arabidopsis COR gene expression. Plant Physiol 128:1169–1172

Tuteja N, Mahajan S (2007) Calcium signaling network in plants: an overview. Plant Signal Behav 2:79–85

Vadassery J, Reichelt M, Hause B et al (2012) CML42-mediated calcium signaling coordinates responses to Spodoptera hervivory and abiotic stresses in Arabidopsis. Plant Physiol 159:1159–1175

Vadivoo AS, Joseph R, Ganesan NM (1998) Genetic variability and diversity for protein and carbohydrate contents in finger millet [Eleusine coracana (L.) Gaertn] in relation to grain colour. Plant Foods Hum Nutr 52:353–364

Viridi AS, Singh S, Singh P (2015) Abiotic stress responses in plants: Roles of calciumregulated-proteins. Front Plant Sci 6:600

Wang TZ, Zhang JL, Tian QY et al (2013) A Medicago truncatula EF-Hand family gene, McCaMP1, is involved in drought and salt stress tolerance. PLoS ONE 8:58952

Xiong L, Zhu JK (2002) Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ 25:31–139. https://doi.org/10.1046/j.1365-3040.2002.00782.x

Xu J, Tian YS, Peng RH et al (2010) AtCpk6, a functionally redundant and positive regulator involved in salt/drought stress tolerance in Arabidopsis. Planta 231:1251–1260

Xu G, Rocha PSCF, Wang M et al (2011) A novel rice calmodulin-like gene, OsMSR2, enhances drought and salt tolerance and increases ABA sensitivity in Arabidopsis. Planta 234:47–59

Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E (2005) Vacuolar Na+/H+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca2+- and pH-dependent manner. Proc Natl Acad Sci USA 102:16107–16112. https://doi.org/10. 1073/pnas.0504437102

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264
Zeng H, Xu L, Singh A et al (2015) Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. Front Plant Sci 6:600
Zhang HY, Mao XG, Jing RL et al (2011) Characterization of a common wheat (*Triticum aestivum* L.) TaSnRK2.7 gene involved in abiotic stress responses. J Exp Bot 62:975–988
Zielinski RE (1998) Calmodulin and calmodulin-binding proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 49:697–725

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.